EFFECT OF HEAVY METAL CO-CONTAMINATION ON THE BIODEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN AN URBAN SOIL WITH HIGH ORGANIC CARBON CONTENT

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Abstract

Biodegradation is a commonly used approach for the removal of organic contaminants from soil, relying on naturally present microorganisms that utilise the pollutants as an energy source. Often these sites are co-contaminated with heavy metals and the aim of the current research was to investigate how this affects the biodegradation of 16 US EPA priority polycyclic aromatic hydrocarbons (PAHs), both in terms of removal rates and the overall functioning of the soil microbial community.

Soil samples were obtained from a Greenfield site in Newcastle upon Tyne. The soil had a high organic content (11.0 %) and also contained elevated lead concentrations as a result of past atmospheric deposition from adjacent industrial activities. PAHs were applied to the soil using a coal tar source dissolved in acetone, giving a total PAH concentration in the spiked soil of 2166 mg kg^{-1}. Individual PAH concentrations ranged from 1.44 mg kg^{-1} (acenaphthylene) to 325 mg kg^{-1} (benzo[b]fluoranthene); the benzo[a]pyrene concentration was 255 mg kg^{-1}. The effect of heavy metal co-contaminants on the biodegradation was investigated using separate amendments of cadmium and lead to give respective total concentrations ranging from 133 to 620 mg kg^{-1} and 340 to 817 mg kg^{-1}. Mercury amendment was used to give an abiotic control. The study was carried out over 40 weeks.

For all treatments, the degradation of PAHs was observed to be biphasic. A novel kinetic model was developed to explain this dependence. In the absence of metal amendment, it was found that PAHs comprising two and three benzene rings generally degrade at a faster rate than four- five and six-membered rings. In the presence of metal amendments, overall % biodegradation after 40 weeks is relatively unaffected for two to four-ring PAHs but shows significant impairment for five and six-ring PAHs. Nevertheless, degradation rates generally decrease with increasing metal concentration, as do soil respiration rate, Shannon Diversity Index, and microbial biomass content. Lead appears to exert the greatest inhibitory effect. The novelty of this study arises from the integrated approach to investigating the effect of metal co-contaminants on the biodegradation of all 16 US EPA priority PAHs together with parameters relating to the functioning and diversity of the soil microbial community.

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Selected Abbreviations

AAS	Atomic absorption spectroscopy
AWCD	Average well colour development
BOD	Biological oxygen demand
CCA	Copper chromium arsenate
CRM	Certified reference material
CLPP	Community level physiological profile
DCM	Dichloromethane
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
ED-XRF	Energy dispersive x-ray fluorescence
EI	Electron Impact
EPA	Environmental protection agency
EU	European Union
FID	Flame Ionization detector
FL	Fluorescence
GC-MS	Gas chromatography-mass spectrometry
GN	Gram negative
GP	Gram positive
HACA	Hydrogen abstraction acetylene addition
HMW	High molecular weight
HOC	Hydrophobic organic contaminants
IARC	International agency for research on cancer
ISQG	Interim sediment quality guideline

LWM	Low molecular weight
MSE	Methanolic saponification extraction
PAHs	Polycyclic aromatic hydrocarbons
PCA	Principal component analysis
PCR	Polymerase chain reaction
PFE	Pressurized fluid extraction
PLE	Pressurized liquid extraction
PLFA	Phospholipids fatty acid
POPs	Persistent organic pollutants
PTV	Programme temperature vaporizer
RSD	Relative standard deviation
SD	Standard deviation
SF-N2	Spore-forming gram negative
SF-P2	Spore-forming gram positive
SIM	Selected Ion monitoring
SSL	Split splitless injector
T-RFL	Terminal restriction fragment length polymorphism
USEPA	United States Environmental Protection Agency
WHC	Water holding capacity

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Declaration

I declare that the work contained in this thesis has not been submitted for any other award and that it is all my own original work. I also confirm that this research fully acknowledges opinions, ideas and contributions from the work of others. The work was carried out under the supervision of Dr. Michael Deary.

Name: Chinedu C. Ekumankama

Signature:

Date

Chapter 1: Introduction aims and objectives

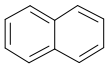
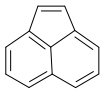
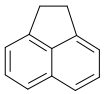
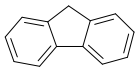
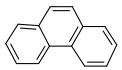
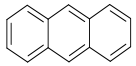
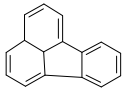
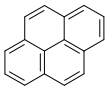
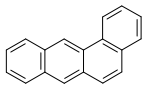
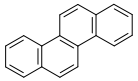
1.1 General introduction

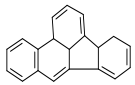
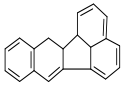
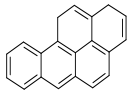
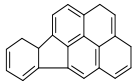
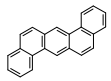
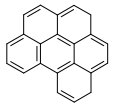
Polycyclic aromatic hydrocarbons (PAHs) are part of a large group of persistent organic pollutants (POP) that are commonly found in the environment. There are several reports on various environmental matrices that are contaminated with PAHs [2]. For example, the concentration of benzo[a]pyrene in different environmental media can be seen in Table 1.1. PAHs are described as hazardous organic compounds that consist of two or more fused benzene rings arranged in either linear, angular or cluster arrangements, consisting of carbon and hydrogen atoms [3, 4]. They may, in addition, incorporate other ring structures, as well as alkyl substituents. Those PAHs comprising entirely of fused benzene rings such as naphthalene (2-rings) or phenanthrene (3-ring) (as shown in Table 1.2) are known as alternant PAHs, whereas those incorporating other ring structures, such as fluorene and benzo[b]fluoranthene (Table 1.2) are non-alternant [5]. The behaviour of PAHs in the environment has been researched for more than twenty years because of their prevalence, persistence and toxicity [6]. Their environmental persistence and toxicity with several being identified as carcinogens and mutagens [7] have led both the European community and U.S. Environmental Protection Agency, (US EPA) to register 16 PAHs as priority pollutants that must be monitored in the environment [1, 8]. Table 1.2 summarises the structures of the USEPA priority list of 16 PAHs. These compounds whilst important in terms of toxic potential and the mass fraction of PAHs they contribute in contaminated sites represent only a fraction of different PAH structural forms that can be found on such sites [5]. Various theories have been advanced as to the mechanism of PAH formation during incomplete combustion processes, the most favoured of which is the hydrogen abstraction acetylene addition (HACA) mechanism; this proposes that polycyclic structures are built up as a result of repetitive addition of acetylene molecules to hydrocarbon radical sites that have been activated by hydrogen abstraction[9]. Ring closure reactions give successively larger polycyclic structures.

Table 1.1: Concentrations of benzo[a]pyrene in different environmental medium [10].

MEDIA	CONCENTRATION
Air	1.3 - 500 ng m^{-3}
Soil	0.8 ng kg^{-1}-100 mg kg^{-1}
Tap water	2.5 - 9 ng L^{-1}
Surface water	130 - 500 ng kg^{-1}
Plants	Up to 150 µg kg^{-1}
Food	0.1 - 20 µg kg^{-1}

Table 1.2: 16 Priority USEPA PAH structures, properties and toxicity classifications

PAH	Structure	Empirical Formulae	Ms ion for Quantitation	Boiling point ^{0}C	Melting point ^{0}C	Solubility in water at 25 ^{0}C mgl^{-1}	Log K_{ow}[a]	Biodeg. rate k_B(day^{-1})[d]	EPA group B2 Carcinogens	IARC group 2 Carcinogens
Naphthalene		$C_{10}H_8$	128	218	80.5	31-34	3.4	0.337	No	2B
Acenaphthylene		$C_{12}H_{8v}$	152	270	80-83	3.93	4.07[b]	0.02	No	ND
Acenaphthene		$C_{12}H_{10}$	154	279	96.2	3.5 -7.4	3.92	0.01	No	ND
Fluorene		$C_{13}H_{10}$	166	295	116	1.9 -1.98	4.18	0.015	No	3
Phenanthrene		$C_{14}H_{10}$	178	338	101	1.29	4.57	0.0447	No	3
Anthracene		$C_{14}H_{10}$	178	340	216	1.29	4.54	0.0052	No	3
Fluoranthene		$C_{16}H_{10}$	202	383	107	0.265	5.22	0.0018	No	3
Pyrene		$C_{16}H_{10}$	202	393	150	0.16 -0.0032	5.18	0.0027	No	3
Benzo[a]anthracene		$C_{18}H_{12}$	228	435	162	0.0044	5.79	0.0026	Yes	2B
Chrysene		$C_{18}H_{12}$	228	441	255	0.006	5.98	0.0019	Yes	3

Benzo[b]fluoranthene		$C_{20}H_{12}$	252	481	168	0.0012	6.12[b]	0.0022	Yes	2B
Benzo[k]fluoranthene		$C_{20}H_{12}$	252	481	217	0.00055	6.06	0.0024	Yes	2B
Benzo[a]pyrene		$C_{20}H_{12}$	252	496	179	0.003	6.5[b]	0.0005	Yes	2A
Indeno[123-cd]pyrene		$C_{22}H_{12}$	276	524[c]	164	0.062	6.58[b]	0.001	Yes	2B
Dibenzo[a,h]anthracene		$C_{22}H_{14}$	278	539[c]	267	0.0005	6.86	0.0024	Yes	2A
Benzo[g,h,i]perylene		$C_{22}H_{12}$	276	545[c]	222	0.00026	7.10	0.0019	No	3

[à][11], [b][12], [c][13], [d][14]

Evaluation of risk according to US EPA [14], International Agency for Research on Cancer (IARC) [15], EPA group B =probable human carcinogens, 1=Carcinogenic to human; 2A= probably carcinogenic to human; 2B= possibly carcinogenic to human; 3= not classifiable as to its carcinogenicity to humans, ND= Non Detected

1.1.1 PAH sources:

PAHs can be released into the environment from two major sources which are natural (biogenic/biosynthetic and geochemical/petrogenic) and anthropogenic (pyrogenic) [2, 3, 16]. Natural sources of PAHs can include volcanic eruptions and forest fires [17]. PAHs may also be produced during natural processes such as thermal geologic reactions and plant fossilisation (petrogenic origin) [18]; this leads to the possibility of natural contamination, for example as a result of petroleum reservoir seepages, or from organic-rich shales [19]. It is well known that petroleum based fuels, oils and coal naturally contain PAHs and they provide the largest natural source of PAHs in the environment [3].

PAHs can also have geochemical origins (petrogenic origin) during pyrolysis which involves the exposure of sediments (organic substances in sediment) to high temperature during sediment diagenesis. The aromatic compounds formed through this process are more stable than the original organic compounds, usually alkylated benzene rings. The alkyl groups can be of sufficient length to allow cyclization and then with time these cyclized moieties become aromatized. The temperature at which this process occurs determines the degree of alkyl substitution on the PAH; the higher the temperature, the less substituted the resulting PAH become. For example, the coking of coal (high temperature) produces a material that is composed of a relatively simple mixture of unsubstituted hydrocarbons. While with the slow burning of wood (lower temperatures) on the other hand, alkylated hydrocarbons survive, producing a more complex mixture of hydrocarbons. Another example is the formation of crude oil over millions of years under low temperatures (100 to 150 ^{o}C) and consequently, the alkylated PAHs would far exceed the unsubstituted PAHs. When PAHs are produced, their aromatic ring arrangement determines their environmental persistence and hence their mobility in the environment. For example anthracene and naphthalene that have linearly arranged benzene rings are the least persistent and are usually not commonly detected in the environment except when they are sequestered into certain organic or inorganic matrices. The aromatic ring arrangement that is most persistent is the angular types [20] which can be found in phenanthrene and chrysene structures. These PAHs can abound where organic materials have been exposed to high temperatures [3]. Thiele *et al.* [21] in their study also stated that PAHs can form under certain biological processes such as the formation of perylene under anaerobic conditions in sediments and PAH formation through enzymatic reactions under oxygen deficiency in soil. The concentrations of individual PAHs compounds in soil that are produced by natural processes have been estimated to be in the range of 1-10 µg kg^{-1} [22] and as most of the naturally produced PAH are cycled through the environment at

low concentrations, it can then be assumed that many bacteria and fungi have developed the capabilities to derive carbon and energy from them and hence their degradation [3, 23].

Anthropogenic sources are by far the major cause of PAH environmental pollution, with approximately 90 % of PAH emissions, estimated to be from this source [2, 22, 24]. Anthropogenic (pyrogenic origin) sources are of two general types. One is the result of crude oil spillage and disposals of coal tar, creosote and petroleum products in the environment. PAHs are also found in significant concentrations in disposed materials such as asphalt, asphalt sealants, new and used motor oil, tire dust, brake lining particles and vehicle exhaust fumes [25]. The high concentrations of PAHs in the environment are usually associated with the disposal of this hydrocarbon material or petroleum residues [26] because though the contaminated area is small in size the level of contamination concentration in such areas are usually high.

The other type of anthropogenic source is from the incomplete combustion of organic material (hydrocarbons) for example wood burning, waste incineration, automobile emissions and industrial discharges and coal gasification [4]. The less efficient the burning process the more PAHs are released into the air and the optimal temperature of PAHs formation are in the range of 660 - 740 ^{o}C [25]. The release of PAHs in air leads to its wide distribution over a short and long distance becoming the main route of PAH distribution. It has been reported that wet/dry atmospheric deposition is one of the main sources of PAHs in soil and marine environment [27]. These types of PAHs (atmospheric) can partition between the particulate and the gaseous phases [24, 28]. PAHs consisting of 5 - 6 aromatic rings (classed as carcinogenic) are mostly associated with the particle phase. The atmospheric PAHs tend to adsorb to particulate materials which can be deposited on the underlying sediments leading to their high accumulation and concentration [29]. The particulate matter can be less than 10 µm in diameter [29]. It is these anthropogenic sources of PAHs that have brought about the huge interest in PAHs, and has resulted in many environmental clean-up programs, and subsequently the development of effective bioremediation technologies [3]. Pyrogenic sources generally result in the formation of high molecular weight PAHs, whereas petrogenically produced PAHs are generally low molecular weight [30].

1.1.2 PAH properties:

In the study of the bioremediation of PAHs, it is essential to understand the properties of these compounds in the environment in order to help explain the observed behaviour and trends. PAHs have a broad range of properties which differ in molecular weight,

structural configuration, water solubility, number of aromatic rings, volatility, and sorption coefficients [31, 32]. Their high persistence in the environment is due to their hydrophobicity and low water solubility [17, 33, 34] as can be seen in Table 1.2. PAHs generally are divided into two categories low molecular weight comprising of two to three rings structures and high molecular weight compounds comprising of four or more rings structure. At ambient temperature PAHs in a pure state are usually coloured crystalline solids [35] and the physical properties vary with their molecular weight and structure as can be seen in Table 1.2. Their low water solubility is confirmed by their high octanol-water partition coefficient (K_{ow}) which shows that they have a relatively high potential for adsorption to suspended particulates in the air, water and for bioconcentration in organisms [35]. Their solubility at 25 ^{o}C decreases with increasing molecular weight which is in contrast to their octanol-water partition coefficient that increases with increasing molecular weight. They have a low to moderately high vapour pressure as can be seen in their boiling point (Table 1.2.). The low molecular weight compounds (example; naphthalene b.p. 218 ^{o}C) are more volatile than the high molecular weight compounds (example; benzo[g,h,i]perylene, b.p. 545 ^{o}C). The physicochemical properties of low molecular weight PAHs indicate that their transfer and turnover will be more rapid than the high molecular weight compounds. The low molecular weight PAH compounds are predominantly part of the atmospheric PAH burden [6].

1.1.3 PAH environmental distribution and transport

The PAH properties that were discussed in Section 1.2 govern the fate, distribution and transportation of PAH in the environment. Understanding the environmental fate of PAHs is very important in a bioremediation study because the loss of PAHs are governed by these events and need to be taken into consideration when planning bioremediation studies. When PAHs are released into the environment they are subjected to chemical oxidation, photolysis, hydrolysis, volatilization, bioaccumulation, biodegradation, adsorption to soil particles and leaching [36, 37]. Figure 1.1 is a representation of these PAH environmental fate processes. Heavy molecular weight PAH which has low aqueous solubility and high vapour pressure, tend to be associated with particle surfaces. For this reason, they may be less likely to be affected by different fate processes such as volatilization, photolysis, and biodegradation.

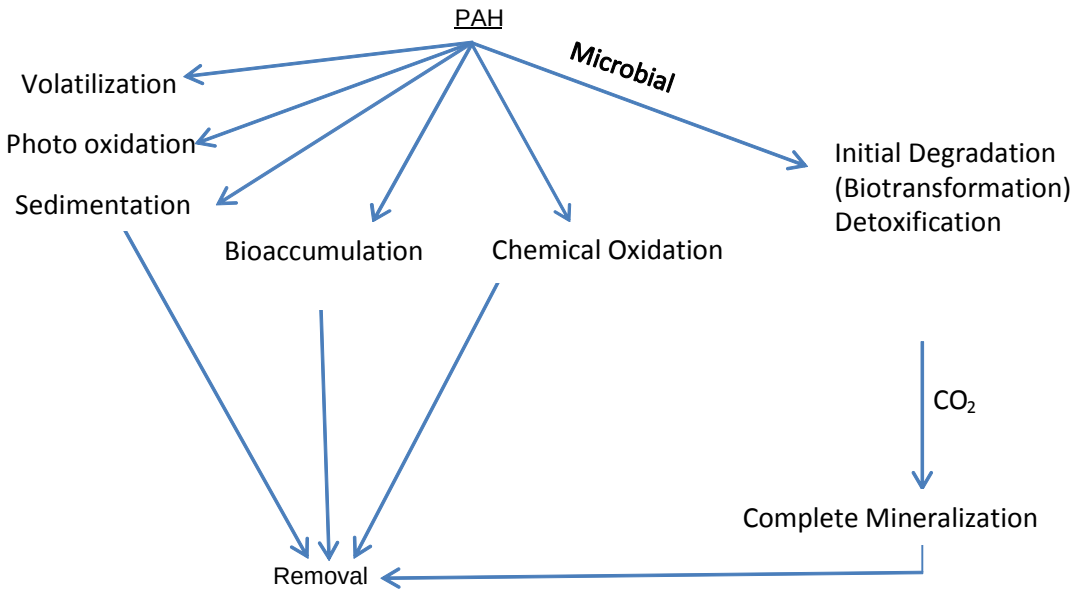


Figure 1.1: Schematic representation of the environmental fate of polycyclic aromatic hydrocarbons [36]

PAH redistribution in soil from contaminated sites occurs slowly and is always heterogeneous, with some of the oily phase spreading on soil particle surfaces and some existing as free product droplets lodged in the interstices of the soil [3]. Their subsequent movement will then depend on their local environmental conditions. Environmental factors such as; contaminant volume and viscosity, temperature, land contour, plant cover, soil composition (e.g. organic matter content) and the influence of natural transporting agents (e.g. flooding, storm water runoff, leaching, etc.) can affect the horizontal and vertical movement of PAHs [38]. Their vertical movement in soil can occur as a multiphase flow that will be controlled by soil chemistry, structure, pore size and water content [3]. Their movement through soil has also been shown to be associated with leachate or with the movement of soil particles [39]. It is generally assumed that when they are adsorbed to soil organic matter they become immobilised but studies [22, 40, 41] have shown that soil water contains some dissolved organic carbon and some amphiphilic substances which have solubilizing capabilities. The amphiphilic substances are constituents of every biological cell membrane, which can consist of solubilizing compounds such as fatty acids, protein, fulvic and humic acids tannins or phospholipids [40]. Some of these amphiphilic substances may be products from anthropogenic sources such as surfactants, soaps, detergent foams, shampoos or other emulsion forming substances which find their way into the environment (natural soil water) [40]. These substances increase the solubility of the PAHs, enhancing their mobility. The dissolved organic substances transport PAHs by forming mobile associates with them. Thus, the impact of dissolved organic carbon on total PAH

mobility ranges from reduction by cumulative sorption or co-sorption to enhancement by co-transport [41, 42]. The mobility of PAH in the soil can also be enhanced by the biosurfactant production capability of bacteria [3]. This biosurfactant increases PAH solubility thereby enhancing their mobility. Weigand *et al.* [41] discovered that increasing dissolved organic matter enhances total PAH mobility. Chi *et al.* [43] also suggested that the process by which dissolved natural organic matter solubilises PAHs in groundwater and enhances its transport in aquatic environments can be used to remediate hydrophobic organic compounds in contaminated soils or aquifer.

1.1.4 PAH toxicity

PAHs are known to have cytotoxic, mutagenic and carcinogenic effects on mammals and other living organisms as earlier mentioned. Their toxic nature has made it an issue of great concern when dealing with contaminated soil/sites. Benzo[a]pyrene was the first PAH for which carcinogenicity was demonstrated [7, 44] and is still the most significant PAH from a regulatory perspective. It is metabolised by cytochrome P450 enzymes in the liver, forming diol epoxides via a ring opening mechanism; these can then form covalent adducts with deoxyribonucleic acid (DNA), which may eventually lead to tumour formation [4]. Other important carcinogens on the USEPA priority list include benzofluoranthene, benzo[a]anthracene, dibenzo[a,h]anthracene and indeno[1,2,3-c,d]pyrene [6] though it should be borne in mind that these compounds exist in complex mixtures in the environment, possibly giving rise to co-carcinogenic or chemopreventative effects [7]. Table 1.2 shows the list of 16 PAH compounds toxicity classification by both the USEPA and the international agency for research on cancer (IARC). The seven PAH compounds listed by the USEPA as probable human carcinogens were categorised on the basis of evidence from animal studies [14] and the IARC has more classifications on 14 of the compounds. The exposure of humans and animals to PAHs are mainly through environmental (air, water), dietary and occupational sources and also from cigarette smoke [10]. Exposure to PAHs constitutes a significant health risk for people living in industrialised areas of the world [4].

When PAHs are ingested, they rapidly undergo absorption into the gastrointestinal tract of humans and animals because of their high lipid solubility [45]. These PAHs can disrupt endocrine signalling leading to reproductive system abnormalities and immune system deficiencies and increased rate of specific cancers. Low molecular weight compounds are also considered to be more toxic to aquatic organisms because of their high water solubility, their toxicity includes mortality and sublethal effects which can

occur at concentrations above a threshold concentration stipulated by Canadian Interim Sediment Quality Guideline (ISQG) [46]. An example of this toxicity was explained using phenanthrene concentrations of 50 µg kg^{-1} and 82 µg kg^{-1} which were above fresh water ISQG of 41.9 µg kg^{-1} and this was observed to be significantly toxic to *Hyalella Azteca*, an amphipod, however, phenanthrene concentrations of 7.75 µg kg^{-1} and 9.8 µg kg^{-1} which were below freshwater ISQG was not observed to be significantly toxic to the same organism [46]. The high molecular weight compounds, of which some are considered carcinogenic, were not acutely toxic to the aquatic organism in this study [46].

PAH-contaminated sites, particularly land associated with former gas works, can have a total PAH concentration ranging between a few hundred and several thousand mg kg^{-1} [47-49]. Given the large number of such sites [6], the fact that these are often in close proximity to population centres and the introduction of a more robust, risk-based, regulatory framework for management of contaminated land [50], there is an urgency to remediate such sites.

1.1.5 PAH bioremediation

Bioremediation can be described as a process of degrading pollutants (organic and inorganic) biologically under controlled conditions into a harmless state in the environment. Bioremediation can also be described as the biological treatment of organic/inorganic waste in order to eliminate their harmful effects. In recent years, bioremediation has proved to be a successful method for remediation and has a cost and technical advantage over chemical techniques [51, 52]. Bioremediation is one of the safest, efficient, eco-friendly and economic means of decontaminating the environment.

Bioremediation of PAHs in contaminated soils, as earlier mentioned, is desired because these compounds have toxic, mutagenic and carcinogenic properties. This process can be accomplished in a variety of ways, for example *in situ* treatment or *ex-situ* method such as bio-piling and composting. The main principle of this technique is the use of indigenous microbial community of the contaminated site to degrade/convert the pollutant into a harmless state. The strategies are developed to promote the microbial metabolism of contaminants and easy accessibility of contaminants to degrading microorganisms. They can be optimised by adjusting the water, air, nutrient supply and by the addition of surfactants. Biostimulation, such as the addition of a bulking agent such as wood chips and/or nutrients such as nitrogen, phosphorus, potassium (N/P/K) can be employed to aid the process, as can bioaugmentation, which

involves the addition of inocula of microorganisms with known pollutant transformation abilities [33]. The bioremediation efficiency can be significantly influenced by microbial ecology, system microbiology (presence of co-substrate, genetics of the relevant organisms and enzyme stability and activity), process operation conditions (microenvironment and configuration) and the presence/absence of indigenous microbial activity.

Bioremediation (biodegradation process) has now become the most common remediation method and there are several good reviews of practical applications [53] and the underlying microbial mechanisms [4, 54, 55]. A wider range of remediation technologies *including* bioremediation is reviewed by Gan *et al.* [56]. Bioaugmentation with cultures of bacteria and fungi isolated from contaminated sites has been shown to be an effective way of increasing biodegradation rates [57].

Biodegradation of PAHs can be carried out by a wide variety of bacteria and filamentous fungi: bacteria generally utilise the PAHs as carbon sources whereas fungal action is part of a detoxification process [4]. Ring cleavage is an essential part of the degradation process for bacteria, such as *Pseudomonas aeruginosa,* and is accomplished by the action of the dioxygenase enzyme on the aromatic ring to yield a dihydroxylated cis-Dihydrodiol, an intermediate that is subsequently oxidised via dehydrogenase to catechol which then undergoes ring cleavage by further enzymic action [4]. Non-ligninolytic fungi such as *Cunninghamella elegans,* utilise cyctochrome-P450 monooxygenases to form an arene oxide that spontaneously rearranges to a phenol which may become conjugated with glycoside, glucuronide, sulphate, xyloside or methyl moieties. Finally, ligninolytic fungi such as *Phanerochaete chrysosporium*, *Berkandera adusta* and *Pleurotus ostreatus*, utilise extracellular lignin peroxidases that catalyse a one-electron oxidation of the PAH to the corresponding quinine which subsequently undergoes ring fission and mineralisation [4, 54, 55]. Figure 1.1 shows a schematic representation of biodegradation of PAH by both bacteria and Fungi. Whilst there have been a great number of studies on individual and mixed cultures it is important to appreciate that in the soil environment, degradation will be carried out by a diverse community of all of these microorganisms in which co-metabolism and detoxification processes are thought to play an important role in overall PAH removal [55, 58]

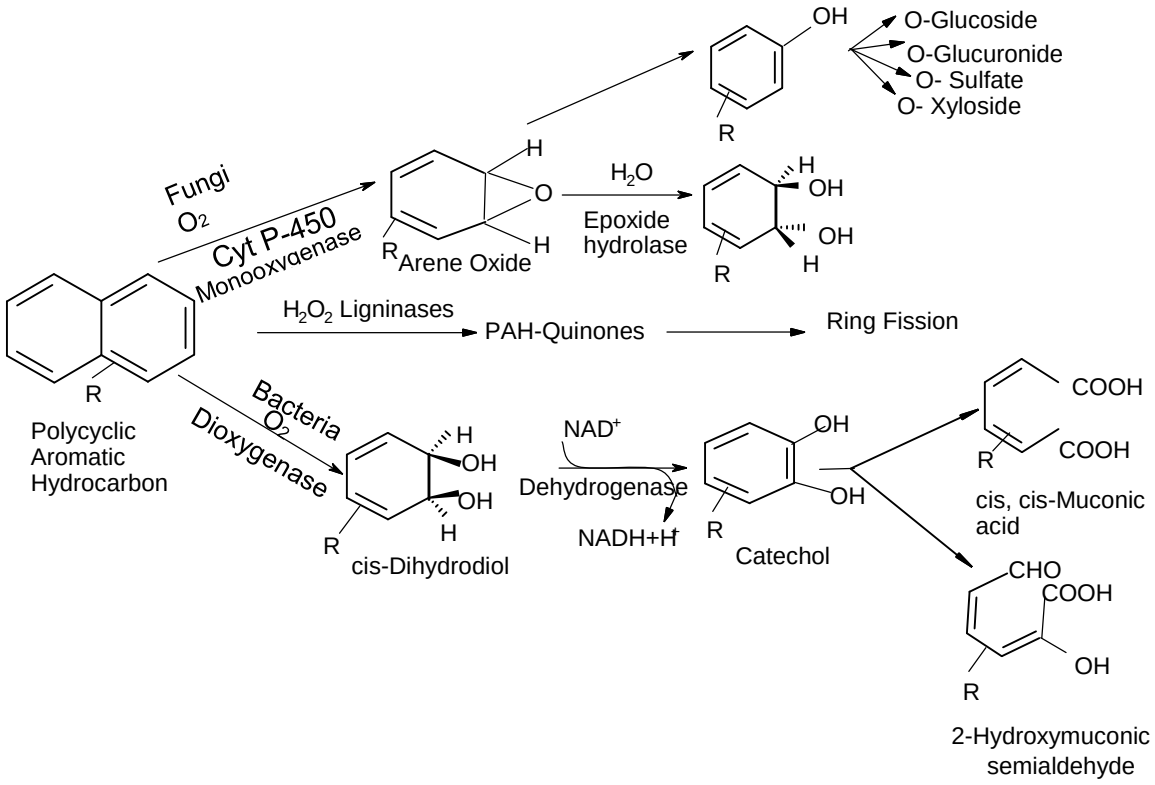


Figure 1.2: Schematic representation of PAH Biodegradation pathway [59]**.**

1.1.5.1 Factors affecting PAH bioremediation efficiency

Bioremediation of PAHs can be greatly influenced by many physical, chemical and microbial factors but the pertinent factors are discussed in the following sections.

1.1.5.1.1 Bioavailability:

Bioavailability in soil refers to that fraction of a substance that can be bioassimilated or biotransformed or transferred freely into or onto living organisms in the soil [1, 60]. It can then be defined as the accessibility of a substance for bioassimilation or the environmental mobility of a substance (e.g. organic compounds) towards microorganism for biodegradation [8]. The two vital factors that determine the quantity of bioavailable compound are: ‘’(i) the rate of transfer of the compound from the soil to the cells of living organisms (mass transfer) and (ii) the rate of absorption and metabolism (the inherent action of the cell)’’ [1, 61]. Bioavailability is one of the most important factors influencing PAH biodegradability in the soil. This is because PAHs, especially the higher molecular weight compounds (HMW), exhibit very low water solubility and therefore, tend to partition onto soil mineral surfaces and to sorb firmly to available organic materials. When this happens the PAHs become physically unavailable to resident microorganisms and are therefore protected from microbial

catalysis. However, some microorganisms (e.g. bacteria) possess properties (such as enhanced cell-surface hydrophobicity, biofilm formation, surfactant production, motility and chemotaxis) which are capable of improving microbial access to PAH compounds [62]. For example Miller *et al*. [37] indicated that *Pseudomonas sp.* growing on phenanthrene and naphthalene produced biosurfactants which increased the solubility of the substrate. Thus, the primary remediation strategy for microorganisms that biodegrade PAHs would be to release biosurfactants and extracellular polymeric substances in order to increase the availability of PAHs, especially the higher molecular weight compounds. The specific microorganisms that carry out this metabolic function are then able to use the PAHs as a source of carbon and energy for biomass production (growth and multiplication) and CO_2 formation [63]. Many soils and sediments polluted with PAHs host active populations of PAH-degrading bacteria [63, 64].

Physical sorption of PAHs is one of the primary factors that affect chemical persistence and thus greatly impacts the effectiveness of bioremediation efforts for soil, sediment and aquifers. Physical factors that impact bioavailability include: soil type, texture and organic matter content. Studies have shown that bacterial degradation can be greatly altered by the physical and chemical properties of the heterogeneous microbial environment [61]. Amellal *et al.* [61] observed that phenanthrene degrading bacteria in the sand aggregate size fraction were present in higher numbers than in the silt aggregate fraction (sand fraction increased from 1×10^3 to 1×10^8 bact./g dry weight of aggregate after 6 months of incubation and contamination while the silt fraction increased from 1×10^3 to 1×10^6 bact./g dry weight of aggregate after 6 months). The reason was that the sand fraction provided more favourable conditions for growth of bacteria that degrade phenanthrene than the silt aggregate; in particular, PAHs were more bioaccessible in the sand fraction than in the silt fraction [61]. The PAH concentration in the sand fraction (0.5 mg kg^{-1}) as a result of the increased microbial activity was less than the PAH content of the silt and clay fraction after 1 year. The biodegradation of PAHs in this instance was controlled by the inaccessibility of PAHs for the bacteria. The clay fraction had the highest PAH concentration (108 mg kg^{-1}) and also the highest organic matter content (32 %) which implies that the PAHs may have been adsorbed by the organic matter content and the large surface area of the clay smectites fraction in the soil.

Li *et al.* [65] also observed that the highest total PAH concentration (40.18 mg kg^{-1}) occurred in the 250-500 µm soil size fraction of a contaminated site; the finest size particle fraction of <50 µm had a medium total PAH concentration of 24.44 mg kg^{-1} while the 50-75 µm particle size had the lowest total PAH concentration (6.27 mg kg^{-1}).

The reason was that the percentage organic carbon content in the soil controlled the PAH concentrations obtained from the different size fractions. The particle size with the highest total PAH concentration had a higher organic carbon content of 0.59 % while the particle size with the lowest total PAH concentration had the lowest organic carbon content of 0.31 %. The organic carbon content of a soil is involved in PAH sequestration and sorption, thereby reducing PAH bioavailability and biodegradation.

1.1.5.1.2 PAH structure:

Structure has an important bearing on PAH biodegradability both from the perspective of the range of organisms that can metabolise specific PAHs, particularly the higher molecular weight ones [55], but also bioavailability (the subtleties of defining bioaccessibility and bioavailability in the context of contaminated soil have been deftly argued by Semple *et al*. [66]). It is observed in the soil that PAH degradation rates are inversely related to the number of rings in the structure [55]; however, interestingly, when biodegradation experiments are carried out in solution, (two to four rings), this relationship does not hold [67]. These observations almost certainly reflect the bioavailability of the PAH compounds when adsorbed onto soil [67]. For bacteria in particular, in order for metabolism of the PAH to take place, it must be available in solution so that transfer can take place into the bacteria cell (Figure 1.3). Higher molecular weight PAHs have low solubility and so will have a low soil pore water concentration, thus affecting the rate at which they are metabolised. Moreover, the bacteria may be physically constrained from contact with pore water adjacent to adsorbed PAHs when the pores are smaller than 0.2 - 0.8 µm [55] (as can be seen in Figure 1.3). Ligninolytic Fungi whilst also being physically constrained by small pore sizes, do have the advantage of being able to grow directly on soil particles containing the adsorbed PAHs; they also possess extracellular lignin peroxide enzymes that can act directly upon the adsorbed phase. Indeed, the non-specificity of these enzymes means that they are able to metabolise a much greater range of PAH structural types than bacteria. Gramss *et al.* for example, have shown that ligninolytic fungi significantly degrade 5- to 7- ring PAHs in soils, whereas limited biodegradation of these compounds takes place in the presence of bacteria alone [68]. Johnsen suggests that the limited number of bacteria that can be grown in pure cultures on PAHs containing five or more aromatic rings is a consequence of low solution availability, and thus the restricted evolution of suitable enzymic pathways for their metabolism [55].

Nevertheless, some bacteria, such as species of *Nocardia, Pseudomonas* and *Mycobacterium* are able to form biofilms, and thus, grow directly on the soil particles [55] and some such as *Pseudomonas aeruginosa* are able to produce rhamnolipid biosurfactants which may solubilise the PAHs, thus increasing biodegradation rates [55, 69]. One final note on the importance of structure is that PAHs possessing alkyl groups and five-member rings (non-alternant) are found to degrade at a lower rate than alternant PAHs, both in soil and solution phase [67].

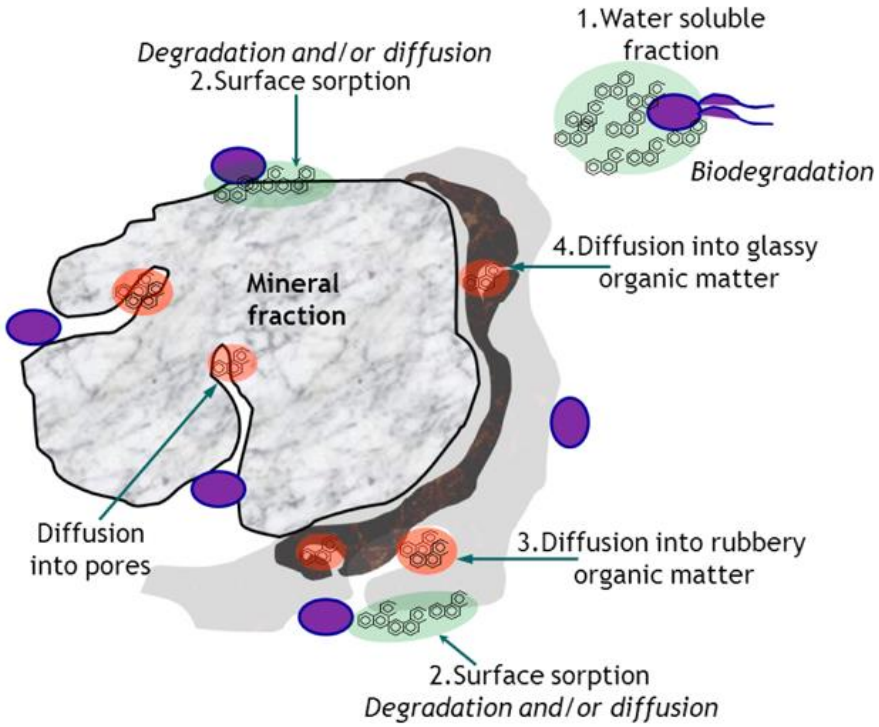


Figure 1.3: Schematic of the different processes of PAH interaction with soil minerals, soil organic matter and microbial community [70]. The rubbery organic matter and the glassy organic matter are forms of organic matter containing dissolution sites. The glassy phase contains more rigid cavities (holes) where contaminants can interact with organic matter and be sequestered [1]. The rubbery phase contains the more mobile and soluble organic matter that can absorb (dissolve) the PAHs.

KEYS:

- PAH-degrading microorganisms
- PAHs compounds diffusing into organic matter and mineral pore
- PAHs that are bioavailable
- Glassy organic matter
- Rubbery organic matter

1.1.5.1.3 Sorption/desorption kinetics

Since biodegradation of PAHs are known to be essentially governed by bioavailability, it is therefore, important to understand the forms that nonbioavailable PAHs can take in soil. PAH sorption and desorption processes exhibit biphasic stages within the soil (Figure 1.4). Initially, a portion of the PAH is rapidly sorbed to the external surfaces of soil, (in minutes to a few hours upon contact to soil surface) primarily to organic materials, the remaining fraction will then be sorbed more slowly over time (weeks or months) [1]. But this rapidly sorbed fraction also becomes readily available for rapid desorption, volatilization, biodegradation by microorganism and extraction by organic solvents [71]. The rapid desorbing fraction will then decrease in amount due to loss by the above-mentioned mechanism and by the diffusion of the initial and later sorbed fractions into intraparticle micropores of the soil environment, where they undergo a slow conversion process, become sequestered and unavailable [3, 71]. This then explains a very important phenomenon termed 'soil ageing', a process whereby PAHs adsorbed onto the soil matrix migrate to increasingly inaccessible soil phase, ultimately to phases that are neither accessible to soil microorganisms nor extractable by robust extraction methods such as soxhlet or accelerated solvent extraction [71]; in effect, the soil is physically self-detoxifying. Factors that can influence ageing include; soil organic matter quantity and quality (in particular the organic carbon fraction), the inorganic composition (e.g. clay content), soil microbial activity, wetting and drying cycles and contaminant concentration [72]. Studies have shown that it is the organic phase of the soil that is most important in the process of soil ageing, with this being nicely demonstrated by Weissenfels *et al.* who also showed that biodegradation was significantly inhibited in soils containing high levels of organic carbon (13.6 %) as a result of reduced bioavailability [36]. Hatzinger and Alexander have shown similar results [73]. The extent of this sequestration process was demonstrated by Northcott and Jones [71], in an ageing study carried out on sterilised soil (2.1 % organic carbon) using ^{14}C labelled phenanthrene, pyrene and benzo[a]pyrene. The amount of benzo[a]pyrene extractable by Soxtex (dichloromethane (DCM), 5 hours) reduced to 83.1 % by day 10, 68.1 % by day 259 and 60.3 % by day 525. The reduction in extractability of the lower molecular weight PAHs, phenanthrene (90.5 %, 89.8 % and 88.9 % respectively) and pyrene (90.4 %, 84.2 % and 82.4 % respectively), whilst smaller in effect, were still nonetheless significant [71]. Only by using methanolic saponification extraction (MSE), were significant extra PAHs released from the soil. MSE causes hydrolysis of labile ester bonds in the macromolecular humic network, causing limited breakdown of the structure, releasing PAHs from this phase. It also breaks any hydrolysable bonds between PAH and soil organic matter. Nevertheless, full recovery was still not attained by this method suggesting that some proportion of

the PAHs were sequestered into organic matter associated with soil humin (recalcitrant organic matter not extractable by dilute alkali [74]). Rates of loss of PAHs to the non-extractable phase were 2.73 to 7.23 x 10^{-3} Bp g^{-1} day^{-1} for pyrene, 2.73 to 3.33 x 10^{-3} Bp g^{-1} day^{-1} for benzo[a]pyrene and 0.38 to 3.87 x 10^{-3} Bp g^{-1} day^{-1} for phenanthrene [71]. Semple *et al.* [39] also observed a fairly rapid sequestration of PAHs (^{14}C labelled fluorene and benzo[a]pyrene) during a 158 month lysimeter study with non-sterilised soil; the non-extractable phase (Soxtec with DCM), determined from ^{14}C activity, was formed within 4 months of spiking (introduction of the PAH substrate) and remained fairly constant during the remaining 148 months of the study. The importance of organic carbon content in determining the extent of non-extractability and non-bioavailability has been demonstrated by Chung and Alexander [75] who measured these properties for phenanthrene (and atrazine) for 16 soils, ranging from 0.07 to 11.0 % organic carbon. A 55 % reduction in extractability was observed for the soil with 11.0 % OC, with OC content representing the most significant factor in multiple linear regression analysis on soil properties. Similar results are reported by Maliszewska-Kordybach [76].

Although PAHs are bound and sequestered in soil, there is the likelihood that they may still be released from soil and sediment because of the changes soil and sediment organic carbon undergoes continuously. There is evidence to suggest that bound PAH residues become remobilized into soil solution or that they do undergo further break down because of microbial interaction and chemical reaction (Figure 1.4). It can be said that organic matter and environmental particles are constantly undergoing alteration caused by environmental, chemical and biotic processes [77] which can weaken the bonds between the PAH and the adsorbing sites thereby releasing the PAH into the environment again. Wu *et al.* [60] observed an increase in the concentrations of 6-ring PAHs (Indeno[123-cd]pyrene and benzo[g,h,i]perylene) after 8 months incubation (at the end of their experiment) in a PAH spiked soil amended with compost. They explained the observed phenomenon by suggesting that the strength of the bonds between the PAH initially adsorbed to the compost was weakened during organic matter mineralisation which led to an increase in the bioavailable PAH that was not bioavailable earlier [60]. Amellal *et al.* [61] also observed that the recovered concentration for PAHs (such as chrysene, dibenzo(a,h)anthracene and benzo(g,h,i)perylene) from their PAH spiked soil were higher after 1 year of incubation and biodegradation than after the initial 6 months of incubation. For example, the recovered concentration of chrysene after 6 months was 40 mg kg^{-1} whereas after 1 year was 100 mg kg^{-1}. The concentration of dibenzo(a,h)anthracene after 6 months was 39 mg kg^{-1} and after 1 year its recovered concentration was 95.8 mg kg^{-1} For

benzo(g,h,i)perylene the recovered concentration after 6 months was 40 mg kg^{-1} but it's recovered concentration after 1 year was 85.3 mg kg^{-1}. The reason being that the PAH compounds were strongly associated with soil constituents at the initial stage (6 months) but after a longer incubation and biodegradation (1 year) the nature of their association with soil constituents were modified which made them extractable once more.

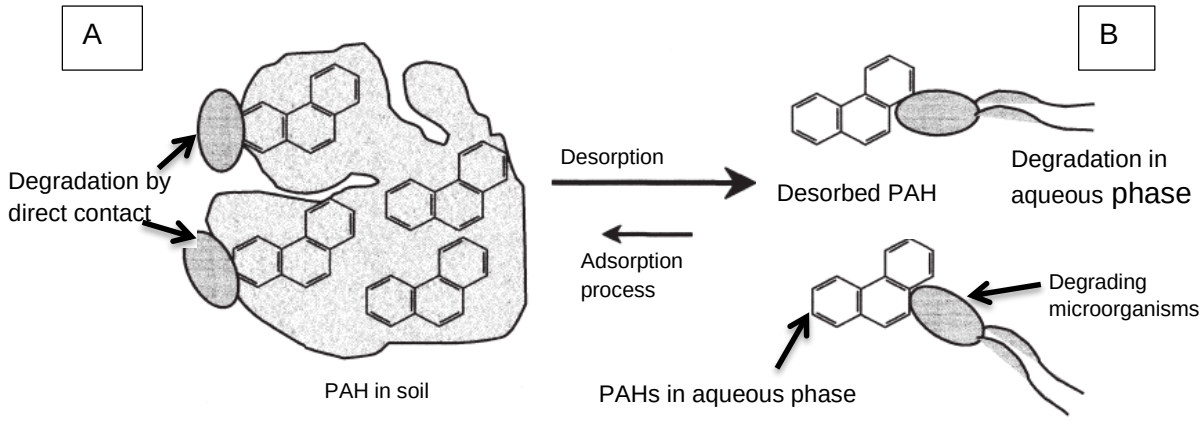


Figure 1.4: Schematic of microbial attack on PAHs in soil: A represents direct contact and B represents degradation in the aqueous phase. The direction of the arrows represent the interaction (sorption-desorption) between the contaminant, the soil and the pore water [1]

PAHs are often found in sites that are co-contaminated with heavy metals, particularly former wood treatment plants where chromated copper arsenate (CCA) and creosote may both have been used [78] but also in former municipal gas or tar works where close proximity to other industries may have resulted in significant contamination by atmospheric deposition. Other occurrences of co-contamination can be in sites such as railway yards, and petrol stations [79]. Such co-contamination raises the question of how the metal toxicity influences biodegradation [47]. In sites with long term co-contamination there is the likelihood that the microbial community will have developed tolerance to the metals and indeed such tolerant bacteria can be cultured and used to bio augment biodegradation in other sites, though this can also be achieved by selective culturing (metal ion containing culture medium), of PAH-degrading bacteria isolated from PAH-only contaminated sites [47, 80]. In order to understand PAH biodegradation in the presence of heavy metal co-contaminated sites, it is important to first understand the interaction of heavy metals in the environment and with the microbial community (biodegradation agent).

1.1.6 Heavy metals

Heavy metals, apart from occurring naturally, can also be introduced into the environment through anthropogenic sources such as mining, smelting, sewage sludge disposal application of pesticides, inorganic fertilizers, steel production and atmospheric deposition [81]. They become very toxic to both natural and man-made environment ecosystems when their concentration exceeds their trace level [82]. Their toxicity and their persistence in soil have received increasing attention, which made many European countries introduce measures during the 1970s that limited metal loading rates of soil due to the use of sewage sludge in agricultural practices. There have also been subsequent European Union (EU) obligatory limits that were established to forestall the accumulation of metal concentrations in agricultural soils [83].

Heavy metal toxicity to microorganisms is through their ionic properties which bind to cellular ligands thereby, displacing native essential elements from their normal binding sites [83] (e.g. arsenate displacing phosphate in the microbial cell). The binding of metals to sulfhydryl groups disrupts the functions of protein and also the binding of metals to phosphate or hydroxyl groups disrupts the function of the microbial deoxyribonucleic acid (DNA). The metal causes a structural change in the proteins and the DNA compounds thereby disrupting their functions. For example cadmium (Cd), when it displaces cellular zinc (Zn) can bind to DNA inducing single strand breaks. This metal-microbe association diminishes; microbial growth, causing abnormal morphological changes and inhibits biochemical procedures in microorganisms.

Long term heavy metal contamination in soil can cause changes in soil microbial community structure and population because of the selective pressure (stress) exerted by high heavy metal concentrations [82, 84]. The changes in microbial community structure and population are usually associated with increased metal tolerance which may also result in loss of microbial functions if the microbial populations possessing a given function are entirely lost from the soil [84]. For example, heavy metal contamination of soil can cause a decrease in microbial diversity responsible for PAH degradation which might cause the bacterial communities to lose part of their PAH degradation capability [85].

Many heavy metals are presently emitted into the soil by human activities as stated earlier but Cd and lead (Pb) are the heavy metals that were used for this present study. Pb and Cd are environmentally concerning elements that have been often reported to cause contamination of soil, sediment, water, and food chains [86-88]. Thavamani *et al.* [79] observed that the concentrations of Pb (concentration range 66 - 671 mg kg^{-1}), Cd

(concentration range 8 - 112 mg kg^{-1}) and Zn (concentration range 64 - 488 mg kg^{-1}) were higher than the concentrations of other metals (which were below the background level) in a manufacturing gas plant site also contaminated with high concentrations of PAHs. Maliszewska-Kordybach *et al.* [89] observed that a coke plant with high PAH contamination also had a Pb and Cd contamination of 350 mg kg^{-1} and 20 mg kg^{-1} respectively.

Cd and Pb may enter the soil as impurities of fertilizers and some black shales may contain cadmium concentrations in excess of 200 mg kg^{-1}. Use of biosolids and composts has also been reported to increase total amounts of Pb and Cd in the soils [90]. The mobility and availability of these metals (which enhances their toxicity in the environment) are controlled by many chemical and biochemical processes such as precipitation-dissolution, adsorption-desorption, complexation-dissociation, and oxidation-reduction and all these processes are affected by soil pH and biological processes [86].

1.1.6.1 Cadmium

Cadmium (Cd) is one of the most toxic metals in the environment and is both carcinogenic and teratogenic and it can be absorbed by plants and enter the food chain [91]. Cadmium exists in nature in the valence state of +2. Cd can affect microbial growth and community diversity and it can be complexed by organic matter or oxides of iron and manganese thereby making it inert biologically. Precipitation of Cd can occur in a low organic matter or sandy soils. In a study on Cd containing soils that were co-contaminated with PAHs, it was observed that biological metabolic activities were low when compared to the control soil [92]. Nevertheless, there were still bacterial strains that were able to potentially survive a Cd concentration of 2 mmol/L during the investigation [92].

1.1.6.2 Lead

Lead (Pb) is also one of the toxic heavy metals that are found in the environment. It exists in the oxidation state of +2 and +4 in the organolead compounds [92]. Lead affects microbial community diversity and activities by inhibiting the nitrification process, nitrogen mineralization, and microbial synthesis of soil enzymes oil enzymes. The complexation of Pb with organic and inorganic minerals (e.g. organic matter, clay minerals) reduces the toxicity of Pb to soil microorganisms [92].

1.1.6.3 Soil microbial interaction with metal

The effect of metals generally, on microbial communities has been extensively reviewed by Bååth [93]. A complex range of effects was observed that is compounded by the range of different approaches and methodologies, with abiotic factors such as pH likely to have had some influence on certain studies. Overall, the relative order of toxicity was Cd>Cu>Zn>Pb, though there were significant variation within individual studies. Overall, several main indicators of microbial activity, including soil respiration, and nitrogen mineralisation, appeared to be relatively unaffected by low concentrations of metals (<100 µg g^{-1}), with increasing inhibition at higher levels; there was also some evidence of stimulation at lower concentrations of metals. More sensitive measures were nitrification and the activities of enzymes such as phosphatase and urease, with metal levels less than 100 µg g^{-1} (mixture of metals) shown to produce inhibitory effects. There is evidence that overall biomass levels (fungi and bacteria) are relatively unaffected up until high hundreds of µg kg^{-1}, but that microbial diversity is decreased i.e. that metal tolerant species increase in abundance. Significant effects on microbial diversity have been demonstrated to occur at Cd concentrations as low as 30 µg g^{-1} when assessed using community level physiological profiling (BIOLOG microplates) [94]. There is also evidence of differential effects on fungi and bacteria, which is important to the present study given the fact that ligninolytic fungi, in particular, are able to biodegrade higher molecular weight PAHs; Bååth *et al.* have shown evidence that in the presence of Zn or Cu (up to 8000 ppm), fungi are less affected and even stimulated, at least in the short-term (37 days), whereas bacterial activity is significantly reduced [95].

However, several bacterial strains in polluted sites have been found to adapt to the heavy metal pollution [92, 96]. There are several metal resistance mechanisms that can be adopted by the microbial community (e.g. bacteria) such as cellular exclusion mechanisms or the binding of the metal ions to the microbial biomass cells as seen in Figure 1.5. This resistance can also be due to the action of the microbial plasmids that limits the intracellular accumulation of metals [96]. For example, resistant cells of *Staphylococcus aureus* have a very efficient chemiosmotic efflux system specific for Cd^{2+} ions, as a result of two separate plasmid genes. The same plasmid gene could confer resistance to multiple metals or a plasmid may contain separate genes for the individual metals [92]. Another example is a study [92] which shows that a cell-free extract of a *Pseudomonas sp* was able to adsorb lead ions more effectively than whole cells from aqueous solutions. Lead can be transformed into organometallic compounds through microbial methylation and this methylation increases the volatility of the metals and its potential loss [92].

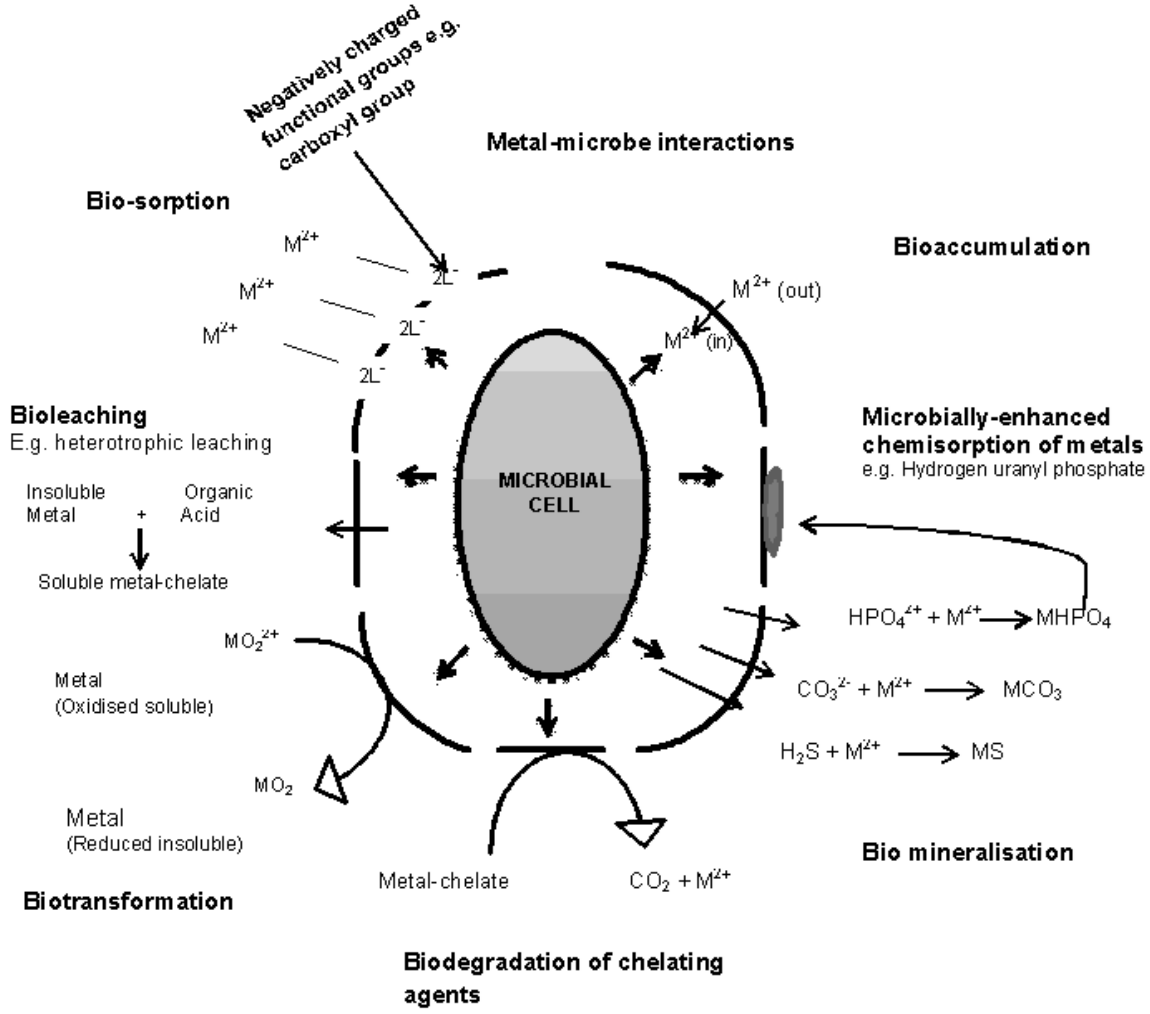


Figure 1.5: Schematic diagram of the different processes involved in the metal-microbe interaction (M^{2+} represents metal ions) [96]

In terms of the specific effect of metals on PAH degradation, Atagana found that in soil spiking studies that degradation of wide range PAHs (phenanthrene, chrysene benzo(a)pyrene, benzo(a)fluoranthene and benzo(a)anthracene) by ligninolytic and non-ligninolytic fungi was unaffected by cadmium and nickel levels of up to 100 µg g^{-1}, but proportionately decreased above these concentrations (up to 500 µg g^{-1}); enzyme activity (Mn-dependent peroxidase activity) was, however, impaired at the lower metal concentrations [97]. Zinc has been shown to marginally stimulate phenanthrene degradation at a concentration 140 µg g^{-1} but causes significant impairment at higher concentrations (720 and 1440 µg g^{-1}) [98].

Although heavy metal can have a toxic effect on the microorganisms that biodegrade PAHs, the co-contamination of heavy metal and PAH may also have a synergistic effect which can both be positive or negative on the microbial community depending on the concentrations and type of both heavy metals and PAHs [99]. This synergistic

effect can either improve or reduce the biodegradation of PAH. Knowledge of this interaction is necessary for a good understanding of PAH bioremediation.

1.1.7 Effect of PAH and heavy metal on soil microbial community

As stated earlier, soils contaminated with PAHs have also been discovered to contain a large variety of other contaminants such as heavy metals, which are often derived from the same source as the PAH [100]. Sources from anthropogenic and natural activities introduce PAH and heavy metals co-contamination into the soil. PAHs and heavy metals are two of the most abundant and harmful pollutants found in contaminated soil.

Most eco toxicologically-based risk assessment methods evaluate contaminated soils by using information generated from the toxicity data of a single pollutant, by that, neglecting mixture effects [101]. These assessments (single pollutant toxicity data) however enable the acquisition of fundamental knowledge about individual pollutants under carefully controlled conditions, but they do not reflect real environmental exposure [102]. In reality, living organisms in the environment are usually exposed simultaneously or sequentially to a variety of pollutants through multiple exposure channels. These varieties of pollutants may possess a combined effect which may be similar (additive) or stronger (synergistic, more than additive) or weaker (antagonistic, less than additive) than expected effects from single pollutant exposure. The effect of this variety of pollutants may depend on the composition of the mixture and may vary significantly from one co-contamination to another.

PAHs and heavy metals both have strong and significant interactions in the environment and also different toxic modes, for example, metals usually bind to reactive cellular macromolecules which interfere with physiological processes such as respiration while in contrast PAHs disrupt membrane function due to the depression of its biological activity induced by PAHs and also many PAHs are potent mutagens, carcinogens and teratogens [88]. The heavy metals and PAH synergistic toxicity phenomenon, as suggested by different studies, is based on the assumption that PAHs may interact with lipophilic components of cytoplasmic membrane of bacteria, thus affecting their permeability and structure which allows the easy penetration of heavy metals into microbial cells to disrupt the cell's functions [79, 103, 104].

However, several studies of the above-mentioned interaction of heavy metal and PAH suggest that this can have both negative and positive effect [99]. For example Guoquing *et al.* [102] observed that heavy metals exhibit toxic activity towards soil biota which led to the decrease in the number and the activity of soil microorganisms and

reduction of PAH microbial degradation rate but another study observed the positive effect of heavy metals and PAHs by suggesting that the presence of Cd and B(a)P increased the levels or activities of pollutant detoxifying enzymes [101].

Gogolev *et al.* [105] observed that low concentrations of Cd (0.1 mg kg^{-1} to 1 mg kg^{-1}) and Fluoranthene (0.05 mg L^{-1} to 0.5 mg L^{-1}) did not reduce the growth and activity of bacteria when added independently in soil, but the addition of both the heavy metal (Cd) and PAH (Fluoranthene) at the same concentrations in soil significantly reduced bacteria growth and activities. In contrast to this synergistic effect, low cadmium concentrations actually enhanced bacteria growth by 38 % when added independently to the soil [105]. Thavamani *et al.* [47] also observed that the presence of Cd (5 mgL^{-1}) stimulated the degradation of a PAH mixture (50mgL^{-1}) that consisted of lower molecular weight (anthracene and phenanthrene) and higher molecular weight (pyrene and benzo(a)pyrene) PAHs. The lower molecular weight PAHs were degraded completely after 60 days and the higher molecular weight PAHs of benzo(a)pyrene and pyrene were degraded to 100 % and 94 % respectively [47]. Khan *et al.* [99] in their study on the degradation of pyrene in soil observed that though pyrene (30 mg kg^{-1}) removal was higher for the soil cultivated with ryegrass (*Lolium multiflorum L.)* than in the uncultivated soil, in the presence of Pb (300 mg kg^{-1}), pyrene, dissipation was accelerated for both the cultivated and the uncultivated soils. In the ryegrass cultivated soil, pyrene removal was much higher for the Pb/pyrene amended soil than in only pyrene amended soil [99]. This then implies that Pb stimulated bacterial growth which enhanced pyrene degradation.

Sokhn *et al.* [106] also observed that increasing the concentration of copper (Cu) from 700 mg L^{-1} to 7000 mg L^{-1} resulted in decreasing degradation of phenanthrene whereas no significant difference in phenanthrene degradation was observed for the control and for the soil with 70 mg L^{-1} Cu. The toxicity exhibited by Cu at 700 and 7000 mg L^{-1} concentrations was evident by the reduced bacterial populations and microbial activities in these treatments. This can be explained as the effect of Cu on microorganisms which inhibits the actions of enzymes involved in the degradation of the intermediates in the phenanthrene degradation pathways. The other notable observation in this study [106] was the degradation of phenanthrene which occurred even at toxic Cu levels of 700 to 7000 mg L^{-1}, implying that highly adapted Cu-resistant species were still performing degradation on the parent phenanthrene molecules [106].

Lu *et al.* [91] also observed that microbial biomass increased in the presence of pyrene alone but decreased in the presence of Cd alone and with pyrene and Cd combination as the concentration of Cd increased. Pyrene could serve as a substrate for microbial

growth and thus enhance microbial biomass in the soil while the toxicity of Cd to microorganisms increased with increasing level of Cd spiking [91]. This result demonstrates that pyrene and Cd affected the microbial biomass production. In general, pyrene and Cd applied together exhibited a significantly greater biocidal effect on their metabolic quotient (qCO_2) than either pyrene or Cd applied alone. Thavamani *et al.* [79] observed the decline in microbial biomass in soils contaminated with both heavy metal and PAHs, where there was a severe inhibition of the dehydrogenase and urease activity. The mixed contamination of PAHs and heavy metals also reduced the bacterial diversity in the soil. These results demonstrated that microbial populations in polluted soils were influenced by the complexity of chemical mixtures present. Jiang *et al.* [94] used Ecoplate average well colour development (AWCD) as an indicator of microbial activity. They observed that phenanthrene and Cd had a negative effect on the AWCD. It delayed the onset of AWCD increase, reduced the rate of AWCD and decreased the maximum AWCD compared to the control. The presence of Cd and phenanthrene had a significant negative effect on microbial metabolism of L-serine, glycogen, and D-cellobiose, L-threonine, D-galacturonic acid, L-asparagine and itaconic acid in the Ecoplate well [94]. It supports the assumption that lipophilic cyclic hydrocarbons such as PAHs interact with the membranes microorganisms including bacteria and yeasts. These interactions lead to changes in the structure and function of the membranes. Increases in permeability to protons and ions can then be observed [107]. Ibarralaza *et al.* [8] noted that Chromium (Cr) (VI) concentration in the range of 500 to 2600 mg kg^{-1} reduced drastically the number of PAH-degrading bacteria present, whereas at low concentrations of 25 to 50 mg kg^{-1} there were no significant changes in the microbial community. Since the effect of metals on soil microorganism depends on their availability in the soil solution, therefore, the increase in Cr (VI) concentrations from 25 mg kg^{-1} to 100 mg kg^{-1} retarded the degradation of phenanthrene and dehydrogenase activity because PAH-degrading bacterial counts were also reduced. The co-contamination of phenanthrene and Cr (VI) caused significant and long-lasting changes in the structure of the microbial soil community which was different from the structural changes produced on soil microbial community when only one of these pollutants were present [8]. The lowest concentration of Cr (VI) established a similar microbial community with the control but also stimulated the increase in the PAH-degrading population which was a positive effect. Guoquing *et al.* [102] observed an inhibition to soil urease activity by the interaction of both PAH and heavy metal. They assumed that heavy metal binds to sulfhydryl groups of the enzyme's active site, forming metal sulfide equivalents. Organic chemicals may denature the entire protein structure. There was also increased urease activity in this study after 14 days of incubation which later declined after 21 days of incubation. The

increased urease activity observed after 14 days was attributed to the increase in the abundance of tolerant microorganisms in the polluted soil which can be due to changes in their physiological adaptations which involved no alterations in their genotype or the replacement of pollutant sensitive species with species that already are tolerant to the pollutant. Handa *et al.* [87] observed that the addition of fluoranthene (250 ng L^{-1}) to soil caused an inhibition of enzyme activity, especially at high fluoranthene concentrations (5 µg L^{-1}) but the addition of fluoranthene and heavy metal (Cd at 29.3 ng L^{-1}) increased the enzyme activity. Contrarily benzo(a)pyrene and indeno[1,2,3-cd]pyrene addition to soil alone showed a significantly strong influence on ethoxyresorufin-O-deethylase (EROD) enzyme activity but when benzo(a)pyrene/indeno[1,2,3-cd]pyrene combined with heavy metal, the enzyme activity was inhibited.

PAHs compounds can both stimulate microbial activity and the biodegradation of each other for example pyrene degradation was enhanced when phenanthrene was added as a co-substrate and benzo(a)pyrene, which can only be degraded by a few microorganisms, had a rapid degradation when phenanthrene was added as co-substrate compared to benzo(a)pyrene alone [47]. Guoqing *et al.* [102] also observed a combined stimulatory activity of phenanthrene, benzo(a)pyrene and fluoranthene on soil microbial activity which they attributed to the gradual adaptation of microorganisms to the pollutants and the utilization of the organic pollutant. They observed an increase in respiration intensity, enzyme activity, and development of microorganisms and a gradual decomposition of the pollutants [102].

However, PAH compounds and their degradation intermediary products can also exhibit toxicity towards microorganisms, thereby retarding the complete bioremediation of PAHs in soil. Gogolev *et al.* [105] observed that the growth of *A.oligospora* was reduced by 14 % at a concentration of 1 mg kg^{-1} of fluoranthene, with 10 and 100 mg kg^{-1} fluoranthene being more toxic. PAH toxicity effects were also demonstrated in another study that showed that eluates of a PAH contaminated soil exhibited a strong biotoxic effect in a bioluminescence assay with *Photobacterium phosphoreum* where a solution containing only 2.6 % of the original eluate reduced the bioluminescence by 20 % [105]. Oxygenated PAHs (an intermediary product of photodegradation) can also be toxic to metabolic processes in microbes, plant and animals. For example anthracene in the photoxidized form was found to be extremely toxic to *Lemna gibba* (a plant) by inhibiting the photosynthetic electron transport and several hydroxyquinones of anthracene also inhibited the growth *L. gibba* [107].

It is known that the solubility of PAHs enhances their biodegradation but inhibition is also common when a PAH is more water soluble because the toxicity of PAHs to microorganisms is related to their water solubility as has been shown in previous case studies above. This toxicity potential can lead to an inhibition of biodegradation especially in a mixture of PAH compounds. For example, naphthalene is strongly toxic in PAH mixture of compounds and can inhibit degradation of other compounds that would normally be biodegraded. This toxicity may be due to its relatively high water solubility (about 30 mg L^{-1}). Fluorene with a solubility of 2 mg L^{-1} and phenanthrene at 1 mg L^{-1} are also frequently inhibitory [92].

Therefore, the bioremediation of a mixture of 16 PAH compounds in the soil (as it is in this present study) was expected to be characterised by both positive and negative interactions of PAH with the soil microbial community. This different level of interaction will be closely monitored over the period of this present study. There is still a paucity of data in this regard because most of the studies on PAH biodegradability have been performed with relatively few PAHs (between one and five compounds) in a simplified model system in order to minimize the number of variables. In real systems, PAHs are generally present as mixtures in a complex environmental condition, therefore, substrate interactions need to be considered during PAH bioremediation planning [61].

The synergistic effect of PAHs and heavy metals can be influenced by soil properties. Soil properties such as organic matter content and in some cases clay content can adsorb the contaminants thereby reducing their bioavailability and mobility, hence their toxicity [108]. The bioavailability of these co-contaminants in the soil is influenced by the competition among the pollutants for sorption sites which results in one pollutant displacing the weaker competing pollutant from the soil particle into the soil solution. The amount of pollutant concentration in the soil solution is thus different from the amount of total pollutant concentration in soil at different times. The distribution of pollutants over the soil phases is of crucial importance to the interaction between PAH and heavy metals towards soil microbial activity [101] because only the metal concentration in the liquid phase is considered available to them. The mobility of heavy metals also depends on various factors such as pH and the other physicochemical parameters which affect solubility and binding to the soil. A high total metal concentration in soil may not be toxic to microbes if it is not bioavailable. Irha *et al.* [108] in their study observed that when a soil has a low affinity for heavy metals in terms of its organic matter content and mineral compositions, the presence of heavy metals will decrease soil microbial activity thereby increasing the persistence of PAH compounds in the soil. It suggested that organic matter content and mineral

compositions are important in reducing the fraction of contaminant that is soluble and available to microorganisms, which may affect the persistence of organic pollutants [108]. Maliszewska-Kordybach *et al.* [89] in their study observed that increased organic substances and acidity of the soil reduced the toxic effect of PAH amendments in the soil as evidenced by the intensity of respiration that had no change compared to the control. They noted a smaller enhancement in the dehydrogenase activity of soil contaminated with PAH and heavy metal than in uncontaminated soil. The suggested reason was that when heavy metals are in high concentrations (Zn^{2+} = 1000 mg kg^{-1} , Pb^{2+} = 500 mg kg^{-1} , Cd^{2+} = 3 mg kg^{-1}) their non-specific binding to bacterial cell surfaces may preclude interactions of PAHs with bacterial membranes leading to the decrease of their toxic effects [89].

The strong and complex impact of PAHs and heavy metal co-contamination on soil microbial community can mostly be observed by monitoring microbial activities such as soil respiration, soil microbial biomass and soil microbial community structure. The knowledge of these microbial communities, their catabolic activities and how they respond to contaminants is essential for the assessment of their biodegradation potential and for bioremediation or transformation of PAHs [109].

1.1.8 Monitoring bioremediation:

This is an important aspect of bioremediation study that helps to determine and understand the remediation processes of PAHs taking place in the soil.

The general strategy for demonstrating that bioremediation is taking place includes measuring the loss of PAH contaminant from the soil and also laboratory assays on microbial catabolic activity in the soil sample. It is also important to determine and maintain optimum laboratory conditions that will enable the bioremediation process to continue. It is suggested that a bioremediation monitoring plan should allow a distinction between biotic and abiotic processes [92]. Abiotic losses (losses that are not due to microbial degradation) such as sorption to soil, volatilization, photooxidation etc. are accounted for based on a sterilised control test.

The major agents of bioremediation are the soil microorganisms. They are the most sensitive part of the soil ecosystem. Measurements of soil microbial community parameters have often been used for soil quality evaluation [92, 110], and for environmental monitoring at contaminated sites [91]. The most commonly used indicators of microbial community status include gross microbial indices such as

microbial biomass, soil microbial respiration rate, metabolic quotient and enzyme activities.

Several studies have been carried out on polluted/contaminated sites based on the information derived from the indigenous microbial community in the polluted area. Microbial community quantity (such as their biomass which is the quantity of the living and active part of soil organic matter [111, 112]) and functional abilities (such as their basal respiration rate, enzymatic activity and carbon source utilisation) have been used to characterise, identify and monitor polluted areas and their effect on the environment [110]. The effect of pollution on microbial community based on their quantity and functional abilities are easily identified through the reduction in number, respiration and other metabolic activity of microbes which also brings about changes in the diversity of microbial community in these areas. Belen *et al.* used enzyme activities such as β-glucosidase, arylsulfatase, urease, acid and alkaline phosphatase to determine the effect of heavy metals pollution from a pyritic mine on microbial community in agricultural soil and sediments along a river. They also used this form of microbial biological indicator to measure the effectiveness of remediation action taken [113]. Hofman *et al*. [114] used microbial biomass level and basal respiration as bioindicators of adverse inputs of heavy metals and persistent organic pollutants from a highway soil.

Assessment of the effect of pollution on microbial community should always be done using more than one measurement of microbial parameters as can be seen in the previous paragraph because using a single microbial parameter may not give the exact effect on the composition and functions of microbes. Hofman *et al.* [115] stated that using only microbial biomass level for pollution effect measurement on soil microbes may be insufficient because of the lack of a benchmark value brought about by high variability of the parameters and the ambiguous relationship with many environmental factors [114, 115]. For example Chander *et al.* [116] observed that microbial biomass may be affected by environmental factors other than increasing concentrations of heavy metals such as the amount and quality of carbon input by plant. Rajapaksha *et al.* [95] also noted that respiration as an indicator of pollution may give varying results especially at low concentrations of pollutants.

This led to the suggestion that a combination of parameters may give a more precise, complete and accurate information about effects of pollutants on the microbial community. Ghosh *et al*. [117] in their study confirmed that microbial biomass and its activity (soil respiration, enzyme activity) measurement seems to provide more sensitive indications of soil pollution by heavy metals than either activity or population

measurements alone. Megharaji, *et al.* [118] also used microbial biomass and soil enzyme activity (dehydrogenase and urease activity) to confirm the effects of petroleum hydrocarbon on microalgae and on the microbial community.

The measurement of these microbial parameters provides information on the presence and activity of viable microorganisms as well as on the intensity, kind and duration of the effect of pollutants on soils metabolic activity. These measurements serve as a good index of the impact of pollution on soil health and soil quality [115, 119]. Changes in these parameters can serve as a warning of decreasing soil quality [114].

The monitoring strategy that was employed in the present study involved measuring the loss of PAH contaminants from the soil through chemical analysis; monitoring the activities of the soil microbial community using microbial biomass and soil respiration; and also monitoring the change in soil microbial community diversity during the bioremediation process using a community level physiological approach. The community-level diversity approach that was used was the BIOLOG Ecoplate measurement which allows the determination of microbial community structure without determining the particular species composition [120].

1.1.8.1 Soil respiration:

One of the most commonly used biological parameters in measuring microbial activity is that of respiration activity. Soil respiration measurement represents the measure of the overall activities of microorganisms which reflects their mineralisation of organic matter (substrates) in the soil [121] [88, 122]. It is one of the major parameters that are measured in toxicity, biodegradation and other soil microbial community study. The information gained from the respiration measurement are used in environmental risk assessment to identify polluted areas and the effect of pollution on the ecosystem [123]. Soil respiration involves the evolution of CO_2 by respiring microorganism, from the soil to the atmosphere which can be as a result of their decomposition of soil organic carbon in order to obtain energy for their growth and functioning [124, 125]. Soil respiration measurement can be performed either *in situ* measuring the cumulative contribution of organisms involved in CO_2 release or *in vitro* as a laboratory measurement where plant roots are excluded and the CO_2 evolved gives an estimate of the total biological activity [121]. Soil respiration is influenced by several environmental factors such as moisture, temperature, available carbon substrate, microbial biomass and seasonal variations (changes in climatic conditions) heavy metal pollution and PAH contamination. Soil temperature and soil moisture content are the

two major abiotic influences on soil respiration [126] and when the two are kept constant, changes in soil respiration are related to the chemical and biological properties of the soil [122]; the respiration under these conditions is known as basal respiration [127].

Low soil moisture content can limit soil respiration by limiting microbial contact with the available substrate as a result of limited diffusion of nutrients in the soil pore space thereby limiting the microbial physiological performance which can lead to dormancy and/or death of microorganisms [128, 129]. Soil moisture and soil temperature can have a confounding effect on soil respiration. Conanta *et al.* [129] showed that the effect of temperature increase on soil respiration is reduced by low soil moisture. They noted that soil respiration increased under wet incubation conditions and that the difference in soil respiration rate among all incubated soils were dependent on their moisture content with the wetter conditions giving rise to a higher respiration rate [129]. Several other studies have shown the same effect [130-132]. Campbell *et al.* [132] also noted that soil respiration is more likely to be less than that predicted by temperature alone when the soil moisture is below 50 %. But soil moisture can also negatively affect soil respiration when it becomes too high because it can cause poor aeration (reduced oxygen concentration) and reduced CO_2 diffusivity which can lead to suppressed microbial activity [131]. Higher soil moisture can lower soil temperature by changing the energy balance, leading to more energy dissipated as latent heat (for evapotranspiration) and less as soil heat flux (for soil warming). The low soil temperature caused by this process could lead to suppressed soil and microbial activity, thus low soil respiration [133].

Several studies have also demonstrated that increasing soil temperature can cause an increase in soil respiration [125, 130, 134]. Soil temperature greatly influences the rate of soil organic matter decomposition which helps to sustain microbial activity in the soil. The increase in soil temperature (for example 15 $^{\circ}$C to 25 $^{\circ}$C) can increase soil respiration rates but as the temperature increases still further the soil respiration rate decreases because an increase in temperature causes the rate of depletion of the labile carbon pool to increase leading to a shortage of accessible carbon to soil microorganisms. Frey *et al.* [135] in their study noted that soil warming over a long period will result in a significantly lower level of microbial biomass likely due to a reduced availability of labile carbon compound. The reduced biomass observed in their study represents reduced number of active microorganism which leads to a low soil respiration. On the other hand, higher soil temperature may stimulate decomposition of recalcitrant carbon pools which could increase the labile carbon but would not increase respiration rate to its initial value because of the reduction in the microbial biomass.

Reduction in the biomass is due to the death of some part of the microorganism community that is not able to withstand the increase in competition for the reduced labile carbon pool. The substrate dependence of microbial activity is demonstrated by the 2 - 6 fold increase in soil respiration immediately after the addition of readily available substrate (labile carbon) in the form of glucose during substrate-induced respiration [136].

Studies have shown that soil respiration can be positively correlated to the soil microbial biomass [127]. The microbial biomass carbon which is the total physiologically active or living part of the microflora [133, 137, 138] plays a vital role in nutrient recycling and performs other important functions in the soil. The reduction of the microbial biomass leads to the reduction in the activities performed by microorganisms which lead to low respiration. Reduction of the microbial biomass may occur as a result of stress caused by biotic and abiotic factors such as high temperature, low moisture, the presence of pollutants and the reduced quality and quantity of substrates as already discussed.

Seasonal variations such as rainfall, soil water content, air and soil temperature can have a great influence on soil respiration. Soil respiration changes seasonally with soil temperature and often decreases with decreasing soil water in the summer [130]. The microbial community tends to adjust to different seasonal conditions; their activity either becomes less or more active depending on the season. Lee *et al.* [131] observed a low soil respiration during winter due to low temperature, with the respiration increasing sharply during the summer; it then decreased in autumn and was moderate during spring. The changes are due to varying temperature and moisture condition during each season. Bekku *et al*. [139] also noted that the response of microbial respiration rate of soils from different regions such as temperate, tropical and arctic were different from their initial respiration rate when they were exposed to different temperature conditions. They observed that microbial respiration rate of soils from the temperate region became lower at a higher incubation temperature while the microbial respiration rate of soils from the arctic region and tropical region increased with increase in incubation temperature [139]. Vanhala *et al.* [122] also observed that sampling time had a significant effect on soil measurements due to the several environmental factors that can affect the structure and functions of soil microorganisms at a given season. They noted that autumn was the best period for soil sampling for soil respiration studies because there was no variation in the chemical properties (amount of organic matter N or organic carbon) of the soil during this period that can affect soil respiration rate. The respiration rate will be based on environmental pollution.

Therefore to overcome the problems of temperature and soil moisture content during soil respiration study it is best to keep the soil temperature and soil moisture constant; the respiration rate will be calculated based on the effect of pollution or nutrient content (especially the nitrogen or organic carbon content) of the soil [122].

There have been conflicting reports on the effects of heavy metal and PAHs on soil respiration. While some studies seem to show that soil respiration is not affected by xenobiotics such as heavy metals [140] and PAHs [101] other seems to show a strong negative effect of heavy metal [95, 127] and PAHs [108] on soil basal respiration. Rajapaksha *et al.* [95] observed that there was no effect of low concentrations of heavy metal on soil respiration in their study but the highest respiration decline (about 30 % reduction) effect was observed when the concentration of heavy metal Cu and Zn was increased to 4 mmol kg^{-1}. Fang *et al.* [140] observed an increase in the soil respiration rate with increasing heavy metal content. There have also been reports of heavy metals stimulating microbial respiration rate at low doses, as observed by Silvia *et al.* [88]. They deduced that the increase in respiration rate can be a sign of metabolic stress on the soil microbial community. This explanation does not provide a clear answer to the complex interaction between heavy metals, PAHs and microorganisms.

The increase in basal respiration under environmental stress or pollution could also be as a result of the increase in the more tolerant microbial community species when the most sensitive species have died because of the contaminants or environmental stress in the soil [95, 141, 142]. Rajapaksha *et al.* [95] observed that soil respiration rate was only a slightly affected by heavy metal addition even at the highest concentration because of the increase in fungal activity, which was unexpected. They noted that fungi growth was more affected by carbon limitation than by heavy metal stress and the dead bacteria cells provided extra carbon for the fungi thereby enabling them to overcome the adverse effect of heavy metal.

Therefore, in the present study, the effect of both heavy metal and PAH on soil respiration was closely observed in order to provide information on how soil microbial community can respond to co-contamination of these pollutants. The effect of other environmental factors such as moisture content, temperature, soil laboratory handling on soil respiration was also carefully examined in the present study in order to determine how this factor can influence soil microbial activity. This was confirmed by Bååth *et al.* [143] when they observed that the influence of other factors such as level of nutrients and acidity can make it difficult to isolate the effect of heavy metals on soil respiration, microbial biomass and the ratio of microbial biomass to organic carbon.

1.1.8.1.1 Microbial metabolic quotient (qCO_2)

The metabolic quotient is the ratio of basal respiration to microbial biomass, (Equation 1.1) and ‘’is inversely related to the efficiency with which the microbial biomass uses the indigenous substrates’’ [144]. It has been used mainly as a sensitive indicator to show the effects of heavy metal toxicity to microorganisms in soil [144].

$$\text{Metabolic Quotient } (qCO_2) = \frac{\text{soil basal respiration}}{\text{soil microbial biomass carbon}} \quad (1.1)$$

This is another microbial characteristic that can be monitored during bioremediation in the presence of contaminants such as metals and PAHs because it provides details on the stress on microorganism as a result of these contaminants in the soil [140]. Yao *et al.* [144] observed that the correlation between heavy metal content and qCO_2 was better than that between heavy metal content and basal respiration or microbial biomass carbon, which revealed that metabolic quotient can be used as an important indicator of soil quality and its values can be associated with soil pollution. They noted that the metabolic quotient was six times higher in the most polluted soil than in sample taken 5 km away from a smelter, and this qCO_2 was significantly correlated with both Cu and Zn concentrations in all soils. Zhang *et al.* [81] also noted that heavy metal contaminated soils had significantly higher qCO_2, which indicates a greater energy requirement for maintenance, a decline in substrate quality and eventually a decrease in microbial metabolic efficiency. They also confirmed the significant and positive correlation between qCO_2 and heavy metals. The high qCO_2 value of microorganisms in the presence of heavy metals and other contaminants could be best explained as the diversion of energy into physiological adaptations necessary to tolerate heavy metals. The consumed carbon substrate under this condition is then released as CO_2 with less being built into organic components or used for growth [81, 142, 145].

1.1.8.1.2 Measuring soil respiration:

Soil respiration measurements have been carried out in different ways. The amount of CO_2 released or oxygen consumption by microorganisms during soil respiration has been used to monitor the rate of respiration. The various methods used in soil respiration are: determination of CO_2 release by titration in a static system [146-150]; determination of CO_2 release using an infrared gas analyser in a flow-through system [116, 134, 151, 152]; determination of CO_2 release using gas chromatography in a flow-through system and a static system [129, 153-155]; and determination of soil respiration by pressure measurement in a static system (Manometric method) [151,

156-159]. Among these methods, the manometric method can be used for both soil respiration and biodegradation (bioremediation) studies. The principle of the manometric method according to British Standard-International Standard method (BS ISO 16072:2002) [160] is based on oxygen being consumed by microorganisms while carbon dioxide is formed at the same time. When this process takes place in a closed vessel containing, in addition to the solid substance (example soil) a sufficiently large air filled gas phase as well as an absorbent for CO_2, the O_2 consumption will lead to a reduction in the gas pressure. In a closed system, the pressure reduction takes place independently of the atmospheric pressure. The change in pressure is proportional to the mass of O_2 consumed. An important condition that is necessary for this measurement is that the temperature must be kept constant during the measurement. Temperature changes lead to pressure fluctuations that could render an oxygen consumption measurement impossible.

The OxiTop (WTW, Weilheim, Germany) respirometry system is a good example of a manometric measurement system. It has been successfully used for degradation studies in oil contaminated soils [157, 159]. It has also been used for respiration studies on heavy metal contaminated soils [123].

The biologically degradable organic substances are degraded to carbon dioxide with the consumption of oxygen under aerobic conditions as illustrated by Equation 1.2 [161]:

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O \qquad (1.2)$$

The oxygen consumed during the biological process produces approximately equimolar quantities of carbon dioxide that are absorbed by sodium hydroxide as represented in Equation 1.3 [161]:

$$CO_2 + 2\ NaOH \longrightarrow Na_2CO_3 + H_2O \qquad (1.3)$$

1.1.8.2. Soil microbial biomass

Microbial biomass, which represents the living component of the organic matter of soil usually makes up less than 5 % of soil organic matter and builds up with increased accumulation of organic matter [162, 163]. Soil microorganisms carry out many functions in the soil ecosystem, such as participation in the transformation of C, N, S and P in soil, and play an active role in the degradation of xenobiotic organic compounds. The soil microbial biomass helps to release readily available soil nutrients for plant growth and this can influence the amount of available nutrients in the soil

[163]. They also help in the mobilization and immobilisation of heavy metals and participates in the formation of soil structure [88]. Microbial biomass carbon is controlled by a variety of environmental factors such as low soil nutrient, low pH and low moisture [164] and soil factors such as the quality of soil organic matter the physical and chemical characteristics of soil and the presence and activity of plants and animal [144]. This implies that heavy metals and PAHs may not be the only major factor affecting microbial biomass [164] and these factors also influence the amount of available pollutant to which microbes are exposed. Microbial biomass correlates better to internal enzyme activity or to measurements of overall activity such as respiration.

Microbial biomass though an important ecological parameter, may often seem to be insensitive to heavy metals especially when they are present at low concentrations but the insensitivity may just represent the pollutant-resistant pollution which may have increased in abundance as a result of reduced competition in the microbial community [118, 164]. This then could produce a change in microbial community structure as a result of reduced microbial diversity which would still need to be investigated further in order to substantiate microbial insensitivity to heavy metal/PAH-polluted soil [144]. Megharaji *et al.* [118] observed that microbial biomass carbon can be a relatively less sensitive parameter to pollutants especially when they are present at low concentration. This was also confirmed by Chander *et al.* [116] who noticed that microbial biomass carbon does not necessarily decrease with increasing heavy metal content which may be due to other environmental factors such as differences in the amount and quality of carbon input by plants.

Several other studies have also described microbial biomass as a very sensitive parameter in the study of the effect of heavy metal pollution in soil [81, 90]. Knight *et al.* [165] noted a reduction in the microbial biomass at low metal concentrations. Zhang *et al.* [81] observed that there was a decrease in the microbial biomass (C, N and P) at a relatively modest and sometimes even at a surprisingly low metal load. The decrease was consistent with an increase in the heavy metal concentrations. They reasoned that microorganisms under heavy metal stress divert energy from growth to cell maintenance functions, thereby requiring more energy to survive under this condition. This implies that a higher percentage of energy is lost and less C, N and P are incorporated into organic components. They suggested the use of the ratio of the microbial biomass carbon (Cmic) to organic carbon (Corg) as an indicator of the adverse effects of heavy metals on the functioning of a soil ecosystem (soil biomass) and the increase of this ratio would signify a decline in the size of microbial community and a reduction of C mineralization. Castaldi *et al.* [90] observed a distinctly negative effect of total heavy metals (Cd, Zn, and Pb) on microbial biomass carbon and also on

the ratio of biomass carbon and the organic carbon (Cmic to Corg ratio). They then proposed the use of biomass, and biomass:organic carbon ratio as a good index for the detection of the effect of heavy metals on soil microbial community, though they acknowledged that other environmental variables may affect soil microbial indices to a greater extent, making it difficult to infer the changes that are in response to stress from heavy metal pollution.

Kamitani *et al*. [164] observed a functional resilience of microbial community to copper concentrations up to 1000 µg g^{-1} in a soil that was rich in nutrients with high pH and moisture conditions which enhance biomass and diversity of microorganisms. They observed that though there was an initial decrease in the ability of the microbes to utilize carbon substrate at the highest Cu concentration, the utilization level did not fall to the level observed in the soil with low nutrients, low pH and low moisture properties. The microbial community in the richer soil had a more active microbial population and the favourable conditions in the soil enhanced their functional resilience and developed a tolerance to Cu during their long period of exposure to heavy metal pollution.

This present study, therefore, considered the influence of PAH and heavy metals on the microbial community biomass. Studies have shown that PAHs can serve as an organic substrate for the microbial community [88], and based on Kamitani's study, the microbial community would be expected to develop functional resilience in the presence of PAH co-contamination. There is still a paucity of study in this area of investigation.

1.1.8.2.1. Soil microbial biomass measurement:

Soil microbial biomass is the quantity of the living and active part of soil organic matter and can be measured using three standard ways. The three standard methods of measuring microbial biomass are: chloroform fumigation incubation, chloroform fumigation extraction and substrate-induced respiration.

Chloroform is used because it is an effective biocide (i.e. gives a near complete kill of the soil microbial population) and does not solubilize non-microbial soil organic matter or render it decomposable [166]. Another reason is that it leaves neither decomposable nor toxic residues in soil. The disadvantage of using chloroform fumigation incubation method is that it can give an underestimation of soil microbial biomass in a low pH soil and in waterlogged soil [167]. It also cannot be used in an air dried soil because air drying renders non-biomass carbon decomposable as well as killing an appreciable

fraction of the biomass, thus giving an erroneous CO_2 emission during incubation for both the fumigated and unfumigated soils [168].

The measurement of microbial biomass in this present study was carried out using two methods; substrate-induced respiration and chloroform fumigation extraction methods. The preliminary phase of this study was carried out using substrate-induced respiration (a physiological method) according to the protocols of Andersona *et al.* [169] whereby the respiratory activities of substrate supplemented microcosm/habitat were used to estimate relative proportions of actively metabolising biomasses. In subsequent phases of the bioremediation study, microbial biomass estimation was carried out using chloroform fumigation extraction method. However, an advantage of using the substrate-induced respiration is that there is no need for the extra determination of organic carbon concentration. The carbon concentration is derived from its respiration using a model calculation but the disadvantage is that it can also give a low estimation of the soil microbial biomass [170]: the readily available substrate used may not be easily decomposed by all the microbial species in the soil. The chloroform fumigation extraction method requires the extracted organic carbon concentration to be determined using an additional protocol. The protocol used for the organic carbon determination was the Hach-Lange total organic carbon method. The advantage is that it is independent of soil respiration.

1.1.8.3 Community level physiological profile

Another important microbial parameter that is influenced by the presence of xenobiotic compounds in the soil apart from their quantity and activity is the microbial community structure. The community structure provides information on the microbial diversity and their functions in the environment. The community structure has a major influence on the community's functional diversity. Microbial community structure refers to the actual catabolic activity expressed while the functional diversity shows the potential activity such as the capability of the community to adapt metabolism (catabolism) [171]. The microbial functional diversity is essential to the understanding of the role of microbial communities in the soil. The change in functional diversity also plays a key role in the understanding of the influence of soil disturbances on microbial community structure

The knowledge of the community structure is essential for a comprehensive environmental risk assessment [110]; however, it is still difficult to achieve, because of the technical limitations in describing the dynamics and roles of the microbial community in the ecosystem [172].

Several researchers have tried to characterize the soil microbial community structure according to functional diversity, but one of the major challenges they faced was on how to assess changes or differences in microbial diversity in soils with respect to community composition and species distribution [172]. Recently, the development of a molecular technique which is also known as fingerprinting technique [173] has gained acceptance in microbiological and ecological studies. This technique allows the determination of microbial community structure without determining particular species [110] and is based on the analysis of the genetic structure or the phenotype of the microbial community present. This technique has been approached in a number of ways such as: Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis which is based on the extraction of deoxyribonucleic acid (DNA) from the soil and then using specific oligonucleotide primers to amplify the DNA marker of the community by polymerase chain reaction (PCR) process [173]; qualitative analyses of ester-linked phospholipids fatty acid (PLFA) composition of the soil, since different subsets of microbial community have different PLFA patterns [165, 174]; and denaturing gradient gel electrophoresis (DGGE) analysis which is based on the separation of 16S rDNA and then analysing the generated profiles based on their DNA sequence [175, 176]. These methods eliminate the bias associated with previous methods which were based on culturing microorganisms and covered only the aerobic heterotrophic fraction of the total bacterial population capable of forming colonies on a solid media [176, 177]; however, most molecular community level techniques are time-consuming and laborious.

This led to the development of a rapid community level cultural approach called community level physiological profiling (CLPP) by Garland and Mills [177]. This is based on the utilization pattern of a number of sole carbon substrates by microbial communities [110, 171]. Their carbon substrate utilization pattern gives information on the functional ability or the functional diversity of microorganisms from environmental samples [171]. This approach involves direct inoculation of environmental samples into the microplates and use of the resulting response to describe differences in microbial communities [177]. CLPP was commercialised by BIOLOG Inc (BIOLOG CA. USA) and they produced the BIOLOG microplates. There are a number of BIOLOG microplates that have been produced such as the Gram negative (GN) microplates which were developed specifically for Gram negative bacteria, Gram positive microplates developed for Gram positive bacteria, SF-N and SF-P which are used for assessment of fungal activity [171] and Ecoplates which recently have been produced for environmental microbial community study. The BIOLOG plates contain 96 wells with 95 wells containing sole- carbon substrates and one well serving as the control containing

only water. The Ecoplate only have 31 sole carbon substrates in its 96 wells, the other 65 wells are replicates of the first 31 carbon substrates but three out of the 65 wells are control wells that only contains water.

The BIOLOG plate substrates contain a colourless redox-sensitive tetrazolium dye which is reduced to a violet insoluble formazan through the dehydrogenase activity of respiring bacteria cells [171, 177]. The insoluble formazan accumulates inside active bacteria cells and it is used as an indicator of respiration and bacteria utilization of the sole carbon source [178, 179].

The pattern of substrate utilization based on colour formation is monitored and analyzed using a microplate reader. The information obtained from the analysis is used to classify the structural pattern and functional diversity of microbial communities from different environmental samples.

BIOLOG microplates with the help of the information provided by Garland have been successfully used to differentiate metabolic activities and functional diversity of microbial community from various environmental samples such as soils, freshwater, rhizospheres and groundwater [178-180]. It has shown to be a sensitive and powerful ecological low-cost analytical tool to describe the differences or changes in soil microbiological characteristics [172].

1.1.8.3.1. BIOLOG Ecoplate:

Among the various BIOLOG plates produced, the Ecoplates provide a complete assessment of the microbial community structure because of the number, type and the diversity of substrates they contain.

The BIOLOG Ecoplates contain substrates that are more ecologically relevant and they were specifically produced for bacteria community analyses of environmental samples [181]. They contain three replicates of 31 environmentally applicable carbon substrates of which at least nine are considered as constituents of plant root exudates [171]. The substrates are based on the ecological functions the microbial community perform within the ecosystem [171, 181]. The replicates in the Ecoplates accounts for the variability in inoculum densities that are obtainable from environmental samples and it also serves as a check for repeatability, reproducibility, and accuracy of substrate utilization pattern of microbial community [181, 182].

Choi *et al.* [183] in their study to compare the abilities of BIOLOG plates (GN2 and Ecoplates) to distinguish among aquatic microbial community from freshwater and

saltwater noted that though the plates have equal capacity to distinguish among microbial communities, Ecoplates optimise the discrimination among the bacterial community more than the other plates because of the type of substrate the Ecoplate contains. Classen *et al.* [181] in their study on the influence of incubation temperature on different BIOLOG plates also noted that Ecoplate substrates were sufficient to distinguish between microbial communities found in different environmental samples. They noted that because of the replication of substrates in Ecoplates there is a greater likelihood that the community level physiology profile data generated is a true representative of the soil samples assessed. This also acts as the advantage Ecoplates has over the other types of BIOLOG microplates.

The Ecoplates have been successfully used to assess the structures of bacteria community that exposed to different concentrations of copper treatment, the outcome of which confirmed the differences in metabolic capabilities of the microbial community based on copper treatment [184]. The Ecoplates have also been used to access the tolerance of microbial communities exposed to mercury contamination; the communities were separated based on their different ability to utilise the carbon substrate when they were exposed to further mercury contamination [185]. Soluble carbon sources in the sample can cause additional colour development. Table 1.2: shows eco plate substrates, their well numbers and their corresponding guilds grouping

Table 1.3: Ecoplates substrates, their corresponding well numbers and associated guild grouping [181]

Substrate	**Plate Code**
Control:	
Water	A1
Amino Acids (n=6):	
L-Arginine	A4
L-Asparagine	B4
Glycly-L-Glutamic Acid	F4
L-Phenylalanine	C4
L-Serine	D4
L-Threonine	E4
Carbohydrates (n=10)	
D-Cellobiose	G1
i-Erthritol	C2
D-Galactonic acid y-lactone	A3
N-Acetyl-D-glucosamine	E2
Glucose -1-phosphate	G2
β-Methyl-D-glucoside	A2
D,L-α-Glycerol phosphate	H2
α-D-Lactose	H1
D-Mannitol	D2
D-Xylose	B2
Carboxylic acids (n=9)	
y-Hydroxybutyric acid	E3
α-Ketobutyric acid	G3
D-Galacturonic acid	B3
D-Glucosamine acid	F2
Itaconic acid	F3
D-Malic acid	H3
Pyruvic acid methyl ester	B1
2-Hydroxybenzoic acid	C3
4-Hydroxybenzoic acid	D3
Amines (n=2)	
Phenylethylamine	G4
Putrescine	H4
Polymers (n=4)	
α-Cyclodextrin	E1
Glycogen	F1
Tween 40	C1
Tween 80	D1

1.9 Aims and objectives of the project:

The main aim of this study was to investigate the effect of heavy metal on the biodegradation of 16 US EPA priority list PAHs and the objectives are as follows:

- To investigate the optimal soil handling, spiking and storage conditions in which to carry out laboratory-based biodegradation projects.

- To investigate the biodegradation of 16 US EPA priority list PAH compounds over an extended period of time under laboratory conditions.

- To determine the effect of different concentrations of heavy metal co-contaminants on the extent and rate of the biodegradation process.

- To determine the effect of PAH compounds and heavy metal contaminants on soil metabolic rate, biomass concentration, and the diversity of the soil microbial community.

- To improve the knowledge on the adaptability of soil microbial communities to different pollutants concentrations and the implications this has for potential bioremediation projects

Chapter 2: A study on soil microenvironment suitable for PAH bioremediation

2.1 Introduction:

In the last chapter, it was reported that bioremediation efficiency in soil can be significantly influenced by the interaction of microorganisms and its soil environment [33]. Studies have reported that bacterial degradation activity is altered greatly by the physical and chemical features of the heterogeneous soil microbial environment [133]. It has also been reported that the ability of soil microbial communities to carry out functions such as degradation can be influenced not only by pollutants such as heavy metals but by other environmental factors such as soil pH, temperature, moisture, and soil organic matter quality (for example carbon and nitrogen content) [149].

The soil environment is a complex, variable multi-component system that serves as a reservoir of microorganisms and has a range of different types of contaminants, mineral matrices and different organic compounds co-existing in different physical and chemical forms [186]. It can also be described as a complex interactive biogeochemical reactor and a major compartment of the terrestrial ecosystem under the influence of anthropogenic activities [162]. Its spatial and temporal variability often poses a challenge during laboratory investigations and this is exacerbated by the fact that biological processes often operate at the level of microsites. The heterogeneity of these microsites often generate discrepancies between results observed under field and laboratory conditions [133]. In order to carry out an efficient bioremediation study a good understanding of soil chemical, physical and biological properties and processes that interact within the soil environment needs to be acquired [162].

The bioremediation of PAHs in soil involves a complex interaction between soil microbial community, soil organic matter and soil minerals. The soil microbial community is responsible for the conversion of PAHs into an environmentally harmless state. The interaction of soil organic matter and soil minerals with PAHs can affect their microbial degradation because of adsorption processes. The interplay between these soil components can either enhance or hinder the biodegradability of PAHs. For example, PAHs can be intricately adsorbed into the humus component of organic matter thereby, reducing the bioavailability of PAH for microbial degradation. Organic matter has also been reported to enhance desorption of PAHs from soil [187]. Clay minerals surfaces can also bind antimicrobial compounds such as heavy metals which might otherwise be detrimental to microorganisms. Clay minerals may also concentrate organic substrates at their surfaces allowing microorganisms to grow in what would

otherwise be unfavourable conditions [126, 188]. These major soil components can play a major role in the fate of PAHs in the soil environment. The basic soil components, the influence of soil organic matter and clay mineral on soil microbial degradation of PAH in the soil environment are discussed in the following sections.

2.1.1 Basic soil composition and soil chemistry:

The soil environment as earlier stated, are open, variable multicomponent and biogeochemical systems, which contains solids, liquids and gases [189]. The open system means that soils can exchange both matter and energy with their surrounding atmosphere, biosphere and hydrosphere. These exchange of matter and energy in soils are highly variable in time and space, but they are the main fluxes that bring about the development of soil profiles and also control the patterns of soil quality [189]. Soils are created at the land surface interface through rock weathering processes mediated by biological, geological and hydrological phenomena. The rock weathering releases the mineral materials which can be grouped into primary minerals and secondary minerals. A mineral can be characterized as a characteristic inorganic compound with well-defined physical, chemical, and crystalline properties [190]. Primary minerals are released rock minerals that are still chemically unchanged. Secondary minerals are minerals that have been derived from the weathering of primary minerals. The primary minerals are broken down and altered into secondary minerals under the influence of climate and biological activity. During the weathering of these primary minerals chemical constituents which consist mainly of silicon (Si), iron (Fe), aluminium (Al), magnesium (Mg), potassium (K) and calcium (Ca) are released. These are able to recrystallize to form secondary minerals referred to as clay minerals. These resulting products (clay minerals) are generally in equilibrium with the newly imposed physicochemical environment [191].

The composition of soil minerals is very variable and depends on the composition of the rocks. The rocks from which the minerals originate are composed mostly of the elements oxygen (O), Si, Al, Fe, Ca, Mg, Na, and K. The soil minerals are therefore made up of these elements. Oxygen and silicon are the two most abundant elements in soils and these two combine chemically to form the 15 silicates. The resistance of these minerals to decomposition by weathering can be positively correlated with the Si-to-O molar ratio of their fundamental silicate structural unit, as a larger Si-to-O ratio signifies greater stability within their structural unit, because there is a reduced need to incorporate metal cations into the mineral structure in order to neutralise the oxygen anionic charge. This type of mineral tends to have a greater degree of covalence bonding within its structural arrangement which makes the mineral to further exclude

metal cations and become more resistant to decomposition in the soil environment [189].

These chemical elements making up soil minerals are mainly found as ionic species with a unique and stable electron configuration that is not affected by any other ions that may be present in a mineral structure. Most of the minerals are either silicates or oxides. The Si in soil silicates is present in the form of silica tetrahedrons, which constitute the basic units of the crystals. There are six types of soil silicates (kaolinite, halloysite, smectite, illites, vermiculite and chlorite) that are mostly recognised based on the arrangement of the SiO_4 tetrahedral in their structure [192].

The released rock minerals finally become soil when organic matter is incorporated into it. The organic matter comprises the remains (residues) of plants and animals. In some cases, these residues are recent additions to the soil while others may be many years old. The organic matter and the rock minerals still undergo further chemical and physical transformations through mineralisation and weathering (respectively) which breaks them down into smaller units. It is the combination of these transformed units (organic matter and mineral materials) that gives soil its unique properties. Together they make up 50 % of the soil volume and the remaining 50 % is pore space filled with either air or water depending on the level of the soil wetness [190, 191]. The soil system is therefore made up of four major components namely: the inorganic components (mineral material), organic matter components, water and air. Their concentrations may differ from soil to soil or from horizon to horizon [193]. The influence of percolating water and living organisms in soil then helps to produce the vertical stratification (the soil horizons) that is seen in soil and also the soil organic matter alters the physical nature of the soil by binding soil particles together into discrete units called aggregates. The stability of these aggregates is used as an indicator of soil structure [194]. Aggregation is also supported by the activities of microbial community (example: polysaccharide capsule formation by bacteria colonies), ionic bridging (e.g. metal ions), clay and carbonates. Soil structure can then be referred to as the size, shape, arrangement of the solids and voids, the continuity of the formed pores and voids, the capacity of the pores to both retain and transmit fluids, organic and inorganic substances and also its ability to support root growth and development [194].

2.1.1.2 Soil inorganic components

The inorganic soil components (primary and secondary minerals) are composed of rock fragments and minerals of different sizes and compositions. They can be classified into three different fractions according to their sizes (particle diameter): sand silt and clay.

Table 2.1 shows the various international classifications of the different mineral particle sizes. This size distribution of the mineral particles after dispersion are commonly used to classify soils under investigation into several textural groups (e.g. sandy soil, clayey sand, sandy-clayey soil etc.) [195]. Soil texture alludes to the ratio of sand, silt and clay in the soil. It plays an important role in soil fertility because the proportion of sand silt and clay along with organic matter largely determine the soil's capacity to store and supply plant nutrient. It gives an idea of the water retaining property of the soil through its clay and sand content [191].

The particles with a diameter larger than 2 mm are gravel, stones and boulders and are not considered soil constituents. They are rock fragments that upon weathering may still yield sand, silt and clay [192]. The sand fraction (largely composed of resistant quartz grains) has the largest particles 0.06 - 2 mm and the clay fraction is the smallest < 2 µm. Sand and silt particles are the same as the mineral material in the parent rock mainly composed of resistant minerals such as quartz. Clay-sized particles, on the other hand, are made of the secondary mineral because unlike primary minerals they have undergone one more phase of chemical weathering which has altered their physical and chemical composition [191]. Sand grains are irregular in size and shape and are not sticky and/or plastic when wet. Their presence in soil promotes a loose and brittle condition that allows rapid air and water movement. They are chemically inert and do not carry electrical charges, hence, have low water holding and cation exchange capacities. Silt particles are intermediate in size and possess characteristics between those of sand and clay. Some silt particles may be capped or coated by clay films as a result of the weathering of the silt surfaces. Silt may, because of this, exhibit some plasticity, stickiness and adsorptive capacity for water and cations. Clay, with the smallest particle size, has colloidal properties. It carries a negative charge and is chemically the most active inorganic constituent in soils. The presence of clay gives to the soil a high water holding and cation exchange capacity. Clay is also sticky and plastic when wet.

Table 2.1 Classification of soil particle sizes in the UK, US and International system [191]

Fraction	UK System	US System	International System
Stones/gravel	>2.0 mm	>2.0 mm	>2.0 mm
Coarse sand	2.0 – 0.2 mm	2.0 – 0.2 mm	2.0 – 0.2 mm
Medium sand	0.6 – 0.212	0.5 – 0.25 mm	0.5 – 0.25 mm
Fine sand	0.2 – 0.06 mm	0.2 – 0.05 mm	0.2 – 0.02 mm
Fine Silt[a]	0.006 – 0.002 mm	0.005 – 0.002 mm	0.02 – 0.002 mm
Medium silt[a]	0.02 – 0.006 mm	0.02 – 0.006 mm	0.02 – 0.006 mm
Coarse silt[a]	0.063 – 0.02 mm	0.063 – 0.02 mm	0.063 – 0.02 mm
Clay	<0.002 mm	<0.002 mm	<0.002 mm

[a][196]

2.1.1.2.1 Primary soil minerals

A primary mineral, as earlier explained, is a mineral that is still chemically unchanged since its deposition and crystallization from molten lava [190, 192, 193]. Table 2.2 shows the lists of the major primary minerals that are found in soil. Primary minerals are mainly found in sand (2–0.06 mm particle diameter) and silt (0.063–0.002 mm particle diameter) fractions of soils but may also be found in slightly weathered clay-sized fractions. They comprise 59.5, 30.0, and 11.5 % by weight of the igneous rock, shale, and sandstone, respectively.

The weathering of primary silicate adds to the local fertility and electrolyte content of soils. The fundamental building block in the atomic structures of most of these minerals is the silica tetrahedron [189] which is shown in Figure 2.1.

Table 2.2 Rock weathering primary minerals [193]

Primary mineral		**Chemical composition**
1	***Quartz***	SiO_2
2	***Feldspar***: Orthoclase, microcline Albite (plagioclase)	 $KAlSi_3O_8$ $NaAlSi_3O_8$
3	***Mica***: Muscovite Biotite	 $H_2KAl_3Si_3O_{12}$ $(H, K)_2 (Mg, Fe)_2 (Al, Fe)_2 Si_3O_{12}$
4	***Ferromagnesian***: Hornblende Olivine	 $Ca (Fe, Mg)_2 Si_4O_{12}$ $(Mg, Fe)_2 SiO_4$
5	***Magnesium silicate***: Serpentine	 $H_4Mg_3Si_2O_9$
6	***Phosphate***: Apatite	 $Ca_5(PO_4)_3 (F, Cl, OH)$
7	***Carbonates***: Calcite Dolomite	 $CaCO_3$ $CaMg(CO_3)_2$

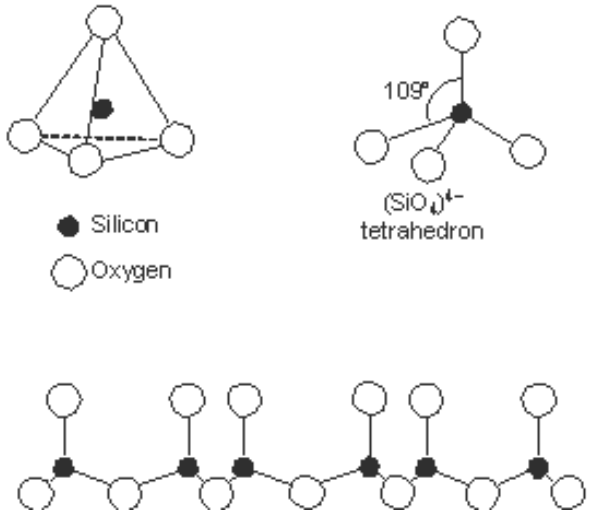


Figure 2.1: Top: Schematic structure of a single silica tetrahedron. Bottom: The arrangement of a large number of silica tetrahedral into a sheet by mutually sharing oxygen atoms [192].

2.1.1.2.2 Secondary soil minerals

Secondary minerals also known as phyllosilicates (clay minerals) are minerals that have been derived from the weathering of a primary mineral, either by a change in the structure or from re-precipitation of dissolved product of a primary mineral [190, 192, 193]. Table 2.3 shows a list of the major secondary minerals and their properties (e.g. cation exchange capacity (CEC) and layer type) that are found in soil. The best known in the list are the crystalline silicate clays but the recent progress in clay mineralogy has led to the discovery of many other types of clay minerals in the soil e.g., amorphous clays, sesquioxide (e.g. Fe oxide and Al oxide), clays and silica minerals. The amorphous clay listed in Table 2.3 are called amorphous because they are amorphous to x-ray diffraction analysis which shows that they exhibit featureless x-ray diffraction patterns [193]. The secondary minerals are primarily found in the clay fraction of the soil but can also be located in the silt fraction [190]. Clay minerals are assemblages of silica tetrahedral and aluminium octahedral sheets. The ratio of silica to the other elements largely determines which type of clay mineral will form. The structure of clay minerals can be simplified by saying that clay minerals are made up of sheets of interlocking silica which are interspersed with the sheets of aluminium oxide [191, 193] as seen in Figure 2.2. It can be divided further using the number of silica to aluminium. The Si–O bond and the O–O bond in the tetrahedral are arranged in such a manner that all the tips are pointing in the same direction with their bases in the same plane. They (tetrahedral) are usually are bonded point-to-point as seen in Figure 2.2. The O–O bond in the aluminium octahedral sheet and the OH–OH bonding occurs through the edges. When one tetrahedral sheet is bonded to one octahedral sheet a 1:1 clay mineral is formed (Figure 2.3). The chemical formula for an ideal 1:1 clay

would be $Si_4^{IV}Al_4^{VI}O_{10}(OH)_8$, where the superscripts represent four and six-fold coordination in the tetrahedral and octahedral sheets, respectively. The 2:1 clay minerals also form when two tetrahedral sheets are coordinated to one octahedral sheet as seen in Figure 2.3. The chemical formula for a 2:1 clay mineral is suggested to be $Si_8^{IV}Al_4^{VI}O_{20}(OH)_4$, e.g. montmorillonite and illite [190]. They are other groups of silicate clay minerals as shown in Table 2.3 but the 1:1 and 2:1 clay minerals are the most studied group [191]. Clay minerals during formation may have other atoms similar in size to silicon combine with oxygen to form part of the silica sheet. However, their chemistry would be different from that of silicon though they may be similar in size. As an example, Al^{3+} usually substitutes for Si^{4+} in the tetrahedral sheet. A similar substitution can also occur in the octahedral sheet where Fe^{2+}, Fe^{3+}, Mg^{2+}, Ni^{2+}, Zn^{2+}, or Cu^{2+} can all substitute for Al^{3+}. This is a process that is known as isomorphic substitution. This process occurs in the crystal lattice without the interruption of the crystal structure of the mineral and therefore, the size of the cationic radius determines the type of cation substitution that can occur within the tetrahedral and octahedral sheets. A cation with a coordination number of 4 could substitute for Si^{4+} in the tetrahedral sheet and a cation of coordination number 6 could substitute for Al^{3+} in the octahedral sheet.

Table 2.3 Major clay minerals in soils [193]

Major minerals	**Layer type**	**CEC cmol (+)/kg**	**Major occurrence**
Amorphous/paracrystalline clays:			
Allophane		35	Volcanic ash soils, spodosols
Imogolite		35	Volcanic ash soils, spodosols
Crystalline silicate clays:			
Kaolinite	1:1	08	Ultisols, oxisols, alfisols
Halloysite	1:1	10	Oxisols, ultisols
Smectite	2:1	70	Vertisols, mollisols, alfisols
Illites	2:1	30	Mollisols, alfisols
Vermiculite	2:1	100	Accessory mineral in many soils
Chlorite	2:2	000	Accessory mineral in many soils
Sesquioxide clays:			
Geothite, α-FeOOH		03	Oxisols, ultisols
Hematite, α-Fe_2O_3		03	Oxisols, ultisols
Gibbsite, $Al(OH)_3$		03	Oxisols, ultisols
Silica minerals:			
Quartz, n(SiO_2)		-	Accessory mineral in many soils
Crystobalite, n(SiO_2)		-	Accessory mineral in volcanic ash soils

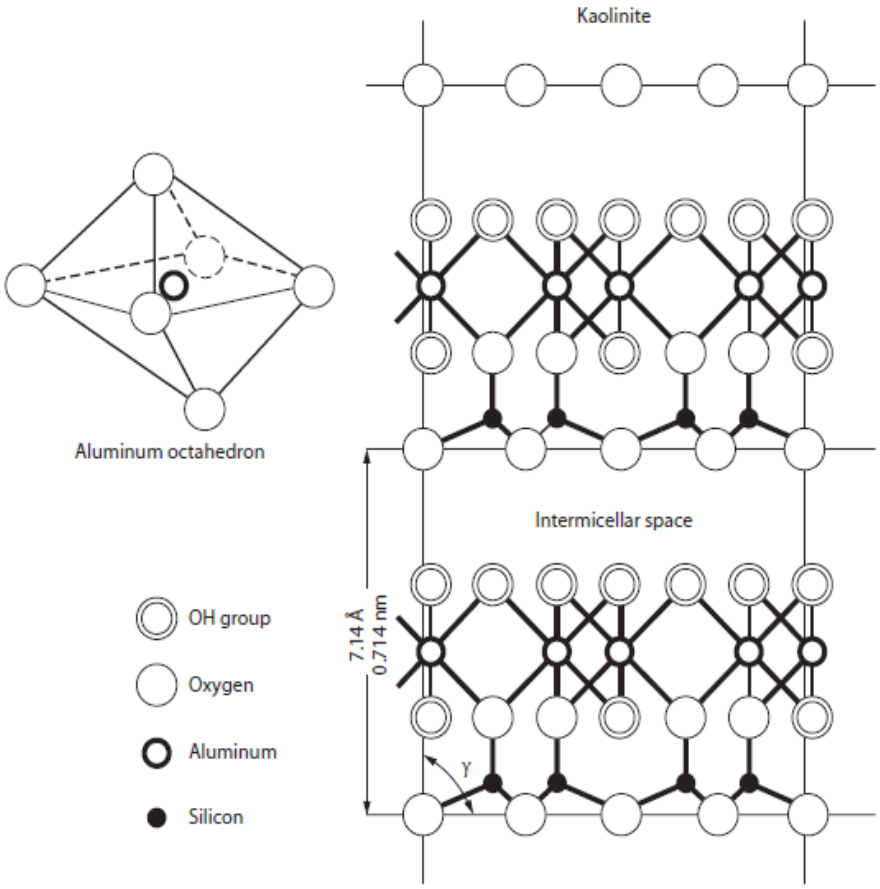


FIGURE 2.2: The structure of kaolinite composed of silica tetrahedron and aluminium octahedron sheets looking down the (b) direction. Unit cell formula is $[Al_2(OH)_4(Si_2O_3)]_2$; a = 5.14 Å, b = 8.93 Å, c = 7.37 Å; α = 91.8°, ß = 104.5°, γ = 90° [192]

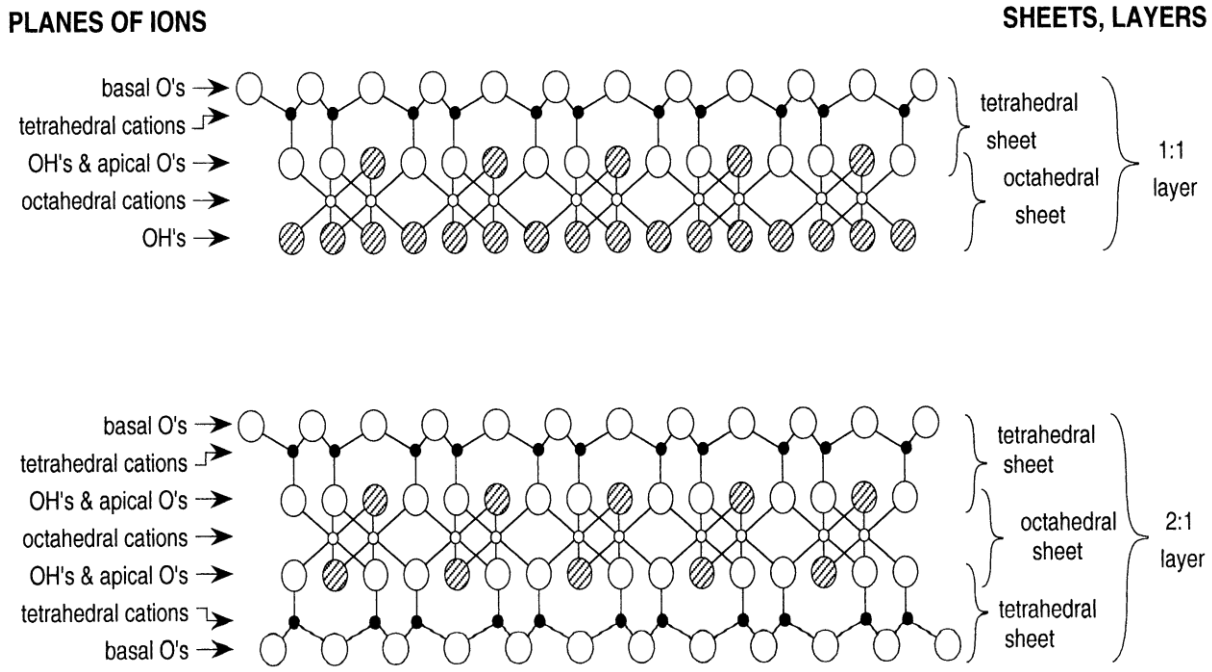


Figure 2.3: Clay mineral structure showing 1:1 and 2:1 layers of silica tetrahedral and aluminium octahedral bonding [190]

Isomorphous substitution often produces a net negative charge associated with the 6 O^{2-} or OH^- of the octahedrons and with the 4 O^{2-} of the tetrahedrons [190]. For example, when one Al^{3+} substitutes for one Si^{4+} in the tetrahedral sheet of an 1:1 clay, $Si_4{}^{IV}Al_4{}^{VI}O_{10}(OH)_8$. The resultant clay now has a new formula $(Si_3Al_1)^{IV}Al_4{}^{VI}O_{10}(OH)_8$. The total negative charge on this new formula is –28 and the total positive charge is +27. The net charge on the clay will then be –1, which would be neutralised by the presence of cations near the outer surface of the 1:1 clay [190]. The type of clay mineral would also determine if the substitution would occur in the tetrahedral or the octahedral sheet. Another example is in the case of illite where a great part of the substitution takes place in the tetrahedral sheet (Si^{4+} is substituted) which creates a moderately confined negative charge that would be neutralised by the presence of potassium ions. This process links adjacent clays tightly together. Whereas in the montmorillonite the substitution occurs in the octahedral sheet ($Al(OH)_3$ sheet is heavily substituted), thus giving rise to a more delocalised charge that allows water to penetrate between individual minerals allowing them to shrink and swell depending on the water content. This also allows montmorillonite to adsorb cations on it's internal as well as its external surfaces. Isomorphic substitution is not a significant feature of 1:1 clays which is why they have only small amounts of permanent charge while the 2:1 clays has more permanent charge [191].

These clay minerals are still subject to further weathering. Under changing physicochemical conditions clay minerals can be converted from one type to another a process referred to as alteration. Two main processes of alteration are distinguished transformation and neoformation. Transformation occurs without changing the basic layer structure of the clay. Desilicification and silicification are the two main trends of transformation which occur under warm and humid conditions. Desilicification is a lateralization process which takes place in the soil when drainage is not restricted and Si can be leached. This gives rise to the formation of ultisols and oxisols rich in kaolinite. A continued desilicification process would lead to the formation of bauxite. Under silicification process, drainage will be more restricted and silica will not be leached but will be added. This favours the formation of smectitic minerals. Neoformation is the synthesis of clay minerals from other secondary minerals with different structure and/or from primary minerals by pseudomorphic replacement or by direct crystallization from solutions or colloidal gel e.g. the conversion of imogolite into kaolinite pseudomorphs and eventually into discrete kaolinite units [193].

2.1.1.3 Soil organic component

The soil organic matter (SOM) refers to the sum-total of all carbon-containing substances in soils. SOM can be described as a combination of plant and animal residues at various stages of decomposition, or it can also be described as substances synthesized microbiologically and/or chemically from disintegrated products of bodies of live and dead microorganisms, small pets or animals, and their decomposition remains [190, 197]. The organic matter content of surface minerals soils is usually only about 0.5 to 5 % weight but ranges up to almost 100 % for some organic soil [198, 199]. Soil organic fraction affects the physical, chemical and biological conditions in the soil. Physically it increases the organic matter content, imparts the darker colors to soils and decreases bulk density with an increase in organic carbon content. It is involved in the formation and stabilization of soil aggregates, promoting soil aeration, moisture retention, resistance and resilience to compaction and thermal properties which improve the soil structure. Chemically, it increases the cation exchange capacity and the water holding capacity. Its cation exchange far exceeds that of clay minerals and it's the reason for the soil's high buffer capacity. It is also the principal sorbent of hydrophobic organic compound (HOCs) affecting their transport and bioavailability [200]. Biologically the soil organic matter is the principle source of nutrient and energy for soil organisms. The fungal and bacterial population increases and decreases in relation to rising and declining organic matter content respectively [193].

The SOM in the various stages of decomposition can be further grouped into non-humified and humified substance. The non-humic substance has recognisable physical and chemical properties and consists of unaltered or partially decomposed compounds such as carbohydrates and related compounds, proteins and their derivatives, fats, lignins, tannins, organic acids, waxes and various partial decomposition products or other forms of living organisms synthesised products [193, 201]. These compounds are easily attacked by soil microorganisms and persist in the soil just for a brief time [190]. The humified compounds (humic substances) are products that have been synthesised from these non-humified substances by a process called humification as seen in Figure 2.4. The humic substances (HS) have non-specific chemical and physical properties associated with well-defined organic compounds [190, 191, 201]. They are characterized as being black, yellow or brown in colour, moderately high molecular weight, partly aromatic, amorphous, polyelectrolyte and refractory [190, 201, 202]. They have a wide variety of functional groups including carbonyl, phenolic hydroxyl, carbonyl, ester and possibly quinone and methoxy groups as illustrated in Table 2.4. These moieties enable their chemical interactions with other class of compounds metals, metal oxides and clay minerals (cation bridging [194] and hydrophobic bonding)

in the soil [203] as illustrated in Figure 2.5. Humic substances make up 70-80 % of the soil organic matter content of most mineral soils [198]. Since humic substances make up the bulk of soil organic matter content, changes in the total organic matter reflect principally changes in the amounts of humic material present.

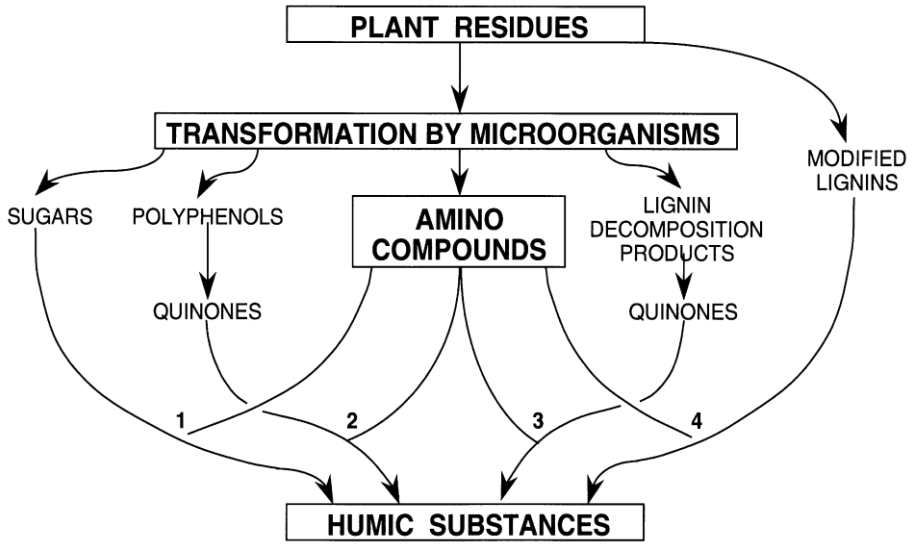
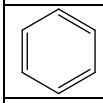


FIGURE 2.4. Mechanisms for the formation of soil humic substances.Amino compounds synthesized by microorganisms are seen to react with modified lignins (pathway 4), quinones (pathways 2 and 3), and reducing sugars (pathway 1) to form complex dark-colored polymers [201]

Table 2.4 Humic substances functional groups and their related chemical interactions [204].

Functional groups of humic substances	**Types of related chemical interaction**
-COOH	Ion-exchange, complexation
-OH	Complexation, hydrogen bonding
>C = O	Reduction-oxidation
	Donor – acceptor interaction (charge transfer complexes)
$-CH_n$	Hydrophobic interaction

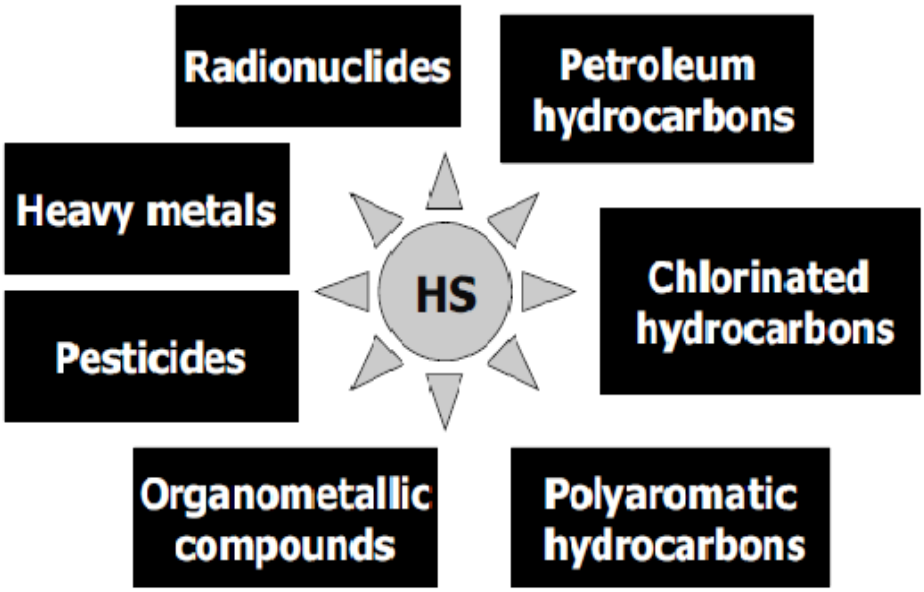


Figure 2.5 The different soil contaminants that can interact with humic substances. For example, HS can form stable complexes with heavy metals and radionuclides; HS can produce adducts and charge transfer complexes with hydrophobic organic compounds; HS can mediate redox reactions of transition metals, chlorinated and nitrated hydrocarbons [204].

The non-humic and humic substances are collectively called soil humus. The humus content in soils worldwide changes efficiently with climate, with aggregation being favoured by low temperature and high precipitation [193]. For instance, an average increment in humus content of a dessert soil is by about one order of magnitude as the mean yearly surface temperature drops fivefold. The typical humus content of tropical forest soils increases roughly threefold as the mean yearly precipitation increases around eightfold. In many soils, the microbial breakdown of litter and humus is the procedure through which carbon (C), nitrogen (N), sulphur (S) and phosphorus (P) are discharged to the coterminous aqueous phase (the soil solution) as inorganic ions which are predisposed to uptake by the biota or loss by three processes such as leaching, erosion (runoff) and emission [193]. Both the humic and non-humic substances are important to the soil environment. Non-humic materials provide short–range effects such as sources of native soil fertility. Humic substances provide long–range effects such as maintaining good soil structure and increasing soil sorption capacity, and water holding capacities [201]. The elemental content of humus is typically 44 - 53 % C, 3.6 - 5.4 % H, 1.8 – 3.6 % N and 40 - 47 % O.

Humic substances can be subdivided into three main fractions through a fractionation process which is based on their solubility in acid or metal salt (alkali) [202, 205, 206] as illustrated in Figure 2.6. A typical fractionation (Figure 2.6) involves precipitation of HS by adjustment of pH and salt concentrations, the addition of organic solvents, or

addition of metal ions [207]. Alkali extraction is usually performed with 0.1 – 0.5 M NaOH and Na_2CO_3 solutions [207]. After extraction, the humic acid precipitate is usually frozen and thawed to remove water and then freeze-dried for subsequent use [207].

The three main fractions separated are; (i). Humin, which is insoluble in alkali and acid (at any pH value), (ii) Humic acid, which is soluble in alkali (e.g. in 0.1 mol L^{-1} NaOH) and insoluble in acid (at pH 1 - 2) and (iii) Fulvic acid that is soluble in both acid and alkali (at all pH values) [202, 205, 206]. These substances are not distinct chemical entities because their separation only depends on the ability of the material to form intermolecular bonds [207]. They are merely considered as a continuum of compounds varying in molecular weight, carbon content, O content, acidity and sorption capacity [201]. Humic acid can still be separated further (Figure 2.6) into hymatomelanic acid (soluble in alcohol), brown humic (soluble in NaCl) and grey humic (insoluble in NaCl) [192].

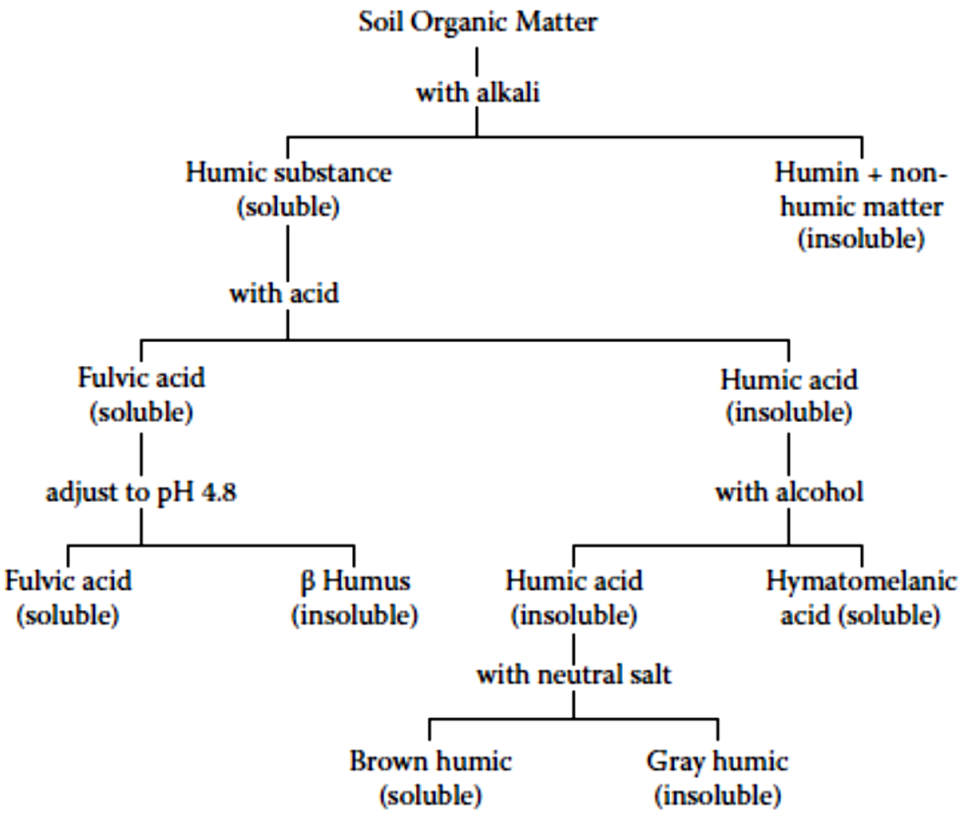


Figure 2.6. The most common fractionation procedure for soil organic matter and humic substances [192]

It has been reported that fulvic acid which is the smallest (in size) of the humic compounds has a molecular weight that is between 10^3 to 10^4 Daltons (Da), humic acid which is slightly bigger has its molecular weight range between 10^4 to 10^5 Da and

humin which is the largest has a molecular weight of 10^7 Da [192, 207]. Humic acid and fulvic acid are the two most investigated humic substances. Compared to soil humus in general, humic and fulvic acids are depleted in N. Their C-to-N molar ratio is 30 % to 50 % bigger than that of soil humus, demonstrating their more prominent imperviousness to net microbial mineralization [189]. However, humic acid and humin (especially) are extremely resistant to biodegradation [205, 208]. Their half-decay time ($t_{½}$) can amount to thousands of years in the soil [207]

Humic acid as seen in Figure 2.7 consists of polymeric units with a basic aromatic ring structure linked by – O -, NH, - N – and – S – bonds. It also contains carboxyl groups that contribute to most of the acidity. The molecules vary in structure and density of functional groups from soil to soil though remaining approximately the same in their basic structure. The fulvic acid fraction as shown in Figure 2.8 contains less carbon and more nitrogen-oxygen than humic acid. It has a smaller proportion of aromatic units and greater peripheral aliphatic chains though the structure is similar to that of humic acid. Also, the carboxyl functional groups appear to be more evident than in humic acid and probably heavily substituted on the aliphatic chains [194]. Humins, which receive the least attention probably because of its low organic carbon content are said to be humic acid type of compounds that are adsorbed onto minerals [201, 209]. In a pyrolysis (methylation) GC-MS analysis performed by Leinweber *et al*. [209] on the three fractions of humic substances, they observed that both humic acid and humin fractions produced a partly similar pyrolysis products. Their most similar products were molecules from plant and microbial carbohydrates, lignin building blocks, long-chained aliphatics (alkanes, alkenes, carboxylic acids, esters) and nitrogen (N) containing compounds [209].

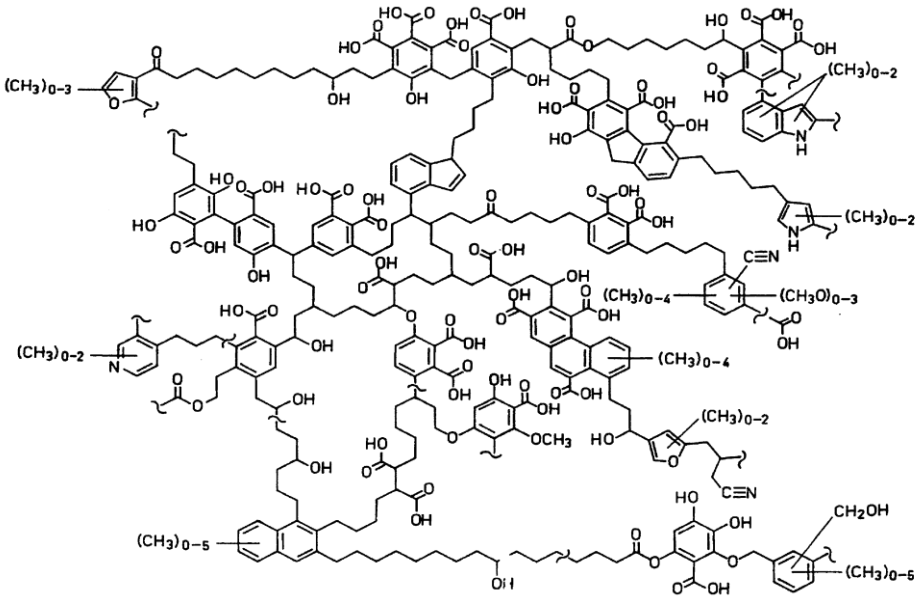


Figure 2.7. Model structure of humic acid [190]

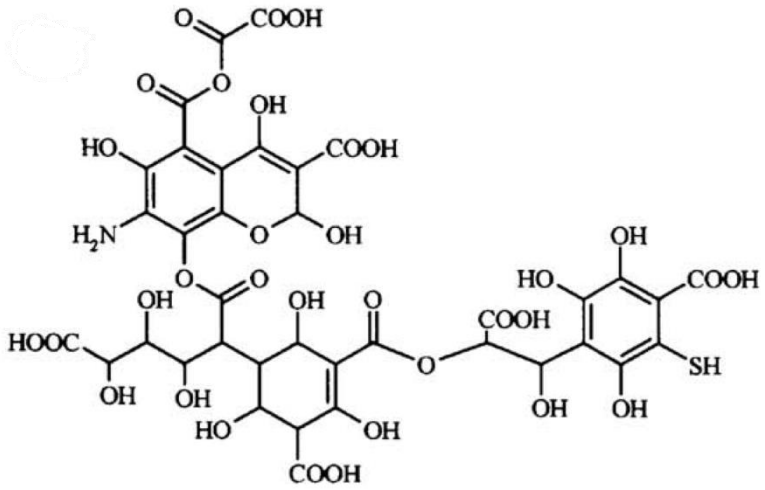


Figure 2.8. The model structure of fulvic acid [210].

These humus substances characterise the organic matter content of the soil as they influence the chemical and physical properties of the soil. They are able to do this due to their high surface area and adsorptive capacities that are as a result of their ability to form chelate complexes with metallic ions. They are also able to form these complexes due to its functional group (e.g. carboxylate) composition. The surface area may be as high as 800 to 900 $m^2\ g^{-1}$ and its exchange capacity could range from 150 to 300 me $100^{-1}\ g^{-1}$ of material [193]. They also provide considerable buffering of soil pH over a wide range value due to the presence of various weak acid functional group [193]. They alleviate the unfavourable structural characteristics of high clay content by promoting aggregation of small soil particles when they are in a fine textural soil.

Organic matter has also been classified into several pools. This concept of pool was based on their different ability to resist microbial attack, hence, these pools exclude the microbial fraction and are composed only of the dead organic fraction [193]. The three most recognized pools are: (1) active (labile), (2) slow and (3) stable or passive pool [193, 211]. The active pool is the least resistant fraction and is composed of compounds most readily accessible for food to microorganisms. It can consist mainly of material in transition between fresh plant residues and stabilized organic matter [198]. It accounts for 2 - 17 % of the total soil organic carbon (SOC) in surface soils [199] and it is considered to be the driving force in soil respiration [198]. Much of it is plant residues and microbial tissue at varying stages of decomposition. It's comparable to plant litter but unlike plant litter that is a residue on the soil surface, this active pool is mixed within the soil. Examples include particulate organic matter, soluble carbon, potentially mineralizable carbon that is extractable with various reagents, microbial biomass carbon and enzymes present in soluble and sorbed forms. The slow pool of organic matter is intermediate in nature between the active and stable (passive) pools. Stable pool comprises of materials that are very impervious to microbial degradation

because of their chemical structure and/or relationship with soil minerals [201] and it has the longest residence time compared to the other types of organic matter [193]. Residence time expresses the age of organic matter as determined by ^{14}C dating [193]. It consists mainly of humic substances [200, 212] produced from the products of biological degradation of plant and animal residues by microorganisms [199]. These humic substances are also responsible for the ageing of sorbed PAHs which makes it inaccessible to soil microorganisms nor extractable by robust extraction methods such as soxhlet or accelerated solvent extraction [71].

2.1.1.4 Organic matter-mineral soil fractions interactions

The interaction between soil organic matter (SOM) and soil mineral fraction are vital in the soil environment. In a typical mineral soil (by weight 80 % inorganic material and 20 % or less organic material [192]), about 50 - 100 % of SOM is associated with the mineral matrix, forming organic mineral complexes [197]. The extent of mineral-associated SOM depends on the soil formation process, soil texture and management. This association governs soil chemistry, affects soil biology and the physical behaviour of soil [197]. Their association is essential for soil structure and the production of more natural organic matter. This is because organic matter cements soil particles into aggregates which increase the stability of the soil and improves the permeability of water and gases into the soil [213, 214]. Together they (clay minerals and organic matter) form the soil colloidal material which has a large surface area to volume ratio or specific surface area [191]. They possess electrostatic charge properties which can only be attributed to clay sized fraction (clay minerals, hydrous oxides of iron and aluminium) and soil humus. This electrostatic charge property enables them to hold onto certain elements (e.g. Ca, K Mg) or compounds (e.g. PAHs), which explains their ability to store nutrients, toxins and change soil pH [191]. Their presence in the soil controls the storage or release of these elements or compounds. The electrostatic charge properties of both clay minerals and humus differ. The 2:1 clay possesses both a permanent and a variable charge, whereas 1:1 clay minerals, hydrous oxides of iron and aluminium and humus have mainly a variable charge. Permanent charges occur because of isomorphic substitution which takes place mostly in the 2:1 clay minerals, causing a charge imbalance and resulting in a permanent negative charge. While the variable charge is as a result of the dissociation of hydroxyl group and protonation which can occur on the surfaces of organic compounds possessing the R'-OH group.
The variable charge on 1:1 clay minerals (e.g. kaolinite) and secondary metal oxides (e.g. aluminium oxide) is because of proton adsorption and desorption and not an isomorphic substitution. The permanent charge is not affected by soil acidity and is

nearly always negative. The variable charge can be positive, negative or zero depending on the acidity of the soil solution (strongly influenced by soil pH) [191]. These differences in electrostatic charges make the soil colloidal material to have either a permanent or variable charge property. The surface charged particle on the soil colloidal material also gives it the ability to adsorb microorganisms (e.g. bacteria) on its surface [206] which can mineralise the active organic carbon pool in the soil. However, it has been reported in several studies [194, 204, 215-218] that SOM can be physically protected from microbial mineralisation because of its (SOM) association with soil mineral fraction (clay minerals). The adsorption of organic matter by clay minerals can be related to their swelling properties. Clay minerals can protect organic matter by adsorbing them into their interlayers (Figure 2.9) thereby enclosing the organic matter in its micropores which prevent the entrance of microorganisms to the organic matter [204]. The adsorption process by clay minerals takes place through cation exchange for positively charged organic compounds, hydrophobic bonding for neutral organic compounds and polyvalent cation bridges for negatively charged organic compounds (organic anions). Soils with a high point of zero charge (PZC) may also be positively charged at ambient conditions and therefore, can directly adsorb organic anions through anion exchange [204]. The functional groups of the organic matter interacting with the clay minerals are mainly carboxyl groups [204]. The protecting effect also depends on the kind of clay mineral that adsorbs the organic matter. Smectites (2:1 clay mineral) are very effective protectors, whereas the protective effect of kaolinite (1:1 clay mineral) is rather weak [204]. This physical protection is generally evidenced by the flushes of SOM mineralization observed when soil aggregates are disrupted. The ability of different levels of soil structure to protect SOM against decomposition has been generally assessed [217]. The adsorbed organic matter associated with clay- and silt-size sedimentation fractions are impervious to physical disruption by ultrasound and to leaching during sedimentation/decantation, and are subsequently firmly associated with the mineral matrix [197]. The organic matter adsorbed to the clay minerals also helps to reduce clay dispersion caused by wet and dry cycles. It has also been reported that wetting events in soil may likely cause de-absorption of the organics leading to their de-protection [217]. The oxides of Fe and Al can also adsorb organic compounds within the soil mineral (Figure 2.9) through mechanisms such as electrostatic attraction, hydrogen bonding, and ligand exchange reactions forming a stable complexation, thereby protecting the organic compound from mineralisation by microorganisms [204].

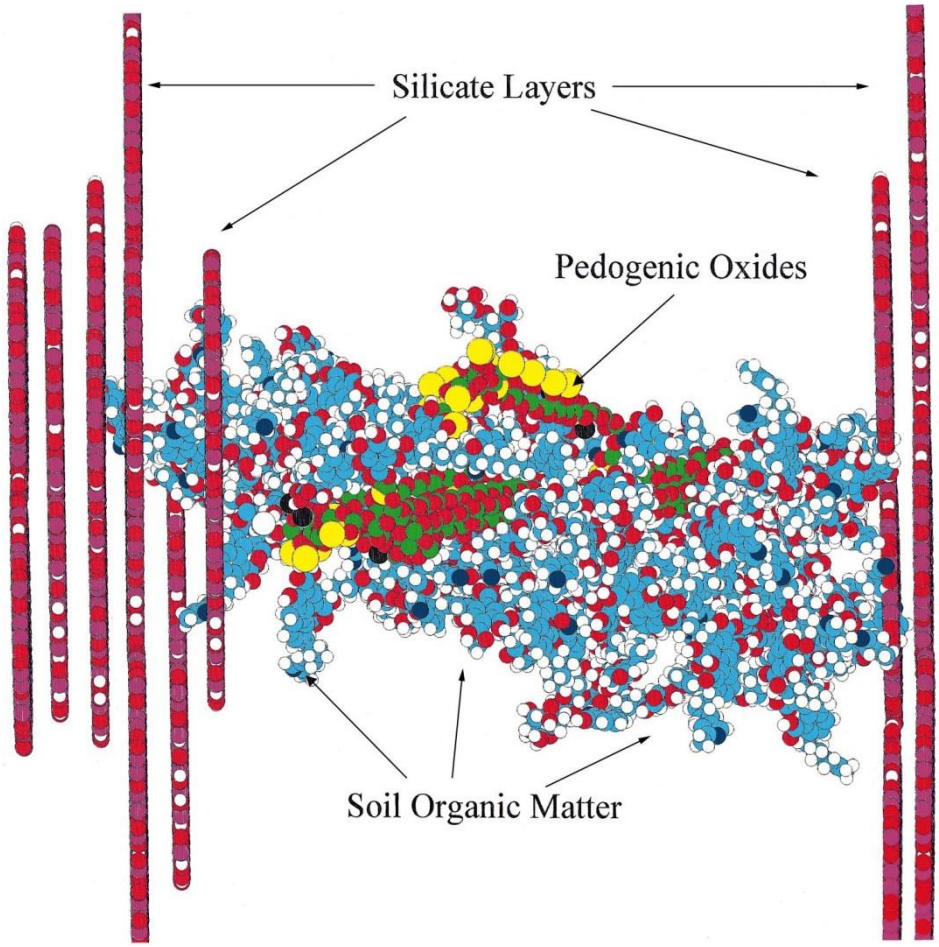


Figure 2.9. Model of a mineral-bound organic particle consisting of the following structural components: (a) mineral matrix of silicate layers, (b) bound soil organic matter complex of humic acids with (c) trapped labile carbohydrates and peptides, (d) pedogenic Fe oxides, and (e) alkali and earth alkali cations. The element colors are: hydrogen, sodium and potassium, white; carbon, cyan; oxygen, red; nitrogen, blue; silicon, violet; iron, green; calcium, yellow; and magnesium, black [209]

2.1.1.5 Soil chemical reactions:

Soil chemical reactions are controlled mostly by the electrochemical properties of soil colloids. This refers to the acidity or alkalinity of the soil. The degree of acidity or alkalinity of a soil is determined by the hydrogen ion, (H^+) concentration in the soil solution. In acidic soil the H^+ concentration is greater than the hydroxide ion (OH^-) concentration whereas in alkaline soils the H^+ concentration is smaller than the OH^- concentration. In a soil with a neutral reaction $H^+ = OH^-$. These values are expressed in pH values ranging from 0 to 14. The p refers to negative log and the H means the H ion concentration in grams L^{-1} [192, 193].

Soil becomes acidic through different mechanisms some of which are natural like the acidity produced by the breakdown of soil organic matter and some of which are caused by humans such as pollution from industries. Soil acidity indicates the high concentration of H ions in soils solution and it can also indicate the likely cations to be held in the greatest concentrations in the exchange sites [191]. For example, acidic soils have high concentrations of exchangeable aluminium, unlike alkaline soils where exchange sites are more likely to be dominated by Na, Ca and Mg.

The soil pH influences the surface charge on soil mineral colloids and organic matter thereby controlling soil reactions such as the cation exchange capacity (the ability of charged soil particles to adsorb a cation), anion exchange (the ability of soil particle to adsorb anions). Soil pH controls this surface adsorption and precipitation reactions through their point of zero charge (PZC) of the soil colloid. The PZC of soil is defined as the suspension pH at which the surface of soil particle (clay minerals and humus) has a net charge of zero. If the measured pH of a colloid is lower than the PZC, the surface is net negatively charged. This surface can then be able to adsorb metal ions through ionic exchange and complexation.

2.2. Effect of soil organic matter on PAH bioremediation

In the soil environment, organic material is associated with particles in the bed sediment (POC – particulate organic carbon) but also exists in a 'dissolved' form in the water phase (DOC - dissolved organic carbon). Dissolved organic carbon (DOC) which is a product of physical chemical and biological breakdown of soil organic matter [140], serves as a substrate for microbial respiration [140, 219]. The sorption of hydrophobic organic compounds such as PAHs to organic material is one of the main processes determining their bioremediation, toxicity and fate by affecting their speciation and transport in the aquatic and subsurface environments [219]. Partitioning of PAHs to DOC may be an important process that determines its mobility because DOC-associated compounds can be easily transported in soil systems. For compounds with low volatilization rates and high sorption partition coefficients such as the PAHs used in this study, sorption to DOC has a large influence on its fate in the soil systems. PAHs might be transported attached to colloids, which act as carriers. Apart from inorganic suspended solids, such as clay minerals, colloids of organic origin play an important role as carriers. Under these circumstances, the factors controlling the mobility of particles are essential for release and transport of PAHs. Colloids and particles can be mobilised during rain events by a decrease in the soil solution’s ionic strength and by increased hydrodynamic forces caused by large flow velocities [100]. In this study, for example, the mobility of colloids can be increased through watering of soil sample to

maintain a stable moisture condition during the study. Depending on the origin and fate of the carriers, the concentration of PAHs in the pore water might either be enhanced or reduced due to the presence of sorbents. This then implies that the amount of soil organic matter in a soil needs to be determined in order to understand how they can influence the bioremediation of PAHs by reducing or increasing their bioavailability to microorganisms.

2.3. Soil mineral surface effect on microbial community and PAH bioremediation:

Soil minerals play a stabilising role in the organic matter. These minerals are the most reactive fraction of soil inorganic components in view of their huge specific surface areas and high charge density attributes [200]. Being endowed with ions, water and organic matter in respect to the bulk soil, the surface of mineral colloids serve as a favoured habitat for soil microorganisms [162]. The surfaces of both mineral colloids and bacteria cells are also negatively charged. However, bacteria have the inclination for creating extracellular polysaccharides which can simultaneously bind to cell and clay surfaces through cation bridging involving polyvalent cations [162]. Mineral colloids have an invigorating effect on the liveliness of adhering bacteria by keeping the pH of the microhabitats within the best physiological range for growth [162]. Microbial activity can likewise be stimulated by mineral colloids through their capacity to sorb metabolites that may otherwise have an adverse effect on microbial growth [133, 198]. This can occur when these toxic metabolites like antibiotics and pesticides become adsorbed by the mineral surfaces, thereby preventing their toxic interaction with microorganisms.

Several studies have shown that PAHs can adsorb to clay minerals, thereby restricting their mobility and biodegradation by soil microorganism [220] but there are other studies that have demonstrated that PAHs can be degraded even in the presence of clay minerals. Huang *et al*. [221] in their study showed that diverse taxa of bacteria can form biofilms on the clay minerals, thereby enhancing the biodegradation of adsorbed PAHs [221]. Non-polar molecules, such as PAHs, are only able to interact with the mineral surface through Van der Waals and dispersive forces, and as such are out competed for the surface sorption sites by water molecules [222]. Meleshyn *et al.* in their modelling study demonstrated the detachment of phenanthrene from montmorillonite surface in the presence of water molecules [223]. Adsorption of PAHs onto mineral surfaces is negligible if the soil also contains organic matter. PAHs tends to adsorb or associate more with organic matter due to the different adsorption

mechanisms used by organic matter on PAHs which includes partition mechanism, surface adsorption and micropore filling [222].

Therefore, it is necessary to determine the soil structure when undertaking a PAH bioremediation study in order to understand how PAHs and the soil microbial community can be affected in the course of the study. Soil structure is known to determine the flow of energy and the distribution and composition of soil microhabitats [162, 201]. The associations between soil mineral particles, natural matter, and microorganisms can happen at a wide range of size scales on the grounds that these materials have an expansive size extent in soils

2.4. Effect of soil handling and storage on PAH bioremediation

Soil treatment and handling after sampling can affect PAH bioremediation studies by changing and reducing the size of the indigenous microbial community that would carry out the biodegradation activity. Soil handling such as drying (loss of soil moisture) and sieving can change the soil structure (particle size distribution) which can expose the microbial community to adverse environmental conditions leading to the death of sensitive soil microorganisms. However, drying of soil has been a conventional method for soil handling in most laboratory investigations [224, 225]. It helps to diminish the variability emerging from the soil's inherent heterogeneity, ease handling and processing, enhance sample blending efficiency and also the distribution and homogeneity of the spiked compounds or metals [226].

Soil storage prior to and during laboratory investigations is also an important aspect for bioremediation studies because environmental conditions such as temperature and moisture content need to be regulated in order to avoid the introduction of artefacts in the experimental results. Temperature and moisture conditions could introduce artefacts in experimental data by affecting the behaviour of the soil microbial community positively or negatively which could be misinterpreted as a pollutant effect. Therefore, there is a need to maintain an optimum temperature and moisture condition that would sustain the microbial community's biodegradation activity.

It is very difficult to recreate the soil's natural environment during laboratory studies but soil handling and laboratory conditions should be such that the influence of external factors (e.g. temperature and moisture conditions) are minimised. Therefore, it is very important to determine suitable environmental conditions and soil handling techniques that would optimise the biodegradation of PAH throughout the duration of the study.

This chapter only covers the experiment and results that were carried out in order to determine an adequate soil handling treatment and environmental conditions that can sustain microbial community during the PAH bioremediation study. It aims to highlight the challenges that are inherent in laboratory investigations involving soil samples, for example, maintaining a sustainable period of soil microbial activity. It then provides information on the different data sets that could be generated when experiments are carried out under natural environmental conditions and under laboratory conditions using the same soil samples.

2.5. Experimental methods

2.5.1 Overview

This study was carried out using different soil handling techniques (drying and sieving) and under different soil storage conditions (temperature and moisture content). The three analytical approaches that were used for this study were the soil respiration rate test, the community level physiological profile test and the microbial biomass carbon test. Each category of experimental analysis were carried out with soil samples treated for different experimental durations ranging from 0 days to 70 days

The soil respiration test was carried out using the OxiTop manometric method and equipment (WTW, Weilheim, Germany) and it was done in five different stages of soil treatment and storage conditions. The five stages of respiration experiments were: (a) to determine the minimum period for which reliable soil respiration rates could be obtained; (b) to determine the influence of soil handling (drying and sieving of soil) on three soil samples spiked with three different concentrations of copper (10 mg kg^{-1}, 100 mg kg^{-1} and 1000 mg kg^{-1} respectively) in an experiment with a 70 day duration; (c) to determine the effect of different storage conditions (moisture regulation) on the soil microbial community in a 42 day experiment; (d) to determine the effect of different environmental conditions such as change in temperature and moisture content in a 35 day experiment; and (e) to optimise soil respiration technique using different sizes of OxiTop manometric sample vessels and undried soil samples with experiment duration for 2 days.

A community level physiological profile (CLPP) evaluation (BIOLOG Eco-plate) was carried out in two stages: (a) determining the effect of soil handling (drying and sieving) on microbial functional diversity of soil spiked with three different concentrations of copper (10 mg kg^{-1}, 100 mg kg^{-1} and 1000 mg kg^{-1}) derived from $CuSO_4.5H_2O$ and (b)

determining the microbial functional diversity on soil exposed to different environmental conditions such as change in temperature and moisture content.

A microbial biomass analysis was carried out on the soil samples spiked with three different copper concentrations; 10 mg kg^{-1}, 100 mg kg^{-1} and 1000 mg kg^{-1} using the substrate-induced respiration method. The evaluation of heavy metal analysis techniques on the three different concentrations of copper (10 mg kg^{-1}, 100 mg kg^{-1} and 1000 mg kg^{-1}) spiked soil was carried out using EDXRF method to determine the total concentrations of copper in the spiked soil samples.

2.5.2 Soil sampling:

Soil samples were collected from Armstrong Park situated in the Northeast area of Newcastle upon Tyne, approximately 2 km from Newcastle city centre. The specific location is a Greenfield site that is currently wooded. Significant industry, including lead works and lead paint works operated to the south of the site, contributing to historically elevated lead and other metals (Zn and Cr) levels in this area [227].The sampling was carried out in a random pattern in which soil was collected by the use of auger/trowel over a shallow depth of 20 cm. A total of 20 kg of soil sample was collected from the sampling site at a time. Sampling was repeated twice for the different soil handling technique analysis. The collected soil sample was transferred to the laboratory using a polyethylene bag in order to maintain the original soil characteristics.

2.5.2.1 Sample handling:

The soil was homogenised and half of the soil sample (10 kg) was air dried to complete dryness for 72 hours and was then sieved with a 2 mm sieve. The other half (10 kg) was not completely dried and was wet sieved with a 2 mm sieve. An additional 1 kg of the soil was used for soil characterisation.

2.5.3 Materials and apparatus

D-glucose 'AnalaR' from BDH (Poole England) was used as a readily available carbon substrate for substrate-induced respiration in accordance with BS ISO 16072. Sodium hydroxide (NaOH) pellets purchased from BDH (Poole England) was used to absorb carbon dioxide (CO_2) during the soil respiration study. Pure quality cupric sulfate ($CuSO_4.5H_2O$) obtained from Fisher scientific Ltd (Loughborough, UK) was used for the heavy metal spiking studies. The binder used in EDXRF analysis was Licowax C Micro powder PM (FLUXANA GmbH & Co,Sommerdeich, Germany). BIOLOG Ecoplates (BIOLOG Inc., 3938 Trust Way, Hayward, CA 94545, U.S.A.) purchased from Techno-

path distribution Ltd, Ballina, Tipperary, Ireland, was used for community physiology profile analysis. NaCl laboratory reagent from Fisher Scientific UK was used for extraction of microorganism from the soil. The pH meter used was Jenway 3020 pH meter supplied by SH Scientific Ltd Northumberland, U.K. The rotary shaker (Stuart Rotator Shaker SB 3) was used to shake soil samples for eco plate analysis. The Centrifuge (Beckman Allegra 6R centrifuge) was used for soil microbial extraction during the eco plate analysis. Ultra-pure water of conductivity 18.2 MΩ cm^{-1} at 25 ^{o}C was produced by a direct QTM Millipore system (Molsheim, France).

2.5.4 Instrumental technique:

2.5.4.1 Microplate absorbance reader:

El x 808 (Bio-Tek Instrument Inc) microplate absorbance plate readers is a multichannel reader that accommodates 96 well microplates. The read modes include endpoint, fast kinetics and linear scanning. The wavelength range is from 380 - 900 nm or 340 - 900 nm (UV model). The instrument was used to read the Ecoplate wells. The instrument measured the wells colour absorbance at 595 nm wavelength and at endpoint mode. The intensity of absorbance depends on the degree of colour developments in the wells as shown in Figure 2.10. The Ecoplates were read at room temperatures (usually between 18 - 24 °C). The instrument was also programmed to shake the Ecoplates before measuring in order to eliminate any trapped bubbles in the wells and the software it used for window data reduction is KC junior software. The plate was manually read at the required times (every 24 hrs).

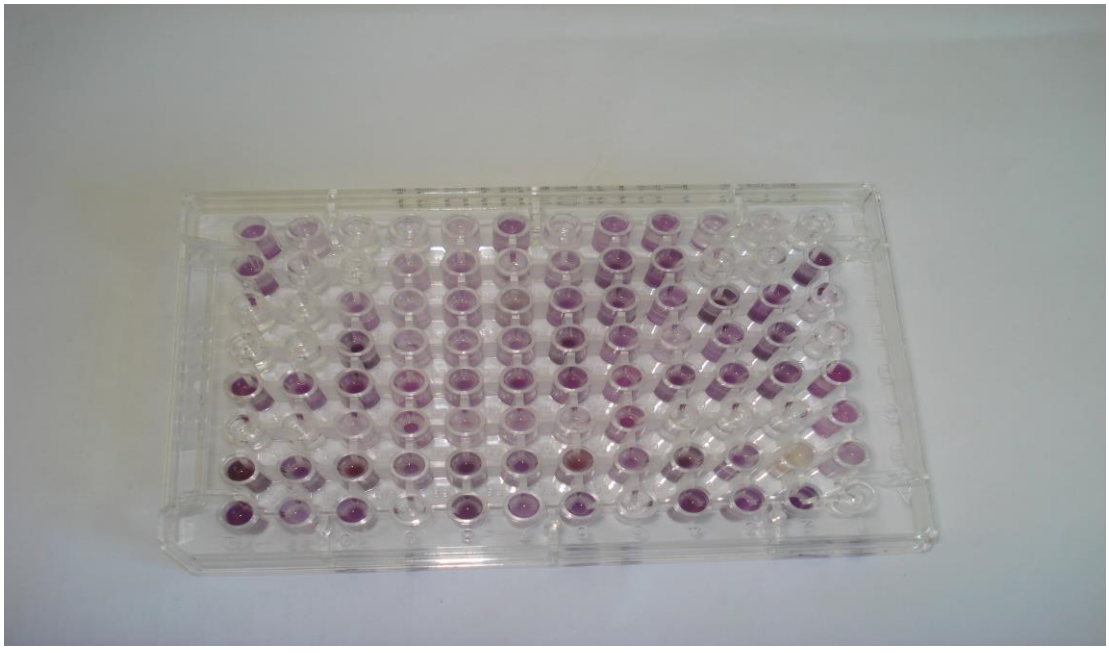


Figure 2.10: BIOLOG Ecoplate with different degrees of colour development in the 96 wells

2.5.4.2 OxiTop respirometry system

The OxiTop system consists of an incubation chamber, six sample vessels, measuring heads and OxiTop controller as seen in Figure 2.11. The OxiTop (WTW, Weilheim, Germany) respirometry system can be used for manometric measurement and it was used in this study to measure the pressure reduction caused by an absorbing agent (NaOH) in the OxiTop vessel during soil respiration.

The oxygen consumed during soil microbial activity produces approximately equimolar quantities of carbon dioxide that were bound by sodium hydroxide an absorbing agent which caused a pressure reduction in the OxiTop vessel. The resulting time-dependent pressure profile is stored in the OxiTop measuring head and this is read by the OxiTop controller through an infrared interface. The data is subsequently uploaded onto a computer for further Excel data analysis using the OxiTop ACHAT OC PC communication software (WTW, Weilheim, Germany). The OxiTop control system records 180 to 360 measured values depending on the total measuring duration (0.5 hours to 99 days) and this enables a detailed examination of the measured pressure reduction curve. The pressure reduction data extracted from the controller was then converted to respiration rate during data analysis. The OxiTop system thermostatic chamber temperature was set at 20 ± 2 °C for the duration of the respiration studies.

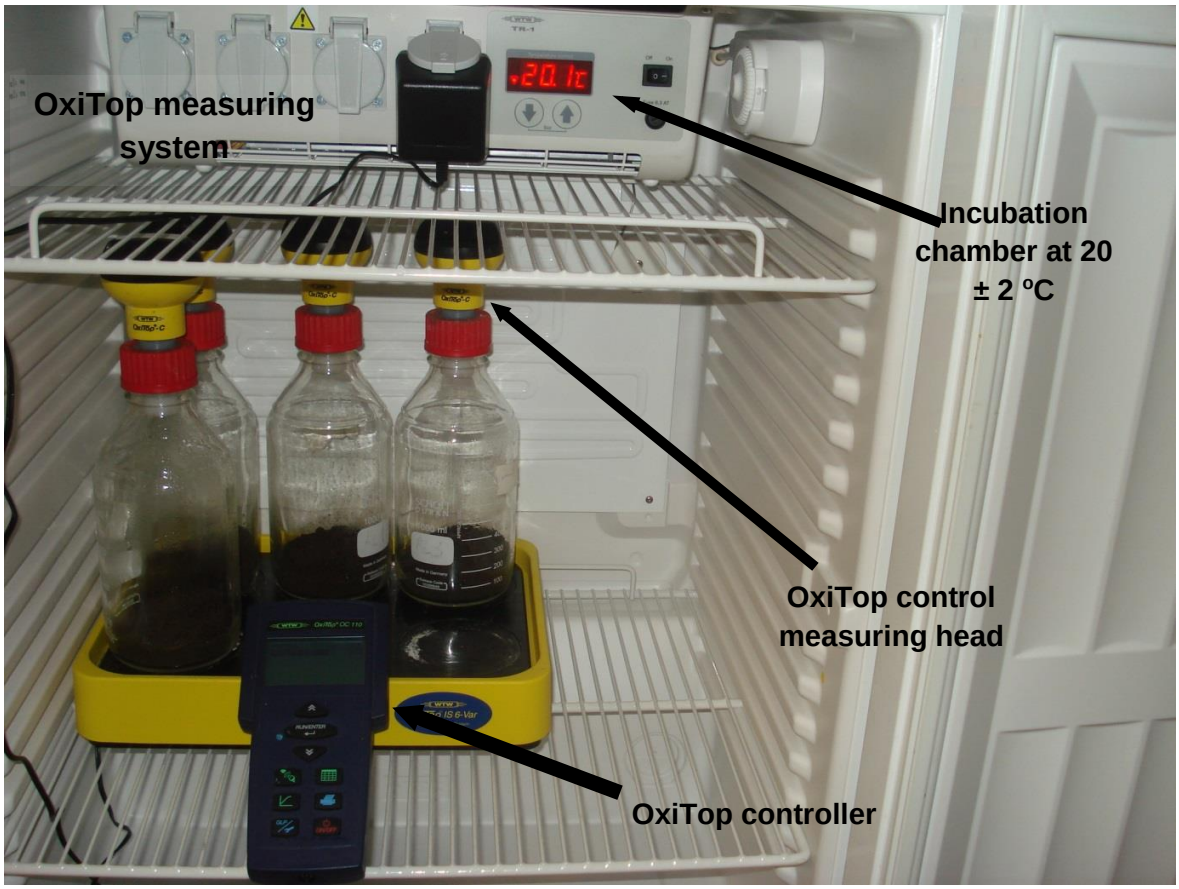


Figure 2.11: An OxiTop incubation chamber with the OxiTop head, bottles and OxiTop measuring control unit

2.5.4.3 Energy dispersive x-ray fluorescence (EDXRF):

The EDXRF instrument (Spectro Analytical X-Lab 2000) fitted with a Gresham Si (Li) detector was used to analyse the pressed soil pellets to confirm the different heavy metal concentrations in the spiked soil. The instrument uses polarised radiation (about 50 KeV) to bombard the sample pellets placed in the carousel thereby stimulating fluorescence. Each element present in the soil sample emits a characteristic radiation which is detected and the total concentration is determined based on the intensity of the radiation. The program used for the analysis was the 5 target geology program [228].

2.5.5 Characterization of soil:

2.5.5.1 Soil pH determination:

Soil pH was determined with a pH probe (Jenway 3020 pH meter) using 1:2.5 ratio of soil to distilled water [229]. The soil solution was stirred thoroughly using a glass rod for

about 2 – 3 minutes and the soil was allowed to settle for about 2 minutes. The pH of the resulting supernatant was then measured using a pH probe.

2.5.5.2 Loss on ignition method:

Organic matter content of the soil was determined using the standard procedure for loss on ignition. 5 g of soil sample was weighed into a small ceramic crucible (pre-weighed crucible). The total weight of the soil and the crucible were determined and recorded. The crucible was placed in a preheated muffle furnace (800 °C) for half an hour. The sample was removed and allowed to cool in a desiccator. The sample was re-weighed again after cooling to determine the final weight. The final weight was subtracted from the initial weight. The value was used to calculate for percentage organic matter as shown in Equation 2.1, where % OM is the organic matter composition and W_i and W_f are the initial and final weights respectively:

$$\%OM = \frac{W_f}{W_i} \times 100 \tag{2.1}$$

The determined percentage organic matter content was multiplied by 0.58 to give the total organic carbon based on the assumption that soil organic matter comprises 58 % organic carbon [147, 230, 231].

2.5.5.3 Soil moisture content:

The moisture content was determined by weighing 10 g of wet soil sample into an evaporating dish, weighing the soil and the dish together and drying the soil in the oven at 105 °C for 24 hrs. The final weight of the dry soil and the dish was subtracted from the initial weight of the wet soil and the dish to determine the moisture content as percentage dry mass.

2.5.5.4 Soil water holding capacity:

The water holding capacity of the soil was determined by weighing 100 g of oven dried soil (24 - 48 hrs at 105 °C) into a cylinder with screen and filter paper at the bottom. The weight of the cylinder, filter paper, and dry soil was then measured. The cylinder and soil was dipped into a basin of water and allowed to absorb water for 2 hrs [121]. After the soil has absorbed water to its maximum the soil was drained till water stopped coming out from the base; this is usually within 2 hrs. The weight of the cylinder, filter

paper and saturated water were re-determined. The weight of the saturated soil was measured and subtracted from the weight of the dry soil to give the weight of water in the saturated soil. The final percentage was calculated using Equation 2.2, where %WHC is the percent water holding capacity and Wa and Ws are the weights of the added water and the saturated soil respectively.

$$\%WHC = \frac{W_a}{W_s} \times 100 \qquad (2.2)$$

2.5.5.5 Particle size determination:

Soil particle size determination was carried out using a Malvern Instrument Mastersizer *S.* The Mastersizer separates the finer proportion of the sample into percentage clay, fine silt, medium silt and coarse silt. The instrument calculates the percentages based on the total amount of soil sample that passed through it. The protocol used for this particle size analysis is as follows:

Approximately 50 g of soil sample was weighed out into a beaker with a known weight. 100 mL of hydrogen peroxide was added to the soil and the mixture was allowed to stand for 2 days until the effervescence process in the soil stopped completely which shows that the organic matter in the soil has been completely digested by the H_2O_2. The soil was dried in the oven at 105 ^oC overnight and reweighed. The dried soil sample was ground using a pestle and mortar and 100 mL of Calgon solution (40 g of $(NaPO_3)_6$ dissolved in 1 litre of distilled water) was added to the soil and sonicated for 5 mins at room temperature. The mixture was sieved with a 63 µm sieve and the sieved portion was introduced into the Mastersizer while the residue was oven dried overnight. The residue was then sieved with 600, 212 and 63 µm sieves to separate the sand fraction into coarse, medium and fine categories. The Mastersizer analysed the sieved extract into percentage clay, fine silt, medium silt and coarse silt.

The final soil sample fraction percentage was calculated using Equation 2.3

$$\text{Percentage soil size fraction} = \frac{\text{weight of sieved soil sample}}{\text{total weight of dried soil}} \times 100 \qquad (2.3)$$

2.5.5.6 Determination of organic carbon by potassium dichromate or Walkley-Black method [231]:

0.4 g of finely ground soil was weighed into a wide-mouthed conical flask (500 mL). 10 mL of a 1 N solution of potassium dichromate was added and 20 mL of concentrated

sulphuric acid was also added. The mixture was swirled gently for 1 minute to mix. The mixture was allowed to stand for 45 minutes in the fume cupboard. 170 mL of deionised water and 10 mL of phosphoric acid was added to the mixture after 45 min. Two drops of diphenylamine indicator (0.5 g indicator powder in a mixture of 20 mL distilled water and 80 mL concentrated sulphuric acid in a beaker) were added and the mixture was swirled. Titration was carried out on the solution using 0.5 N ammonium ferrous sulphate (0.5 N solution: 196.06 g $FeSO_4$ $(NH_4)_2SO_4.6H_2O$ was dissolved in deionised water, 100 mL concentrated sulphuric acid was added and made up to 1litre with deionised water).

The end point of the titration was noted at the point when the solution changes from blue to green colour. The total dichromate used, subtracted from half of total ammonium ferrous sulphate added is equal to the volume of dichromate reduced (consumed) during the reaction. 1mL of potassium dichromate used in the reaction is equal to 4 mg carbon in the soil. The percent organic carbon is calculated according to Equation 2.4.

$$\% \text{ organic carbon} = \frac{\text{amount of dichromate consumed x 4}}{\text{400 mg (of soil)}} \text{ x 100} \qquad (2.4)$$

2.5.5.7 Determination of nitrogen content was determined by the Kjeldahl method:

Two samples of 2 g of dry soil were weighed into 2 separate Kjeldahl flasks; two selenium catalyst tablets and one copper catalyst tablet were added into each flask. Approximately 15 mL of concentrated sulphuric acid was also slowly added to each flask. The tubes were mixed gently by swirling and then placed in a heating block at 440 °C for 30 minutes or until bright green colour appears. The samples were then allowed to cool at room temperature. The tubes were placed into Kjeldahl apparatus which uses sodium hydroxide to react with ammonium sulphate formed in the tubes as a result of the acid digestion and heating. The resulting ammonia gas produced was distilled off and bubbled into a solution of boric acid containing two drops of methyl red indicator. This indicator turns from blue/purple to green as the distillation proceeds if nitrogen was present in the soil.

The solution was titrated against 0.05 M HCL (in burette) and the end point is corrected to the nearest 0.1 mL. The following calculation is used to determine the percentage nitrogen present in the soil:

1 mL of 0.05 M HCl is equivalent to 0.0007 g N

Hence N (g) = titre (mL) x 0.0007

$$\% \text{ Nitrogen} = \frac{\text{N (g)}}{\text{Dry sample weight (g)}} \text{ x } 100 \qquad (2.5)$$

2.5.6 Soil spiking with copper

A total of 6 kg of the air-dried and sieved (2 mm sieve) soil was divided into three groups of 2 kg of soil for each group. The 2 kg of soil for each group was spiked with different concentration of copper solution to give additional respective copper concentrations of low (10 mg kg^{-1}), medium (100 mg kg^{-1}) and high (1000 mg kg^{-1}) on a dry weight basis.

2.5.6.1 Spiking procedure:

$CuSO_4.5H_2O$ was used as the source of Cu metal. The soil spiking for low (10 mg kg^{-1}), medium (100 mg kg^{-1}) and high (1000 mg kg^{-1}) of copper concentrations required 0.078 g, 0.7803 g and 7.803 g of $CuSO_4.5H_2O$ respectively, each was dissolved in 427.32 mL of ultra-pure water needed to adjust the soil to 60 % of its water holding capacity. The soil (2 kg) was spread out thinly on a plastic tray and the dissolved copper solution was applied on it using a water spraying vessel. The wet soil was then thoroughly mixed with a hand trowel and the resultant mixture was expected to yield the above Cu concentrations or more in soil dry weight.

The three spiked 2 kg soils were each transferred into a 3 L volume of plastic vessel with a perforated lid in order to allow an easy circulation of air. A control soil was also made up by applying ultra-pure water onto a thinly spread out soil and mixing the wet soil thoroughly using a hand trowel. The control sample was then transferred into a plastic vessel the same as those spiked with different copper concentrations.

The four pots of soil were stored in a plant room at approximately 20 ^{o}C. Loss of moisture from the pot samples was monitored and maintained at 60 % water holding capacity by adding the required amount of distilled water once every week. The loss of moisture was monitored gravimetrically by the moisture content method as described above.

Three (10 g) soil samples from each pot was air-dried and analysed for total copper concentration using EDXRF. The analysis was carried out in order to check and confirm the copper concentration in the pots.

2.5.7 Determination of the total copper concentrations by EDXRF

Soil samples were air dried in the fume cupboard. Soil subsamples were powdered in an agate ball mill. 4 g of the powdered sample was weighed out and mixed with 0.6 g Hoechst wax (Licowax C micro powder) binding powder using an orbital shaker for 1 minute. The soil/binder mixtures were compressed into pellet forms using a hydraulic press that applied 10 tonnes of pressure for 30 seconds. The formed soil pellets were analysed in the EDXRF using a geology 5-target program

2.5.8 Soil respiration experiments

2.5.8.1 Determination of the minimum soil respiration period:

It was essential to determine the minimum soil respiration period because of the anticipated high number of soil samples and the number of times soil respiration measurement would have to be carried out. Soil respiration equipment (OxiTop system) only contains six chambers for sample incubation and so from a capacity perspective, it is important to know the minimum number of days it takes for a reliable respiration rate to be determined, thus allowing the maximisation of the number of samples subject to respiration analysis over a given time. Two types of soil respiration measurements were used for this test; the basal respiration and the substrate-induced respiration. Basal respiration involved using the soil without the addition of any form of an artificial substrate (nutrient) while substrate-induced respiration as the name implies requires the addition of artificial substrate (nutrient) which serves as a readily available substrate that microorganisms can mineralise [160]. The two different soil respiration methods were tested in order to determine the minimum period for a significant pressure change for both.

The six OxiTop vessels (1 L volume) were divided into three replicates for each of the two respiration methods. A glucose ($C_6H_{12}O_6$) concentration of 5 g L^{-1} was prepared for the substrate-induced respiration test. 100 g of dry soil was used for the three replicates of basal respiration and also for the replicates of substrate-induced respiration. The 100 g of dry soil for the basal respiration was adjusted to 50 % of its water holding capacity using 17.95 mL of distilled water and that for the substrate-

induced respiration was adjusted with 17.95 mL of the glucose solution (5 g L^{-1}). The wet soils were transferred into OxiTop vessels and the OxiTop measuring head was then set up to measure the pressure reduction in each vessel for 5 days in the OxiTop incubation chamber (incubation temperature; 20 ± 2 °C).

2.5.8.2 Determination of the influence of soil handling (complete drying and sieving) on soil spiking:

The two main reasons for the basal soil respiration measurement on the Cu spiked soils: to investigate the influence of soil handling on spiking procedure by evaluating the respiration measurements on both the control soil and the spiked soils; to determine the effect of the different copper concentrations on soil microbial community.

100 g of soil from the un-amended Cu soil (control sample) and the copper amended soils maintained at 60 % (low, medium and high) were collected for soil respiration measurements. The respiration measurement on the soil samples were carried out on 1, 7, 14, 28, 42, 56 and 70 days after the experimental set up. The OxiTop measuring heads at each time was set up to measure pressure reduction in the OxiTop vessels for 2 days and the respiration rate were calculated from the data extracted from the OxiTop controller.

2.5.8.3 Determination of adequate storage conditions (loss of moisture and air circulation) for laboratory soil study

The storage conditions that were monitored in this test using soil basal respiration were moisture regulation through air circulation. The soil used for this test was also completely dried and rewetted to 60 % of its WHC before it was transferred into their respective storage vessels. Two plastic storage pots each containing 2 kg of dry soil that has been adjusted to 60 % of its water holding capacity was used for the study. One of the storage pots was stored without a lid but was enclosed in a bigger plastic vessel that has enough room for air circulation. This bigger plastic vessel also had two 200 mLs beakers filled with distilled water and wet paper towels placed inside it, to regulate the loss of moisture. The lid on the bigger vessel was perforated six times. The other storage pot was stored with a lid on it which was perforated four times and it was not enclosed in the bigger plastic vessel. The two pots were stored in the laboratory plant room with a temperature between 18 and 20 °C.

100 g of soil from each of these soil pots were collected for soil respiration measurements on the 1, 7, 14, 28, 35 and 42 days after the experimental set up. The OxiTop measurement was set up to measure the pressure reduction for 2 days and data analysis followed subsequently.

2.5.8.4 Determination of adequate environmental conditions (temperature and moisture) for soil microbial study

This study was to determine the adequate temperature and moisture conditions that would be suitable to sustain soil microbial activity during the course of biodegradation investigations. The soils used for this study was the wet sieved soil which was not dried completely. The environmental conditions that were tested are: **(a)** *Unregulated temperature and moisture conditions***:** soil pot with 2 kg of wet soil stored on the open roof of the University building top with no shelter. Moisture conditions for this soil sample was maintained through natural conditions (rainfall); **(b)** *unregulated temperature with regulated moisture conditions*: soil pot with 2 kg of wet soil stored in a shelter on the rooftop with the moisture conditions maintained twice a week by adding 41.75 mL of distilled water to it. This amount of water was the calculated local weekly average rainfall; **(c)** *regulated temperature and moisture conditions:* the third soil pot with 2 kg of wet soil was stored in the laboratory plant room (temp: 18- 20 °C) with the moisture content maintained at 80 % of it WHC.

All the soil pots stored at the three locations were without lids; soil samples taken for respiration measurements (100 g) were collected from the whole length of the vessel using a soil corer. Respiration measurements were taken at 1, 7, 14, 21, 28 and 35 days after the experimental setup. The OxiTop measurement was set up to measure the pressure reduction for 2 days and data analysis followed subsequently.

2.5.8.5 Determination of an improved respiration rate resolution using two different OxiTop vessels

Soil respiration measurement was carried out on two different OxiTop vessel capacities (250 mL and 1000 mL); this was in order to determine the vessel with an optimised the pressure reduction measurement.

100 g of wet soil sample maintained at 80 % of its WHC was used for each OxiTop vessel for soil respiration measurement. The soils were collected randomLy (top and bottom) from the same soil vessel stored in a shelter on the rooftop. The OxiTop measurement was set up to measure pressure reduction for 2 days.

2.5.8.6 The OxiTop set up procedure:

100 g of soil for each of the treatment maintained at their respective water holding capacity was collected at every measurement period and transferred to an OxiTop measuring vessel. Two pellets of NaOH were transferred into the OxiTop control measuring head pellet housing unit. The bottle was closed with the OxiTop control measuring head (finger tight) and transferred into the OxiTop incubation chamber. The temperature in the incubation chamber was maintained at 20 °C ± 2. The soil in the bottle was allowed to stabilise for about 10 minutes, then the respiration measurement was commenced for two days.

2.5.8.7 Soil respiration data analysis:

The soil respiration results were determined using the oxygen consumption equation [161] as shown in Equation 2.6. The values of oxygen consumed were used instead of the amount of carbon dioxide evolved because manometric respiration method principle determines the amount of oxygen consumed by the microorganism during biological activities. The amount of oxygen consumed was determined based on the rate of pressure change in the OxiTop vessel and the rate of change in pressure is proportional to the mass of oxygen consumed.

$$BA = \frac{M_R(O_2).V_{FR}}{R.T.M_{BT}} \times \Delta p \qquad (2.6)$$

Where;

BA = soil respiration [in $mgO_2\ kg^{-1}\ TS^{-1}$]

$M_R\ (O_2)$ = molar mass of oxygen: 32000 mg mol^{-1}

V_{FR} = free gas volume [in L]

R = general gas constant: 83.14 L mbar $mol^{-1}\ K^{-1}$

T = measuring temperature [in K]

m_{BT} = mass of dry soil substance in the measuring preparation

Δp = reduction in pressure of the measuring preparation [in mbar].

The rate of pressure change value was obtained from the soil respiration pressure graph as illustrated in Figure 2.12 and 2.13. The section of the graph showing the

linear region (184 to 1880 min.) chosen for the soil respiration rate calculation is illustrated in Figure 2.12.

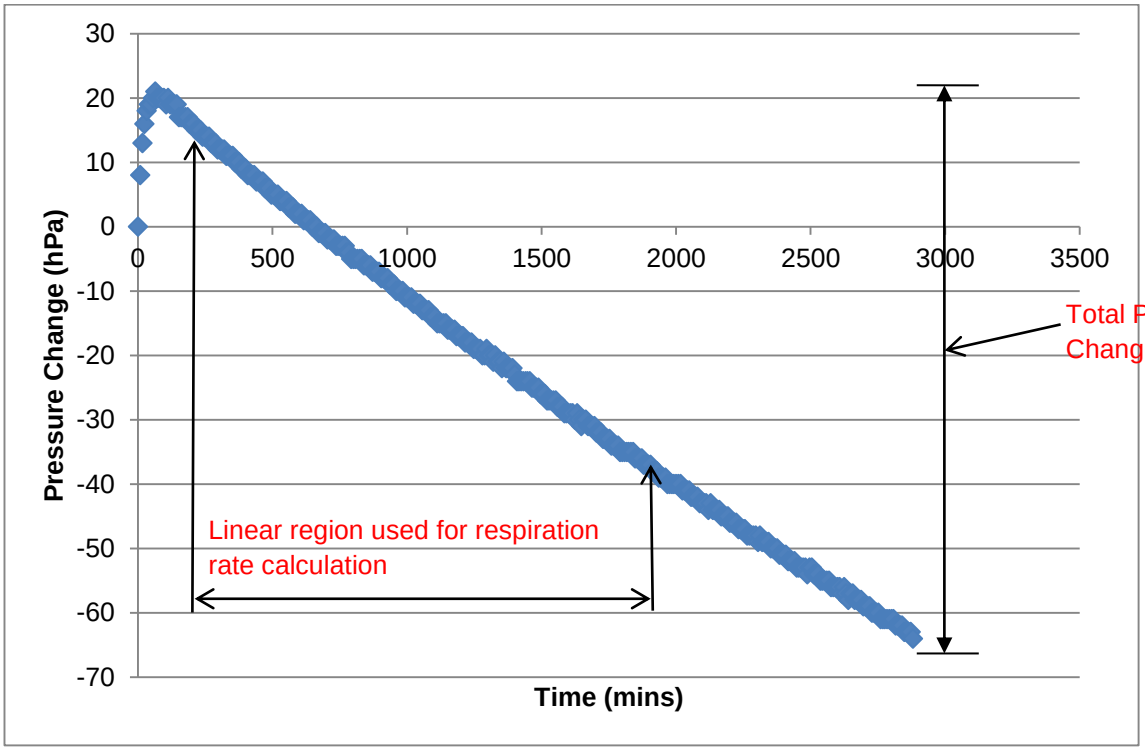


Figure 2.12: A typical soil respiration pressure change graph

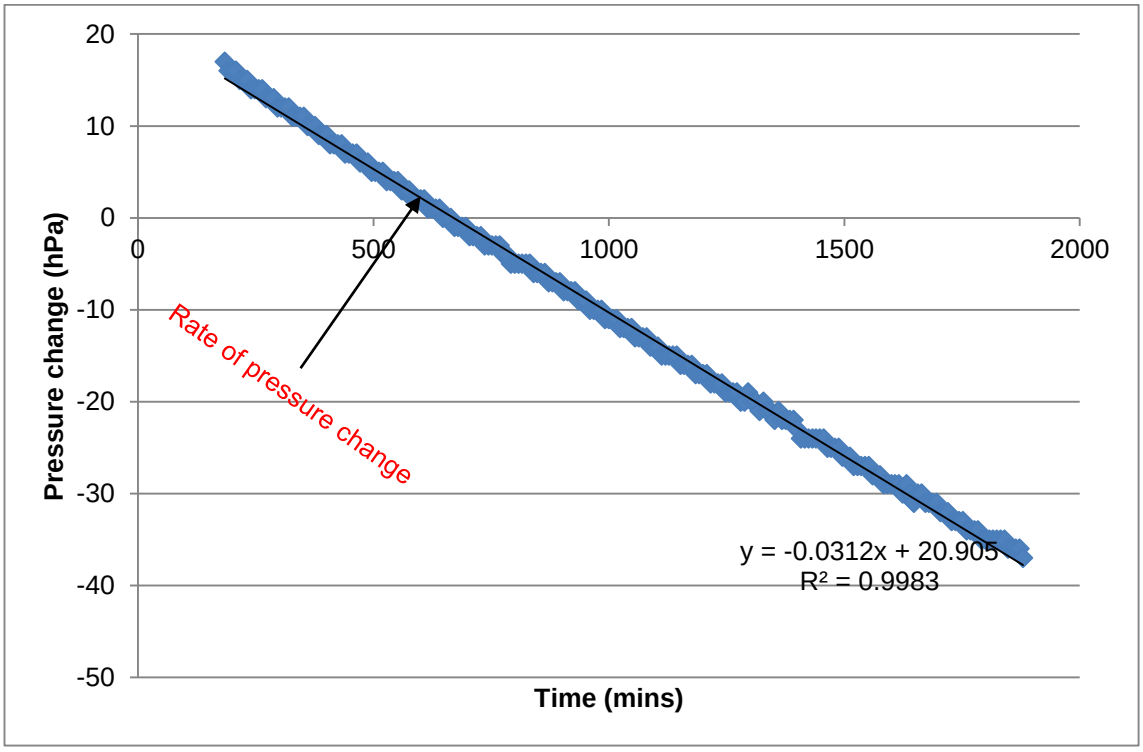


Figure 2.13: The rate of pressure change derived from the equation of a straight line graph. Times between 184 mins-1880 mins were used to obtain the straight line equation for all the soil respiration calculations.

2.5.9 Microbial biomass carbon concentration measurement

The microbial biomass carbon for the Cu spiked pot samples in Section 2.5.8.2 was determined using the substrate-induced respiration method introduced by Anderson and Domsch's [169]. The test was carried out at the beginning and end period of the experimental set up (1 and 70 days).

100 g of soil was adjusted to 80 % of its WHC by adding 10 mL of glucose solution. The glucose solution was prepared according to OECD (2000) guidelines of 4000 mg of glucose per kg of soil [136]. 100 g of soil then required 400 mg of glucose which was dissolved in 10 mL of distilled water and added to the soil. The substrate-induced respiration measurement was set up to measure for two days using the OxiTop method in Section 2.5.8.6

2.5.9.1 Microbial biomass carbon data analysis

Microbial biomass carbon concentration was determined using the data extracted from the substrate-induced respiration measurement. The Equation 2.7 derived by Anderson and Domsch's [169] was used for the calculation

$$C_{mic}\ [mg\ g^{-1}] = 40.04y + 0.037 \qquad (2.7)$$

Where $y = mLCO_2\ h^{-1}\ g^{-1}$

The $mLCO_2h^{-1}g^{--1}$was calculated based on the stoichiometry of O_2 and CO_2 production during respiration: 1 mg of consumed O_2 corresponds to 1.375 mg of respired CO_2 [161].

2.5.10 Community level physiological profile determination by BIOLOG Ecoplate method

The microbial metabolic diversity was measured on the soil experimental set ups described in Sections 2.5.8.2 and Section 2.5.8.4. The Ecoplate measurements were used to determine the effect of soil handling (complete drying and sieving of soil) on soil spiking, different Cu concentrations and environmental conditions on the soil microbial community metabolic diversity.

2.5.10.1 Selection of time from average well colour development values for Ecoplate measurements

Soil microbial community from 3 g of unspiked soil in triplicate were extracted and used to inoculate the BIOLOG Ecoplate. Absorbance measurements were taken every 24 hours for 168 hours and the AWCD at each period of measurement was calculated in order to select an appropriate time for Ecoplate absorbance measurement.

2.5.10.2 BIOLOG Ecoplate experimental procedure

3 g of soil from each soil treatment at their respective analytical periods were placed in a 50 mL cylinder tube and made up the 30 mL mark with 0.9 % strength NaCl solution [149]. The soil mixture was shaken with end over end rotary shaker (Stuart Rotator Shaker SB 3) for 60 mins at 30 rpm at room temperature. The mixture was centrifuged (Beckman Allegra 6R Centrifuge) at 200 rpm for 5 mins to settle solid particles [232]

The resulting supernatant was diluted 1 mL in 100 mL of 0.9 % NaCl (200 µL in 10 mL) and the diluted supernatant of each sample was used to inoculate the 32 well (150 µL per well) Biolog ECO plates. The 96 Ecoplate wells are made up of three replicates of 31 carbon substrate well and 3 blank well.

The plates were incubated at 22 °C in the oven till the time for measurement and the substrate utilization was monitored by measuring the light absorbance using EL 808-ultra microplate reader (Bio-Tek Instrument, INC).

The absorbance measurement for the soil treatments were taken at the 72 hour mark after the well inoculation (based on Section 2.5.10.1 results) and the generated absorbance values were used to calculate their AWCD.

2.5.10.3 BIOLOG Ecoplate data analysis

Average well colour development (AWCD) was calculated for all the experimental data generated by the Ecoplate according to the method used by Garland *et al*. [178, 179] as shown in Equation 2.8. The AWCD reflects the oxidative potential of soil microorganisms developing in the Ecoplate wells and it can be used to assess microbial activity [94, 233].

$$AWCD = \frac{\sum_{i=1}^{31} (A_i - A_0)}{31} \quad (2.8)\ [179, 183, 234]$$

Where A_i represents the absorbance reading of well *i* and A_0 is the absorbance reading of the blank well (inoculated but without a carbon source). Absorbance values for the wells with C sources were blanked against the control well. Negative values were considered as 0 in subsequent data analyses [179].

2.6 Results and discussion

2.6.1 Characterisation of soil sample:

Soil chemical and physical properties were evaluated so that the results from this study can be compared with results from similar literature studies. Table 2.5 shows the obtained values for the soil particle sizes. Table 2.6 shows the UK system of soil particle size classification used for the soil particle size obtained values in Table 2.5. Table 2.7 shows the values of other soil chemical and physical properties evaluated:

Table 2.5 Soil Texture Values

Clay %	Fine Silt %	Medium silt %	Coarse Silt %	Coarse Sand %	Medium Sand %	Fine Sand %
5.95	11.13	14.83	9.3	1.56	15.23	40.33

.

Table 2.6 Classification of soil particle sizes in the UK [235]

Fraction	**UK System**
Stones/gravel	>2.0 mm
Coarse sand	2.0 – 0.2 mm
Medium sand	0.6 – 0.212
Fine sand	0.2 – 0.06 mm
Fine Silt	0.002 – 0.006 mm
Medium silt	0.006 – 0.02 mm
Coarse silt	0.02 – 0.063 mm
Clay	<0.002 mm

Table 2.7 Soil properties values

PARAMETER	RESULTS
% Maximum Water holding Capacity	35.61 %
60 % Water holding Capacity	21.37 %
% Nitrogen	0.37 %
% Organic Carbon by Loss on Ignition	9.176 %
% Organic Carbon by Walkley-Black method	4.97 %
Soil pH	5.58

2.6.2 Spiked copper concentrations:

The obtained average copper concentrations on the spiked soils from EDXRF analysis are shown in Table 2.8

Table 2.8 Measured average copper concentrations in 3 pelletised samples of the spiked and unspiked pot samples

SAMPLES	MEAN CONCENTRATION (n=3) mg kg^{-1} ±SD
Control soil (Unspiked)	57.5 ± 10.6
Low copper amendment (Spiked to an additional 10 mg kg^{-1})	69.8 ± 11.2
Medium copper amendment (Spiked to an additional 100 mg kg^{-1})	209.5 ± 20
High copper amendment (Spiked to an additional 1000 mg kg^{-1})	1366.1 ± 309.2

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2.6.3. Soil respiration study:

2.6.3.1 Minimum soil respiration period experiment

The result of the study carried out to determine the minimum period it takes for a significant change in pressure to occur are shown in Figure 2.14.

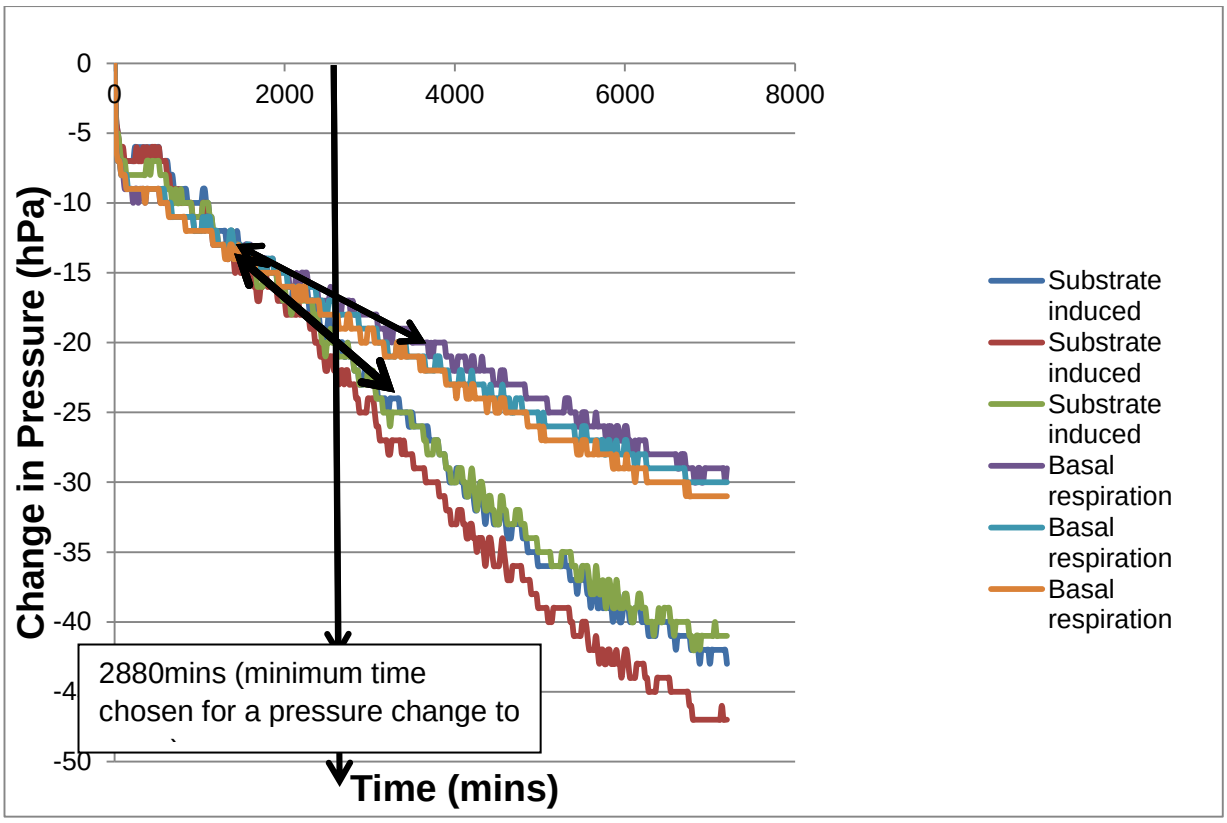

Figure 2.14: Minimum period it takes to obtain the rate of pressure change in the soil respiration study.

The results shows that in both substrate induced and basal respiration the minimum duration to determine the extent of the linear phase similarly occurs at 2880 minutes but as the duration extends the rate of pressure change in substrate induced may increase, more than the basal respiration because of the amount of readily available nutrients (substrates) that microorganism can utilise easily. However, such an increase may not be in a linear phase as seen in Figure 2.14 and the linear phase is the value that is extracted for the soil respiration rate calculation in Equation 2.6

Based on the result shown in Figure 2.14 it was considered that rate of pressure change during basal and substrate-induced respiration can occur within approximately 2 days (2880 mins). All the subsequent basal respiration and substrate-induced respiration period for all other experiments were programmed for 2 days.

2.6.3.2 Influence of soil handling on soil spiking studies: effect of complete drying

The second experiment result on the basal respiration of the spiked samples with the control are shown in Figure 2.15:

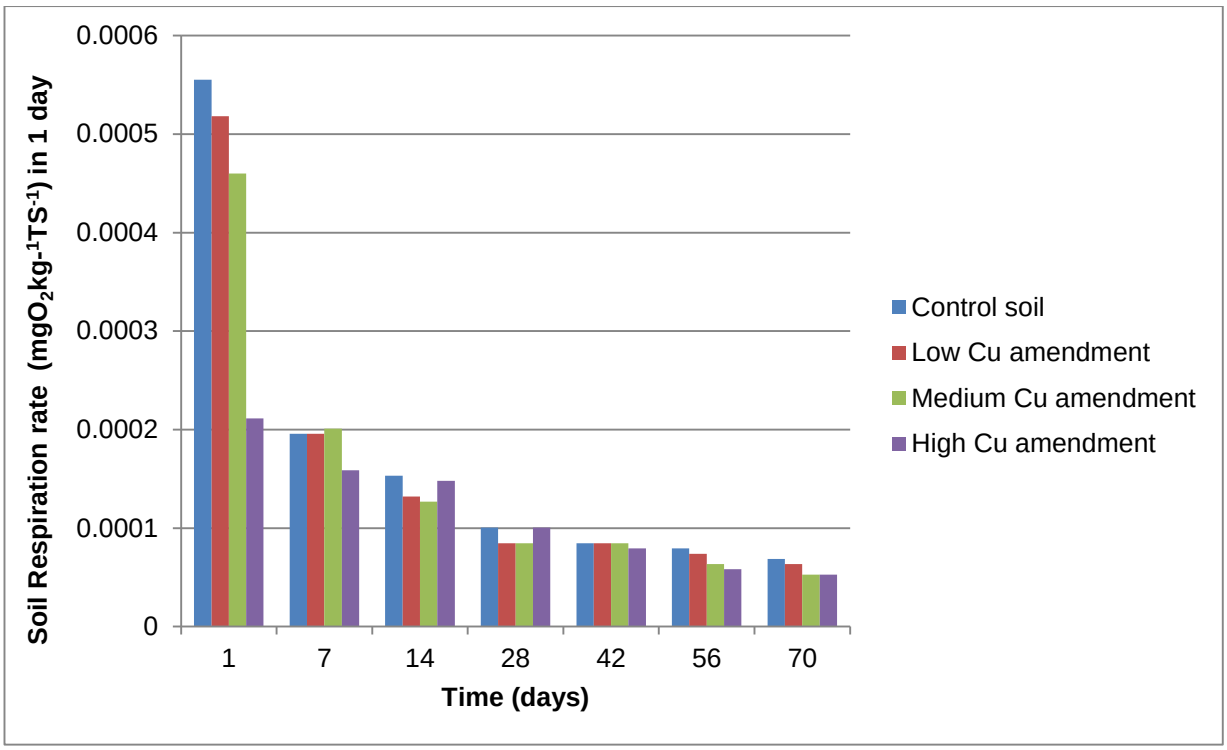

Figure 2.15: Soil respiration rate values for spiked soils over a 70 days period of respiration measurement. The soil had been completely dried prior to spiking.

The results in Figure 2.15 shows that the soil respiration value was very high after the first day of spiking (rewetting the soil after drying) but declined rapidly and steadily from day seven to the end of the measurement period. The effect of the copper concentrations (especially low and medium) on the microbial respiration rate was negligible after the first day of respiration measurement even at the highest Cu amendment and in most cases the respiration rate of the control was similar to the respiration rate of the soils amended with Cu metal. The decline in respiration rate for the control and the Cu-amended soils were very similar with a marginal variation on day 56 and 70 measurement periods.

Heavy metals are known to exhibit a toxic effect on soil microorganisms and the effect of copper concentration on microorganism includes inhibition of cell division, suppression of microbial respiration and growth and ultimately leads to the death of microorganisms [95]. These effects on soil microorganisms were not clearly evident in this respiration measurement because of the soil handling processes used during the experiment, for example, the drying and rewetting of the soil. These experimental processes (drying and rewetting) have been reported by literature studies to reduce sensitive microbial species [226, 236]. It has also been reported that low moisture below 50% level could lead to the death of microorganisms [129] thereby nullifying any significant pollutant effect that can be observed during soil spiking studies. Drying and

rewetting are known to increases the amount of microbial carbon, nitrogen and phosphorus in the soil which generates flushes of available carbon as seen in the respiration rate value for the first day of measurement (Figure 2.15). The flushes generated by drying are attributed to the nutrient released from decomposed soil microorganism killed by drying and subsequently mineralised by living microorganism. These effects (flushes) can be confused with the effect of pollutant spiking (heavy metal) during an experimental investigation thereby leading to an error in result data interpretation.

2.6.3.3 Effect of storage conditions on soil microbial community activity for laboratory-based studies: effect of loss of moisture and air circulation.

The results of the soil respiration study carried out on two soil samples stored under different laboratory conditions to determine the effects of air circulation and moisture regulation on the microbial community yielded the following results represented graphically in Figure 2.16

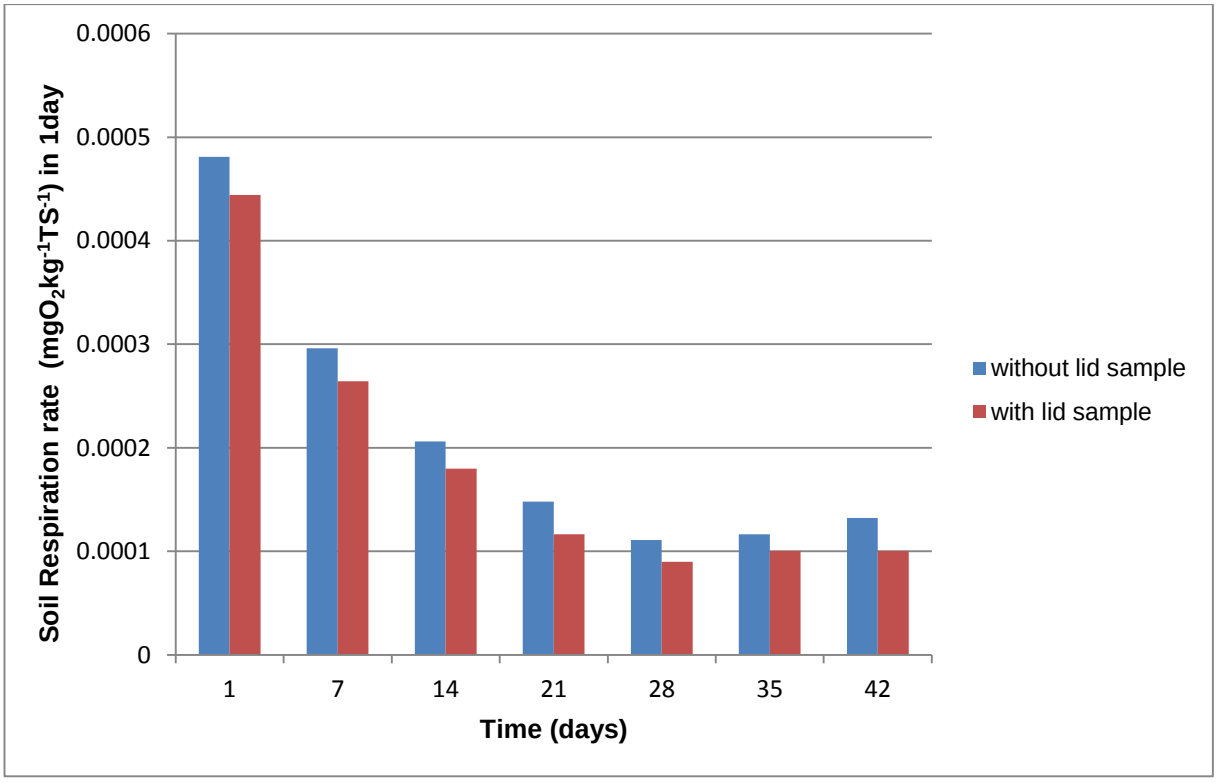

Figure 2.16: Soil respiration rate values obtained from the study conducted on sample stored without a lid and on sample stored with a lid**.**

The result shows that soil sample stored without a lid and enclosed in a bigger vessel (loss of moisture controlled with 200 mLs of distilled water) had a higher respiration

activity for all the period of measurement than the soil samples stored with a lid and without an adequate control for loss of moisture. The respiration which represents soil microbial activity shows that microbial activity was supported more in the samples without a lid (with moisture control) than in the samples with a lid (no moisture control). The decline in the respiration rate at later stages of measurement can be due to other environmental conditions such as a decline in substrate quality (no added nutrient) [122, 237].

2.6.3.4 Determination of adequate environmental conditions for soil microbial study: effect of temperature and moisture conditions

The result of soil respiration experiment carried out on pot samples stored under different temperature and moisture regulations are shown in Figure 2.17.

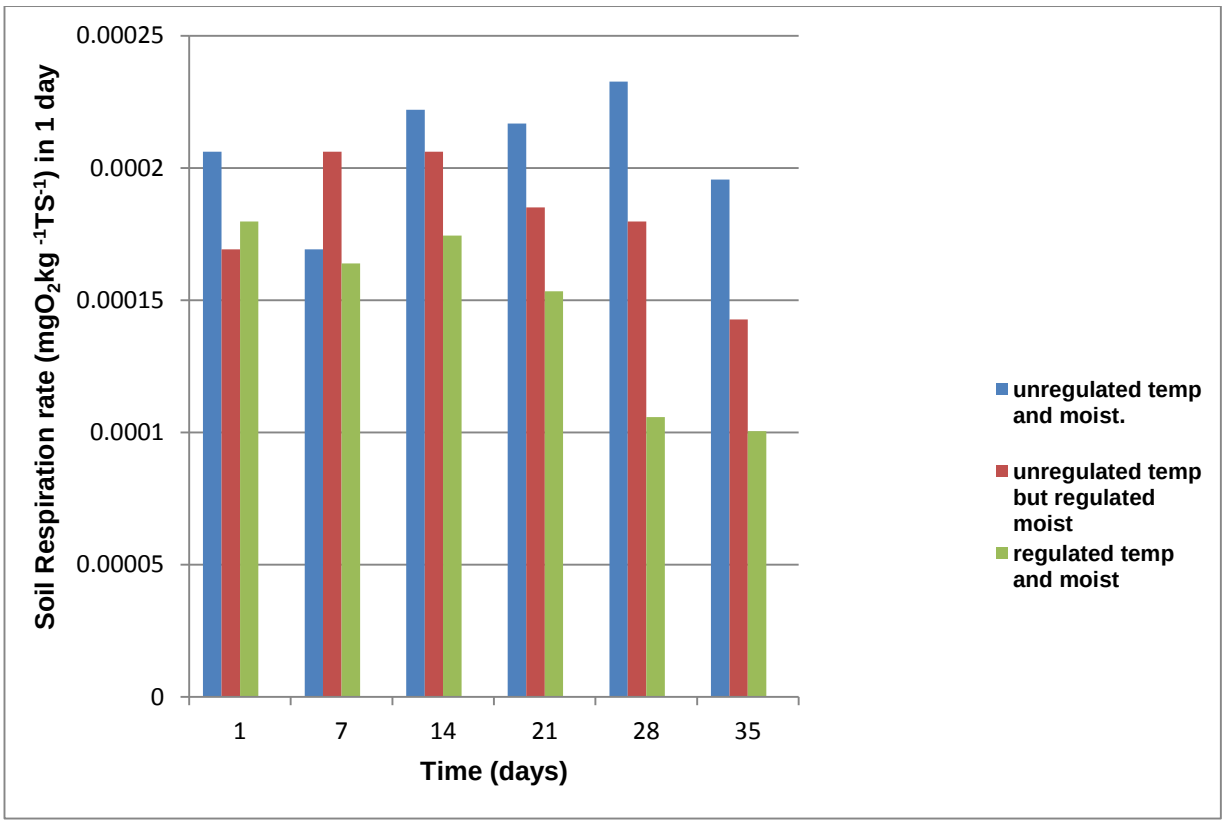

Figure 2.17: The soil respiration rate values for soil sample stored under different temperature and moisture conditions: unregulated temp and moisture; unregulated temperature but with regulated moisture; and regulated temperature and moisture.

The respiration rate results in Figure 2.17 show that the pot samples stored under unregulated temperature and moisture (rooftop without shelter) had the highest and the most stable respiration activity for all the measurement period than the pot samples stored with the other conditions. The varying temperature and moisture conditions for

this pot sample supported the soil microbial activities which are evident in their respiration activity. The respiration rate value of the soil pot samples stored under unregulated temperature but with a regulated moisture condition (rooftop with shelter) was also higher than the respiration value for the pot sample stored under laboratory conditions. The varying temperature condition for this pot sample (rooftop with shelter) seems to have enhanced soil microbial activity more than the microbial activity of pot soil samples stored under constant temperature and moisture condition (laboratory condition).

The results of this investigation show that when samples are treated in a similar process as that which is obtainable under natural conditions respiration rate values would be more consistent and stable. The respiration result obtained through this process will be a true representation of microbial activity in the soil than the result obtained from samples treated under several artificial conditions (laboratory condition). Under these natural conditions, the effect of pollutants (e.g. PAHs and heavy metals) would be evident and distinct than under artificial conditions whereby; temperature and moisture limitations can also add to the stress on soil microbial community.

Nevertheless, whilst maintaining a relatively stable and sustainable respiration is desirable, perhaps of more importance is to maintain controlled experimental conditions, particularly when it comes to looking at PAH degradation rates (covered in later chapters). Related studies in this chapter, i.e. those looking at laboratory storage conditions and the importance of using soil that has not been allowed to completely dry out, have established conditions that will help optimise the stability of the respiration rate in the control soil samples. The effect of natural conditions is, however, a very interesting result.

2.6.3.5 Improved soil respiration rate resolution

The result on the test carried out on two similar unspiked soils and undried soils using different OxiTop respiration vessel sizes; 1000 mL and 250 mL, in order to determine an improved respiration rate resolution are shown in Figure 2.18.

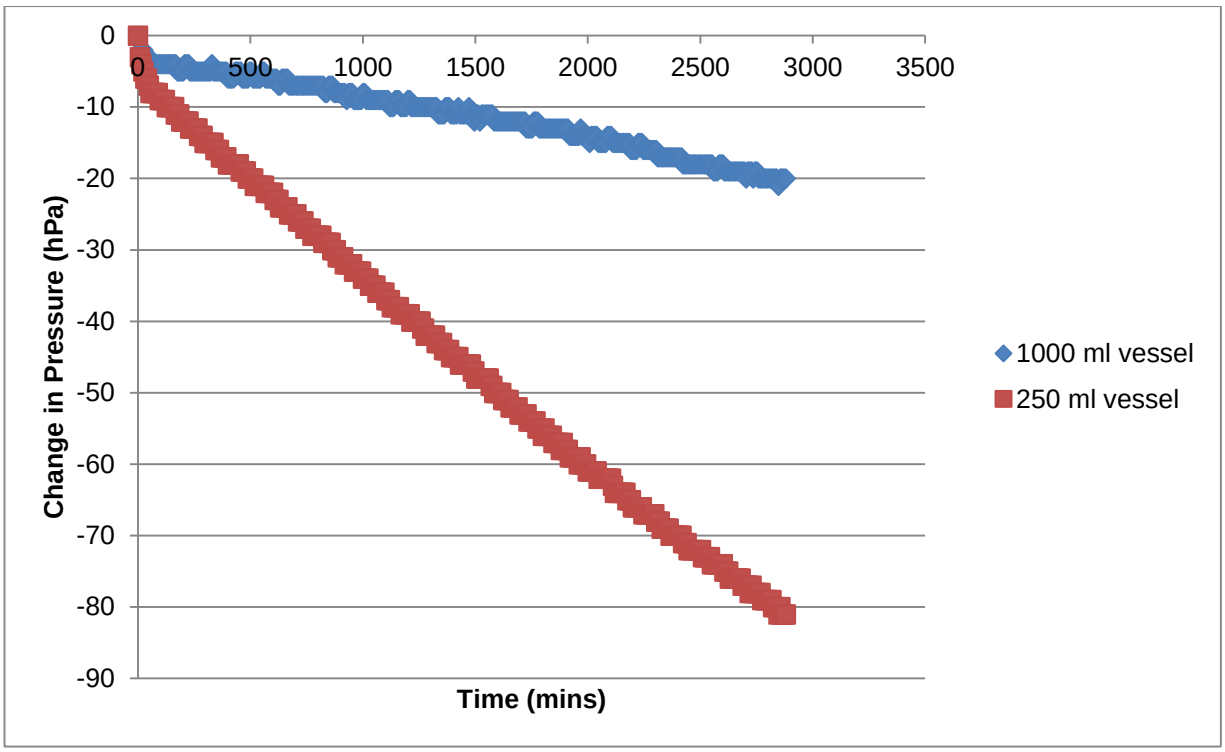

Figure 2.18: Change in pressure values obtained from a 2 day respiration study conducted on soil samples using a 1000 mL OxiTop respiration vessel and a 250 mL OxiTop respiration vessel.

The result shows (Figure 2.18) that the change in pressure due to soil respiration in the 250 mL OxiTop vessel was greater and has a better resolution than the change in pressure observed for the 1000 mL respiration vessel. This is because the 100 g of soil used had a low level of soil microbial activity (no added nutrient) which can only be accentuated in a small size (250 mL) OxiTop vessel than in larger size (1000 mL) OxiTop vessel, i.e. the same carbon dioxide production would result in a roughly four-fold increase in the pressure drop when using the 250 mL vessel. The amount of soil used during soil respiration experiment and the anticipated level of soil microbial activity helps to determine the size of the respiration vessel. When using a small amount of soil with a low level of soil microbial activity it is important to use a smaller respiration vessel which would accentuate the microbial activity in the soil as seen in Figure 2.19. This would enhance any apparent change in pressure reduction caused by contamination effect (e.g. heavy metals).

2.6.4 Soil microbial biomass carbon results:

The microbial biomass carbon value for each of the pot samples at the beginning and end of the incubation period was calculated from substrate-induced respiration, the results for which are shown in Table 2.9.

Table 2.9: The microbial biomass carbon values of the spiked at the initial stage of respiration study (after 1 days of spiking) and at the final stage of the respiration study (after 70 days of spiking)

Samples	Initial biomass (day 1) (mg kg^{-1})	Final biomass (day 70) (mg kg^{-1})
Control	195.4	172.1
Low copper amendment	195.4	128.4
Medium copper amendment	183.7	99.3
High copper amendment	131.3	73.1

The results in Table 2.9 shows a reduction in the microbial biomass carbon concentration for all of the samples. The high copper amended sample had the lowest microbial biomass carbon concentration. The microbial biomass carbon concentration for the low copper and medium copper concentrations were similar to that of the control on the first day of measurement which could be that soil microbial community was more tolerant to that level of copper concentration than at the highest copper concentrations. The observations for the low and medium copper concentration in Table 2.9 can also be explained as an increase in the resistant species (e.g. fungi) of the microbial community because of the reduced competition caused by the death of low resistant microbes (e.g. bacteria) [147]; some studies have shown that fungi are more resistant to heavy metal pollution than bacteria [95, 238].

The decline of soil microbial biomass with increasing concentration of heavy metal as shown in Table 2.9 confirms the result obtained by other studies. Zhang *et.al* [229] in their study noted that microbial biomass carbon decreased with increasing soil Cd content. The microbial biomass carbon mean concentration in some of the cases they studied decreased from 199.2 ± 9.4 mg kg^{-1} to 183.4 ± 5.2 mg kg^{-1}. The decrease in the biomass concentration of the control is also similar to the result obtained by other studies. Wang *et.al* [146] in their study noted that pre-incubation of soil samples from different locations for 7 days at 25 ^{o}C with water holding capacity at 55 % decreased the microbial biomass concentration of the soil samples. The initial biomass concentrations range for the different soils were from 61 - 684 mg kg^{-1} but this was reduced to a biomass concentration range of 34 - 659 mg kg^{-1} after 7 days of incubation. Soil handling processes such as drying and wetting could have also contributed to the decline in the soil microbial biomass of all the samples.

2.6.5 Community level physiological profile using Biolog Ecoplates

2.6.5.1. Selection of time from AWCD analysis for Ecoplate measurements

The calculated AWCD from three soil replicate Ecoplate absorbance measurement as seen in Figure 2.19 shows that at 72 hrs the AWCD seems to be at its mid-point and also seems to be in the linear phase of colour development. The linear phase of colour development is estimated to be the point where more than 60% of the wells have a positive reading after the blank has been subtracted according to literature [179, 180, 239]. The mid-point is also estimated to be a function of both the lag time before colour developed and the linear rate of colour development [164, 179]. Garland et al. [177], in their study, reported that using AWCD reference values between 0.25 - 1.0 would yield a relatively similar CLPP for microbial community analysis. The selected AWCD time (72 hrs) from this result was then used to measure subsequent Ecoplate well absorbance values

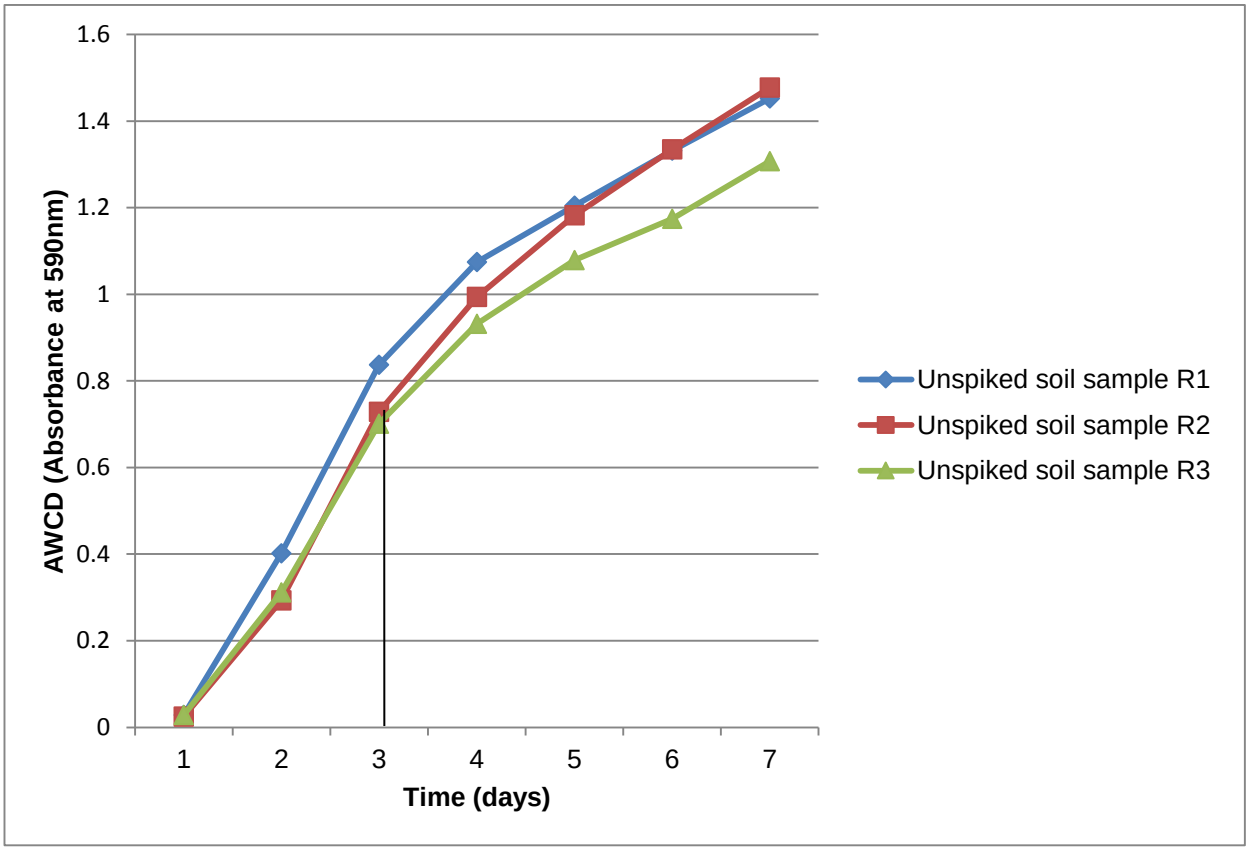

Figure 2.19: Calculated AWCD for triplicate soil samples Ecoplate absorbance values measured every 24 hours over a period of 7 days.

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2.6.5.2. Influence of metal spiking on CLPP in soil spiking studies:

The results obtained from the AWCD analysis on the spiked samples and the control is shown in Figure 2.20.

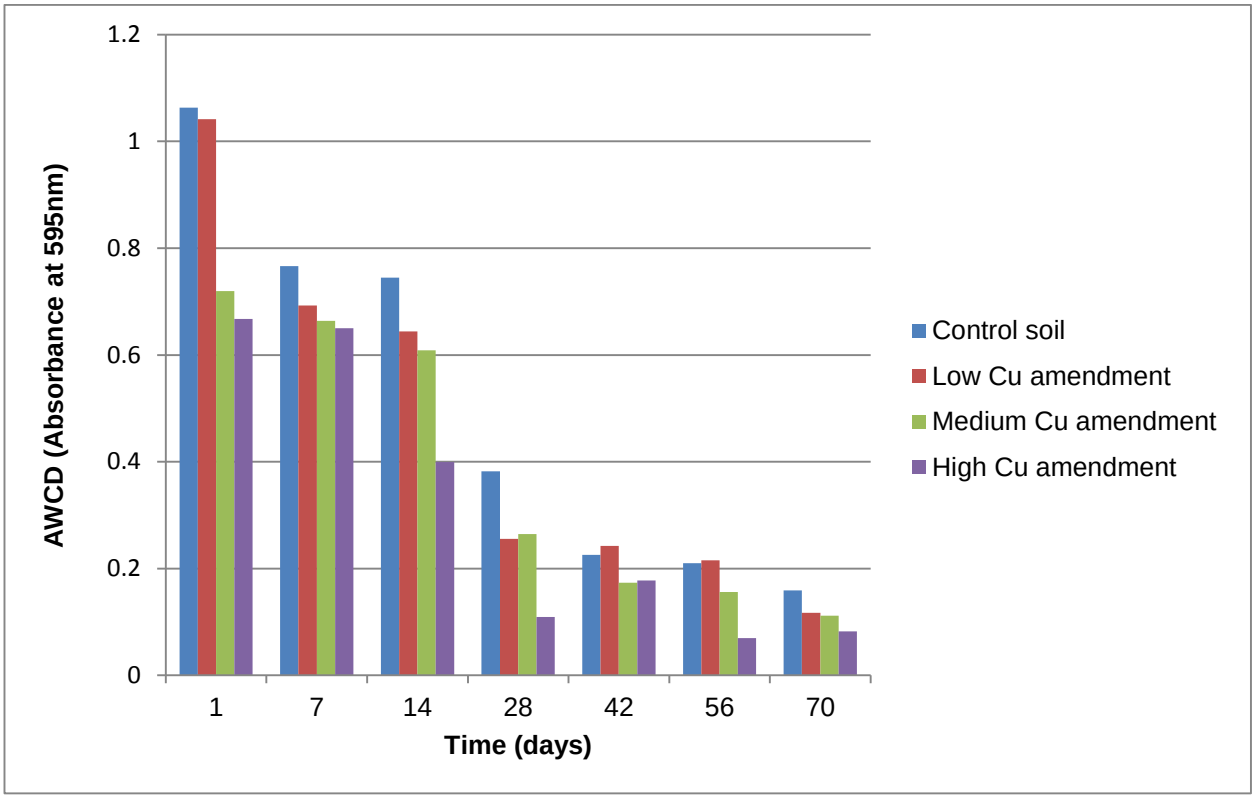

Figure 2.20: Calculated AWCD for the effect of soil handling on the spiked soils over a 70 days period of Ecoplate measurement.

The AWCD was used in this study to represent the metabolic activity of the soil microbial community in the different soil treatments and their ability to respond to carbon substrates [82]. It can be seen in Figure 2.20 that the metabolic activity in the soil with the low copper amendment was similar to the control on the first day of measurement but it declines at the later days of measurement. The AWCD for the soil with the highest copper amendment was the lowest compared to the other treatments as seen in Figure 2.20. An important observation in this result was the sharp decline in the AWCD value for all the samples from 28 days of measurement to the end of the study (70 days).

It seems that the metabolic activity of the soil microbial community in the low Cu-amended soil was not affected by either the soil handling or the added copper metal on the first day of measurement. This could be that the microbial community was able to develop tolerance to the low level of Cu amendment but the metabolic activity declined when other environmental factors added more stress to the community. The soil with

the medium Cu amendment showed a steady decline at the initial stages of measurement but a rapid decline was apparent from the 28 days of measurement to the end of the study which could also be because of the added stress caused by soil handling. The soil handling process of drying and rewetting has been seen to cause a decline in the respiration rate of microbial community (Figure 2.14) and it seems this process has also affected the metabolic activity of the soil microbial community as seen in the AWCD of the control sample. However, this effect (soil handling process) became more prominent in the AWCD value from the 28 days of measurement unlike in the soil respiration rate measurement were it became prominent after the first day of measurement. The reason for this could be that the more tolerant members of the soil microbial community were still able to metabolise the Ecoplate carbon substrate, but after 14 days the number of these tolerant members reduced causing a sharp decline in the AWCD of the Ecoplate. The activities of these tolerant members in the soil could have been sustained by the nutrients released by the dead microorganisms (more sensitive species) [226, 236] but the decline of this nutrient would have also added more stress on the community members leading to the death of these tolerant members of the microbial community. It has been reported by literature that poor quality of soil substrate can lead to a reduction in the activity of microorganisms in the soil [240]. By maintaining a constant temperature and moisture (under laboratory conditions) any effect on the microbial community could be attributed to the poor quality of substrate available to the microbial community or the lack of substrate that can be utilised by microorganisms [122]. Therefore, soil handling process affected the response of microbial community to Cu amendment which made it difficult to evaluate the actual toxicity of the Cu metal on the community.

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2.6.5.3 Effect of environmental conditions (temperature and moisture) on CLPP

The AWCD values obtained from the Ecoplate carbon substrate utilization measurement for the three unspiked samples stored under different environmental conditions are shown in Figure 2.21

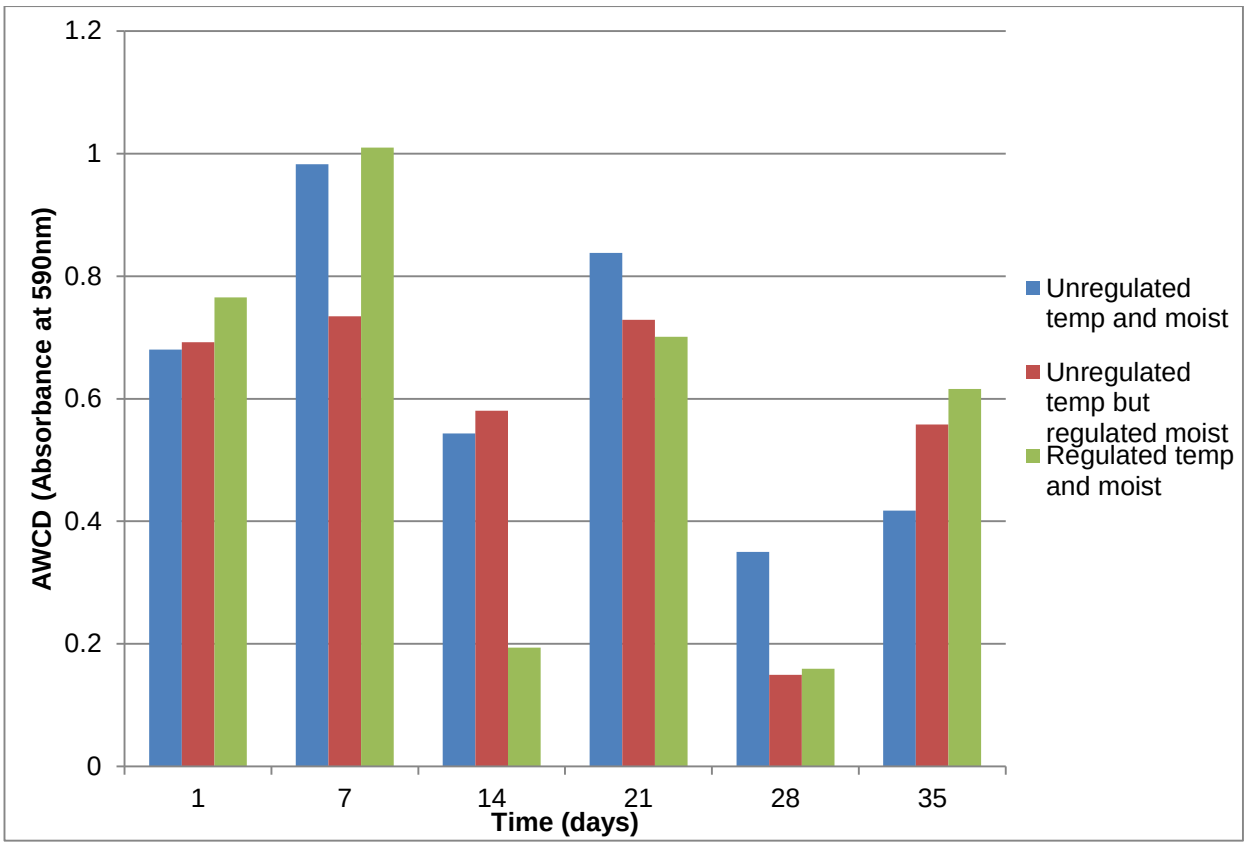


Figure 2.31: Calculated AWCD for soil sample stored under different temperature and moisture conditions: unregulated temp and moisture; unregulated temperature but with regulated moisture; and regulated temperature and moisture.

The AWCD value was also used in this investigation to represent the metabolic activity of the soil microbial community. The result as seen in Figure 2.21 shows a variation in the AWCD value for all the environmental conditions investigated but the soil sample with unregulated temperature and moisture conditions (rooftop) had the least decline in their AWCD throughout the investigation period. The variations in the AWCD for the soil samples could be because of the complex heterogeneous soil microbial community and the environment. Nevertheless, a reasonable microbial metabolic activity was still observed on day 35 because of the highly reduced soil handling processes (no drying out of soil).

2.7 Conclusion

The study has been able to demonstrate that soil microenvironment can affect results from soil laboratory investigations. The results from this study show that soil handling such as drying and rewetting can affect soil microbial activity during laboratory studies. It has also been able to demonstrate that to obtain a more consistent microbial activity during soil investigations it is best to use the natural environmental conditions.

However, natural environmental conditions cannot be recreated in a laboratory investigation and in the study of the effect of pollutant (heavy metals) on soil microbial community it is a standard practice to maintain a constant temperature and moisture conditions in order to reduce the environmental variability factors and accentuate the pollutant effect. Nevertheless, based on the results of this study it is apparent that soils should not be air dried, stored without loss of moisture regulations and stored without a proper air circulation system.

Changes in these soil handling techniques and environmental conditions were subsequently made during the PAH biodegradation study carried out in the later part of this project. In addition, 250 mL OxiTop measuring vessel was also used for subsequent soil respiration measurements based on the observations made in Section 2.6.3.5.

Chapter 3: Bioremediation of polycyclic aromatic hydrocarbons in soil: pilot study

3.1. Introduction

The monitoring and testing of the efficacy of bioremediation processes are essential in determining the effectiveness and cost-effectiveness of this remediation option [241]. The monitoring and efficacy testing requires a careful selection of robust, simple and rapid techniques for determining the decrease of pollutant (PAHs) concentrations in the soil while also assessing the soil quality using specific key information on the abundance of microbial community, microbial processes and activity. Having established the correct environmental conditions and soil sample handling procedures that will reduce the adverse abiotic effect on soil microbial community in the previous chapter, it was now the time to optimise the proposed bioremediation monitoring parameters that will be used during the period of the study.

The extensive nature of the proposed bioremediation study made it necessary to carry out prior tests on the analytical methods and instruments that will be used during the study. To this end a pilot study was conducted in which PAH spiked soil spiking process, PAH spiked soil extraction process, PAH spiked soil concentration quantification process, soil heavy metal concentration determination, soil bioavailable heavy metal concentration determination, soil microbial biomass analysis, soil community level profiling analysis and soil respiration analysis were all tested in detail.

Most laboratory based remediation studies for PAH contaminated soil, involve artificially contaminated soil (spiking) [72, 224, 242]. Soil spiking has enabled the optimization of removal and/or degradation efficiencies of PAH contaminants. It has also become a commonly used tool for determining cause and effect of soil toxicity.

Several methods such as low and high solvent usage, dilution mixing, water spike/mixing, sediment water slurry and shell coating has been used by various studies to spike contaminants into the soil [224, 226, 236] and each method tried to obtain a homogenous mixing after spiking which is necessary for the validity and statistical significance of the result. However, it is intrinsically difficult to obtain a homogenous distribution of spiked compounds because the soil is a heterogeneous multiphase system with a continuous phase of binding sites and micro-regions

[243]. This then has led to the use of a number of practices to improve the distribution and homogeneity of the spiked compounds within the soil. Some of those adopted practises are: soil sieving to obtain uniform particle distribution; drying to improve soil fluidity; spike mixing using large volumes of carrier solvent to wet the soil and distribute the added contaminant, and long mixing times. Some of these practices can introduce changes in the physical and chemical properties of the soil that may affect the remediation process being investigated. The American Society for Testing and Material (ASTM) recommends, that if sieving is necessary, it should be carried out using as large a sieve as possible, no smaller than 1 to 2 mm grid size [226] because sieving can affect both the spiked compound bioavailability and bioaccumulation by changing the particle size distribution of the soil, thereby affecting the assimilation efficiency of soil bound organic contaminants to size-selective feeders [226] .

Drying of soil for spiking experiments [224, 225] on one hand helps to decrease variability arising from the soil's inherent heterogeneity, giving easy handling and processing properties and improving sample mixing efficiency and spiked compound distribution and homogeneity [226]. However, it has been observed (from the spiking experiment in chapter 2) that it may also introduce significant effects into the experiment which can be misinterpreted and attributed to the influence of the spiked compound. Drying and rewetting increase the amount of microbial carbon, nitrogen and phosphorus in the soil which generates flushes of available carbon, nitrogen and phosphorus and other soil nutrients. The flushes generated by drying can be attributed to nutrient release from decomposed soil microorganism killed by drying and subsequently mineralised by living microorganism. It also changes the distribution of soil organic matter (SOM), by increasing the amount of dissolved organic matter [226, 236]. Reid *et al.* [236] in their study observed that spiking protocols where dried soil was used, had high homogeneity of PAH compounds (Relative Standard Deviation was 2.40 % for n = 6) but the lowest microbial number, as established by viable plate counting. When the same protocols were used with wet soil they also had a high homogeneity of PAH compound (RSD 4.1 % for n = 6) but a minimal impact on the microbial population. Using a minimal volume of carrier solvent for spiking and allowing sufficient time for solvent evaporation has also being suggested as a better method of reducing the effect of carrier solvent (e.g. acetone, ethanol etc.) on microbial population [236, 243].

The ideal guiding principle for the adopted spiking procedure would be that spiking should involve minimal soil handling and manipulation. It should not compromise the physical and chemical nature of the soil or the stability of the PAH compounds. It should have minimal or no effect on soil microbial community and the carrier solvent should be removed and not have a persistent presence in the soil.

A good degree of homogeneity of spiked soil should be obtained after spiking because it is essential for the statistical validity of the experimental results and also to avoid introducing zones of higher and lower localized concentrations in the spiked soil [224, 226, 243]. The relative standard deviation for the homogeneity of spiked soil should be < 20 % for the mixing to be considered valid [226].

The carrier solvent used for the PAH spiking process (in this case) should be such that it can solubilise the PAH [244] and enable an even distribution of the PAH in the soil matrix. Acetone was used as a carrier solvent for the dissolution of coal tar pitch because it solubilises PAH, has a high vapour pressure and a low boiling point [243, 244]. Acetone is also a water miscible solvent which is the most popular choice for spiking wet soil and its high vapour pressure means that there is no residual organic carrier solvent after spiking [243]. Other organic solvents can introduce organic carbon in the soil and influence the partitioning of the PAH compounds in the soil [243], this can lead to an erroneous experimental observation. Acetone can serve as a readily available organic substrate for microorganisms [245] and Brinch *et al.* [225] in their study also explained that acetone can have an adverse effect on soil microbial community. They observed that there was a reduction in the total number of bacteria count when acetone was added to the soil [225]. Therefore, the influence of acetone on soil microbial community during spiking needs to be reduced by finding the right percentage of soil that has to be spiked first with acetone before mixing the whole soil with the remaining unspiked soil. This procedure will be used in this study to avoid any overestimation or underestimation of the soil microbial community.

There are different methods that have been used by various studies to estimate the total microbial biomass carbon in the soil [169]. The method that was used in the previous chapter (Chapter 2) for the soil biomass analysis was substrate-induced respiration. This method was considered to have various errors, some of the observed errors were that it can give a low estimation of the total microbial biomass

[170] because not all species of the microbial community can easily catabolise the glucose used as a readily available substrate. This method was also considered not to be useful when basal respiration study is already used in the research because of the similarity in their method of determining soil respiration. Therefore, a different method had to be used for the later part of the research.

The use of BIOLOG Ecoplates to assess the functional diversity of microorganisms from different environmental samples has been recognised as a simple and rapid method of community level physiological profiling. A critical factor with its use is that the right amount of inoculum density needs to be used in order to achieve a reliable and reproducible microbial community data. One of the ways uniformity of the inoculum density can be achieved is through dilution [171, 178]. The right amount of dilution is necessary in order not to eliminate the different species of the microbial population if it is too dilute and also to avoid including artefacts in the eco plate wells that may generate erroneous data. For this reason, it is necessary to determine the right amount of dilution that will give a reproducible data of the soil microbial community.

The BIOLOG Ecoplate has three replicate sets of 31 environmentally applicable substrates which make it easier and economical to do three replicate samples during the experimental process. However, it can be quite difficult to achieve sample replication and reproducibility because of the heterogeneity that is associated with microbial communities in soil [133, 246]. Soil microbial communities are extremely diverse for example the amount of bacteria species present in a Gram of soil can be estimated to be as many as 13,000 species of bacteria [133]. This heterogeneity can also be exacerbated during soil sample handling and pre-treatment such as sieving and drying. The moment a soil sample is disturbed or taken for analysis, the community within it will likely start changing; some of the more sensitive species might reduce in number whilst opportunistic species might expand as a result. There can also be a change in the structural shape of microorganisms (phenotype or genotype change) as they try to adapt to the altered environment [171].

Replication data from the Ecoplate can also be impacted by other physical processes such as during sample dilution, inoculation and manufacture of the plate. Other factors such as soil particles in the well can cause artefacts in the results by increasing optical dispersion, whilst dirt underneath the plate can also cause erroneous data generation. Another source of error could be the development of

colour in the substrate blank well (control well) with the manufacturers suggesting that the colour in the substrate blank well could be due to cell lysis, or utilization of endogenous or extracellular polymers by microorganism [247]

Therefore, there was a need for replication experiment, to check for some of these errors and devise a method of correction and also to determine if there was any variability inherent in the sample processing and the data generation method. The variation among the Ecoplates was also determined using one soil sample extract. The replication experiment was essential in order to ensure that the data is reproducible and that the Ecoplate assessment method can be used to compare microbial communities.

The pilot study involved spiking the soil with coal tar pitch. The coal tar pitch was used as a source of PAH in the soil [6, 13, 248]. PAHs are constituents of coal tar and they are released into the environment during the incomplete combustion of coal [6, 249]. Coal tar pitch is produced as a residue product during coal tar distillation and is used as a binder in the production of pitch coke, carbon fibre and different carbon materials [248]. Extractable PAH content in coal tar pitch based on research studies is approximately 10-16 % [13, 248]. The difference in PAH content of coal tar pitch is based on the grade of coal tar used during distillation. The PAH content of coal tar itself is also influenced by the characteristics of coke batteries and the tar processing technology employed during coke production [13]. The PAH content of coal tar pitch used for the spiking study was determined prior to the pilot study. The PAH concentration in coal tar was also determined and compared with the concentration in coal tar pitch. This was done in order to determine whether this alternative source of PAHs had a greater concentration of PAHs.

3.2. PAH analytical techniques

There have been many established extraction techniques used to isolate and analyse PAHs from various phases.

Several researchers [13, 248-250] have studied the extraction and analysis of PAHs using a variety of methods. The method used depends on the various contamination phases and the test material [248]. All the methods for extraction and analysis have the aim of providing qualitative and quantitative evidence of PAH contamination in various media. The approach for each method is designed to make PAH compounds more accessible and available for extraction and for easy

identification and quantification. Some of this method is also designed on the basis of its economic value, accuracy, speed and portability for *in situ* analysis. Extraction techniques are designed to separate the contaminants from its matrices and generate a total recovery of the analyte in a way that would eliminate the transfer of unwanted analyte/matrix into the analytical separation stage [251]. The analytical method identifies and quantifies the recovered/extracted contaminants.

The methods of extraction can be divided into instrumental and non-instrumental. The instrumental methods include microwave assisted extraction, supercritical fluid extraction, accelerated solvent extraction/pressurised fluid extraction. The non-instrumentation method includes soxhlet extraction, soxtec extraction, shake flask extraction and sonication extraction. Non-instrumentation methods can also be divided into those for which heat is required and those methods for which no heat is added but which utilizes some form of agitation [252].

The common denominator for each of these methods are the choice of an organic solvent which would enhance the recovery of PAHs from the soil (supercritical fluid extraction is the only exception because it combines temperature and pressure with the use of carbon dioxide to enhance recovery [45]). The analytical extraction procedure employed during the present study was accelerated solvent extraction (pressurised fluid extraction). This was based on the comparison of extraction technique by Dean *et al.* [253] as shown in Table 3.1. Itoh *et al.* [254] in his comparison of extraction techniques observed that accelerated solvent extraction (pressurised fluid extraction) techniques produced a better recovery and reproducibility than the microwave assisted extraction (MAE) and soxhlet extraction methods. Wang *et al.* [255], when comparing three extraction methods also observed that using accelerated solvent extraction (ASE) produced the best extraction efficiency for PAH spiked soil extraction than MAE and soxhlet extraction methods. The ASE technique is fully automated, can extract up to 24 samples simultaneously, uses less solvent and is also fast. This chosen technique was very suitable for the pilot study because of the large number of samples that was analysed during the course of the study.

Table 3.1: Analytical figures comparing extraction techniques [253]

	Soxhlet	Shake-flask	Ultrasonic	SFE	MAE	ASE
Sample mass (g)	10	1-10	2-30	1-10	2-5	Up to 30g
Extraction time	6, 12 or 24 h	3 X 5 mins	3 X 3 min	30 $mins^{-1}h$	20 mins (plus 30mins cooling and pressure reduction)	12 mins
Solvent type	Acetone-hexane (1:1, v/v); acetone-DCM (1:1,v/v); DCM only; or toluene-methanol (10:1, v/v)	Acetone-DCM (1:1, v/v); or DCM only.	Acetone-DCM (1:1, v/v); DCM only or hexane only	CO2 (plus organic modifier), Tetra-chloroethane used as the collection solvent for TPHs for determination by FTIR, otherwise DCM	Typically, acetone-hexane (1:1, v/v), The solvent is/are required to be able to absorb microwave energy	Acetone-hexane (1:1, v/v) or acetone-DCM (1:1, v/v) for OCPs semi-volatile organics, PCBs or OPPs; acetone-DCM-phosphoric acid (250:125:15, v/v) for chlorinated herbicides
Solvent consumption (mL)	150-300	10 X 3	100 X 3 (for 30 g)	10-20	25-45	25
Extraction method	Heat	Mixing/agitation	Agitation by sonication	Heat+pressure	Heat + pressure	Heat+ pressure
Sequential or simultaneous	Sequential (but multiple assemblies can operate simultaneously)	Sequential (but multiple assemblies can operate simultaneously)	Sequential	Sequential	Simultaneous (up to 14 vessels can be extracted simultaneously)	Sequential
Method development time	Low	Low	Low	High	High	High
Operator skill	Low	Low	Moderate	High	Moderate	Moderate
Equipment cost	Low	Low	Low	High	Moderate	High
Level of automation	Minimal	Minimal	Minimal	Minimal to high	Minimal	Fully automated up to 24 samples can be extracted
EPA method	3540	-	3550	35560 for TPHs and 3561 for PAHs	3546	3545

TPHs, total petroleum hydrocarbons; PAHs, polycyclic aromatic hydrocarbons

3.2.1 Accelerated solvent extraction (ASE):

The pressurised fluid extraction (PFE) which trades as Accelerated Solvent Extraction utilises organic solvent at high temperature and pressure to extract pollutants from environmental matrices [252]. Its extraction under high pressure (up to 2000 psi) maintains the organic solvent in its liquid state while extracting at temperatures above their boiling point. This process increases extraction efficiency by enhancing solvation ability with a lower viscosity which allows for a higher diffusion rate of the analyte [251].

This system mainly comprises a solvent supply system, extraction cell, oven collection system and purge system, all of which are under computer control. Using a carousel, the system is capable of 24 automated sequential extractions and contains 24 collection vials plus an additional four vial positions for rinse/water collection [253]. The system has been shown to use less solvent than other methods. The major disadvantage of this method is the high capital cost of the system and the need for treatment of high sulphur soil to limit the risk of blocking the tubing between the hot sample cell and the cooler collection vial. Figure 3.1 shows a schematic diagram of a pressurised fluid extraction system.

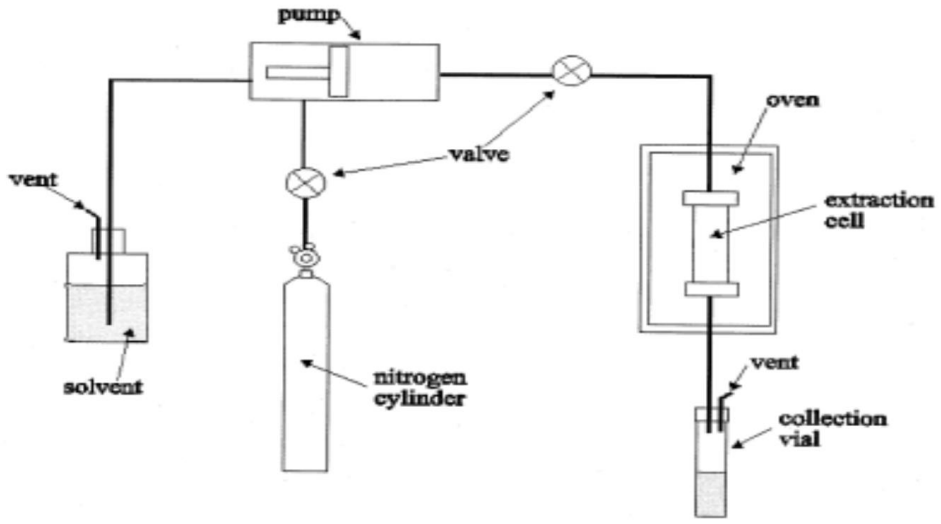


Figure 3.1: The schematic diagram of accelerated solvent extraction system [253]

3.2.2 Gas chromatography-mass spectrometry principle and description

Gas chromatography–mass spectrometry (GC–MS) is an instrument that combines two analytical tools: gas chromatography for the highly efficient gas-phase separation of thermally stable and volatile organic and inorganic compounds in complex mixtures, and mass spectrometry that confirms the identity of these separated components as well as the identity of any unknown component [256]. Gas chromatography is a physical separation method in which the compounds in a mixture are distributed (selectively) between the mobile phase (an inert carrier gas), and a stationary liquid or solid phase, that is retained in a column. The differences in column separation are described as gas-liquid chromatography (GLC) and gas-solid chromatography (GSC) respectively. With the exception of some specialised analysis such as for inorganic gases, GLC is the most used and will be discussed in this study. The chromatographic separation occurs as a result of repeated sorption/desorption of analytes during their movement along the stationary phase by the carrier gas. The achieved separation is because of the differences in the distribution coefficients of individual compounds in the mixture. An essential initial process in the GC separation method involves the volatilization (be in gaseous phase) of the analytes prior to their separation. GC separations can be operated in packed columns as well as open capillary columns, but this study will only focus on the use of capillary column as these are most widely applied in GC–MS.

The GC-MS instrumentation (Figure 3.2) is made up of a gas control unit, an injector or sample introduction system, a column housed in the temperature-programmable column oven, and a transfer line or the interface to the mass spectrometer. The function of the gas control unit is to control the flow-rate of the gas flows through the injector, the column, and also to detect carrier gas and, if required, auxiliary gases. The choice of the carrier gas is determined by constraints such as cost, availability, inertness and detector compatibility rather than its ability to influence separation because it should be a non-interactive gas, cheap, non-flammable, and environmentally friendly since it is vented at the end of the instrument. However, it can influence resolution through its effect on column efficiency because of differences in solute diffusion rates for various gases. It can also effect analysis time and play a role in pressure limiting situations because of differences in gas viscosities. This is the reason why gases such hydrogen, nitrogen and helium are most popular because they don’t effect analysis time and resolution and they are also cost effective. The carrier gas used in this study was

helium (He). The carrier gas is typically supplied from a compressed gas cylinder and applied at a pressure below 0.3 MPa. The carrier gas flow rate is approximately 20 mL min^{-1} for a packed column and 1 mL min^{-1} or 1.5 mL min^{-1} (as is the case for this study) for an open capillary column. The carrier gas is usually cleaned prior to use, by means of a moisture-and-oxygen trap so as to eliminate oxygen, water, and hydrocarbons. This is because the presence of oxygen in the carrier gas detrimentally affects the stationary phase in the GC column.

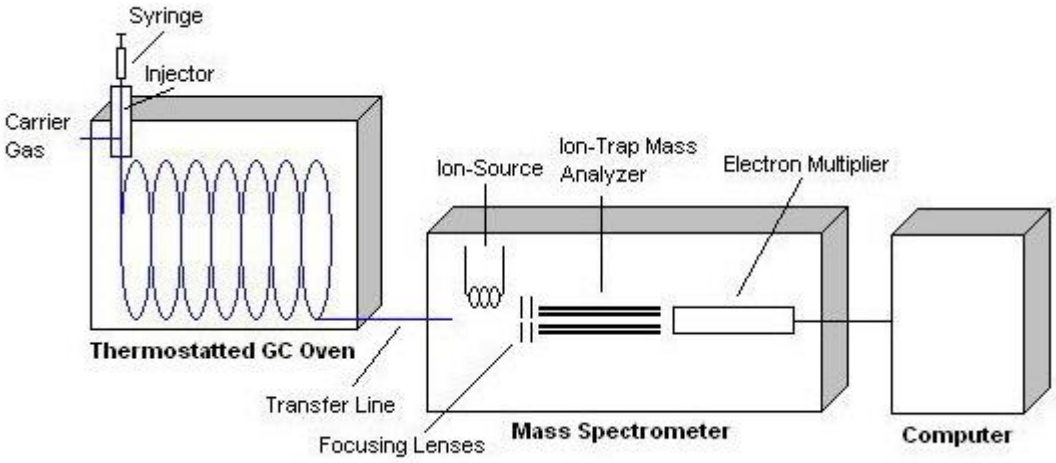


Figure 3.2: Schematic of the Gas Chromatography-Mass Spectrometry system [257]

3.2.2.1 Sample injection:

The sample introduction is a critical step in the operation of GC. The aim is to introduce a representative portion of the sample in a narrow band at the top of the column, i.e., without thermal degradation and/or component discrimination due to differences in volatility and also without overloading the column. Samples are introduced into the injector by means of a hypodermic syringe. The most widely applied injection techniques are split injection, splitless injection, and on-column injection. The split injection allows a larger sample volume to be introduced into the injection port and then “splits” or divides the sample. Due to the sample splitting in the split injector (Figure 3.3) a relatively large sample volume, e.g., 1 µl, can be injected into the heated injection port where it is instantly vaporized, with only a small proportion (0.01 µl) being introduced into the column [258], and the rest being vented to waste. The split ratio (ratio of flow to column) can be of the order of 1:10, 1:50 or 1:100 with the higher split ratio used for the smaller internal diameter column [256]. Smaller sample volume is also used in a splitless and on-column injection [259]. In splitless injection, the splitter vent is closed for a specified time,

typically 50 to 120 s, while the sample flows onto the head of the column. The splitter vent is then opened afterwards to purge the remaining sample and solvent from the injector. In this splitless injection also, the initial column temperature usually depends on the boiling point of the solvent used for dissolving the analytes.

With all these injection techniques the sample is instantly heated (typically up to 230 ^{o}C) to allow the organic solvent and organic compounds to vaporise into a gaseous state. Figure 3.3 shows the diagram of a split/splitless injector. Another type of injector is the programmed temperature vaporizer (PVT) which is similar to the split/splitless injector except that it is capable of introducing a large volume of sample (typically 250 mL). It achieves this, by running a temperature programme within the PTV which allows the removal of the organic solvent that is different to the organic compounds to be analysed. Its temperature control is such that the vaporizer chamber can be heated or cooled rapidly.

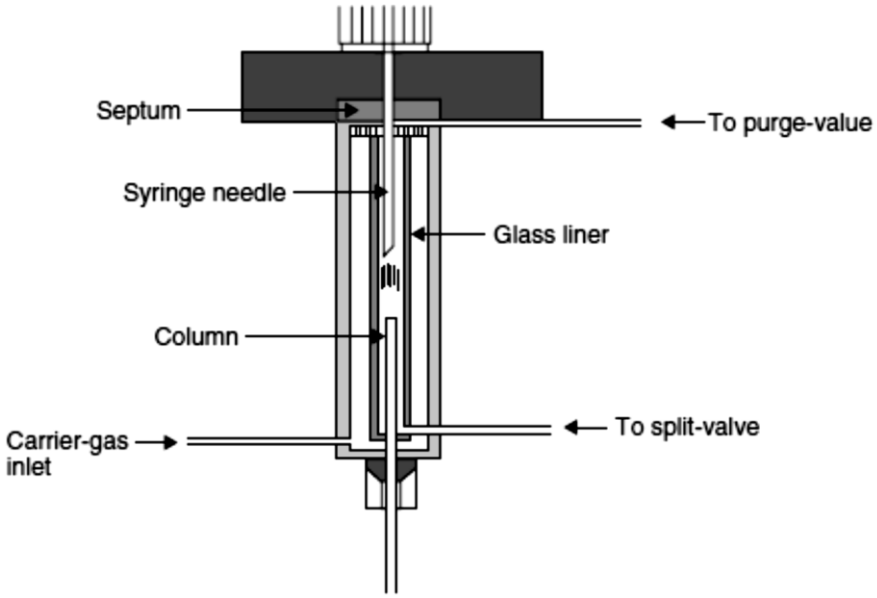


Figure 3.3 An examples of a split/splitless injector [259]

3.2.2.1.2 Automatic sampler:

An automatic sampler duplicates manual samples measurements and injection. Sample vials are a glass with vapour tight septum caps which can be discarded after use. The sampler flushes the syringe with a new sample to remove traces of the previous samples, pumps new sample to wet the syringe completely and eliminate any bubbles, takes in a precisely measured amount of sample and inject

the sample into the gas chromatograph. The automatic samplers are machine reproducible and consistently more precise.

3.2.2.2 Columns

In GC, the column alone determines the selectivity of the separation of compounds because of the inertness of the mobile phase. The columns are in two basic types as earlier stated (packed column and capillary column). The capillary column is widely used because it has an obvious advantage over the packed column. Such advantages are: providing high-resolution efficiency, greater sensitivity (despite the injection of less analyte), inertness and reproducibility.

The two main types of capillary columns are: (1) packed columns with solid particles over the whole diameter of the column (micro packed) and (2) open tubular columns with an open and unrestricted flow path through the middle of the column. The open tubular columns are still divided into three which are: wall coated open tubular (WCOT) columns, support coated open tubular (SCOT) columns and porous layer open tubular (PLOT) [258]. These open tubular capillary columns can be differentiated based on their different methods of stationary phase application in their column walls. In WCOT column (Figure 3.4) the stationary phase is applied as a thin (immobilized) liquid film. A common generic stationary phase comprises of 5 % diphenyl and 95 % dimethylsiloxane (Figure 3.5) which is chemically bonded onto the silica. The SCOT column has a thicker stationary phase film applied, featuring higher sample load ability and increased deactivation of the column wall. The PLOT column has its wall coated with small particles in order to enable gas-solid chromatography [256]. The WCOT is the most frequently used and it was also used for this study in the analytical section. Some of its advantages are: the increase in speed of analysis regardless of separation, shorter retention times, longer life, greater inertness, greater reproducibility, higher efficiency and lower bleed [258].

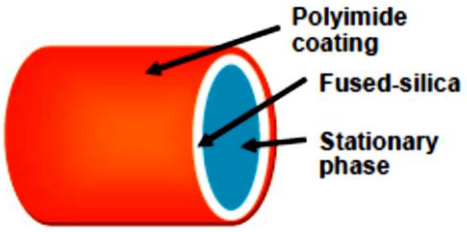


Figure 3.4 An example of a typical GC column [260]

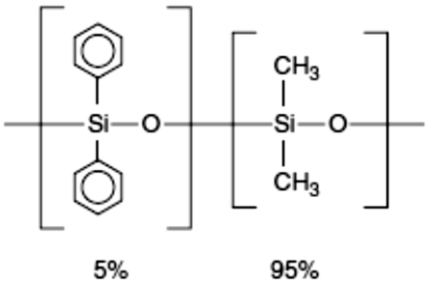


Figure 3.5 The stationary phase of a DB-5 gas chromatography column, consisting of 5 % diphenyl- and 95 % dimethylpolysiloxanes [260, 261]

A typical capillary GC column (Figure 3.4) length may range between 15 – 100 m (e.g.30 m long), depending on the type of application. Shorter columns are utilised for fast analysis, example, for heat-sensitive and high-boiling compounds. Longer columns are utilised in high-resolution separations. The internal diameter of the capillary column may range between 0.1 – 0.53 mm (e.g. 0.25 mm internal diameter) and its stationary phase (film thickness) between 0.25 – 5 mm (e.g. 0.25 mm). The disparity in the physical dimensions of a column and choice of stationary phase for GC can affect the separation of compounds. Therefore, the choice of stationary phase should be based on maximizing the difference in selectivity between the analytes towards the phase. The separation is expanded by exploiting the solute-stationary phase interactions that impede the progress of some solutes relative to others in order to increase their retention. The types of interactions that can take place between solute-stationary phases are: London or dispersion forces, which are weak and nonspecific; and dipole-dipole interactions or dipole–induced dipole interactions. As the sample is injected at the top of the column, and with the carrier gas, the analyte groups move at different distinctive rates through the column and elute in a steady progress one after another from the column. The average rate at which an analyte travel depends on the fraction of the time spent in the stationary phase, and thus on its affinity with the stationary phase. The ideal stationary phase liquid should have a low vapour pressure, high thermal and chemical stability, low viscosity, nonreactivity toward sample components and a wide temperature operating range (e.g. -80 °C to 450 °C). The phase must exhibit reasonable dissolving powers for the solutes in order to ensure symmetrical peaks. The stationary phases can be divided into nonpolar, polar and specialty phases. These differ in their ability to interact with solutes of different structure, i.e. their selectivity. The nonpolar phase contains no functional groups capable of specific interaction with the sample. The interaction between the compounds and the stationary phase is limited to dispersive forces and components are separated

according to their volatility with elution order following their boiling points. Compounds that cannot be separated on the basis of their boiling point (i.e. have similar boil point) require a different stationary phase for separation. Under this condition, a polar phase containing groups capable of specific interaction with the samples may be used. The elution order will now be based on the combination of volatility and the specific polar to polar interactions.

The GC column can be operated in two modes in a temperature programmable column oven. The modes are isothermal (i.e. fixed temperature during the GC run) or temperature programmed (i.e. the temperature is varied during the GC run). The temperature programmed mode allows a low initial temperature to be maintained which enables the separation of high-boiling-point analytes; which is then followed by a stepwise or linear temperature increase to separate analytes with lower boiling points. A typical isothermal operating temperature may be 100 ^{o}C whereas in temperature programmed the oven is linearly expanded at a rate of normally 4 to 20 °C min^{-1}. Therefore, components with higher boiling points and/or a stronger retention to the stationary phase are progressively released. The maximum operating temperature usually depends on the type of stationary phase in use. Operating the column close to its maximum operating temperature usually results in more serious column bleeding, which thus prompts a more accelerated contamination of the MS ion source. Toward the end of the temperature program, a period is required (normally few minutes) to permit the temperature to come back to its original starting temperature before the injection of the following sample.

Other than the mass spectrometer detection can also be done with detectors such as Flame ionization (FID), Electron Capture (ECD), Photo-ionization (PID), Flame photometric (FPD), NPD (nitrogen-phosphorous detector), and thermal conductivity (TCD). The type will be chosen according to the type of compounds that are analysed, such as organic, phosphorous, nitrogenous, halogenous, and aromatic compounds, etc. The most universally used detector is the FID and the mass spectrometer (such as quadrupole, ion trap or time of flight).

3.2.2.3 Electron impact ionization

Electron impact ionization takes place in the mass spectrometer (MS) and this was the type ionization used in the GC-MS analytical section of this study. The MS

principle is to produce a gas phase ions that are separated according to their mass to charge (*m / z*) ratio. This allows for both the structural identification of the compounds and quantitative data analysis to take place. The separated vaporised compounds from the GC is introduced into the MS through a transfer line from the open capillary chromatographic column (open split coupling) and in order to ionize these separated compounds an ion source is required. The ion source converts the separated vaporised compounds into an ion. The electron impact (EI) ionisation source (Figure 3.6) is one of the most common ion sources that are used in MS operation.

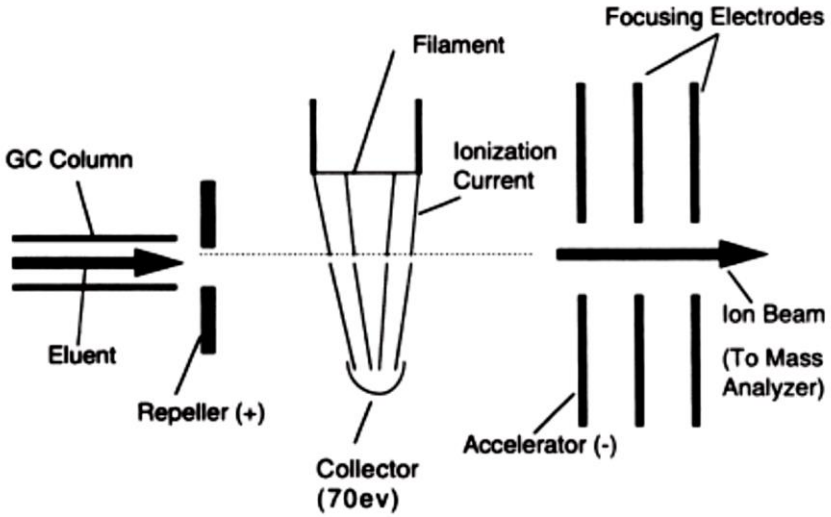


Figure 3.6 Electron impact ionization (EI) source [262]

In EI, the analyte vapour is subjected to a barrage of energetic electrons (normally 70 eV) produced from a heated tungsten or rhenium filament (cathode), which accelerates towards an anode, thereby colliding with the analyte vapour. Some of the released electrons would be elastically scattered, others would cause electron excitation of the analyte molecules upon collision. These excitations can cause the complete removal of an electron from the molecule which would then, produces a (positively) charged ion denoted as M^+ and two electrons. The M^+ ion is known as the molecular ion. It's *m / z* ratio relates to the molecular mass M_r of the analyte. The positively charged ions are then separated by the MS. Equation 3.1 expressed the electron bombardment process. Electron ionization is usually carried out in a high-vacuum ion source (typical pressure ≤ 10^2 Pa), which helps to prevent intermolecular collisions. Electron ionization spectra are also highly replicable.

$$M + e^- \longrightarrow M^+ + 2e^- \qquad (3.1)$$

3.2.2.4 Mass analyser

After leaving the ion source, the charged ions are repulsed and attracted by charged lenses into the mass analyser. Here the ionic species are isolated by their mass - to - charge proportion (*m / z*) by either magnetic or electrical fields. Regular mass analyzers for GC – MS are quadrupoles, quadrupole ion traps and time of flight. Other types of analyzers are: single - focusing magnetic sector and double - focusing magnetic sector (high resolution, more expensive). The quadrupole ion trap was the analyser used in the analytical section of this study and it is the mass analyser that would be discussed in this section.

The quadrupole ion trap mass analyser (Figure 3.7) can be said to be a three-dimensional ion trap which suggests that ions move in the x and y directions but in this case there is no z direction, the ions oscillate around a fixed point [263]. It consists of a cylindrical ring electrode with two end caps to which a radio frequency (RF) (applied at alternating current (AC) voltage of 1 kV approximately and at 1.1 MHz) is applied [263]. The RF then induces the oscillatory motion of the ions. The top end cap contains openings for bringing ions or electrons into the trap while the base end cap contains openings for ions ejected toward the electron multiplier. Ions that are produced either inside the trap itself or remotely from an ion source are stored in the trap. A comparatively high pressure of helium gas (0.1 Pa) is available in the ion trap with the aim of stabilizing the ion trajectories. Increasing the RF causes the ion trajectories to increase and to approach the end caps. As this RF is scanned upwards the ions are ejected from the trap in order of increasing *m / z* and those that emerge through apertures in the lower end cap are detected by the electron multiplier. Quadrupole ion traps are simple in design, less expensive, and competent in rapid scanning for GC – MS applications. It shows a similar sensitivity with the quadrupole mass analyser when it is operated in selected ion mode (SIM) but ion traps are typically 20 - to 50 – fold more sensitive than quadrupoles in scanning mode [262].

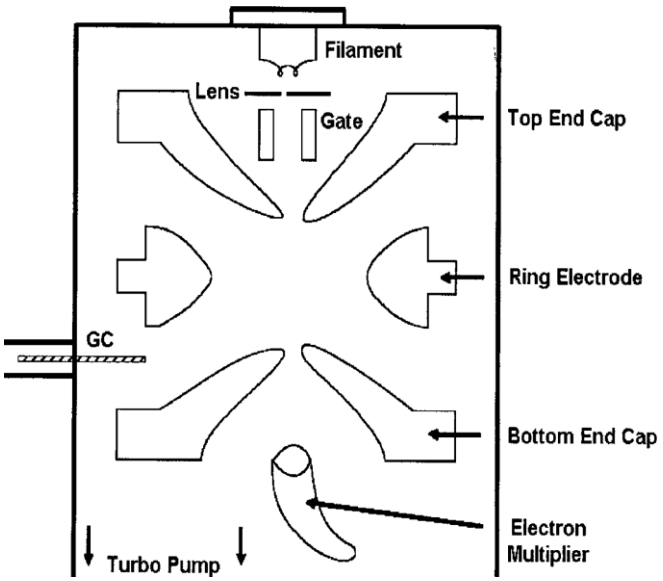


Figure 3.7 Schematic diagram of an quadrupole ion-trap mass spectrometer [256]

3.2.2.5 Ion detection

The resolved ions after passing through the mass analyser, sequentially strike a detector. There are several types of detectors such as the faraday cup collector, photographic plate, channel electron multiplier array, and electron multiplier [258]. There are two types of electron multiplier: electron multiplier with a conversion dynode and electron multiplier with a continuous dynode. The electron multiplier with a continuous dynode (Figure 3.8) is the most commonly used detector and it was the type used for this study. The continuous dynode electron multiplier tube is used to tally the ions and produce a mass spectrum. It comprises of an entrance, within which is covered with lead oxide semiconducting material. The cone is biased with a high negative potential (e.g. - 3 kV) at the entrance and held at the ground close to the collector. Incoming positive ions from the mass analyser are attracted towards the negative potential of the electron multiplier tube. On impact, the positive ion releases a cascade of (secondary) electrons. These secondary electrons are attracted towards the ground collector within the electron multiplier tube. In addition, the initial secondary electrons can also collide with the semiconductive surface causing a further cascade of electrons to be released. This multiplication of electrons proceeds until every one of the electrons (up to 108 electrons) are gathered. This discrete pulse of electrons is further aggrandized (amplified up to 1 million-fold) remotely and recorded as a number of ion ‘counts per

second'. The electron multiplier also has a restricted lifetime dictated by the aggregate collected charge, which is monitored [260, 262].

The entire MS operation is carried out under high vacuum. This is a crucial prerequisite to prevent the loss of the charged species by collision with other ions, molecules, or surfaces. The *mass spectrum was* a plot of the ion abundance as a function of *m* / *z.* Under controlled conditions, the proportions of ion abundance and the definitive *m* / *z* species present are attributes for each compound. These attributes are then used to determine the molecular weight and the chemical structure of each compound.

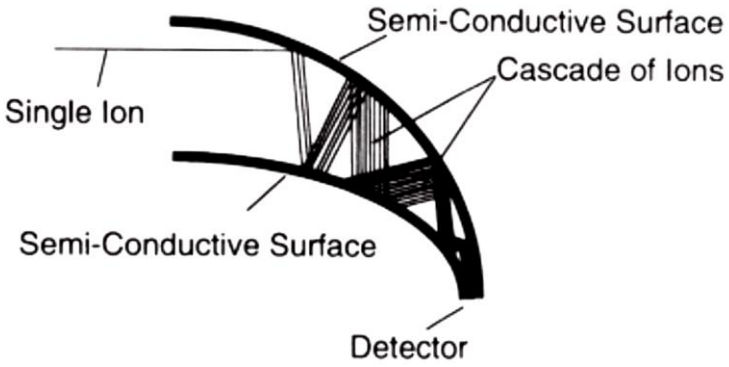


Figure 3.8 Electron multiplier (continuous - dynode version) [262]

3.3. Experimental methodology

3.3.1. Overview

This chapter gives the full details of all the experimental and instrumental analysis that was carried out during the pilot study. The study was also carried out to observe and determine the challenges and the experimental errors that might be encountered during the main bioremediation study.

This study was carried out for 4 weeks using the various techniques and instruments that were later used in the full bioremediation study; the objective was to test the reliability, efficiency and accuracy of these methods.

The PAH experimental analysis was carried out in different stages: the coal tar and coal tar pitch analysis; calibration and optimisation of the instruments using PAH standard solution and certified reference materials; and a preliminary PAH spiking

test using PAH standard solution and coal tar pitch spiking study. The coal tar pitch (PAH) spiked soil was also spiked with two different heavy metals: Pb and Cd. The concentration of the heavy metals was 250 mg kg^{-1} each.

The soil respiration experimental evaluation was carried out in two stages; (1) the effect of acetone on soil respiration and (2) the soil respiration of the coal tar pitch spiked and unspiked soil.

The community level physiological profile (CLPP) evaluation (BIOLOG Ecoplate) was carried out in five stages: (1) test of inoculum dilution, (2) selecting a specific time point for Ecoplate assessment, (3) determining the effect of acetone on microbial community level physiological profile using Ecoplate (4) Ecoplate replication and reproducibility and (5) the coal tar pitch spiked soil CLPP.

The microbial biomass experimental evaluation using the total organic carbon method was carried out in two stages; (1) test of accuracy using an organic carbon standard solution of a known concentration and (2) microbial biomass determination on the coal tar pitch spiked soil using total organic carbon method

The evaluation of heavy metal analysis techniques in coal tar pitch spiked soil was carried out in two stages: (1) total heavy metal concentration determination using EDXRF and (2) determining the bioavailable metal concentration using EDTA method.

3.3.2 Soil sampling:

Soil samples were collected from Armstrong Park situated in the Northeast area of Newcastle upon Tyne, approximately 2 km from Newcastle city centre. The sampling was done in a random pattern and in an intrusive sampling technique where soil was collected by the use of auger/trowel over a shallow depth of 30 cm. A total of 20 kg of soil sample was collected from the sampling site. The collected soil sample was transferred to the laboratory using a polyethylene bag in order to maintain the original soil characteristics.

3.3.3 Sample preparation:

The soil was homogenised, air dried for 24 hours to reduce the excess moisture content but not completely dried and was then sieved with 2 mm sieve. 1kg of the soil was used for soil characterisation.

3.3.4 Materials and reagents:

PAH standard solutions of 2000 µg mL^{-1} were purchased from Thames Restek U.K Ltd., Buckinghamshire, UK (2000 µg mL^{-1} in dichloromethane). sonicator (Bransonic Ultrasonic Cleaner 2200) was used to warm and sonicate PAH standard solutions before use. Aluminium Oxide (Al_3O_3) purchased from Sigma-Aldrich Ltd (Dorset, UK) was used as a clean-up reagent to remove endogenous compounds such as fatty acids, cholesterol or steroids by adsorption chromatography. 4,4'-difluorobiphenyl used as an internal standard was purchased from Sigma-Aldrich Ltd., Dorset, UK. All the solvents (example: dichloromethane, acetone) used during the experiment were analytical reagent grade and were purchased from Fisher Scientific Ltd. (Loughborough, UK). High purity hydromatrix (diatomaceous earth) was purchased from Varian Inc. (Harbor City, CA, USA). Certified reference materials for PAH spiked soil (LGCQC3008 Sandy soil) were purchased from LGC Standards, Teddington, UK. The binder used in EDXRF analysis was Licowax C Micro powder PM (Fluxana GmbH & Co,Sommerdeich, Germany). Filter papers (ASE200) made from glass fibre cellulose were obtained from Dionex Corporation (Sunnyvale, USA). Ethylenediaminetetraacetic acid (EDTA) was purchased from Fisher scientific Ltd (Loughborough, UK). Chromacol Silanized auto sample vials and metal salts $PbCl_2$ and $CdCl_2.2½H_2O$ were obtained from Sigma-Aldrich Ltd Dorset, UK. Coal tar pitch and Coal tar were supplied by Liver Grease, Oil and Chemical Co. Ltd Liverpool, UK.

The ultra-pure water of conductivity 18.2 MΩ-cm at 25 ^oC was produced by a direct QTM Millipore system (Molsheim, France). Concentrated hydrochloric acid (HCl) at least 98 % purity was supplied by Fisher Scientific Ltd. (Loughborough, UK). Alcohol-free Chloroform solvent ($CHCl_3$) (Sigma-Aldrich UK) and potassium sulphate (K_2SO_4) were Sigma-Aldrich reagents, Anti-bumping granules by BDH laboratory (Poole England), Whatman No.1 filter paper by Sigma-Aldrich Ltd. (Dorset, UK) was used to filter the extracted samples. Total Organic Carbon (TOC) was analysed using a direct method mid-range Test 'N' Tube which comprised of; acid digestion solution vials mid-range TOC, pH 2.0 Buffer solution, sulphate funnel, micro indicator ampule for mid-range TOC tubes, TOC persulfate powder pillows, pH paper and TOC 1000 mg L^{-1} C standard solution. The test kit was purchased from Hach-Lange Ltd Salford, U.K. D-glucose also from BDH laboratory (Poole England) was used as a readily available carbon substrate for substrate-induced respiration in accordance with BS ISO 16072. Sodium hydroxide pellets purchased

from BDH chemical laboratory Ltd. (Poole England) was used to absorb carbon dioxide (CO_2) during the soil respiration study. BIOLOG Ecoplates (BIOLOG Inc., 3938 Trust Way, Hayward, CA 94545, U.S.A.) purchased from Techno-path distribution Ltd, Ballina, Tipperary, Ireland, was used for community physiology profile analysis. NaCl laboratory reagent from Fisher Scientific UK was used for extraction of microorganism from the soil.

3.3.4 Instrumentation and laboratory equipment

3.3.4.1 Microplate absorbance reader and energy dispersive x-ray fluorescence (EDXRF):

The details on the use of microplate absorbance reader for the Ecoplates and the EDXRF analysis for the heavy metal spiked soils can found in sections 2.5.3.1 and 2.5.3.2 respectively.

3.3.4.2 Accelerated solvent extraction:

Accelerated solvent extraction (ASE), ASE200 instrument (Dionex UK Ltd., Camberley, Surrey) was used for soil PAH extraction. The extraction cell volume used was 22 mL The extraction conditions were a temperature of 100 °C, a pressure of 2000 psi, an extraction time of 10 mins (5 mins equilibration plus 5 mins static) and an additional time of about 3 minutes for rinsing with fresh solvent and $N_{2,}$ making a total of approximately 13 minutes per a sample. A single static flush cycle was applied. The solvent used for the extraction was 50 % dichloromethane and 50 % acetone.

3.3.4.3 Gas chromatography-mass spectrometer (GC-MS):

The GC-MS (Thermo Electron Corporation, UK) was based on the method of Afanasov *et al.* [248]. It was operated in a single ion monitoring (SIM) mode with a split injection volume of 1 µL. The system was controlled from a PC with Xcalibur™ 1.4 SR1 software. Separation of the 16 US EPA PAH compounds were carried out using a capillary column DB-5 MS (5 % diphenyl- 95 % dimethylpolysiloxane, 30 m x 0.25 mm ID x 0.25 µm film thickness) supplied from Thames Restek (UK). The injector port, detector and transfer line temperature were set at 280 °C, 270 °C and

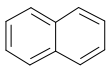
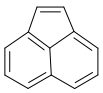
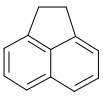
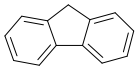
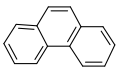
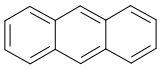
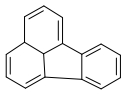
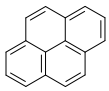
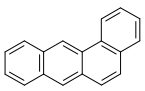
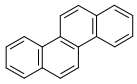
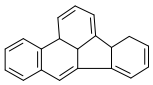
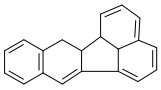
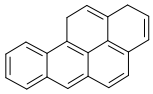
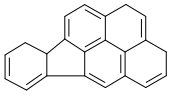
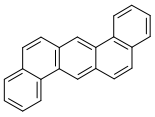
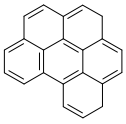
300 °C respectively. The temperature programme used for this analysis was initial temperature, 70 °C with a holding time of 2 mins. The temperature increases at a rate of 7 °C min^{-1} until it reaches 180 °C for the first ramp and then increases again at the rate of 3 °C min^{-1} until it reaches 280 °C for the second ramp with a final holding time of 3 mins. The separation of the PAHs compounds in the sample was achieved in about 54.05 mins. Standards for the 16 USEPA PAH and solvent blanks were run with every 10 soil extract samples to validate the accuracy and precision of the instrument [47]. Blanks were used to control the presence of compounds after every set of runs and also to check for the purity of the solvents used for GC-MS analysis. The chosen operating conditions for the GC-MS are shown in Table 3.2. The selected ion monitoring (SIM) mode used for the GC-MS was set up based on the elution times of the 16 USEPA PAH standards, run in full scan mode. Table 3.3 shows the parent ions for each individual PAH and their retention times.

Table 3.2: GC-MS operating conditions and acquisition parameters

Operating Conditions	Acquisition Parameter
Injector Mode (GC)	Split
Carrier gas flow (GC)	1.5 mL/min
Split flow (GC)	15 mL/min
Split ratio (GC)	1:10
Temperature injector (GC)	280 °C
Injection volume (GC)	1 µL
Ion source temperature (MS)	270 °C
Start time (MS)	4 mins
Scan Mode (MS)	Selected Ion Monitoring (SIM), the *m / z* values scanned for are in Table 3.3
Damping gas flow (MS)	0.3 mL/min

GC= gas chromatography; MS = mass spectrometer

Table 3.3: 16 USEPA PAH standard and their retention times

PAH	Structure	Empirical Formulae	Ms ion for Quantitation	Retention time (minutes)
Naphthalene (NAP)		$C_{10}H_8$	128	9.69
Acenaphthylene (ACY)		$C_{12}H_8$	152	14.80
Acenaphthene (ACE)		$C_{12}H_{10}$	154	15.39
Fluorene (FLU)		$C_{13}H_{10}$	166	17.14
Phenanthrene (PHE)		$C_{14}H_{10}$	178	20.69
Anthracene (ANT)		$C_{14}H_{10}$	178	20.90
Fluoranthene (FLUH)		$C_{16}H_{10}$	202	26.78
Pyrene (PYR)		$C_{16}H_{10}$	202	28.04
Benzo[a]anthracene (BaA)		$C_{18}H_{12}$	228	36.20
Chrysene (CHY)		$C_{18}H_{12}$	228	36.42
Benzo[b]fluoranthene (BbF)		$C_{20}H_{12}$	252	43.53
Benzo[k]fluoranthene (BkF)		$C_{20}H_{12}$	252	43.72
Benzo[a]pyrene (BaP)		$C_{20}H_{12}$	252	45.49
Indeno[123-cd]pyrene (IDP)		$C_{22}H_{12}$	276	52.14
Dibenzo[a,h]anthracene (DBA)		$C_{22}H_{14}$	278	52.46
Benzo[g,h,i]perylene (BgP)		$C_{22}H_{12}$	276	53.47

3.3.5 Characterization of soil:

Soil pH determination, organic carbon by loss on ignition, soil moisture content determination, soil water holding capacity and soil particle size determination were all carried out according to the procedures used in sections 2.5.5.1, 2.5.5.2, 2.5.5.3, 2.5.5.4 and 2.5.5.5 respectively.

3.3.6 PAH analytical instrument optimisation and quality control:

It is generally a good laboratory practise to calibrate, optimise and check instrument reliability before carrying out the analytical procedure with the instrument. In order to carry out soil PAH extraction and quantification using the ASE and GC-MS respectively, there were prior essential steps that were taken to ensure an optimal performance of the analytical method and a good result quality.

The steps taken to optimise the analytical method of the ASE were: using a certified soil reference material containing 16 US EPA priority PAH compounds to carry out repeated extraction (cell size 22 mL) to test out clean up procedures and optimum temperature and pressure settings. An extraction procedure with a preliminary soil spiking using 16 USEPA PAH standard solutions with a known concentration of the required 16 PAH compounds was also carried out with the ASE. The protocol used in optimising GC-MS for analysis involved using a known concentration of PAH standard solution to select the right GC-MS parameters that would produce a good quality PAH compound separation and quantification. Such parameters are: (i) temperature program, ion source temperature, transfer line temperature, time of analysis, (ii) selection of ions using mass spectrometer parameters (either full scan or SIM (selected ion monitoring mode)), (iii) choice of the injection volume in the GC, (iv) autosampler parameters (sampling positions and rinsing after sampling), (v) injection of blank solvents . The next step was to choose the PAH standard concentration range for calibration curves, choosing the right concentration of internal standard necessary for quantification. The other step was to run the extracted PAH certified reference material and the preliminary soil spiked sample for quality assurance and accuracy. The extracted CRM and soil was run in triplicate and a standard check (PAH standard solution) was also used during each analysis.

Observations and modifications of these parameters were still made during extractions, by taking care of the sample solution injected (clean-up), the appearance of chromatograms after injections (peak tailing and column-bleeding), and the consistency of standard quantitation when analysing samples. The efficiency of clean-up based on the colour of the extract and the influence of the adsorbent was also monitored during analysis.

The quality and precision of the entire analytical procedure with extraction was quantified based on the obtained accuracy of % recoveries with spiked soils and CRM. After the optimisation procedure was carried out, the developed method was then used to analyse and quantify the unknown PAH concentration in the coal tar pitch spiked soil

3.3.6.1 Calibration standard:

Six PAH standard (in dichloromethane) solutions of 0 mg L^{-1}, 1 mg L^{-1}, 2 mg L^{-1}, 5 mg L^{-1}, 10 mg L^{-1} and 15 mg L^{-1} were prepared from a 2000 mg L^{-1} PAH standard (Thames Restek U.K Ltd). These standards were used to optimise the GC parameters and also obtain a calibration curve for subsequent PAH analysis. Fresh standard solutions of 5 mg L^{-1} and 10 mg L^{-1} were also prepared and used simultaneously with every 10 samples analysis in order to check the stability of the calibration and standards.

3.3.6.2 Certified reference material analysis

Three 10 g test portion of a CRM with appropriate certified concentrations of 16 PAH compounds were extracted in order to compare the obtained values with the certificate values. The 10 g used was based on the certificate's recommended soil weight for extraction. 2 g of alumina was also used as an in-situ ASE clean-up and hydromatrix was used as an absorbent of excess moisture. After the ASE, the extract was reconstituted in 50 mL of dichloromethane (DCM), 10 mg L^{-1} of internal standard (4,4'-difluorobiphenyl) was added to the diluted extract and 2 mL of the diluted solution was transferred into a 2 mL Silanized (Chromacol; Sigma-Aldrich) GC auto sample vials for GC-MS analysis

3.3.6.3 Preliminary soil spiking and extraction:

20 g of soil was placed in a beaker and 10 mL of dichloromethane containing 0.5 mL of PAH standard solution (2000 mg L^{-1}) was added to the soil. The soil was mixed thoroughly with the solvent using a glass rod. The spiking was carried out in triplicate and the three spiked 20 g samples of soil were air dried for 24 hrs for the solvent to evaporate prior to ASE. The spiked soil was ground and sieved using a < 250 µm sieve. 10 g of soil from the sieved soil sample was weighed out and used for ASE. After the ASE the extracted solution was reconstituted in 100 mL and 10 mg L^{-1} of internal standard (10 mg L^{-1} 4, 4'-difluorobiphenyl in DCM) was added to the extracted solution for GC-MS analysis. 2 mL of the diluted solution was then transferred into a 2 mL Silanized (Chromacol; Sigma-Aldrich) GC auto sample vials for GC-MS analysis. The expected 16 PAH compounds concentration in the extracted spiked soil was 5 mg L^{-1}. This spiking was carried out in order to check accuracy and quality of the spiking procedure and extraction process.

3.3.6.4 Coal tar and coal tar pitch concentration determination:

The concentration of PAH in coal tar pitch and coal tar was determined in order to obtain PAH content of coal tar and coal tar pitch. This knowledge helped in the decision making of the choice of either the coal tar or the coal tar pitch for soil spiking and also to determine how much of each material would be needed for soil spiking. Two different methods were employed: dissolution in a solvent and accelerated solvent extraction.

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3.3.6.4.1 Dissolved coal tar pitch PAH concentration:

0.625 g of ground coal tar pitch was dissolved in 10 mL of acetone. The mixture was filtered and diluted further in 50 mL of acetone. The solution was also filtered the second time to ensure no solid sediment in the filtrate. The final filtrate was diluted with 100 mL of DCM. 10 mL of the final dilution was taken for GC-MS analysis by adding 10 mg L^{-1} of internal standard to it. 2 mL of the diluted solution was transferred into a 2mL Silanized (Chromacol; Sigma-Aldrich) GC auto sample vials for GC-MS analysis

3.3.6.4.2 Extracted coal tar pitch:

0.05 g of coal tar pitch was extracted using ASE method. The extracted solution was diluted 100 mL dichloromethane and 10 mL of internal standard (10 mg L^{-1}) was added to the solution. 2 mL of the extracted solution with the internal standard was transferred into a 2 mL Silanized chromacol auto sample vial for GC-MS analysis.

3.3.6.4.3 Preliminary coal tar pitch spiking analysis:

0.625 g of coal tar pitch was dissolved in 10 mL of acetone and mixed with 100 g of soil in a bottle. The mixing was done by shaking the coal tar pitch solution and the soil vigorously for about 10-15 minutes. The coal tar pitch was weighed out to obtain an equivalent of 1000 mg kg^{-1} PAH concentration in 100 g of soil. The spiked soil was air dried in a fume cupboard for 24 hrs in order for the acetone to evaporate. After air drying the spiked soil was homogenised, ground and sieved with a < 250 µm sieve. Three replicates of 10 g spiked soil were weighed out and extracted using the ASE method. After ASE, the solution was reconstituted with 50 mL dichloromethane, and 5 mL of internal standard (10 mg L^{-1}) was added to the extracted solution. 2 mL of solution with the internal standard was transferred into a 2 mL Silanized GC auto sample vials (Chromacol; Sigma-Aldrich) and analysed in the GC-MS.

3.3.6.4.4 Coal tar analysis:

6.25 g of coal tar was dissolved in 100 mL of acetone. The mixture was filtered twice using Whatman filter paper. The filtrate was made up to 100 mL of acetone. 10 mL of the filtrate was diluted with 100 mL of DCM. 10 mL of the DCM dilution was taken for GC-MS analysis by adding an internal standard of 10 mg L^{-1} to it. 2 mL of the diluted solution was transferred into a 2 mL Silanized (Chromacol; Sigma-Aldrich) GC auto sample vials for GC-MS analysis

3.3.7 Preliminary soil respiration experiment:

3.3.7.1 Determining the effect of using acetone as a carrier solvent during spiking on soil respiration:

An initial soil respiration test was carried out in order to determine the effect of using acetone for soil spiking on soil respiration rate. This was to determine the

required coal tar pitch soil spiking ratio that will reduce the influence of acetone (coal tar pitch carrier solvent) on soil microbial community.

1.8 kg of soil was divided into three groups of 600 g labelled as A, B and C. Each group was further divided into two replicates subset comprising 300 g of soil each.

Group A: the two replicates of 300 g of soil were used as the control without acetone spiking.

Group B: the 300 g of soil in this group for each replicate was further divided into two halves of 150 g each. One-half was spiked with 30 mL of acetone and the other half was unspiked. The acetone spiked half was air dried overnight to allow the acetone to evaporate. The two halves were homogenised thoroughly using a trowel and 200 g from the homogenised soil was used for soil respiration test. This was also repeated for the replicate

Group C: the 300 g of each replicate was divided into a quarter, 25 % of the soil (25 g) was spiked with 30 mL of acetone and was air dried overnight. The 75 % of the soil (225 g) which was not spiked was homogenised with the spiked 25 % soil after the air drying and used for soil respiration test. The homogenisation was done with a trowel for a thorough mixing.

Spiking Procedure: The spiking procedure used for this experiment was spreading out the soil as thinly as possible and spraying acetone on the soil evenly using an acetone bottle spray. Then the soil was mixed thoroughly using a small trowel and allowed to air dry overnight in a fume hood.

3.3.7.2 Soil respiration experimental procedure:

100 g of soil for each of the treatments maintained at 75 % water holding capacity was transferred to a 250 mL Oxi-Top bottle. Two pellets of NaOH were transferred into the Oxi-Top control measuring head pellet housing unit. The bottle was closed with the Oxi-Top control measuring head (finger tight) and transferred into the Oxi-Top incubation chamber. The temperature in the incubation chamber was maintained at 20 °C ± 2. The soil in the bottle was allowed to stabilise for about 10minutes, then the respiration measurement was set off for two days using the Oxi-Top measuring controller.

The oxygen consumed during the biological process produces approximately equimolar quantities of carbon dioxide that are bound by the absorbing agent sodium hydroxide. The pressure reduction in the vessel due to the CO_2 absorption

is stored in the OxiTop measuring head which is transferred to the OxiTop controller through an infrared interface and this information is transmitted to the computer for further analysis in Microsoft Excel using the OxiTop ACHAT OC PC communication software (WTW, Weilheim, Germany).

3.3.8 Preliminary study on community level physiological profile using Ecoplate

3.3.8.1 Test of Ecoplate inoculum dilution:

30 g of soil samples were divided into three groups of A, B and C.
Group A; comprises of samples that used a dilution factor of 10, Group B; comprises of samples that used a dilution factor of 50 and Group C; comprises samples that used a dilution factor of 100.

The microbial community from 3 g of homogenised soil (three replicates) of each group was extracted and used to inoculate the Ecoplate according to their groups' dilution factors and the plate reading was taken every 24 hrs for 165 hrs.

3.3.8.2 Selecting a specific time point for Ecoplate assessment:

Two methods were used for this assessment during the study; the first method was to determine according to Garland *et al*. [179] a specific average well colour development reference values between 0.25 and 1 that will yield relatively similar CLPPs for use in the community classification [164]. This estimate of AWCD rate, therefore, is a function of both the lag time before colour began to develop and the linear rate of colour production thereafter. The second method was to determine an appropriate incubation time point that will preserve the greatest disparity between well responses while keeping the maximum number of wells within the linear absorbance range [120, 234].

The microbial community from 3 g of soil was extracted in three replicates and used to inoculate an Ecoplate. The Ecoplate absorbance reading was taken every 24 hours for 7 days for the AWCD method and the absorbance reading was done every hour for 80 hrs for the incubation time point method.

3.3.8.3 Determining the effect of acetone on microbial community level physiological profile using Ecoplate:

The test was done on the same acetone spiked soil used to determine the effect of acetone on soil respiration rate. 3 g of soil was collected from the three different groups as described in section 3.4.1.1. **Group A**: control soil without acetone spiking. 3 g of 3 replicates from the control soil was used for microbial community assessment

Group B: Half of soil spiked with acetone and the other halve was unspiked. The two halve were homogenised thoroughly using a trowel and 3 g of 3 replicates from the homogenised soil was used for microbial community assessment

Group C: 25 % of the soil was spiked acetone 75 % of the soil was not spiked and the two parts were homogenised with a trowel for a thorough mixing. 3 g of 3 replicates from the homogenised soil sample was used for microbial community assessment.

3.3.8.4 Determining Ecoplate replication and reproducibility:

This study was carried out for two weeks and a total of 7 kg (6996.8 g) of fresh soil was used for this study. The soil was air-dried for 48 hours and sieved with 2mm mesh sieve.

The approx. 7 kg of soil was adjusted to 70 % field water holding capacity (WHC) by adding 1223 mL of distilled water and vigorously mixing the soil using an electric cement mixer.

The experiment was carried out in three replicates of R1, R2 and R3. Each replicate had three soil samples, each sample had 250 g of wet soil (70 %WHC) contained in a PVC trough with a diameter as 3.2 cm and depth as 30 cm. The total number of PVC trough used was nine; three for each group.

The initial weight of the PVC trough with wet soil was taken and used to maintain the water content of the soil (70 % of its WHC) during the experiment period.

The replication and reproducibility experimental set up were done in such a manner that variability across the replicates R1, R2 and R3 was assessed (E1), variability between Ecoplate was assessed (E2) and also, variability within a replicate was assessed (E3)

The experiment was carried out over a period of two weeks for times; t = 0, t = 1 wk and t = 2 wks. For each analysis, one PVC trough was harvested from each of the three replicates. All the samples had the same type of treatment.

3.3.8.4.1 Ecoplate study:

At each time of analysis, an equivalent weight of 3 g of soil was used to extract the microbial community. The 3 g of soil was obtained from the PVC troughs harvested from the three replicate.

To test for the variability across replicates (E1); 3 g of soil was taken from each of the harvested 3 PVC microcosms. The microbial extract from the soil of each replicate was used to inoculate one Ecoplate.

To test for the variability between Ecoplates (E2); the same extracted microbial community from each of the three replicates from E1 assessment was used to inoculate a second Ecoplate. Therefore, E2 Ecoplate is a duplicate of E1 Ecoplate.

To test for the variability within a replicate sample (E3); 3 g of soil (X3) was taken from one harvested PVC microcosm and the microbial extract from these soils were used to inoculate one Ecoplate. The assessment of variability within replicates was carried out at the three different periods of analysis. The variability within replicate 1 (R1) was done at T = 0, the variability within replicate 2 (R2) was done at T = 1 wk and the variability within replicate 3 (R3) was done at T = 2 wks. Figure 3.9 shows the schematic of the experimental design used for this assessment.

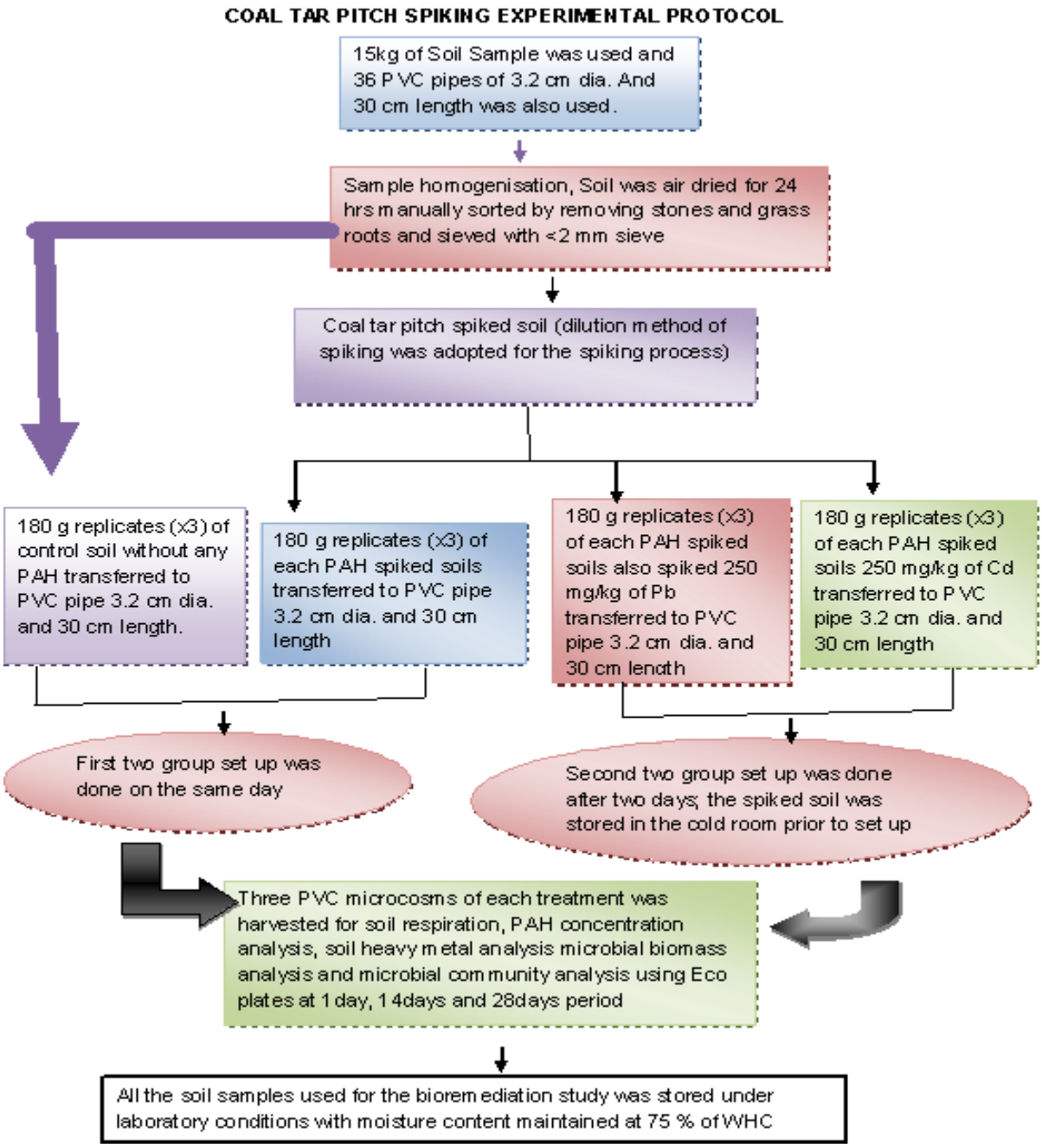


Figure 3.9: Schematic view of protocol used in coal tar pitch spiking experiment

3.3.8.5 BIOLOG Ecoplate experimental procedure:

3 g of soil placed in a 50 mL cylinder tube and made up the 30 mL mark with 0.9 % (w/v) NaCl solution [149]. The soil mixture was shaken with end over end rotary shaker (Stuart Rotator Shaker SB 3) for 60 mins at 30 rpm at room temperature. The mixture was further centrifuged (Beckman Allegra 6R Centrifuge) at 200 rpm for 5 mins to settle solid particles [232]

The resulting supernatant was diluted 1 mL in 50 mL of 0.9 % NaCl (200 µL in 10 mL) and the diluted supernatant of each sample was used to inoculate the 32 well (150 µL per well) Biolog ECO plates. The 96 Ecoplate wells are made up of three replicates of 31 carbon substrate well and 3 blank well.

The plates were incubated at 22 °C in the oven and the substrate utilization was monitored by measuring the light absorbance using EL 808 ultra-microplate reader (Bio-Tek Instrument, INC).

In some of the experiments the absorbance measurements were taken every hour for 80 hours, every 24 hours for 168 hours and some were taken at a determined set point of 50 hours. The time for the absorbance measurements depends on the type of Ecoplate assessment that was being carried out. One of the main advantages ECO plates have is that it contains three replicates of substrates in one plate. Absorbance values for the wells with carbon substrates were blanked against the control well and negative values were considered as zero in subsequent data analysis [179].

3.3.8.6 Data analysis for BIOLOG Ecoplate

The absorbance values of the Ecoplates were measured immediately after inoculating (0 hr measurement). Then for Ecoplates that had precipitation in their wells, the absorbance values of the initial measurement were subtracted from the respective absorbance values of successive measurement [237]. Average well colour development (AWCD) was calculated for all the experimental data generated by the Ecoplate according to the method used by Garland *et al*. [179] as shown in Equation 3.2. The AWCD reflects the oxidative potential of soil microorganisms developing in the Ecoplate wells and it can be used to assess microbial activity [94, 233].

$$\text{AWCD} = \frac{\sum_{i=1}^{31}(\text{A}i-\text{A0})}{31} \qquad (3.2)\ [179, 183, 234]$$

Where A_i represents the absorbance reading of well *i* and A_0 is the absorbance reading of the blank well (inoculated but without a carbon source). Absorbance values for the wells with C sources were blanked against the control well. Negative values were considered as 0 in subsequent data analyses.

The Shannon diversity index is a common ecological metric used to track and understand shifts in communities over space and time. It gives the information about the diversity of metabolised carbon substrates through the generated absorbance data. Using the carbon substrate utilization profile (CSUP) gathered from a single BIOLOG Ecoplate plate, substrate diversity (H) was calculated as:

$$H = -\sum p_i \ln(p_i) \quad (3.3)\ [232, 234]$$

Where:

H - Substrate diversity

p_i - Ratio of the activity of a particular substrate to the sums of activities of all substrates

Activity - chosen metric for analysis (absorbance value at 50hrs and 72hrs)

Two other parameters associated with substrate diversity which can be calculated using the generated absorbance data are substrate richness (S) and substrate evenness (E). Substrate richness can be described as a measure of the number of different substrates utilized by a microbial population. Substrate evenness can also be defined as the equitability of activities across all utilized substrate. Substrate richness was calculated as the number of wells with a corrected absorbance greater than 0.25 [180]. Substrate evenness was calculated as:

$$E = H / H_{\max} = H / \log S \quad (3.4)\ [120]$$

3.3.8.6.1 Data standardization

Standardization of the data involves rectifying of each absorbance value by its corresponding blank value and afterwards dividing by the AWCD for that time point. Standardization can also be described as the normalization of the corrected absorbance value by the AWCD. It allows the comparison of microbial communities from soils described by different inoculum density [237]. The standardized absorbance for well *k* can be calculated as:

$$\overline{A_k} = \frac{A_k - A_0}{\frac{1}{31}\sum_{i=1}^{31}(A_i - A_0)} \quad (3.5)\ [234, 264]$$

Where A_i represents the absorbance reading of well *i* and A_0 is the absorbance reading of the blank well (inoculated but without a carbon source). Where there is a

very little response in a well, negative values of standardized absorbance may occur and, since this is physically meaningless, they are coded as zero for further analysis. Standardised data are usually used for multivariate analysis.

Multivariate statistical techniques were later used in Chapter 6 to compare the generated samples absorbance data given the large number of variables (93) per a single BIOLOG Ecoplate.

3.3.9 Experimental design for coal tar pitch and heavy metal soil spiked main study (pilot):

Thirty-six microcosms made from sawn length of PVC tubes each with a diameter of 3.2 cm and a length of 30 cm was divided into four groups of A, B, C and D with 3 replicates within each group subset. The PVC were filled with 180 g of either coal tar pitch spiked or non-coal tar pitch spiked (control). Some of the coal tar pitch spiked soil was also spiked with lead (Pb) or cadmium (Cd) heavy metal which was represented in the group. The full descriptions of the groups are shown below and Figure 3.9 shows a schematic experimental protocol that was used for the coal tar pitch spiked soil study. Each group microcosm replicate of three was harvested for analysis at 24 hrs, 2 weeks and 4 weeks

Group A = control soil,

Group B = Coal tar pitch spiked (PAH) control soil,

Group C = Coal tar pitch (PAH) soil with 250 mg kg^{-1} of Pb,

Group D = coal tar pitch (PAH) soil with 250 mg kg^{-1} of Cd,

The soil water content was adjusted to 75 % of its water holding capacity and maintained at 75 % during the experiment by monitoring the weight of each PVC microcosms.

3.3.9.1 Coal tar pitch soil spiking experiment:

3.3.9.1.1 PAH spiking with coal tar pitch:

In total 11 kg of coal tar pitch spiked soil was required for the pilot biodegradation experiment. The coal tar pitch was dissolved in acetone as the carrier solvent. A total of 392.81 g powdered (by grinding and sieving to 2 mm) coal tar pitch was

dissolved in 1.1 litres of acetone (100 mL of acetone/kg of soil) [78] in a 2.5 litre Winchester bottle. The coal tar pitch was allowed to dissolve over 48 hours, during which time the bottle was shaken regularly. The adopted method of spiking was dilution method [226] where the solvent was applied to only one-quarter of the total required soil i.e. 2.75 kg in batches using a cement mixer. The spiked soil was allowed to air dry in an open area outside the laboratory for 24 hours for the acetone to completely evaporate. The remaining 9.014 kg was then mixed with the spiked soil using a cement mixer (Figure 3.10) to ensure a thorough mixing of the soil. By initially spiking only one-quarter of the soil with acetone/coal tar pitch any detrimental effects of the solvent on the soil microbial population was minimised. The total concentration of the 16 PAH compounds that was embarked to be accomplished from the coal tar pitch soil spiking process was 2500 mg kg^{-1}. The coal tar pitch control soil was transferred into the PVC pipe that was used for the study.

Figure 3.10: Soil spiking and mixing using an electric cement mixer

3.3.9.1.2 Heavy metal spiking:

3.3.9.1.2.1 Lead (Pb) spiking:

180 g of the coal tar pitch spiked soil was further spiked with 250 mg kg^{-1} of Pb. $PbCl_2$ was used as the source of Pb metal in the coal tar pitch spiked soil. The water spike/mixing method was adopted for heavy metal spiking [226] where 0.0604 g of $PbCl_2$ was dissolved in 50.4 mL of distilled water that was needed to adjust the soil to 75 % of its water holding capacity and the resulting solution was thoroughly mixed with the coal tar pitch spiked soil using the electric cement mixer. The resultant mixture was expected to yield a Pb concentration of approximately 250 mg kg^{-1} dry weight or more in the soil. EDXRF analysis on the spiked soil was carried out immediately to check and confirm the Pb concentration. The Pb-spiked soil was transferred into the PVC pipe that was used for the pilot study.

3.3.9.1.2.2 Cadmium spiking:

180 g of the coal tar pitch spiked soil was also spiked with 250 mg kg^{-1} of Cd. $CdCl_2.2½H_2O$ was used as the source of Cd metal in the coal tar pitch spiked soil. 0.0914 of $CdCl_2.2½H_2O$ was dissolved in 50.4 mL of distilled water and the spiking was carried out using the same method that was used for lead. The resultant mixture was expected to yield a Cd concentration of approximately 250 mg kg^{-1} dry weight or more in the soil. EDXRF analysis on the spiked soil was carried out immediately to check and confirm the Cd concentration. The Cd spiked soil was transferred into the PVC pipe that was used for the pilot study.

3.3.9.2 Incubation and storage:

The unspiked control PVC microcosm, coal tar pitch and heavy metal spiked PVC pipe microcosms was stored in an aerated and moisture regulated plastic box that was incubated at a controlled temperature between 18 °C – 20 °C (plant room). The top lid of the plastic box was perforated to allow for easy air circulation and two beakers of water with a wet paper towel were used to regulate the loss of moisture in the box. The water content of the soil was maintained at 75 % WHC at 7 days intervals by checking the weight difference of the PVC pipes. PVC microcosms for each treatment with its replicates (3 replicates) were harvested for analysis after 1 day, 14 days and 28 days subsequent to spiking. Figure 3.11 shows the set-up for the storage box used to house the microcosms.

Figure 3.11: PVC microcosm storage box used during the pilot study of PAH bioremediation in soil

3.3.10 PAH extraction and analysis

3.3.10.1 Pressurised fluid extraction soil preparation protocol:

The protocol used for the ASE during the study was as follows;
2 g of Alumina (Al_2O_3) was added into an ASE 22 mL extraction cell, on top of filter paper (in-situ clean up), the filter at the outlet was to prevent the clogging of the metal frit. Then 10 g of soil (ground and sieved using <250 µm sieve) was mixed with an equivalent quantity of hydromatrix (Varian) and was added into the 22 mL extraction cell on top of the Alumina. Any free space in the cell was filled with an additional hydromatrix and a final filter paper was placed on top, after which the cell is closed. The 22 mL cell containing the soil sample was then transferred to the ASE extraction chamber where extraction was carried out. After the ASE procedure, the extracted solution was reconstituted in 100 mL, including 10 mL of internal standard (10 mg L^{-1} 4, 4'-difluorobiphenyl in DCM) for the GC-MS analysis. 2 mL of the diluted extract with internal standard was then transferred into a 2 mL

Silanized GC auto sample vials (Chromacol; Sigma-Aldrich) and analysed in the GC-MS.

3.3.10.2 Gas chromatography-mass spectrometer (GC-MS):

The PAHs concentration (mg kg^{-1}) in soil samples were quantified by GC-MS, using an internal standard calibration procedure according to the predetermined extraction conditions. The concentration of the internal standard (4, 4'-difluorobiphenyl) was fixed at 10 mg L^{-1} in the calibration solutions and in the solutions extracted from the spiked soil. Six PAH calibration points were used: 0, 1, 2, 5, 10 and 15 mg L^{-1}. In addition, after each group of 10 samples had been run, a 5 mg L^{-1} and 10 mg L^{-1} PAH standard and the solvent blank were run so as to assess the response of the instrument [47]. All the measured PAH concentrations in the soil were reported in mg kg^{-1}, dry weight.

3.3.11 Experimental procedure for heavy metal analysis:

Total and bioavailable Pb and Cd concentrations were determined for the spiked soil. The total heavy metal concentration was determined using Energy Dispersive X-ray Fluorescence (EDXRF). The soil bioavailable Pb and Cd concentration were determined using a modified version of the ethylenediaminetetraacetic acid (EDTA) extraction method: rather than determining the concentration of the extracted soluble metal, the metal concentration in soil was determined by EDXRF before and after EDTA extraction. The calculation for the bioavailable Pb and Cd in their respective soils was then done by subtracting the soil residue heavy metal concentration from the total heavy metal concentration.

3.3.11.1 Total heavy metal concentration determination by EDXRF

Soil samples were air dried in the fume cupboard. Soil subsamples were powdered in an agate ball mill. 4 g of the powdered sample was weighed out and mixed with 0.6 g Hoechst wax (Licowax C micro powder) binding powder using an orbital shaker for 1 minute. The soil/binder mixtures were compressed into pellet forms using a hydraulic press that applied 10 tonnes of pressure for 30 seconds. The formed soil pellets were analysed in the EDXRF using a geology 5-target program

3.3.11.2 EDTA extraction:

0.05 M EDTA was prepared as ammonium salt solution by adding in a beaker 14.61 g EDTA to 80 mL ultra-pure water. The dissolution was achieved by gradually adding 13 mL of ammonia solution (25 %). The solution was then transferred to a 1litre polyethylene container and approximately 900 mL of ultra-pure water was added. The pH of the solution was adjusted to 7.0 by adding a few drops of HCl as appropriate and the solution was made up to 1litre with ultra-pure water.

2 g of soil sample was weighed into a 50 mL centrifuge tube and 20 mL of 0.05 M EDTA (pH 7.0) was added. The mixture was shaken in an end over end shaker at 30 rpm for 1 hr at room temperature. The supernatant from the mixture was filtered through using a Whatman filter paper. The residue after the filtration was dried and used to determine the heavy metal concentration that was not extracted with the EDTA solution. The heavy metal concentration determination was carried out using EDXRF.

3.3.11.3 EDTA residue soil sample preparation for EDXRF

Soil samples were air dried in the fume cupboard. The soil sample was powdered using pestle and mortar. 1.5 g of the soil sample was weighed out and analysed in the EDXRF using a geology 5-target program.

3.3.12 Coal tar pitch soil spiked respiration:

Soil respiration tests were carried out on the pilot study microcosms at 24 hrs, 2 weeks and 4 weeks after spiking. The soil respiration test was carried out in triplicate for each treatment but the arrangement was staggered to accommodate all the replicates because the respiration incubation chamber can only accommodate six Oxi-Top vessels at a time. The groups were as follows. Group A: coal tar pitch unspiked control; Group B: coal tar pitch spiked control, were both tested the same time. After two days Group C: coal tar pitch spiked soil with Pb metal spiking and Group D: coal tar pitch spiked soil with Cd metal spiking was also tested.

3.3.12.1 Soil respiration experimental procedure:

The same soil respiration experimental procedure used in section 2.5.8.5 was used for this section

3.3.12.2 Data analysis for soil respiration:

The details of the soil respiration data analysis can be found in section 2.5.8.7

3.3.13 Community level physiological profile assessment on coal tar pitch pilot study:

The Biolog Ecoplate was used to assess the effect of heavy metal and coal tar pitch (PAH) on the microbial community functional diversity. The Ecoplate assessment was performed on all the groups detailed in the experimental design section, 3.2.5. Ecoplate assessments were also performed in replicates for each of the groups according to the analytical periods of the study

3.3.13.1. BIOLOG Ecoplate experimental procedure:

The details of the Ecoplate experimental procedure can be found in section 2.5.10

3.3.13.2 Data analysis for BIOLOG Ecoplate

The details of the data analysis can be found in section 2.5.10.

3.3.14 Microbial biomass experiment

The microbial biomass assay was done in two different stages: the first stage; was the soil organic carbon extraction stage and the second stage was the total soil organic carbon concentration determination. The determined organic carbon concentration was converted into soil microbial biomass carbon (SMBC). The first experiment under this section was to test the accuracy of the method in determining the total soil organic carbon concentration (TOC method) using the HACH-LANGE digestion vials and instrument.

3.3.14.1 Test of the accuracy of total organic carbon method

10 mg L^{-1} carbon standard solution was prepared from a 1000 mg L^{-1} carbon stock standard by diluting 10 mL of the stock solution with 1000 mL ultra-pure water in a 1000 mL volumetric flask. 3 mL of the prepared 10 mg L^{-1} carbon standard was added into a mid-range digestion vial. One persulfate powder pillow was also added into the digestion vial and an indicator ampule was inserted into the digestion vial. The digestion vial was heated in a digester for two hours and allowed to cool for one hour. 3 mL of ultra-pure water was added into another digestion vial to serve as a test blank and the same procedure used for the digestion vial containing the standard solution was repeated for test blank. After cooling the digestion vials were inserted into a spectrophotometer (HACH program 427) where the organic carbon concentration was read. The test blank digestion vial was first inserted and zeroed before the digestion vial containing the standard solution was inserted and read.

3.3.14.2 Determination of microbial biomass on the coal tar pitch spiked soil

The microbial biomass carbon determination for the coal tar pitch spiked and unspiked soils involved two processes; the soil organic carbon extraction process and the determination of the extracted soil organic carbon concentration.

3.3.14.2.1. Extraction process:

Two soil samples of 10 g field moist weight were used for each sample in the experimental design groups: one of the 10 g soil samples was subject to fumigation with $CHCl_3$ to kill the microbial biomass and the other was a control. The 10 g field moist soil samples were adjusted to its 60 % water holding capacity.

The 10 g for fumigation was placed in 50 mL glass beaker and placed in a large desiccator (30.5 cm internal diameter)

The desiccator was lined with moist paper and contained a beaker with 20 mL of alcohol-free $CHCl_3$ and a few anti-bumping granules.

The desiccator was evacuated until the $CHCl_3$ boiled vigorously, (usually for 2 minutes) the tap closed and the desiccator was kept in the dark at 25 °C for 24 hrs. (The desiccator was covered with a black plastic bag).

At the end of 24 hrs the beaker of $CHCl_3$ and the paper was removed from the desiccator and the $CHCl_3$ vapour removed from the soil by repeated (5 times) evacuation using a vacuum pump to ensure that all the chloroform was vented.

The sample was extracted in 50 mL K_2SO_4 in a horizontal shaker at 200 rpm for 30 min.

The sample was filtered through a pre-leached (with 0.5 M K_2SO_4) Whatman No.1 filter paper.

A similar extraction protocol was carried out on the non-fumigated soil sample after 24 hrs of being kept in the dark without chloroform. The soil extractant ratio has been shown to have no significant effect on the extractable carbon flush [167]. Tate *et al.* [167] compared extracted carbon from this method with extractable carbon from sonication method and observed an indistinguishable extractable carbon between the two methods for some of the soils they used. The soil extract from both the fumigated and non-fumigated samples were analysed for organic carbon concentration using the total organic carbon direct method protocol.

3.3.14.2.2 Determination of the organic carbon concentration:

10 mL of each soil extract was added to a 50 mL Erlenmeyer flask that contained a magnetic stirrer bar. An additional 0.4 mL of pH 2.0 buffer solution was added to the soil extract solution to adjust the pH 2.0. The flask was placed on a magnetic stirring plate and stirred at a moderate speed for 10 minutes. The COD (chemical oxygen demand) reactor was turned on and heated to 105 °C. Mid-range acid digestion vials were labelled according to each soil treatment and one mid-range acid digestion vial was labelled as a reagent blank. TOC persulfate powder pillows were added to each acid digestion vial (colourless liquid). 1.0 mL of ultra-pure water was added to all sample vials and reagent blank vial using an auto sample pipet. The vials were swirled to give a homogenous mixture and blue indicator ampules rinsed with ultra-pure water and dried with a lint-free wipe was inserted into all the vials. The vials were capped tightly and placed in the COD reactor for 2 hours at 105 °C. The vials were removed from the reactor and placed in a test tube rack to cool for one hour. The cooled vials were measured using a Hach-Lange spectrophotometer.

3.3.14.2.3 The principle of direct method protocol:

This method was used because it is an independent method (independent of soil respiration), unlike substrate-induced respiration, which is based on soil respiration. The principle of operation of this method is to agitate the sample under moderately acidic conditions to eliminate the inorganic carbon. In the external part of the vial, organic carbon in the sample is broken down by the persulfate as well as the acid to form carbon dioxide. During the course of digestion, the carbon dioxide diffuses into the pH indicator reagent in the inner ampule. The adsorbed carbon dioxide is in equilibrium with carbonic acid. Carbonic acid alters the pH of the indicator solution which then alters the colour. The degree of colour alteration is comparable to the original measure of carbon contained in the sample. Absorbance is then be measured at 430 and 598 nm

The microbial biomass carbon was determined by using Equation 3.8

$$\mathrm{C\ (mg/l)} = \frac{EC}{Kec} \tag{3.8}$$

Where:

EC = the difference between the organic carbon concentrations in the fumigated and non- fumigated samples

K_{ec} = soil specific factor for converting extractable carbon to biomass carbon and often estimated as 0.45 [170]; it corrects for the incomplete release and extraction of the microbial carbon and was obtained by calibrating against alternative methods to estimate the microbial carbon [170]

3.3.15 Metabolic quotient (qCO_2)

The metabolic quotient which is conversely identified with the efficacy with which microbial biomass utilises indigenous substrates [140, 144] was determined by dividing the soil respiration values by the soil microbial biomass values as shown in Equation 3.9

$$\text{Metabolic Quotient } (qCO_2) = \frac{\text{Soil basal respiration}}{\text{Soil microbial biomass carbon}} \tag{3.9}$$

3.4 Results and discussion

3.4.1. Characterisation of soil sample:

Soil chemical and physical properties were evaluated so that we can compare our results with similar studies in the literature. The Tables 3.4 and 3.5 show the values obtained for each chemical and physical property evaluated:

3.4.2. Soil texture values:

Table 3.4: The percentage composition of the various soil fractions that make up the soil texture

Clay %	Fine Silt %	Medium silt %	Coarse Silt %	Coarse Sand %	Medium Sand %	Fine Sand %
6.02	11.3	15	9.38	1.49	14.6	38.7

Table 3.5: The result of soil sample properties measured

PARAMETER	RESULTS
% Maximum Water holding Capacity	43.2 %
75 % Water holding Capacity	32.4 %
% Nitrogen	0.37 %
% Organic Carbon by Loss on Ignition	11.1 %
% Organic matter by LOI	19.1 %
Soil pH	6.02

The soil texture values show that the soil has sandy loam characteristic. Sandy loam soil is usually ideal for microbial activity because it allows for easy circulation of air, water and nutrient. The soil also has an appreciable amount of organic carbon. The organic carbon also serves as nutrient for the microbial community because carbon is one of the essential element for microbial growth [94].

3.4.3 Calibration of PAHs:

Calibration curves for PAH standard solutions based on a concentration range of 0-15 mg L^{-1} with 6 calibration data points were determined using GC-MS. The regression coefficients (R^2) obtained for each of the 16 US EPA PAH compounds were greater than 0.999. The high value of the regression coefficient for each 16 PAH compounds as shown in Table 3.6, established the reliability of the GC-MS and the standard solutions for the purpose of identifying and quantifying unknown PAH compounds concentrations. These 16 US EPA PAH standards were used for instrument optimization and after optimizing the GC-MS parameters, a suitable temperature program was found for the analysis of 16 PAHs. Peaks were sharp, isomers were well separated, and peak intensities were good for a 10 mg L^{-1} PAH standard concentration as shown in Figure 3.12.

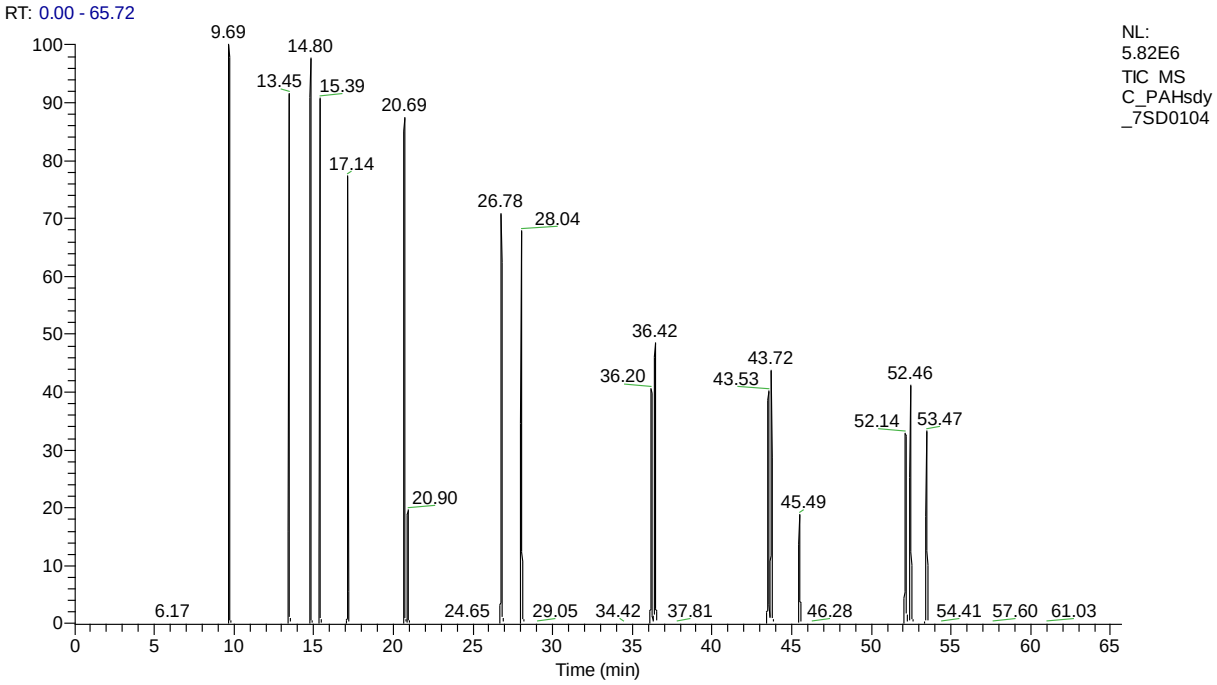
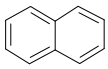
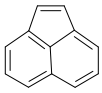
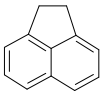
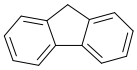
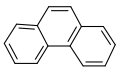
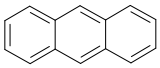
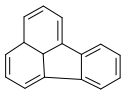
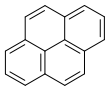
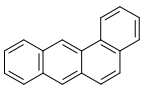
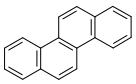
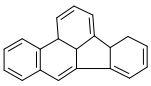
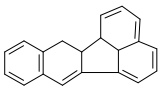
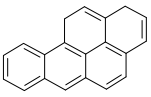
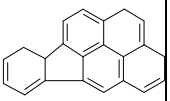
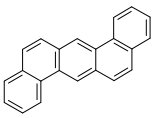
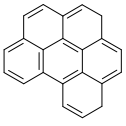


Figure 3.12: Example of chromatogram with 16 separated PAH peaks obtained with a 10 mg L^{-1} PAH standard solution

Table 3.6: GC-MS calibration of 16 USEPA PAHs from a six point graph derived using six PAH standard solutions (0 mgL^{-1} -15 mgL^{-1})

PAH	Structure	Empirical Formulae	Ms ion for Quantitation	Retention time (minutes)	Calibration Regression y=mx +c	R^2
Naphthalene (NAP)		$C_{10}H_8$	128	9.69	0.1024X – 0.0171	0.9997
Acenaphthylene (ACY)		$C_{12}H_8$	152	14.80	0.0988X – 0.0065	0.9996
Acenaphthene (ACE)		$C_{12}H_{10}$	154	15.39	0.0966X + 0.0014	0.9992
Fluorene (FLU)		$C_{13}H_{10}$	166	17.14	0.0729X – 0.00148	0.999
Phenanthrene (PHE)		$C_{14}H_{10}$	178	20.69	0.1204X – 0.0204	0.9997
Anthracene (ANT)		$C_{14}H_{10}$	178	20.90	0.0646X – 0.0013	0.9996
Fluoranthene (FLUH)		$C_{16}H_{10}$	202	26.78	0.1334X – 0.0245	0.9996
Pyrene (PYR)		$C_{16}H_{10}$	202	28.04	0.1424X – 0.0242	0.9997
Benzo[a]anthracene (BaA)		$C_{18}H_{12}$	228	36.20	0.0976X – 0.0225	0.9995
Chrysene (CHY)		$C_{18}H_{12}$	228	36.42	0.0969X – 0.023	0.9995
Benzo[b]fluoranthene (BbF)		$C_{20}H_{12}$	252	43.53	0.0886X – 0.0195	0.9995
Benzo[k]fluoranthene (BkF)		$C_{20}H_{12}$	252	43.72	0.0858X – 0.0175	0.9996
Benzo[a]pyrene (BaP)		$C_{20}H_{12}$	252	45.49	0.0533X – 0.0099	0.9996
Indeno[123-cd]pyrene (IDP)		$C_{22}H_{12}$	276	52.14	0.1119X – 0.0255	0.9995
Dibenzo[a,h]anthracene (DBA)		$C_{22}H_{14}$	278	52.46	0.1353X – 0.0442	0.999
Benzo[g,h,i]perylene (BgP)		$C_{22}H_{12}$	276	53.47	0.1178X – 0.0243	0.9996

3.4.4 Certified soil reference material analysis

The robustness of the analytical procedure (ASE and GC-MS analysis) was tested using one CRM analysed in triplicate. The measured concentrations of 16 US EPA PAH compound based on three replicates are shown in Table 3.7. It can be seen that the measured PAH concentrations compare favourably with the certified values. The closeness of the measured value to the certified reference material materials was an indication that the developed instrument conditions and setting would yield accurate and reproducible results.

Table 3.7: Measured PAH concentration in a certified reference material (CRM LGC QC 3008)

PAHs	**CRM LGC QC 3008 (sandy soil 2)**			
	Measured (+/- SD) n=3 (mg kg^{-1}) RSD		Certificate Value (+/-SD) n=3(mg kg^{-1})	Certificate RSD
Naphthalene	3.9 ± 0.6	16	3.1±0.9	28
Acenaphthylene	2.5 ± 0.7	29	3.4 ±1.6	47
Acenaphthene	1.9 ± 0.3	16	<2	-
Fluorene	6.3 ± 1.1	18	7.7 ±1.7	22
Phenanthrene	34.9 ± 0.8	2	34 ± 7.1	21
Anthracene	6.2 ± 0.2	4	5.9 ±2.1	35
Fluoranthene	33.2 ± 0.9	3	32 ± 6.4	20
Pyrene	23.2 ± 1.0	4	24 ± 6.5	27
Benzo(a)anthracene	9.3 ± 1.5	16	11 ± 2.5	23
Chrysene	7.8 ± 1.9	24	9.9 ± 2.1	21
Benzo(b)fluoranthene	9 ± 0.3	4	9 ± 3.3	37
Benzo(k)fluoranthene	5.3 ± 0.5	10	5.8 ± 2.1	37
Benzo(a)pyrene	6.9 ± 1.3	20	8.2 ± 1.8	22
Indeno(1,2,3-cd)pyrene	4.7 ± 0.8	16	5.2 ± 1.8	35
Dibenzo(a,h)anthracene	1.1 ±0.4	34	<2	-
Benzo(g,h,i)perylene	4.6 ± 1.0	21	5.2 ± 1.8	35

3.4.5 Preliminary spiking analysis

Analysis of soil spiked with 5 mg kg^{-1} 16 US EPA PAH standard solutions gave average recoveries > 50 % for mid-molecular weight PAHs (fluorene to pyrene) and also > 75 % recoveries for the heavier molecular weight PAHs i.e. benzo(a)anthracene to benzo(ghi)perylene. For the lightest, i.e. small molecular weight PAHs, recoveries of 22.8 % for naphthalene, 43.6 % for acenaphthylene and 46.8 % for acenaphthene were obtained (Table 3.8). Typical RSDs for the recovery of PAHs, using the established instrument conditions and method ranged from 1.9 to 15.9 %. The low recovery percentage of naphthalene and the other low

molecular weight PAHs can be as a result of their volatilisation. PAH undergoes volatilisation and chemical oxidation when exposed in the environment [92]. It has also been suggested that in a soil with organic carbon >0.1 % the sorption of non-ionic organic contaminants will be controlled by organic matter [71]. Sawada et al. [224] in their spiking study observed the complete loss of naphthalene in their spiked soil which they attributed to volatilization of the compound and also the organic carbon in their soil which was about 8.8%. The organic carbon in the spiked soil used in the present study was 11.06 %. Therefore, the loss of low molecular weight PAH in the spiked soil could also be due to the rapid adsorption of the PAHs into the organic matter in the soil.

Table 3.8: Measured PAH concentration from spiked soil and its percentage recovery

PAHS	Measured Concentration of PAH spiking standard solution	Measured Concentration of PAH spiked soil (mg kg^{-1})	% Recovery	% RSD n=3
Naphthalene	5.7	1.3	22.8	15.9
Acenaphthylene	5.5	2.4	43.6	6.5
Acenaphthene	5.7	2.7	46.8	3.8
Fluorene	5.8	3.2	55.8	1.9
Phenanthrene	5.4	4.3	78.9	12.7
Anthracene	5.1	4.5	87.2	11.2
Fluoranthene	5.4	4.9	91.0	9.1
Pyrene	5.4	4.6	86.1	8.5
Benzo(a)anthracene	5.0	4.1	81.8	9.5
Chrysene	5.3	4.3	80.3	10.2
Benzo(b)fluoranthene	5.2	4.5	85.9	11.3
Benzo(k)fluoranthene	5.1	3.9	75.1	9.5
Benzo(a)pyrene	5.0	5.0	99.8	8.6
Indeno(1,2,3-cd)pyrene	5.2	4.1	78.8	10.4
Dibenzo(a,h)anthracene	5.2	3.7	72.3	12.7
Benzo(g,h,i)perylene	5.3	4.1	76.8	11.2

3.4.6 Preliminary coal tar and coal tar pitch spiking analysis

The preliminary analysis that was carried out on coal tar (Table 3.9) and coal tar pitch to determine PAH concentrations showed that coal tar contained a high concentration of light molecular weight PAH (naphthalene to acenaphthene) compared to their concentration in coal tar pitch (Table 3.10). Coal tar pitch contains a higher percentage concentration of mid to high molecular weight PAH compared to coal tar. This trend can also be seen in the percentage concentrations of PAHs in both coal tar and coal tar pitch based on literature studies, as shown in Table 3.13 and 3.14. There are always differences in PAH composition and concentration among coal tar and coal tar pitch. The difference in PAH content of coal tar pitch is based on the grade of coal tar used during distillation. The PAH

content of coal tar itself is also influenced by the characteristics of coke batteries and the tar processing technology employed during coke production [13]. The essence of the analysis was to determine the product that had a more comprehensive composition and concentration of 16 US EPA PAH compounds. Based on their respective PAH compositions it can be therefore be said that coal tar pitch has a higher composition and concentration of PAH compounds than coal tar. The extractability of the PAH from coal tar pitch (Table 3.11) and PAH extractability from soil spiked with coal tar pitch (Table 3.12) was also determined and it shows that coal tar pitch still has a high percentage composition and concentration of extractable 16 US EPA PAH compounds. This was the major reason that determined the choice of coal tar pitch as the soil spiking product for the PAH bioremediation study.

The GC-MS chromatogram in Figure 3.13 also shows a high peak for the high molecular weight PAHs compounds extracted from a coal tar pitch spiked soil. The peaks were identified by comparing the mass ions and retention times of eluted compounds against the eluted mass ion and retention times of a 16 PAH standard concentration. The identified compounds were quantified by using a 5 PAH standard solutions calibrating curve for each of the 16 PAH compounds. The higher peak signifies ion abundance of the high molecular weight PAHs (e.g. FLUH, Ms ion = 202) in the coal tar pitch which can be interpreted to mean a high concentration of the high molecular weight PAH compounds present compared to the low molecular weight compounds (e.g. ACE, Ms ion = 154). The retention times for the PAH compounds with higher mass ions (e.g. Bap, RT = 45.48) have more residence time in the GC column than the compounds with a low mass ions (e.g. ACE = 15.39). The retention is measured as the time distance between the sample injection and compound detection [256] and the compounds separation is based on the vapour pressure of the volatilised PAH compounds and also on their predilection with the stationary phase, which enshrouds the internal surface of the silica column support as they pass down the column in a carrier gas (helium).

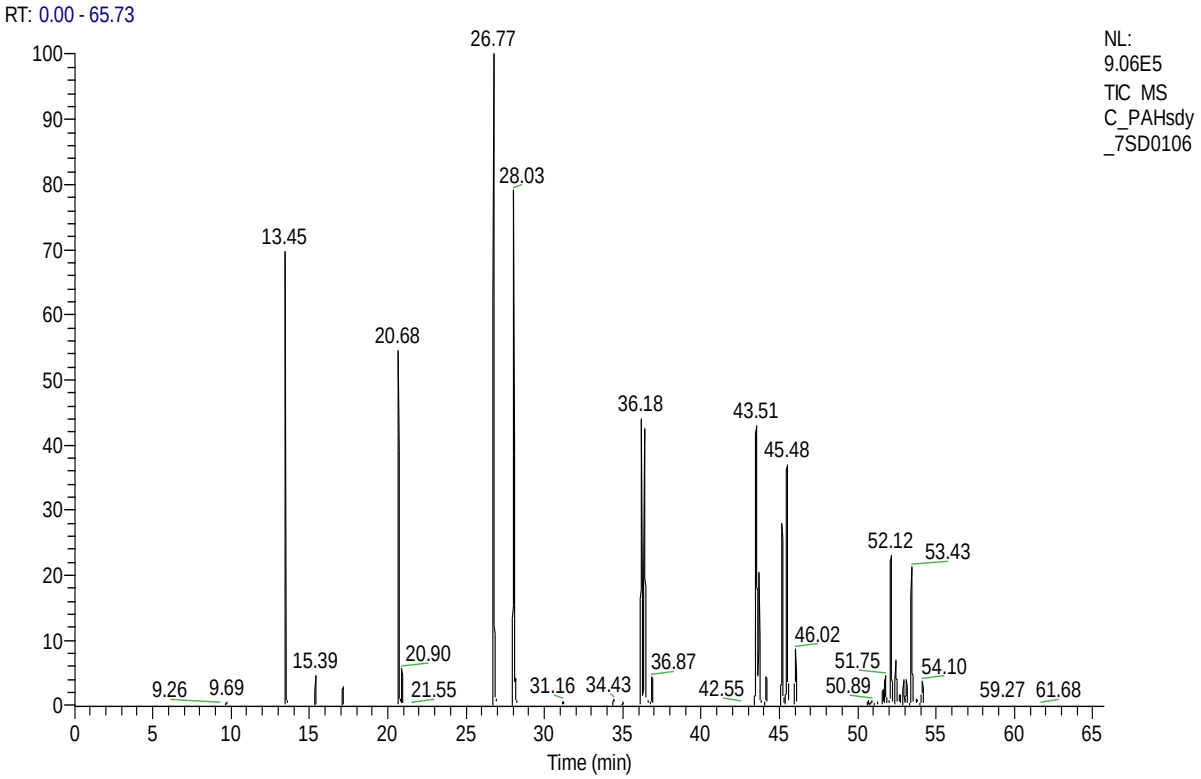


Figure 3.13 An illustration of a GC-MS chromatogram of PAH compounds extracted from Coal tar pitch spiked soil, showing the PAH compounds peak height (later converted to peak areas) and their retention times (times of elution).

Table 3.9: Measured concentration of PAH in coal tar

PAHs	Content of polycyclic aromatic hydrocarbon in coal tar		
	mg kg^{-1}	wt %	RSD % n=3
Naphthalene	4780	0.5	2.0
Acenaphthylene	68	0.0	1.1
Acenaphthene	4160	0.4	1.9
Fluorene	2610	0.3	2.4
Phenanthrene	1200	0.1	1.2
Anthracene	614	0.1	6.8
Fluoranthene	1900	0.2	3.2
Pyrene	1520	0.2	3.2
Benzo(a)anthracene	1280	0.1	13.8
Chrysene	817	0.1	8.4
Benzo(b)fluoranthene	1230	0.1	6.7
Benzo(k)fluoranthene	463	0.1	4.8
Benzo(a)pyrene	1420	0.1	8.1
Indeno(1,2,3-cd)pyrene	663	0.1	11.2
Dibenzo(a,h)anthracene	186	0.0	9.2
Benzo(g,h,i)perylene	518	0.1	11.0

Table 3.10: Measured concentration of PAH in coal tar pitch

PAHs	Content of polycyclic aromatic hydrocarbon in coal tar pitch		
	mg kg^{-1}	wt %	RSD % n=3
Naphthalene	162	0.0	1.6
Acenaphthylene	80	0.0	17.1
Acenaphthene	573	0.1	6.3
Fluorene	639	0.1	13.0
Phenanthrene	3290	0.3	0.1
Anthracene	1590	0.2	6.7
Fluoranthene	9480	0.9	4.8
Pyrene	8940	0.9	3.3
Benzo(a)anthracene	12000	1.2	5.9
Chrysene	7580	0.8	5.6
Benzo(b)fluoranthene	13600	1.4	5.3
Benzo(k)fluoranthene	5550	0.6	5.0
Benzo(a)pyrene	13700	1.4	17.5
Indeno(1,2,3-cd)pyrene	10100	1.0	14.8
Dibenzo(a,h)anthracene	2070	0.2	13.2
Benzo(g,h,i)perylene	7520	0.8	12.6

Table 3.11: Measured concentration of PAH in ASE extracted coal tar pitch

PAHs	Concentration of polycyclic aromatic hydrocarbon extracted from coal tar pitch		
	mg kg^{-1}	wt %	RSD % n=3
Naphthalene	483	0.1	11.2
Acenaphthylene	7540	0.1	1.2
Acenaphthene	2810	0.3	5.5
Fluorene	992	0.1	3.0
Phenanthrene	10900	1.1	3.5
Anthracene	3150	0.3	7.7
Fluoranthene	13300	1.3	3.8
Pyrene	11500	1.1	3.6
Benzo(a)anthracene	19000	1.9	8.3
Chrysene	10700	1.1	1.6
Benzo(b)fluoranthene	18400	1.8	1.1
Benzo(k)fluoranthene	6990	0.7	15.0
Benzo(a)pyrene	18800	1.9	6.2
Indeno(1,2,3-cd)pyrene	10200	1.0	2.8
Dibenzo(a,h)anthracene	4760	0.5	7.2
Benzo(g,h,i)perylene	8710	0.9	5.5

Table 3.12: Measured concentration of PAH extracted from soil for preliminary coal tar pitch soil spiking

PAHs	Concentration of polycyclic aromatic hydrocarbon in Preliminary coal tar pitch spiked soil		
	mg kg^{-1}	SD n=3	RSD %
Naphthalene	0.9	0.1	8.9
Acenaphthylene	2.8	0.3	10.0
Acenaphthene	16.6	1.0	5.7
Fluorene	3.8	0.2	4.7
Phenanthrene	90.9	2.2	2.4
Anthracene	26.2	2.1	8.1
Fluoranthene	128.0	5.4	4.2
Pyrene	114.0	2.9	2.5
Benzo(a)anthracene	209.0	18.1	8.7
Chrysene	113.0	6.6	5.9
Benzo(b)fluoranthene	245.0	9.0	3.7
Benzo(k)fluoranthene	99.9	4.8	4.8
Benzo(a)pyrene	290.0	3.9	1.3
Indeno(1,2,3-cd)pyrene	169.0	1.0	0.6
Dibenzo(a,h)anthracene	66.4	3.5	5.3
Benzo(g,h,i)perylene	128.0	9.6	7.5

Table 3.13: Percentage concentration of PAH in coal tar from literature studies [248]

PAHs	Range of PAH (wt %) content in 4 different coal tar product	
	Coal tar PAH (wt %) Range	Mean (wt %)
Naphthalene	6.7 – 14.3	9.7
Acenaphthylene	0.9 – 1.0	1.0
Acenaphthene	0.2 – 1.0	0.6
Fluorene	0.9 – 1.3	1.1
Phenanthrene	3.0 – 4.8	3.6
Anthracene	0.6 – 0.8	0.6
Fluoranthene	2.0 – 3.3	2.4
Pyrene	1.2 – 2.1	1.5
Benzo(a)anthracene	0.5 – 0.8	0.6
Chrysene	0.6 – 1.0	0.7
Benzo(b)fluoranthene	0.5 – 0.9	0.6
Benzo(k)fluoranthene	0.2 – 0.3	0.2
Benzo(a)pyrene	0.4 – 0.7	0.5
Indeno(1,2,3-cd)pyrene	0.3 – 0.5	0.4
Dibenzo(a,h)anthracene	0.1 – 0.1	0.1
Benzo(g,h,i)perylene	0.2 – 0.4	0.3

Table 3.14: Percentage concentration of PAH in coal tar pitch from literature studies [248]

PAHs	Range of PAH (wt %) content in 5 different coal pitch product	
	Coal tar pitch PAH (wt %) Range	Mean (wt %)
Naphthalene	-	-
Acenaphthylene	-	-
Acenaphthene	0.1 -0.3	0.2
Fluorene	0.1 – 0.2	0.1
Phenanthrene	0.9 – 1.3	1.1
Anthracene	0.2 – 0.3	0.2
Fluoranthene	1.5 – 2.1	1.8
Pyrene	1.4 – 1.7	1.6
Benzo(a)anthracene	0.7 – 0.9	0.8
Chrysene	1.0 – 1.4	1.1
Benzo(b)fluoranthene	1.2 – 1.5	1.3
Benzo(k)fluoranthene	0.4 – 0.5	0.5
Benzo(a)pyrene	0.9 – 1.1	1.0
Indeno(1,2,3-cd)pyrene	0.7 – 1.1	0.9
Dibenzo(a,h)anthracene	0.1 – 0.2	0.2
Benzo(g,h,i)perylene	0.5 – 0.9	0.6

3.4.7 Coal tar pitch soil spiking

The dilution method of spiking adopted for the present study yielded a high homogeneity of the PAH compounds in the spiked soil. The relative standard deviation for the spiking ranged from 0.3 % to 3.6 % for all the PAH compound as shown in Table 3.14. Reid *et al.* [236] adopted this method in their protocol for spiking wet soil with B(a)P and PHE and obtained a high degree of the PAH compound homogeneity with RSD (n = 6) as 4.1 %. This also shows that the spiking method will yield valid and reproducible PAH data [226]

Table 3.15: Measured PAH concentration in coal tar pitch spiked pilot study

PAHs	Initial concentration of polycyclic aromatic hydrocarbon in coal tar pitch spiked soil used for the pilot study		
	mg kg^{-1}	SD n=3	% RSD
Naphthalene	3.9	0.1	2.6
Acenaphthylene	0.2	0.0	2.0
Acenaphthene	15.6	0.4	2.6
Fluorene	14.9	0.4	2.7
Phenanthrene	177.0	5.5	3.1
Anthracene	29.0	0.9	3.1
Fluoranthene	338.0	11.6	3.4
Pyrene	259.0	9.3	3.6
Benzo(a)anthracene	263.0	9.2	3.5
Chrysene	272.0	9.2	3.4
Benzo(b)fluoranthene	374.0	13.3	3.6
Benzo(k)fluoranthene	274.0	9.3	3.4
Benzo(a)pyrene	328.0	11.2	3.4
Indeno(1,2,3-cd)pyrene	198.0	6.4	3.2
Dibenzo(a,h)anthracene	601.0	2.0	0.3
Benzo(g,h,i)perylene	164.0	5.4	3.3

3.4.7.1 PAH degradation in spiked soil

The degradation and loss of PAHs in soils has been extensively studied by various authors [36, 39, 265]. PAHs in soil are subject to chemical oxidation, photolysis,

hydrolysis, volatilization, bioaccumulation, adsorption to soil particles and leaching and mineralisation by the soil microbial community [36]. Microorganisms can totally degrade or partially transform PAHs through enzymic action (dioxygenase, monooxygenase) of individual species of microbes or interdependent communities. However, complete mineralisation of high molecular weight PAH can be achieved by only a limited number of microorganisms. Higher molecular weight PAHs are less bioavailable (low solubility) and are strongly adsorbed into the organic matter content in soil, thus making them more persistent in soil [20].

The abiotic loss of PAH in this pilot study could not be quantified because there was no PAH sterilised soil treatment in the experiment plan. The pilot study could not incorporate this treatment because the aim of the study was to establish an optimum experimental protocol and instrumental method that would be used in the main study. Nevertheless, the result obtained from the PAH loss and degradation in the spiked soil as shown in Figure 3.14 and 3.15 could still be used to explain the effect of heavy metal on the PAH degradation and loss. The percentage degradation for the treatment with heavy metal was reduced compared to the treatment without the heavy metal. The reduction in percentage degradation was greater in the treatment with Pb than in the treatment with Cd. This then implies that Pb has a more inhibiting effect on total PAH biodegradation than Cd.

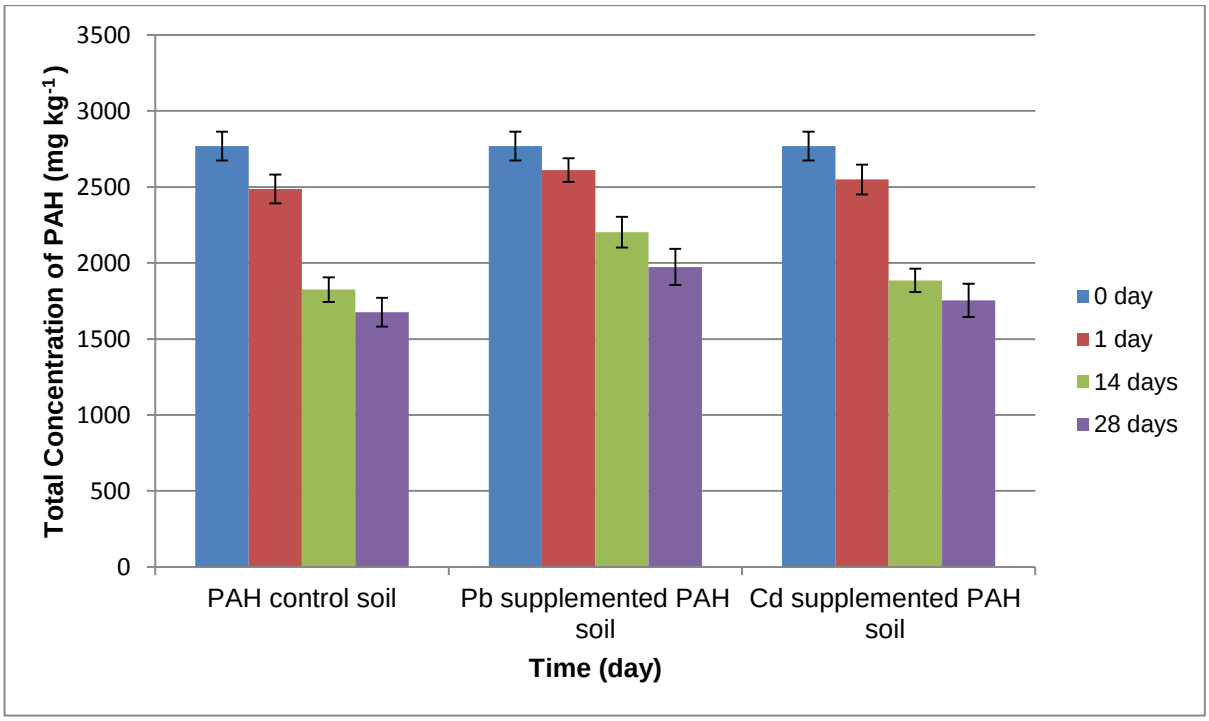


Figure 3.14: Change in total PAH concentrations over a 28 day period in the presence and absence of heavy metal co-contaminants. The error bars represents the SD n = 3

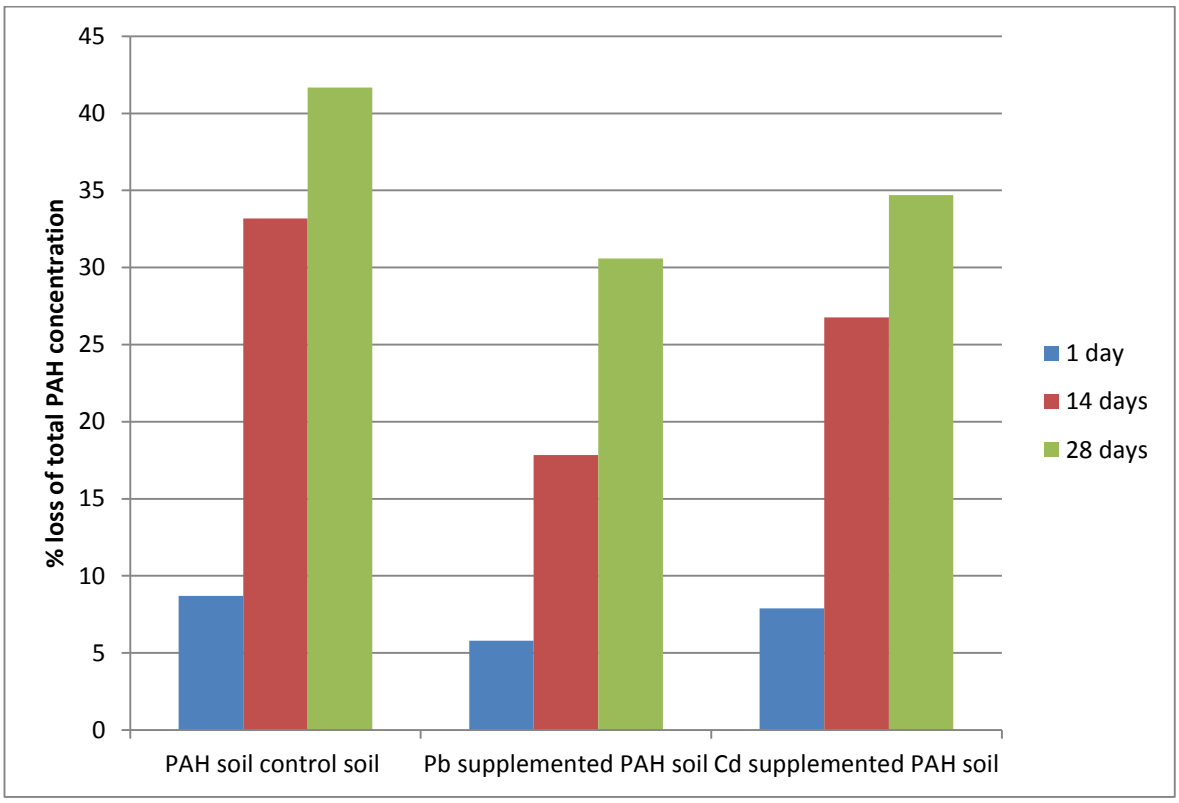


Figure 3.15: Percentage loss of 16 USEPA Priority total PAH after concentration for PAH control soil; Pb supplemented PAH soil; Cd supplemented PAH soil

The percentage loss of naphthalene was observed to be the highest in all the treatments used after the first day of spiking (Figure 3.16). Naphthalene is very volatile and can easily be reduced by abiotic processes such as chemical oxidation and sorption to organic matter content of the soil. Fluoranthene (FLUH) and pyrene (PYR) degradation was higher in the Cd-treated soil than in the control and the Pb-treated soil. Thavamani *et al*. [47] in their study observed the stimulating effect of Cd on the degradation of benzo[a]pyrene (BaP) and PYR. The PAH compounds were degraded to 100 % and 89 % respectively after 60 days of natural attenuation in the presence of Cd. The percentage degradation of acenaphthene (ACE) after 1 day was higher for Pb-treated soil than the control and Cd-treated soil. Pb can also have a positive effect on the degradation of PAH compounds, Khan et al. [99] in their study observed a higher degradation of PYR in the presence of Pb after 8 weeks of incubation than the degradation in PYR alone amended soil. However, pyrene degradation was not stimulated by Pb in this study rather it was stimulated by Cd.

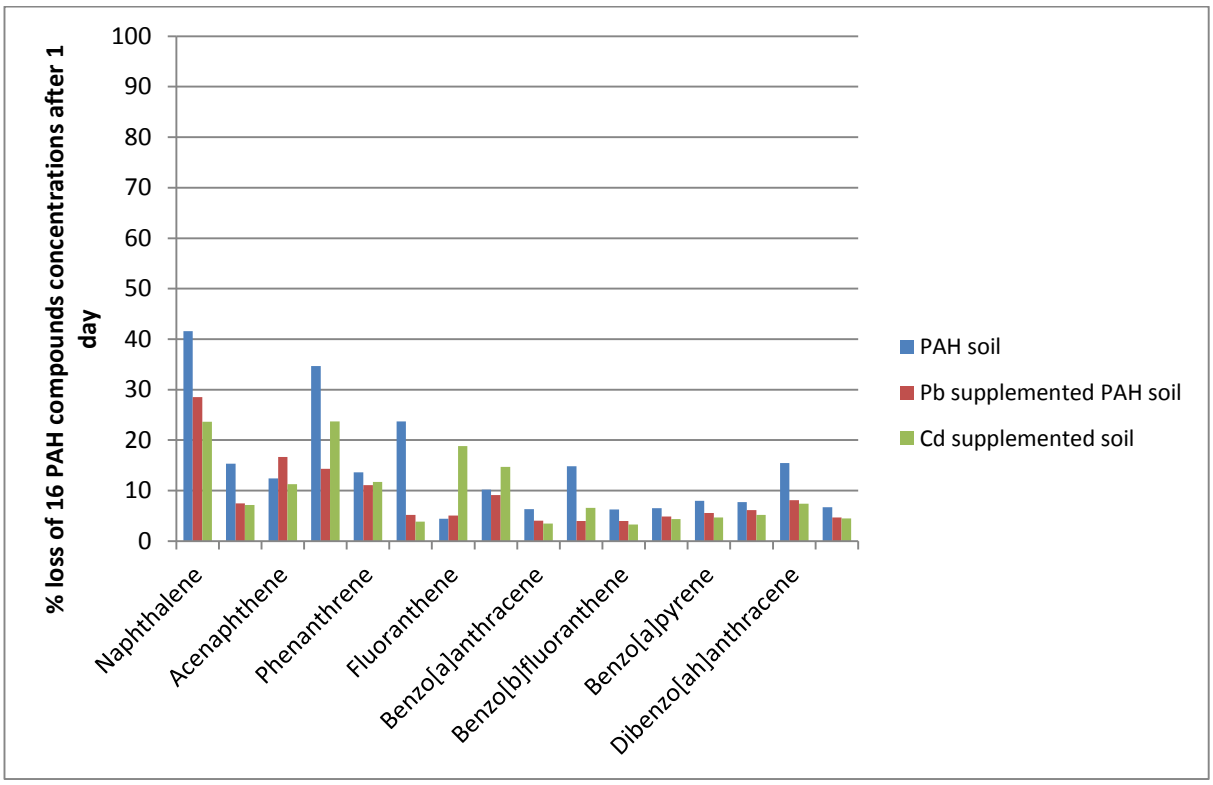


Figure 3.16: Percentage loss of 16 USEPA Priority PAH after 1 day of coal tar pitch soil spiking for PAH control soil; PAH spiked soil with 250 mg kg^{-1} Pb; PAH spiked soil with 250 mg kg^{-1} Cd

The percentage loss of PAH compounds after 14 days (Figure 3.17) was still consistently higher in the Cd-treated soil than the Pb-treated soil for most of the 16 PAH compounds but it was only higher than the control for ACE, PHE, ANT and FLUH. The loss of the lower molecular weight (NAP, ACY) PAH and mid-molecular weight (FLU, PHE and ANT) was higher than the heavier molecular weight (BkF, BaP, IDP, DBA) PAH compounds

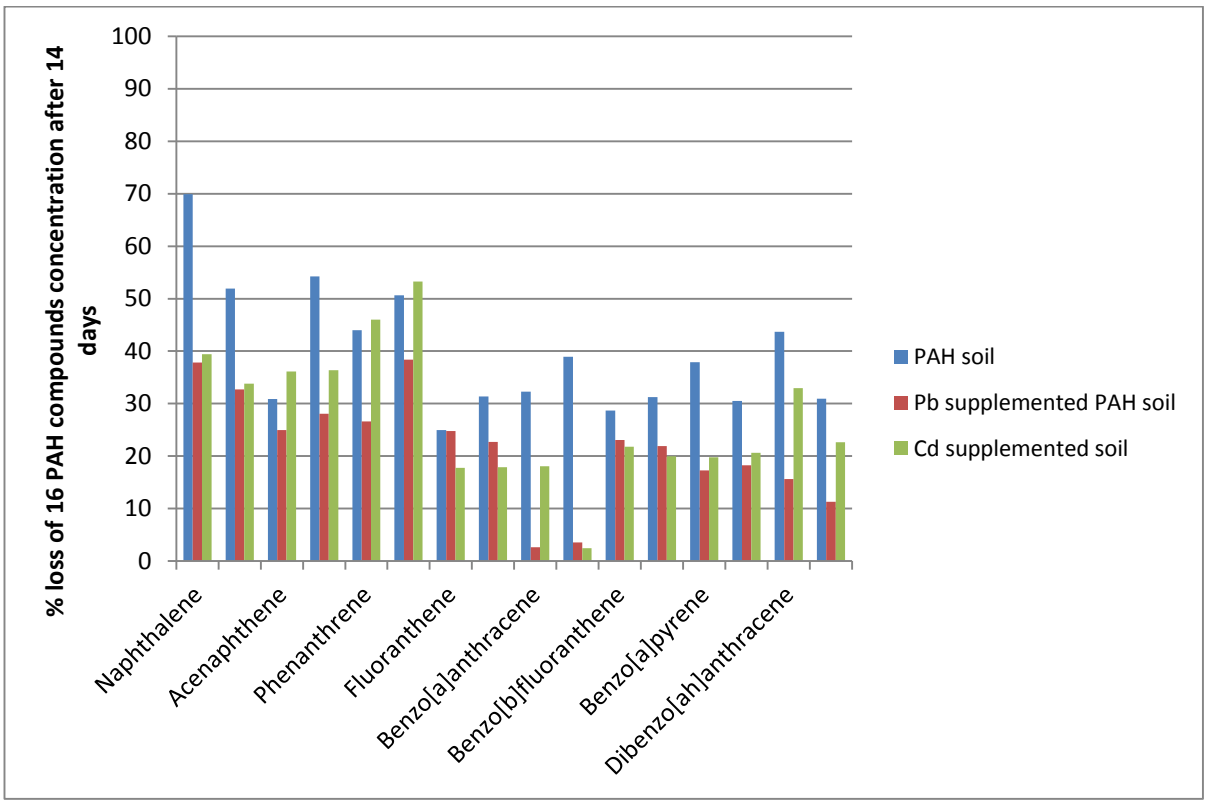


Figure 3.17: Percentage loss of 16 USEPA Priority PAH after 14 days of coal tar pitch soil spiking for PAH control soil; PAH spiked soil with 250 mg kg^{-1} Pb; PAH spiked soil with 250 mg kg^{-1} Cd

The percentage loss of PAH compounds after 28 days (Figure 3.18) was similar to their loss after 14 days for the Cd-treated soil and the control soil. The Cd was still having a stimulating effect on the degradation of ACE, PHE, ANT and FLUH more than the control and Pb-treated soils.

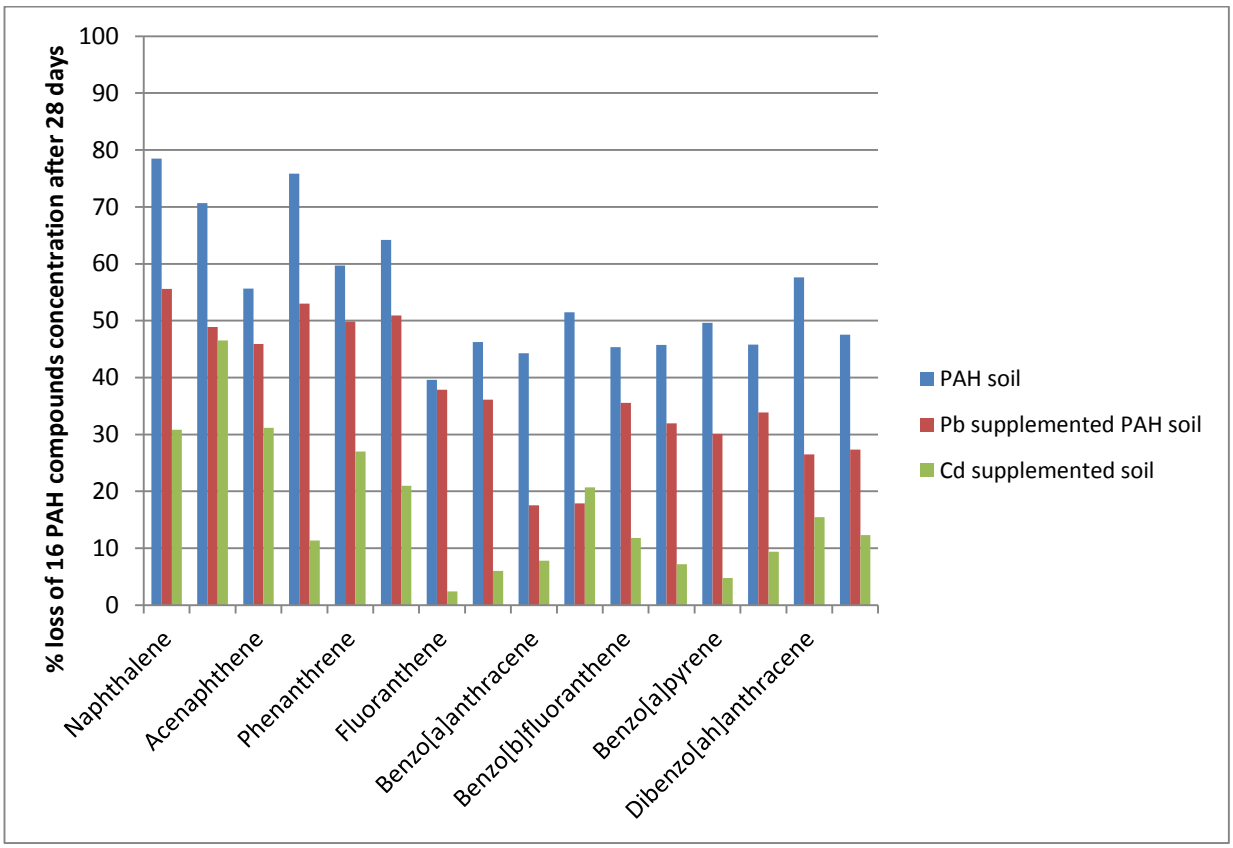


Figure 3.18: Percentage loss of 16 USEPA Priority PAH after 28 days of coal tar pitch soil spiking for PAH control soil; PAH spiked soil with 250 mg kg^{-1} Pb; PAH spiked soil with 250 mg kg^{-1} Cd

3.4.8 Variation in heavy metal concentration in spiked soil during the course of the pilot study

The water spike/mixing method used to spike heavy metals to the coal tar pitch spiked soil produced a homogenous mixture as shown by the RSD (n = 3) in Table 3.16. The RSD (n = 3) percentage range of 0.6 to 1.3 for Pb and 5.1 for Cd shows a high degree of homogeneity. The standard deviation was based on three spiked

replicate samples that were taken differently. The mean added concentration for spiked Pb was higher than the mean concentration for the unspiked of Pb for the sample which shows that the results for the biodegradation and microbial community activity would have been influenced by the spiked Pb heavy metal. The soil used in spiking already has Pb metal background contamination as indicated by the concentration of Pb in the unspiked control soil. The bioavailable Pb and Cd in the soil as shown in Table 3.17 indicates a high concentration of Pb and Cd in solution phase. The bioavailable fraction of the metals is known as the major factor that controls the effect of heavy metal on soil microbial community.

Table 3.16: Measured total concentrations of spiked Pb and Cd soil at the beginning and end of the pilot study

Sample	Measured concentration of Pb (±SD) n=3 (µg/g)				Measured concentration of Cd (±SD) n=3 (µg/g)			
	1day	28days	%RSD 1day	%RSD 28 day	1day	28days	%RSD 1day	%RSD 28 day
Unspiked Control	75.5±14.8	90.1±14.2			0.2±0.3	0.3±0.2		
PAH control	93.3±33.6	73.2±16.2			0.4±0.3	0.4±0.4		
Pb supplemented PAH soil	406.5±2.4	404.2±5.2	0.6	1.3	0.2±0.2	0.1±0.1		
Cd supplemented PAH soil	70.1±1.2	69.8±1.4			380.7±19.6	398.2±20.5	5.1	5.1

Table 3.17: Measured concentrations of Pb and Cd after EDTA extraction at the beginning and end of the pilot study

Samples	Measured concentration of Pb after EDTA Extraction (±SD) n=3 (µg/g)		Measured concentration of Cd after EDTA Extraction (±SD) n=3 (µg/g)		Calculated bioavailable concentration of Pb (µg/g); Total concentration after EDTA extraction		Calculated bioavailable concentration of Cd (µg/g); Total concentration after EDTA extraction	
	1day	28days	1day	28days	1day	28days	1day	28days
Pb supplemented PAH soil	92.4±8.1	85.3±28.1	0.0	0.0	314.1	318.9	0.0	0.0
Cd supplemented PAH spiked soil	34.6±1.5	34±2.3	40.2±6.7	48.3±10.8	35.5	36.8	380.7	349.9

3.4.9 Soil respiration

3.4.9.1 Effect of acetone on soil respiration

In order to determine the spiking method that would be adopted for the coal tar pitch pilot study, an analysis of the effect of acetone on soil microbial community was carried out. Dendooven et al. [245] stated that acetone can liberate soil organic C and can also serve as a readily available substrate for soil microorganisms thereby increasing emission of CO_2. Figure 3.19 shows that soils that were spiked with acetone had a greater respiration rate compared to unspiked soil. Soil respiration measurement represents a measure of the total microbial activities in soil [121, 122]. The results indicate an increased activity of soil microbial community in the presence of acetone. The result as shown in Figure 3.19 implied that there was a need to minimise the effect of acetone on soil microbial community during the spiking process. The outcome from this was that the spiking was carried out using the dilution method which utilised the spiking ratio of 25:75 acetone soils to unspiked soil. The one-quarter acetone spiked soil was allowed to evaporate for 24hrs to ensure that the solvent was completely removed before mixing with the unspiked soil. This method reduces the effect of acetone on the soil respiration as can be seen in the Figure 3.19, though there was still some microbial activity stimulation.

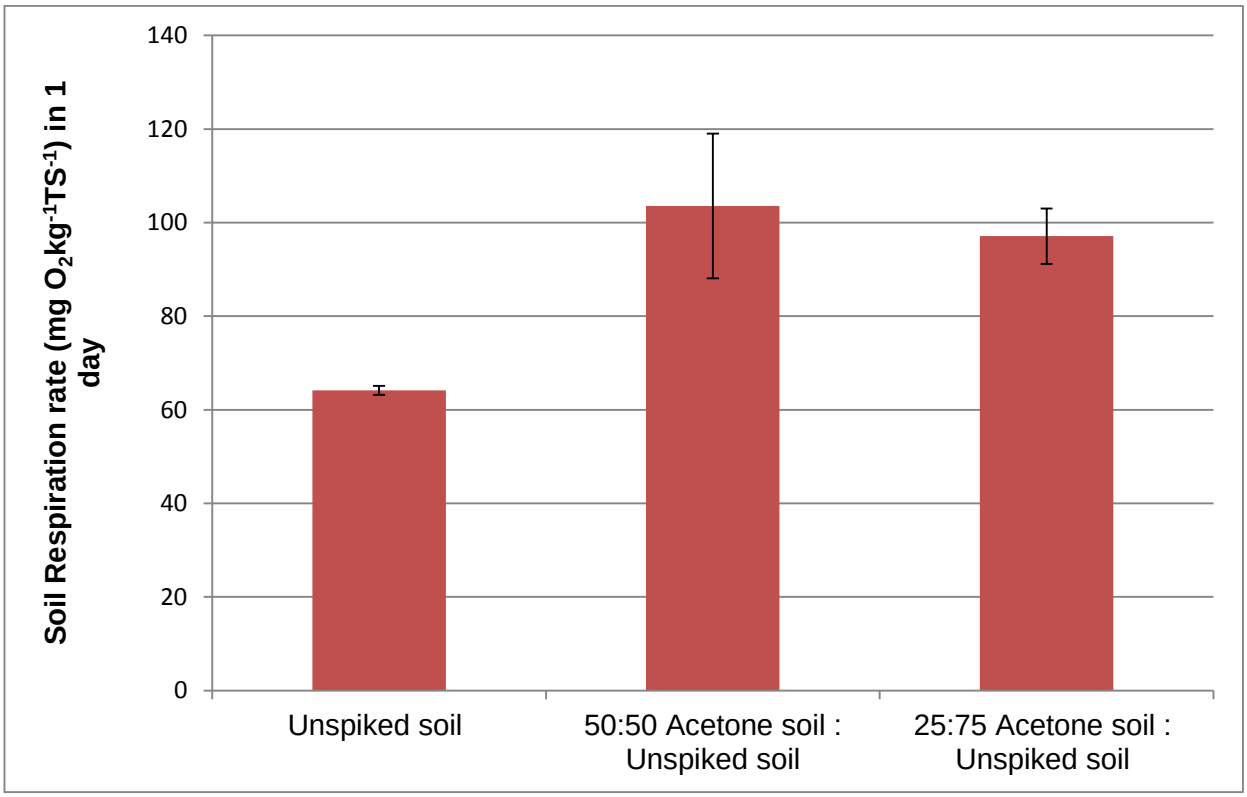


Figure 3.49: Soil respiration of soil spiked with acetone and unspiked control using different acetone: soil spiking ratio. The error bars represents the SD n = 3.

3.4.9.2 Soil respiration of PAH and heavy metal spiked soil

Microbial activity represented by soil respiration measurement for the soils spiked with PAH and heavy metals shows that there was an increase in the soil respiration and respiration rate after the first day as seen in Figure 3.20. As expected the introduction of coal tar pitch and acetone would serve as an organic substrate for the microbial community. PAH compounds can serve as a carbon and energy source for many microorganisms in the soil [23] and it has been suggested that cell growth results from the degradation of two or three ringed PAHs but not the four-ring PAH compounds because the latter are not readily bioavailable. The soils treated with heavy metals (Pb and Cd) also had an increased respiration activity after the first day of treatment. It has been reported that the basal respiration rate of microorganisms was stimulated in the presence of Cd [91] compared to the unspiked control. The increased respiration rate later declined with increasing Cd concentration [91]. Lu *et al*. [91] also reported a significant higher respiration rate ($p < 0.05$) when the co-contaminants Cd and pyrene were present, which they attributed to stress.

It is suggested that under heavy metal stress, the energy utilization efficiency of microbial metabolic processes (growth) is decreased, which then leads to a greater requirement of carbon for cell maintenance which ultimately increases respiration. It can also be suggested that microorganisms in highly polluted soils require more energy to survive (the adverse conditions), thus, a higher portion of the carbon consumed would be lost as CO_2 and a smaller proportion of the carbon would be assimilated [82]. However, the increased respiration rate observed for the heavy metal spiked soil at the initial period, subsequently declined rapidly at the later period, most probably because of the biocidal effect of heavy metal on the microbial community. The respiration rate decline was slightly more with Cd-treated soil than Pb-treated soil as shown in Figure 3.20. The reduction in respiration activity for soils treated with heavy metal and PAH represents the toxic effect PAH and heavy co-contamination can have on the microbial community as also observed in the study carried out by Gogolev *et al*. [105].

The respiration rate of the PAH spiked soil with no heavy metal addition also decreased after 28 days. It has been suggested that PAH can also exhibit toxicity to microorganisms, their toxicity is related to their water solubility and this can occur in PAH compound mixtures. For instance, naphthalene can be strongly toxic in a mixture of PAHs and can inhibit the degradation of other PAH compounds that would have been biodegraded [92]. The soil respiration rate of the unspiked soil increased after the

first day and subsequently which can be as a result of microbial utilization of the available carbon in the soil.

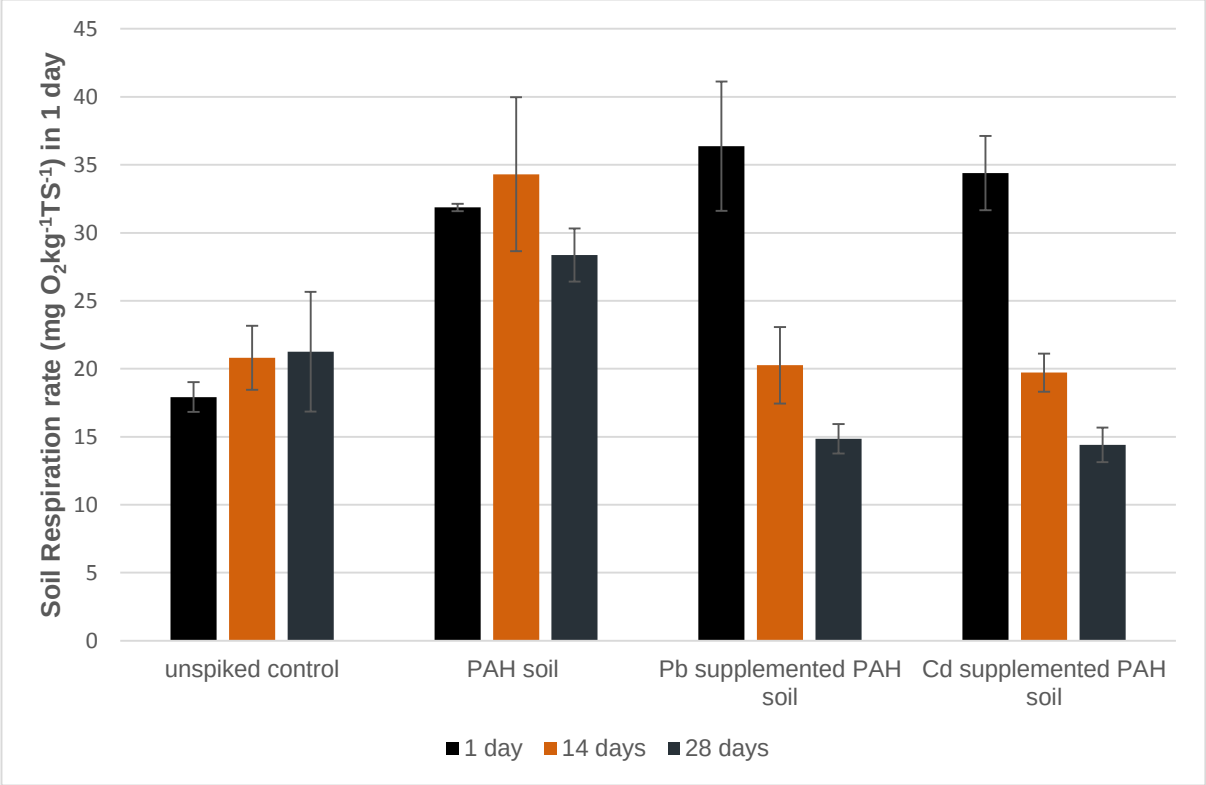


Figure 3.20: Soil respiration of PAH spiked and unspiked soil**.** The error bars represents the SD n = 3

3.4.10 Community level physiological profile (CLPP)

Community level physiological profile (CLPP) was used to compare the microbial community diversity for all the soil treatment; it has advantages over both classic cell culturing techniques and molecular level RNA amplification as these other techniques are time-consuming and require specialised expertise [177]. The CLPP technique is straightforward; it uses an automated measuring apparatus and provides a more meaningful assay of community structure than isolate-based methods. This is because it measures the utilisation of carbon (carbon substrates), which is a major factor that regulates microbial growth and community structure in soil [144]

3.4.10.1 Effect of dilution factor

The dilution of soil extract for Ecoplate inoculation as mentioned in the introduction was necessary for the purpose of normalising the inoculum density of the extracted soil microbial community [264]. Figures 3.21, 3.22 and 3.23 show the different dilutions that were used to determine the right amount needed for the Ecoplate analysis. The reliable

use of Biolog Ecoplate requires samples of approximately equivalent inoculum densities. If this criterion is not met, then the comparison of variation in the overall colour formation among samples will be compromised by the different responses of inocula with different cell densities. Garland *et al.* [178, 179] has recommended dilution as a method of standardizing inoculum density for microbial community comparison. Data transformation (dividing each single corrected absorbance value by AWCD) was another method suggested by the same authors but Kersters *et al*. [247] in their study observed that dilution of samples was the most efficient method because data transformation did not significantly reduce the influence of inoculum density. Adjustments of sample densities prior to inoculation using culturing technique can be laborious especially when dealing with a large number of samples. Dilution then becomes an efficient way of normalising inoculum density as observed by Kersters *et al*.

More diluted inocula will result in a protracted rate of colour development [182] as seen in Figure 3.23 which shows that activities in most wells did not start until after 48hrs. The optimal amount of dilution that was used for subsequent analysis was 1 mL of extract in 50 mL of 0.9% NaCl solution (Figure3.16) because it shows a balance of not being too dilute and too low dilution. The activities in the majority of the well for 1 mL in 50 mL NaCl inocula started just after 24 hrs which are also similar to 1mL in 10 mL 0.9 % NaCl dilution (Figure 3.21).

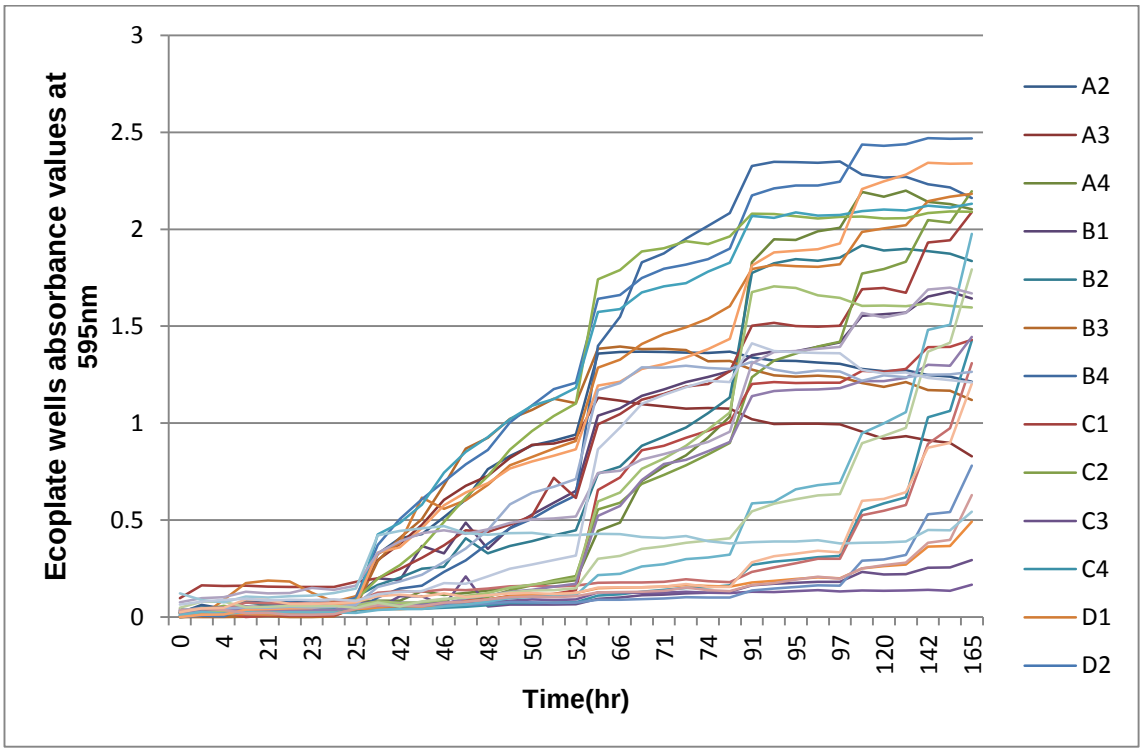


Figure 3.21: Ecoplate absorbance values of 1mL of soil extract in 10 mL of 0.9 % NaCl solution

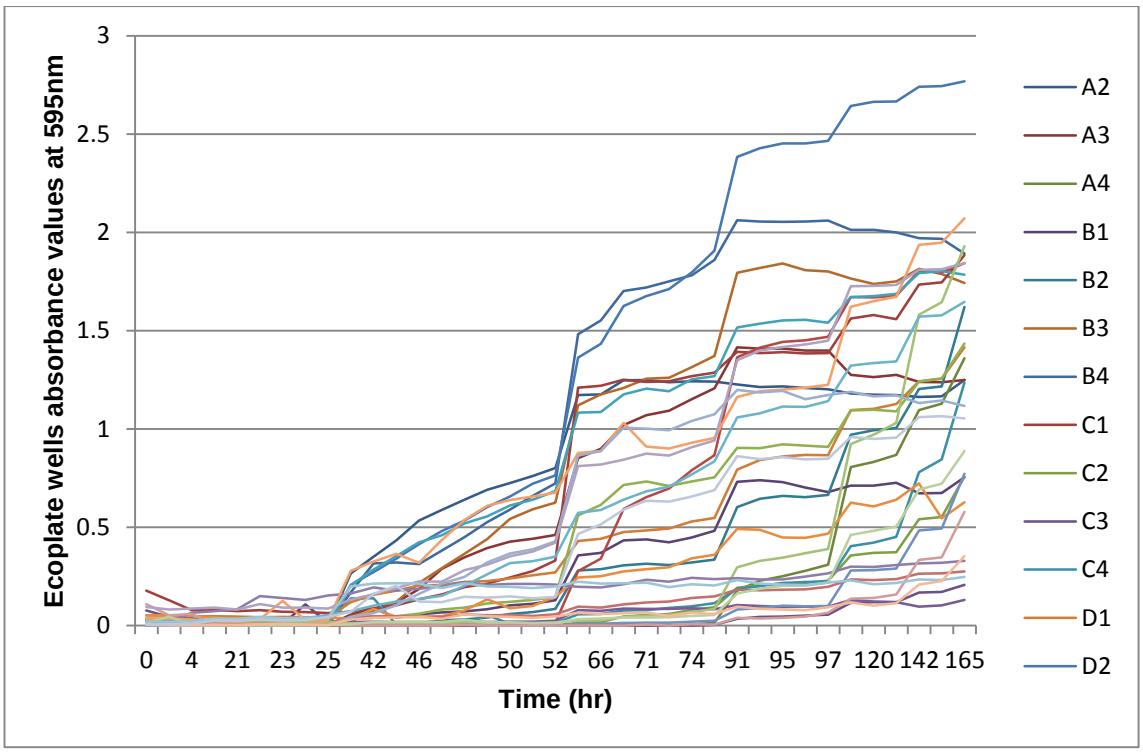


Figure 3.22: Ecoplate absorbance values of 1mL of soil extract in 50 mL of 0.9 % NaCl solution

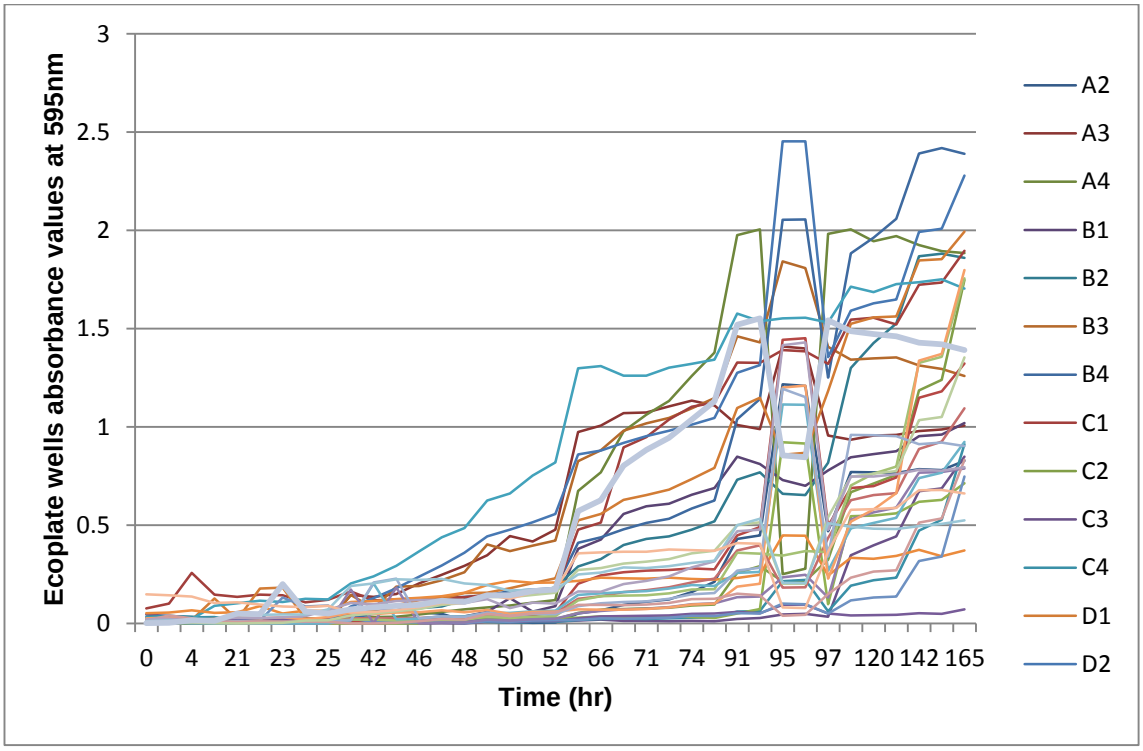


Figure 3.23: Ecoplate absorbance values of 1 mL of soil extract in 100 mL of 0.9 % NaCl solution

3.4.10.2 Selecting the time of Ecoplate analysis

Choosing a set time for analysis was based on the appropriate incubation time point that preserved the greatest divergence between well responses while keeping the maximum number of wells within the linear absorbance range [120, 234]. An expansion in the disparity between well absorbance values demonstrates an increment in the volume of information contained in the data set. Thus, if the incubation time chosen for the absorbance value is too early, there would be little information gained from the data set, because at the early stages of growth the disparity between well absorbance values is insufficient to produce useful information and the colour development in some wells may be missed [120, 171]. But choosing a later incubation time to measure absorbance values would give more information with respect to the CLPP of the microbial inoculum in as much as the values are not above absorbance value of 2 [120]. For example Haack *et al.* [182] observed a false negative result (absorbance value less than the control) at 24hrs of incubation for BIOLOG Gram negative substrate 2, 3-butanediol and thymidine indicative of *Corynebacterium* sp. strain 5, which later became a positive result after 72 hrs of incubation. Absorbance values of above 2 are outside the linear absorbance range as can be seen in Figure 3.18 and it also means that the saturation level of the most of the wells has been reached which can contribute to a measurement error. The set time selected for absorbance measurement from the result in Figure 3.24 was 50 hrs. At 50 hrs majority of the wells have had a considerable activity in it and the absorbance value is still within the linear range. All subsequent analysis on CLPP was carried out with the data set generated at 50 hrs absorbance value. The method used in Chapter 2 was the AWCD reference value of 1.0 (data not shown here) according to Garland *et al.* [177], who showed that using AWCD reference values between 0.25 - 1.0 would yield a relatively similar CLPP for microbial community analysis.

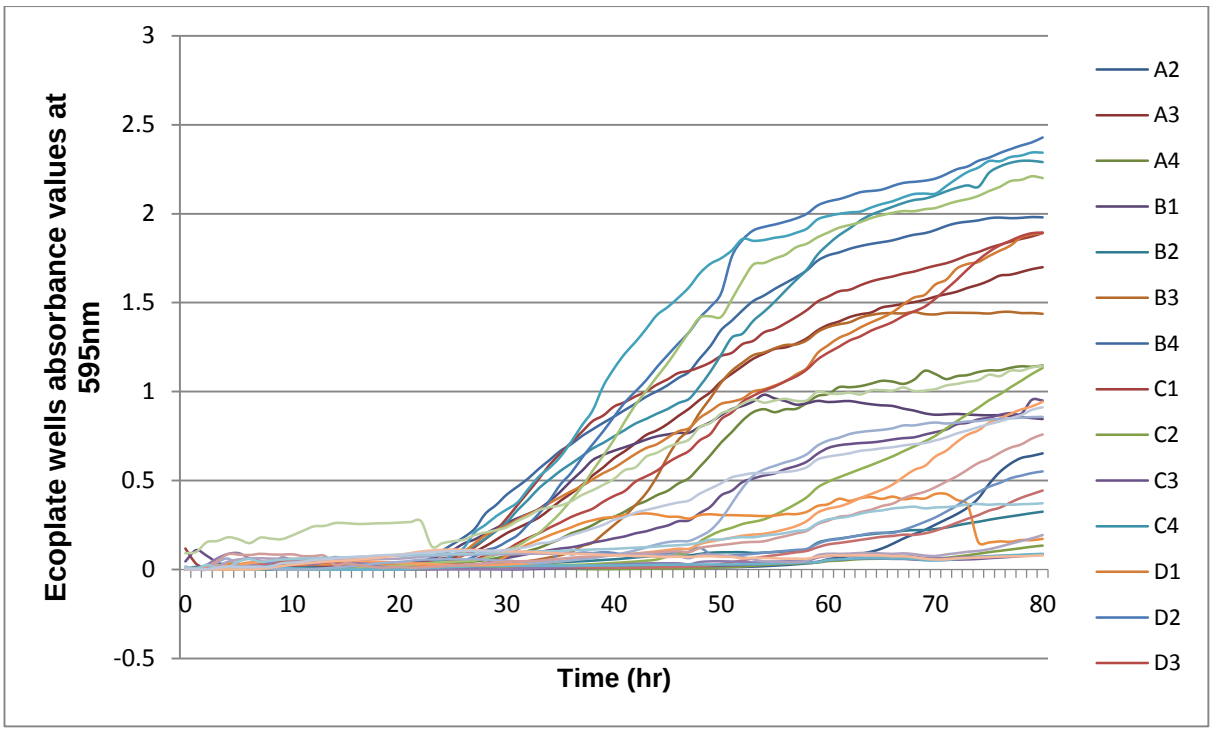


Figure 3.24: Ecoplate absorbance values at 595 nm over 80 hrs. Soil extract dilution was carried out using 1mL of extract in 50 mL of 0.9 % NaCl solution

3.4.10.3 Effect of acetone on community level physiological profile (CLPP)

The effect of acetone on the microbial community was assessed using Biolog Ecoplate. The effect of acetone was carried out on the three different spiking strategies that were tested for the acetone spiking experiment and it showed that acetone can have a more negative effect on microbial community metabolic diversity if the right spiking method was not used. The average well colour development of the wells (AWCD) was used to check this effect and it was calculated by extracting information from the substrate utilization pattern gathered through the Biolog Ecoplate 24 hourly absorbance measurement for 168 hrs (7 days). The AWCD shows the metabolic capacity of soil microorganisms developing in Ecoplate wells and it can serve as an indicator of microbial activity [233]. The AWCD for the unspiked soil shows that the microbial community activity increased over the duration of the measurement as expected. The soil spiking ratio of 25 % of acetone soil and 75 % unspiked soil (dilution method) showed a delayed phase at the beginning of measurement but was later increasing at the same rate as the unspiked soil as shown in Figure 3.25. The soil spiking ratio of 50% acetone soil and 50% unspiked soil did not show a much lower rate of increase in its metabolic activity as expected. The metabolic activity in all the three samples were similar which was different from the results of the studies carried out by both Brinch et al. [225] and Reid et al. [236] which showed that acetone reduced the microbial

number established by plate counting (microbial culturing on a nutrient plate). The metabolised substrate diversity (H) and substrate richness (S) for the unspiked soil and the 25:75 spiking ratio was not much higher (Table 3.18) than the 50:50 spiking ratio. This shows that there were similar metabolic activities in the unspiked soil, 25:75 spiked soil and the 50:50 spiked soils. The substrate evenness (E) for the 50:50 spiked soil and the 25:75 spiked soil did not show any much difference as expected though they were higher in the 50:50 spiked soil as seen in Table 3.18. But the values for substrate evenness for the two acetone spiked soils were higher than that of the unspiked soil which shows that some substrates were used more than some other substrates (more uneven substrate utilization) in the acetone spiked soils. This can imply a reduction of the metabolic diversity of the microbial community. However, the differences are not as much as expected in all the three samples (Table 3.18).

Though these results did not confirm the effect of the two acetone dilution methods on the soil microbial community but the 25:75 spiking ratio was chosen as a better dilution method. The reason is because using 50:50 spiking ratio in the main study where a higher volume of acetone would be needed might produce a significant error in obtained results. Therefore as a precautionary measure, the 25 % acetone and 75 % soil dilution method was used in subsequent studies.

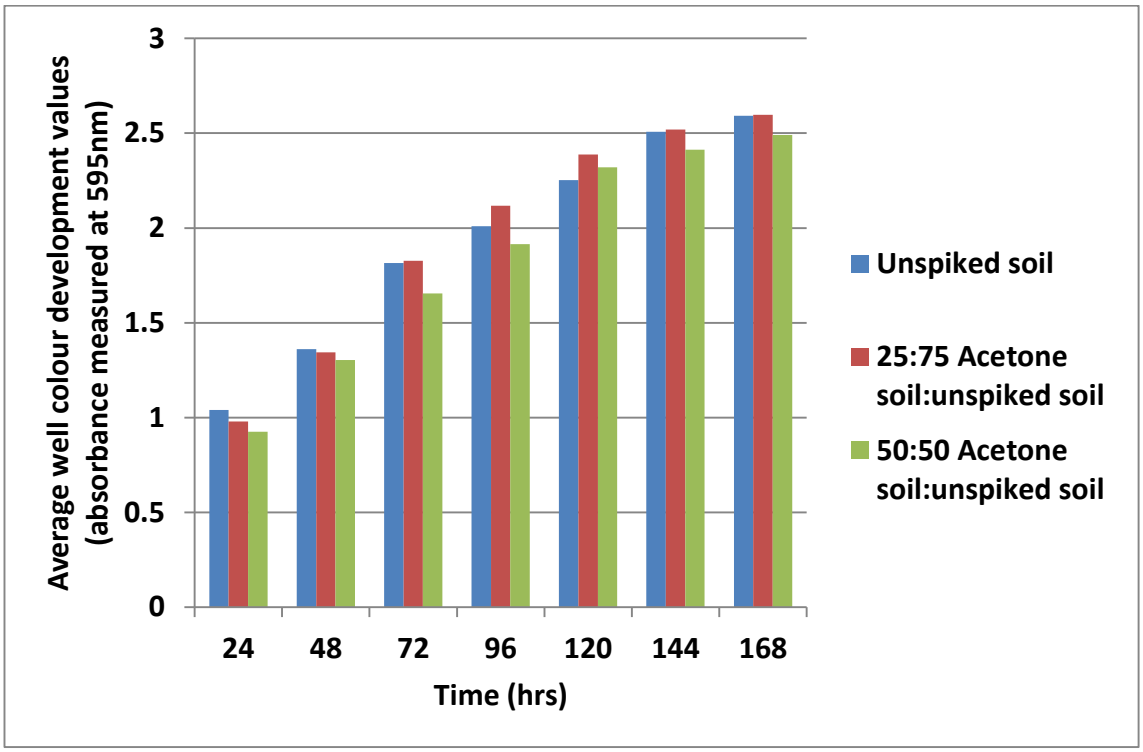


Figure 3.25: Ecoplate Average colour development values for soil spiked with different acetone soil: unspiked soil ratio

Table 3.18: Shannon diversity index, substrate richness and substrate evenness of acetone spiked soil and unspiked soil

Treatment	Shannon diversity index, H	Substrate richness, S	Substrate evenness, E
Unspiked soil	2.49	13	2.24
One-quarter spoil spiked	2.47	10	2.47
50:50 Acetone spiked :unspiked	2.43	9	2.55

3.4.10.4 Replication and reproducibility

The standard deviation and relative standard deviation (RSD n = 3) for the replication experiment show that the CLPP using Eco plate is reproducible. The variation between replicates had a high RSD (n = 3) of 17 % on the first day but decreased to 6.98 % and 7.82 % after 7 days and 14 days respectively (Table 3.19). The variation between Eco plates also had an average standard deviation of 0.0267 across the duration of measurement and the highest RSD (n = 3) as 9.52 % on the 14 days of measurement. The variation within a replicate, which can also serve as a test of variation in the method of data generation [171], also had a low average standard deviation (n = 3) of 0.0267 over the measurement duration. These values confirm the observation made by Calbrix et al. [246] in their study which showed that the average variance between replicates according to different dilutions range from 17 % to 33 % with the less diluted having the lowest variance of 17 %. The low percentage variation showed that low dilution produced the most reproducible result. This statement also confirmed the result obtained in section 3.5.10.1 where an appropriate dilution that would produce a reproducible result was obtained for this study. Kersters et al. [247] using BIOLOG Gram negative (GN) plates observed reproducible data. They observed that calculated standard deviation (n = 5) on the absorbance value for 96 well for communities using a single production batch ranged from 0.01 to 0.25 for *Aeromonas,* (pure cultures) and from 0.01 to 0.50 for *Variovorax* (pure cultures) and from 0.06 to 0.70 for surface water (microbial community extracted from surface water). They also observed a less significant variation among five BIOLOG GN plate production batches for microbial community extracted from surface water. Haack et al. [182] in their study demonstrated that the patterns of positive and negative responses and substrate utilization rate and extent were highly reproducible for simple microbial communities when the inoculum

density was controlled. They observed that replicate microplates inoculated with the same microbial community and plates inoculated with two independently constructed identical model communities exhibited nearly equivalent BIOLOG GN profiles. Calbrix et al. [246] in their study considered a percentage variance to be high if it exceeds 20 percent. The RSD (n = 3) values for all the variation experiment as shown in Table 3.19 was less than 20 % and much lower than 10 % for variability between Ecoplates, which then confirms the reproducibility of BIOLOG Ecoplate results. This then implies that when samples of similar inoculum density exhibit different patterns of positive and negative substrates responses, it probably reflects real differences in community composition [182].

Table 3.19: Mean of AWCD and their standard deviation for reproducibility experiment

Variability Test	**Mean of AWCD at 595nm absorbance value (±SD) n=3**					
	0day	%RSD	7day	%RSD	14days	%RSD
Variability between 3 replicates (E1)	0.35±0.06	17.14	0.43±0.03	6.98	0.34±0.03	8.82
Variability between Eco plates (E2)	0.42±0.02	4.76	0.42±0.04	9.52	0.41±0.02	4.88
Variability within a replicate sample (E3)	0.43±0.02	4.65	0.39±0.02	5.13	0.39±0.04	10.26

3.4.11 Coal tar pitch spiked soil community level physiological profile

The CLPP for the coal tar pitch spiked soil and unspiked soil shows an increased metabolic activity for the coal tar pitch heavy metal treated soil than the unspiked after the first day of spiking as seen in Figure 3.26. This increase was followed by a sharp decline in the metabolic diversity for the heavy metal treated soil than the untreated soil. The microbial community in the heavy metal treated soils seem to have had short-term stimulation [95] as was observed in the soil respiration (Figure 3.20) and the microbial biomass (Figure 3.27) results. The other reason could also be that the concentration of heavy metal that was bioavailable to the soil microorganism was low. The bioavailability of heavy metal in soil influences their toxicity to soil microorganisms. Metals present in soil solution may not necessarily be bioavailable because soil organic matter usually contains large amounts of water-soluble organic matter which sequester the free metal ions and render them less available [149]. The values of the metabolic

diversity, H, for the metal treated soil was not affected after the first day and it remained marginally stable throughout the duration of the experiment but the substrate richness and evenness emphasized the effect of the heavy metal after the first day. The substrate richness for Pb metal was low compared to the other treatments. The substrate richness for the Cd was high after the first day and 14 days, but it declined after 28 days. The evenness for Pb showed a higher uneven substrate utilisation after 14 days and 28 days which was also seen in the decline of their AWCD. The evenness for Cd was low for the first day and after 14 days but became high after 28 days. This implies that there was a difference in the community composition of the Cd and Pb heavy metal treated soil compared to the PAH (with no metal) treated soil and untreated control. The application of the coal tar pitch would have provided more carbon substrate for the soil microbial community resulting in the treated soil AWCD being higher than the AWCD of the untreated control. The substrate richness, metabolic diversity and evenness of the PAH spiked soil (with no metal) was always greater than the untreated control because of the higher carbon input in the coal tar pitch treated soil as shown in Table 3.20. Gomez et al. [266] in their study showed that the application of organic amendment (compost) to the soil stimulated microbial population and it significantly increased their AWCD, metabolic diversity and substrate richness.

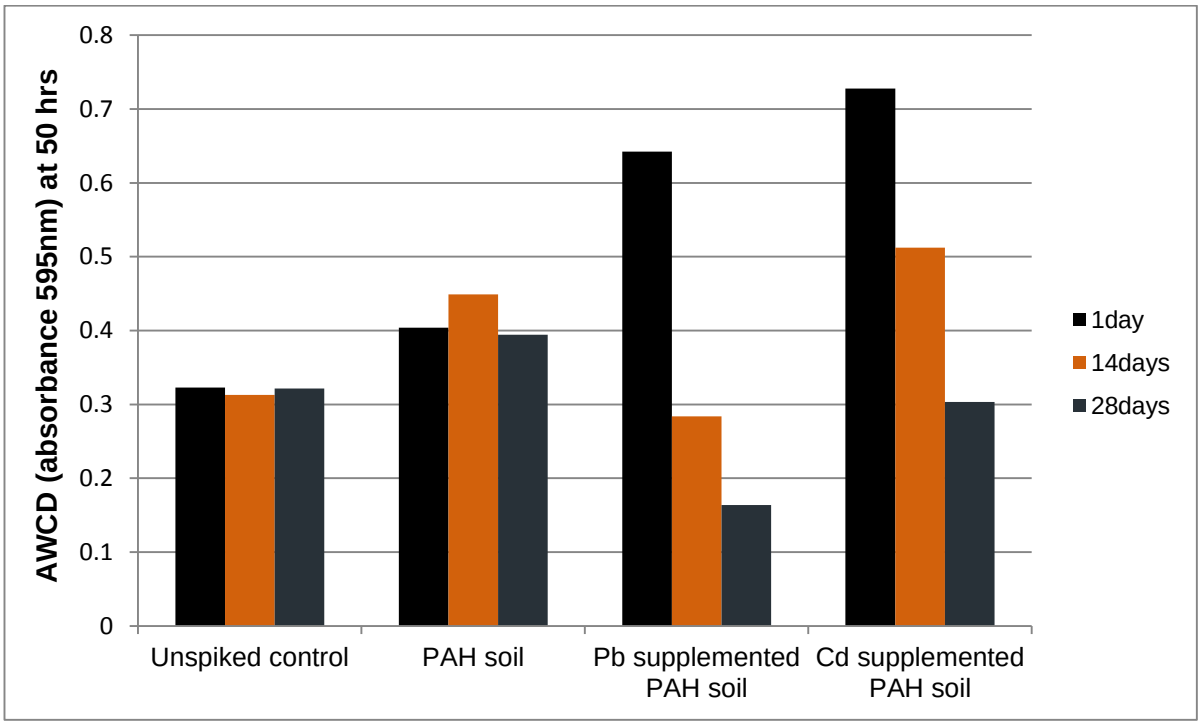


Figure 3.26: Average well colour development values of PAH spiked soil and unspiked control soil.

Table 3.20: Shannon diversity index H, substrate richness and substrate evenness of spiked and unspiked samples

Samples	Shannon diversity index H (SD) n=3 1day 14days 28days			Substrate richness S (SD) n=3 1day 14days 28days			Substrate evenness E (SD) n=3 1day 14days 28days		
Un spiked control	2.6±0.3	2.6±0.3	2.6±0.4	9.6±5.6	9±3.5	9.3±2.1	3.0±0.5	2.9±0.4	2.7±0.3
PAH control	2.7±0.1	2.6±0.02	2.7±0.1	12.3±1.5	13.9±2.1	12.2±3.6	2.5±0.2	2.3±0.2	2.6±0.4
Pb supplemented PAH soil	2.5±0.4	3.1±0.9	2.6±0.1	8.8±4.4	8.8±4.4	5.1±1.2	2.9±0.7	3.4±0.3	3.7±0.5
Cd supplemented PAH soil	2.8±0.05	2.8±0.1	2.7±0.06	22.6±2.7	15.97±1.8	9.4±4.6	2.1±0.04	2.3±0.03	3.1±1.0

3.4.12 Soil microbial biomass

Microbial biomass concentration (the living component of soil) has been used by numerous studies as an indicator of stress on soil microorganisms [82, 144, 149, 237, 266, 267]. It can serve as an indicator of soil environmental quality and it has been employed in national and international monitoring programs [267]. The biomass concentrations shown in Figure 3.27 indicate that the coal tar pitch treated soils (including the heavy metal treated soils) had a higher biomass concentration than the unspiked control after the first day of spiking (24hrs after spiking). This implies that the coal tar pitch stimulated the growth of the microbial community and the Cd spiked soil stimulated the microbial community even more because it had the highest biomass concentration. The effect of heavy metal was not observed after the first day of spiking probably because the concentration of heavy metal that was bioavailable to microorganism was still low. Soil physicochemical features such as pH, organic matter content or dissolved organic matter (DOC) can influence metal solubility and bioavailability without an apparent change in the total heavy metal content of the soil [110]. The metal ions in solution also may still not be bioavailable to the microorganisms because of the dissolved organic carbon which can form complexes with the metal ions [149]. Bååth [93] in his review on heavy metal stated that no effect of heavy metal was observed during the first week of degradation of different litter fraction in a litter decomposition study because of the easily degradable substrate that was still available to the microorganisms. However, a decrease in degradation rate due to heavy metal pollution was observed with time.

The decline in the biomass concentration for the heavy metal treated soil (Figure 3.27) became more apparent after 28 days of spiking which could be as a result of the heavy metal effect or the effect of both heavy metal and PAH [79]. As mentioned earlier in this chapter, a reduction in microbial biomass concentration under heavy metal stress can be due to the reduced energy utilization efficiency of microbial metabolic processes caused by metals which then makes microorganisms use more carbon for maintenance instead of growth [110].

The coal tar pitch spiked soil and the unspiked control, had a marginal decline in their biomass concentration after 28 days compared to the heavy metal treated soils as can be seen in Figure 3.27. This marginal decline can be due to other environmental factors such as pH, temperature moisture content and organic matter quality (example; carbon and nitrogen contents in the soil) [149].

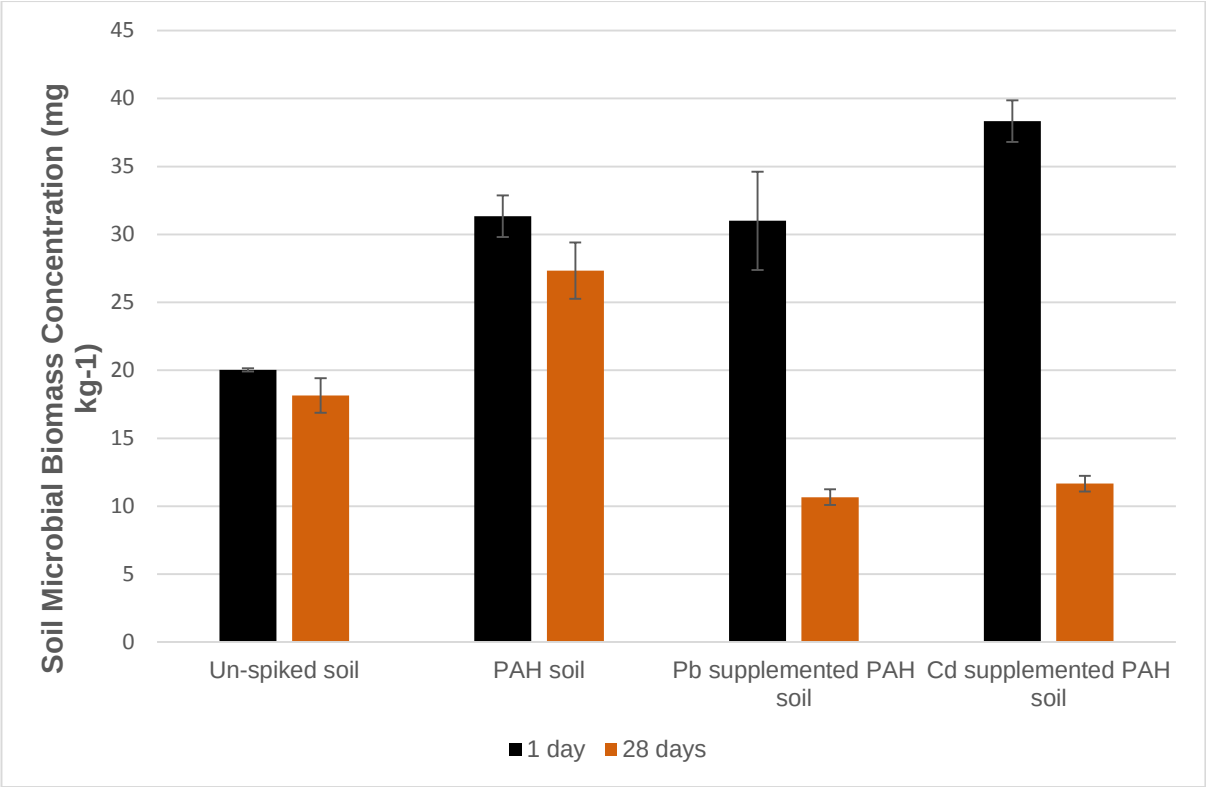


Figure 3.27: Soil microbial biomass carbon concentration of PAH spiked soil and unspiked control soil

3.4.13 Metabolic quotient:

The metabolic quotient value for the soils treated with heavy metal (Pb and Cd) was higher on the 28 days than the unspiked soil and the PAH spiked soil with no metal as shown in Figure 3.28. This is an indication of heavy metal stress on the microbial community. The metabolic quotient is conversely identified with the efficacy with which microbial biomass utilises indigenous substrates [144]. The increase observed for the metal treated soils after 28 days means that there was a reduction in microbial biomass concentration (Figure 3.27) for the metal treated soils. Increase in metabolic quotient (qCO_2) is generally an indication of stress on microorganism caused by contamination and ecosystem disturbances [267]. There was not much effect by the heavy metal on the microbial community after the first day of spiking as shown in the metabolic result. The Cd-treated heavy metal had the lowest value of metabolic quotient which confirms its stimulation on the microbial community. This stimulation effect was observed in their microbial biomass concentration and their metabolic diversity H (Table 3.20). The increase in metabolic quotient observed for the unspiked soil at the same stage (after 28 days) can be attributed to poor quality of the available organic carbon (nutrient content) in the unspiked soil (Figure 3.28) [237]. This negative effect was confirmed by their 28 days microbial biomass concentration shown in Figure 3.27. The PAH spiked

soil had a fairly stable metabolic quotient for the two periods of measurements (1day and 28 days). This means that the coal tar pitch spiking had a marginal effect on their microbial biomass ability to utilise the carbon substrates introduced into the soil through the coal tar pitch.

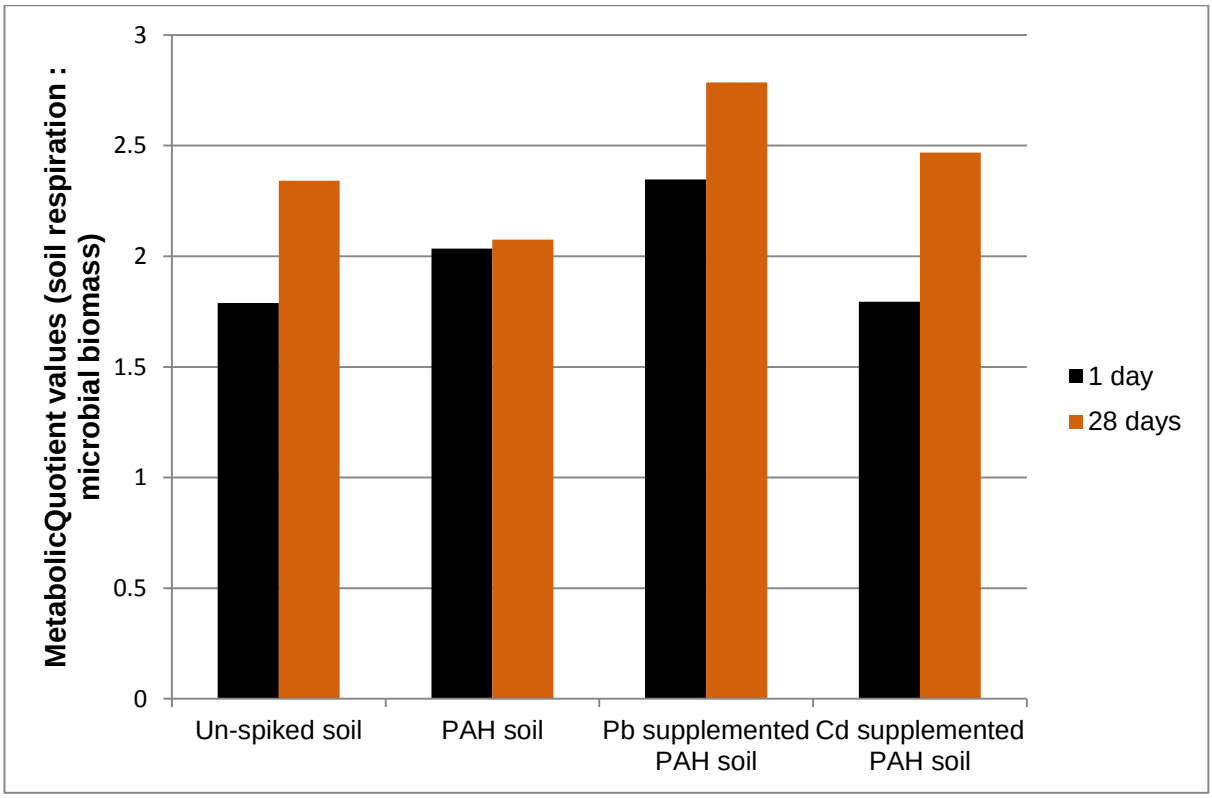


Figure 3.28: Soil metabolic quotient of PAH spiked soil and unspiked control soil

3.5 Conclusion

The results obtained from this study served as the foundation for the subsequent main bioremediation study. The study was used to resolve issues such as: how coal tar pitch spiking should be carried out; the concentrations of the 16 US EPA PAHs in coal tar pitch; the optimum instrumental settings and conditions necessary for PAH analytical extraction and quantitation; the required time to measure BIOLOG Ecoplate absorbance values; the best dilution factor that should be used for Ecoplate soil extract; and the effect acetone can have on soil microbial community.

The study also showed a relationship in the results for the soil respiration, CLPP, soil biomass, and soil metabolic quotient for the coal tar pitch and heavy metal amended soils which support the conclusion derived by Muhammad et al. [82], unlike the findings of Yao et al. [144] which did not show any relationship between the AWCD values, the microbial biomass concentrations and the heavy metal concentrations.

There were further experimental adjustments that were made for the spiking procedure to be used in the main study. They include: (a) changing the experimental plan to have a sterilised PAH spiked and unspiked soil which is required to monitor the effect of abiotic factor such as PAH organic matter sorption, volatilisation, chemical oxidation and other environmental effect; (b) carrying out the same soil handling treatment for both the spiked soil and unspiked soil; and (c) heavy metal spiked soil without PAH was also introduced in order to compare the effects of heavy alone on the microbial community.

The triplicate carbon substrate in the BIOLOG Ecoplate allows a determination of experimental variation to be made. This study has been able to show that Ecoplate generated data and its microbial metabolic diversity assessment was reproducible, although a further statistical analysis would be carried out on the obtained data to test for their statistical significance. The paucity of study on this issue makes this study the first extensive study that has been carried out on the reproducibility of BIOLOG Ecoplate.

Chapter 4: Biodegradation of PAHs

4.1 Introduction

Biodegradation of PAHs as earlier mentioned in Section 1.5 can be carried out by a wide variety of bacteria and filamentous fungi: bacteria generally utilise the PAHs as sources of carbon and energy for biomass production (growth and multiplication) and CO_2 formation [2, 3], whereas fungal action is part of a detoxification process [4].

PAH-degrading microorganisms are universally dispersed in the natural environment, for example, soils (bacteria and non-ligninolytic fungi) and woody materials (ligninolytic fungi). Many PAH contaminated soils and sediment host active populations of PAH-degrading microorganisms [33, 63, 64] which can also use co-metabolism and detoxification processes [55, 58] as their methods of PAH removal. The microbial metabolic mechanism (aerobic) for PAH degradation involves the oxidation of the aromatic ring followed by the systematic breakdown of the compound to PAH metabolites and/or carbon dioxide [1].

The rate at which microbial cells transform PAHs during biodegradation processes hinges on the rate of absorption and metabolism (the inherent action of the cell) and the rate of conveyance to the cell (mass transfer). The biodegradation efficiency is also severely hampered by two main factors, the poor accessibility of PAH compounds to microorganisms (bioavailability) and the toxic effect of other co-contaminant (heavy metals) upon the microorganisms [31]. The PAH bioavailability is often influenced by the biphasic behaviour of PAHs in the soil whereby; PAHs are rapidly sorbed by the external surfaces of soils (organic materials) [1] but this adsorbed fraction also becomes readily available (bioaccessible) for desorption by microorganisms which are able to solubilise PAH via biosurfactant production or degrade the PAH through their extracellular enzymes (Fungi) [71]. The sorbed fraction also goes into a phase of non bioaccessibility because of its (sorbed fraction) diffusion into intraparticle micropores of the soil environment (especially soil organic matter), where they undergo a slow conversion process, become sequestered (ageing process occurs) and unavailable [3, 71] for either degradation, volatilization or extraction by organic solvents. This process (ageing process) decreases the size of the both the bioavailable and bioaccessible fraction but there is always an equilibrium maintained between the aqueous (directly bioavailable) and adsorbed phase with the latter greatly favoured. This adsorbed phase is also in equilibrium with even the less bioaccessible phases.

Poor bioavailability of PAHs is often seen as a mitigating factor to their microbial degradation, some of the microorganisms (e.g. bacteria) that are able to carry out the

degradation of the bioaccessible PAH fraction as earlier mentioned possesses properties (such as surfactant production, emulsifiers production, motility and chemotaxis) which enhance the degradation of non-bioavailable PAH compounds [37, 62]. The abilities of these microorganisms give them an advantage over those without these abilities. An example of this is shown in a study where the *Pseudomonas* growing on phenanthrene and naphthalene was able to produce biosurfactants which increased the solubility of its substrate [268].

The degradation of a mixture of PAH compounds by a microbial community can also be hindered by inhibition to microorganisms caused by increased solubility of some of the toxic PAH compounds (for example Fluorene) [92] as have been discussed in Section 1.7. PAH microbial degradation can also be enhanced through co-metabolism as observed by Guoqing *et al.* [102].

The pertinent factor to this study that can also hinder PAH microbial degradation is the presence of heavy metal co-contaminants which are often found in many contaminated sites. Heavy metals can have a detrimental effect on the microbial community, for example on biomass content and metabolic activities [110, 113, 114, 185] which can affect the degradation of PAHs. Some studies on PAH and heavy metal co-contamination have observed that PAH degradation was reduced in the presence of heavy metals [102, 106], whereas other studies have observed a stimulating effect of heavy metal (especially at low concentrations) on microorganisms which increased degradation of PAHs [47, 99]. There seem to be conflicting evidence on the concentrations at which heavy metals produce this stimulating effect on microorganisms. Bååth *et al.* [93] in his review reported microbial activities (soil respiration, nitrogen mineralisation) stimulation at metal concentrations <100 mg kg^{-1}, Thavamani *et al.* [47] observed that the presence of Cd at 5 mg L^{-1}, stimulated the degradation of a PAH mixture while Khan *et al.* [99] observed increased pyrene dissipation in soil in the presence of 300 mg kg^{-1} of Pb. However, Khan *et al.* used rye grass in a cultivated soil and there is the possibility that rye grass provided enough organic carbon substrate which seemed to cushion the effect of heavy metals on microorganisms (especially Fungi) [95]. Nevertheless, they also observed stimulation by Pb at an uncultivated rye grass though at a lower rate. In the present study, the heavy metal threshold concentration where microbial activity stimulation occurred and its implication on PAH biodegradation will be evaluated.

Microbial activity stimulation can also be due to the development of tolerance within the microbial community (increased metal tolerant community) whereby biodegradation is

carried out at similar rates, though at a much reduced microbial community diversity [84].

This chapter aims to investigate the degradation of the individual 16 USEPA PAH compounds in a high organic matter content soil (11.37 %). The soil organic matter possesses a humin component which is associated with the non-bioaccessible and non-extractable (by accelerated solvent extraction) fraction of PAHs in soil. Therefore, the removal of PAHs through the influence of soil organic matter would be highlighted in this chapter.

The effect of heavy metal co-contamination on the degradation of the individual 16 PAH compounds is evaluated. The heavy metal concentration and the heavy metal (Pb or Cd) that caused microbial activity stimulation were evaluated. Furthermore, the resulting effect on the degradation of the individual 16 USEPA PAH compounds because of the microbial stimulation was also evaluated.

The chapter also intends to develop a kinetic model of the biphasic behaviour of PAH in a high organic carbon soil.

4.2. Experimental methodology

4.2.1. Overview

This was a 40 week spiked microcosm study of the biodegradation of 16 USEPA priority list PAHs in the presence and absence of cadmium and lead co-contaminants at various concentrations.

The biodegradation experiments were carried out in PVC microcosms cut from a water pipe (20 cm long and 3.2 cm diameter). Each soil treatment was divided into 39 separate microcosms allowing replicates of three to be harvested for analysis at 1, 2, 3, 5, 7, 9, 12, 15, 20, 25, 30, 35 and 40 weeks. A sample was also taken immediately that the treatment had been prepared (0 week). Each microcosm contained 250 g of soil and was sealed at the bottom end with polyethylene. Microcosms were stored in a plant room 20 ^{o}C with diurnal light cycle. Soil moisture was maintained at 75 % of the maximum water holding capacity by checking the difference in the weight of the microcosm and adjusting with an appropriate amount of distilled water. The following treatments were set up: PAH only; PAH with 100, 250 and 500 mg kg^{-1} added Cd; PAH with 100, 250 and 500 mg kg^{-1} added Pb; abiotic control in which 500 mg kg^{-1} mercury additions ($HgCl_2$) were repeated at 0 and 7 weeks to maintain sterile conditions.

PAHs concentrations were determined by GC-MS analysis after extraction by accelerated solvent extraction (ASE).

4.2.2 Soil sampling:

Soil used in the study was collected from Armstrong Park situated in the northeast of Newcastle upon Tyne approximately 2 km from the city centre (Grid reference). The specific location is a Greenfield site that is currently wooded. Significant industry, including lead works and lead paint works operated to the south of the site, contributing to historically elevated lead and other metals (Zn and Cr) levels in this area [227]. The sampling was carried out from the top soil to a depth of 30 cm (0- 30 cm) in a random pattern and in an intrusive sampling technique where soil was collected by the use of auger/trowel over a shallow depth. A total of 200 kg of soil sample was collected from the sampling site. The collected soil sample was transferred to the laboratory using many polyethylene bag in order to maintain the original soil characteristics.

4.2.2.1 Sample preparation:

The soil was homogenised, air dried for 24 hours to reduce the level of moisture content but not completely dried and was then sieved with 2 mm sieve. 1kg of the soil was used for soil characterisation.

4.2.3 Materials and reagents:

PAH standard solutions of 2000 µg mL^{-1} were purchased from Thames Restek U.K Ltd., Buckinghamshire, UK (2000 µg mL^{-1} in dichloromethane). sonicator (Bransonic Ultrasonic Cleaner 2200) was used to warm and sonicate PAH standard solutions before use. Aluminium Oxide (Al_3O_3) purchased from Sigma-Aldrich Ltd (Dorset, UK) was used as a clean-up reagent to remove endogenous compounds such as fatty acids, cholesterol or steroids by adsorption chromatography. 4,4'-difluorobiphenyl used as internal standard was purchased from Sigma-Aldrich Ltd., Dorset, UK. All the solvents (example: dichloromethane, acetone) used during the experiment were analytical reagent grade and were purchased from Fisher Scientific Ltd. (Loughborough, UK). High purity hydromatrix (diatomaceous earth) was purchased from Varian Inc. (Harbor City, CA, USA). Certified reference materials for PAH spiked soil (LGCQC3008 Sandy soil) were purchased from LGC Standards, Teddington, UK. The binder used in EDXRF analysis was Licowax C micro powder PM (Fluxana GmbH & Co,Sommerdeich, Germany). Filter papers (ASE200) made from glass fibre cellulose were obtained from Dionex Corporation (Sunnyvale, USA). Ethylenediaminetetraacetic

acid (EDTA) was purchased from Fisher scientific Ltd (Loughborough, UK). Chromacol Silanized auto sample vials and metal salts $PbCl_2$, $HgCl_2$ and $CdCl_2.2½H_2O$ were obtained from Sigma-Aldrich Ltd Dorset, UK. Coal tar pitch and Coal tar were supplied by Liver Grease, Oil and Chemical Co. Ltd Liverpool, UK. The ultra-pure water of conductivity 18.2 MΩ-cm at 25 ^oC was produced by a direct QTM Millipore system (Molsheim, France). Concentrated hydrochloric acid (HCl) at least 98 % purity was supplied by Fisher Scientific Ltd. (Loughborough, UK).

4.2.4 Instrumentation and laboratory equipment

4.2.4.1 Energy dispersive x-ray fluorescence (EDXRF):

The details of the EDXRF procedure can be found in section 2.5.7

4.2.4.2 Accelerated solvent extraction:

The details of the ASE can be found in sections 3.3.4.2 and 3.3.10.1

4.2.4.3 Gas chromatography-mass spectrometer (GC-MS):

PAH analysis was based on the method of Afanasov *et al.* [248] and was performed on a Thermo Electron Corporation GC-MS, fitted with a capillary column DB-5MS (5 % diphenyl- 95 % dimethylpolysiloxane, 30 m x 0.25 mm ID x 0.25 µm film thickness) supplied from Thames Restek (UK). The analysis was carried out in single ion monitoring mode with a split injection and an injection volume of 1µL. Several monitoring regions were set up during the course of the 66 minutes run time, each monitoring a specific set of single ions; these were set up based on the elution times of a 16 USEPA PAH standard, run in full scan mode as shown in Table 3.3 (Chapter 3). Each PAH was calibrated on standards of 0, 1, 2 5 and 10 µg mL^{-1} and a 10 µg mL^{-1} standard was run after each group of 10 samples to validate the precision and accuracy of the instrument [47]. Blanks were also used (as in Chapter 3) to control the presence of residual compounds on the column after every set of runs and also as a check on the purity of the solvents used for GC-MS analysis.

The Instrument conditions for the analyses were; the injector port and the detector temperature were set at 280 ^oC and 270 ^oC respectively. The other instrument conditions are shown in Table 4.1.

Table 4.1: The GC-MS programmed temperature conditions used for the analysis of PAH compounds

Temperature Conditions			
	Rate(^{0}C/min)	Temperature (^{o}C)	Holding Time (minutes)
Initial		70	2.00
Ramp 1	7	180	0.00
Ramp 2	3	300	8.00

The system was controlled from a PC with Xcalibur™ 1.4 SR1 software.

4.2.5 Characterization of soil:

The soil characterization details can be found in section 3.3.5

4.2.5.6 Determination of organic carbon (removal of carbonates) using Flame 200 organic elemental analyser (Thermo Scientific):

2 g of air dried soil sample was sieved with 2 mm sieve and crushed for soil particle uniformity. Approximately 4.000 mg of the soil in triplicate samples were transferred into caps ready for analysis.

The instrument furnace (the part of the instrument which incinerates the sample) was set to 98 ^{o}C and the instrument oven (which houses the detector) was set at 50 ^{o}C. The carrier gas (which picks up the evolved CO_2) was helium.

The instrument was then calibrated using 3 organic carbon standards (aspartic acid) containing 36.09 % organic carbon before the samples were analysed. Sample blank was also analysed at the beginning of every soil sample analysis. Each sample was analysed for 360 seconds in order to obtain a strong organic carbon peak. The calculated soil organic carbon concentrations were obtained from the instrument as percentage organic carbon.

4.3 Experimental design:

A total of five hundred and twenty-eight microcosms made from sawn length of PVC tubes each with a diameter of 3.2 cm and a depth of 30 cm were used for the study. The experiment was divided into four groups of A, B, C and D. Each group had 11 PVC tubes per a treatment with 3 replicates of those 11 PVC tubes treatments, making it a total of 33 PVC tubes for each treatment in a group.

Group A has two treatments and required 66 PVC tubes, group B has two treatments and also required 66 PVC tubes while group C and D has six treatments each and each required 198 PVC tubes for their treatments. The treatments for the groups are;

Group A= Non-PAH spiked control soil (A), Non-PAH spiked control soil amended with mercuric chloride (AS),

Group B= PAH spiked control soil (B), PAH spiked control soil amended with mercuric chloride (BS).

Group C= Non-PAH spiked soil amended with 100 mg kg^{-1} Pb (C1), Non-PAH spiked soil amended with 250 mg kg^{-1} Pb (C2), Non-PAH spiked soil amended with 500 mg kg^{-1} Pb (C3), PAH spiked soil amended with 100 mgkg^{-1} Pb (C4), PAH spiked soil amended with 250 mg kg^{-1} Pb (C5), PAH spiked soil amended with 500 mg kg^{-1} Pb (C6),

Group D = Non-PAH spiked soil amended with Cd 100 mg kg^{-1} (D1), Non-PAH spiked soil amended with 250 mg kg^{-1} Cd (D2), Non-PAH spiked soil amended with 500 mg kg^{-1} Cd (D3), PAH spiked soil amended with 100 mg kg^{-1} (D4), PAH spiked soil amended with 250 mg kg^{-1} of Cd (D5), PAH spiked soil amended with 500 mg kg^{-1} Cd (D6),

The PVC microcosms all had the same amount of soil which was 250 g and all the PVC set up was stored in the laboratory plant room with temperature between 18 ^{o}C and 20 ^{o}C

The PAH concentration of the spiked soil was determined immediately after the spiking of soil with coal tar pitch and the microbial community analysis for the coal tar pitch spiked soil was also determined soon after the spiking.

The heavy metal concentration of the soil was determined by EDXRF using a Spectro Analytical X-Lab 2000 instrument (section 4.2.3.1) operating a geology programme [228], measurement for the treatments that has been amended with heavy metal was done immediately after the heavy metal spiking. The bioremediation study was run for 40 weeks and the analyses for the treatments in each group was carried out in the following order of times; 0 days 1 week, 2 weeks, 3 weeks, 5 weeks, 7 weeks, 9 weeks 12 weeks, 15 weeks, 20 weeks, 25 weeks, 30 weeks, 35 weeks and 40 weeks.

The heavy metal bioavailable fraction in the soil was determined by the EDTA method and quantified using a flame atomic absorption spectrometer. The bioavailable fraction was only measured out for the initial day of spiking and for the final day of the experiment.

Each group and its three replicates were analyzed for residual concentration of PAH and their corresponding heavy metal concentration.

4.3.1 Coal tar pitch soil spiking experiment:

4.3.1.1 PAH spiking with coal tar pitch:

In total 91 kg of coal tar pitch spiked soil was required for the biodegradation experiment. The coal tar pitch was dissolved in acetone as the carrier solvent. A total of 568 g powdered (by grinding and sieving to 2 mm) coal tar pitch was dissolved in 9.1 litres of acetone (100 mL of acetone/kg of soil) [78] divided into four 2.5 litre Winchester bottles; the coal tar pitch was allowed to dissolve over 48 hours, during which time the bottles were regularly shaken The adopted method of spiking was dilution method [226] where the solvent was applied to only one-quarter of the total required soil i.e. 22.75 kg in batches using a cement mixer as shown in Figure 4.1. The spiked soil was allowed to air dry in an enclosed space outside the laboratory for 24 hours for the acetone to completely evaporate. The remaining 68.25 kg was then mixed with the spiked soil using a cement mixer (Figure 4.1) to ensure a thorough mixing of the soil. By initially spiking only one-quarter of the soil with acetone/coal tar pitch any detrimental effects of the solvent on the soil microbial population was minimised. The target concentration of total PAH to be achieved from spiking was 2500 mg kg^{-1}. The coal tar pitch spiked control soil was then transferred into the sawn off PVC microcosms that were used for the study.

Figure 4.1: Soil spiking and mixing using an electric cement mixer

4.3.1.2 Heavy metal spiking:

Heavy metal spiking was done in two categories: the first category of spiking was carried out on non-coal tar pitch spiked soil (non-PAH spiked soil) and the second category of spiking was carried out on coal tar pitch spiked soil (PAH spiked soil).

The purpose of the first category of heavy metal spiking was to evaluate the independent effect of heavy metal on soil microbial community and the purpose of the second category of spiking was to also determine the combined effect of heavy metal and PAHs on soil microbial community.

4.3.1.2.1 Lead (Pb) spiking:

Lead spiking for the non-PAH spiked soil was done in addition to the background Pb concentration (206.6 ± 8.5) and it was carried out in three different Pb concentrations of low (100 mg kg^{-1}), medium (250 mg kg^{-1}) and high (500 mg kg^{-1}). The non-PAH spiked lead spiked soil was grouped under three different groups of C1 (100 mg kg^{-1}), C2 (250 mg kg^{-1}) and C3 (500 mg kg^{-1}) for easy data handling. 9 kg of soil was spiked with Pb

concentrations for each group and its replicates. $PbCl_2$ was used as the source of Pb metal in the pitch spiked soil and the water spike/mixing method was adopted for the heavy metal spiking [226]. The non-PAH spiked soil spiking for the 100 mg kg^{-1}, 250 mg kg^{-1} and 500 mg kg^{-1} of Pb required 1.208 g, 3.02 g and 6.04 g of $PbCl_2$ respectively, each was dissolved in 85 mL of distilled water needed to adjust the soil to 75 % of its water holding capacity. The resulting solution was thoroughly mixed with the soil according to their respective groups using the electric cement mixer. The resultant mixtures were expected to yield either the above-named Pb concentrations or higher Pb concentrations in soil dry weight. 9 kg of PAH spiked soil of which their group comprises of C4 (100 mg kg^{-1}), C5 (250 mg kg^{-1}) and C6 (500 mg kg^{-1}) was also spiked with the same amount of $PbCl_2$, distilled water and the same method that was used for the non-PAH spiked soil. EDXRF analyses on the spiked soils were carried out immediately to check and confirm the added Pb concentrations. All the spiked Pb-spiked soils were transferred into the PVC microcosms and stored in a plant room at 20 ^oC with diurnal light cycle.

4.3.1.2.2 Cadmium spiking:

Cadmium spiking for the non-PAH spiked soil was also carried out using three different Cd concentrations of low (100 mg kg^{-1}), medium (250 mg kg^{-1}) and high (500 mg kg^{-1}). The non-PAH spiked Cd spiking was also grouped under three different groups comprising of D1 (100 mg kg^{-1}), D2 (250 mg kg^{-1}) and D3 (500 mg kg^{-1}). 9 kg of non-PAH spiked soil was used for each group of Cd spiking and $CdCl_2.2½H_2O$ was used as the source of Cd metal in the spiked soil. The non-PAH spiked soil spiking for 100 mg kg^{-1}, 250 mg kg^{-1} and 500 mg kg^{-1} Cd required 1.8282 g, 4.57 g and 9.14 g of $CdCl_2.2½H_2O$ respectively, each was dissolved in 85 mL of distilled water needed to adjust the soil to it 75 % water holding capacity (WHC). The spiking was carried out using the same method that was used for lead spiking. The resultant mixture was expected to yield above named Cd concentrations in dry weight of soil. 9 kg of PAH spiked soil grouped into three groups of D4 (100 mg kg^{-1}), D5 (250 mg kg^{-1}) and D6 (500 mg kg^{-1}) was spiked with a similar amount of $CdCl_2.2½H_2O$, distilled water and the same method that was used for the non-PAH spiked Cd spiked soil. EDXRF analysis on all the spiked soil was carried out immediately to check and confirm the added Cd concentrations in the soil. The Cd spiked soils were transferred into PVC microcosms and stored in a plant room as the other spiked soils.

4.3.1.2.3 Mercury spiking:

Mercury (Hg) spiking was carried out in order to sterilise the soil and monitor the effect of abiotic factors such as soil texture, volatilization and soil organic matter on the removal of PAH compounds from the soil. The mercury spiking was carried out on 9 kg of both the non-PAH spiked soil and the PAH spiked soil to serve as a sterile control for the present study. The sterilised soils were grouped into AS (non-PAH spiked sterilised soil) and BS (PAH spiked sterilised soil) for the purpose of data handling. The two groups were spiked 500 mg kg^{-1} of Hg, using $HgCl_2$ as the source of Hg metal in the soil. The spiking was carried out at the onset of the study and after 7 weeks of the study to maintain sterility.

The spiking for 500 mg kg^{-1} Hg for both the non-PAH spiked soil and the PAH spiked soil required 6.092 g of $HgCl_2$ which was dissolved in 85 mL of distilled water needed to adjust the soil to 75 % of its WHC. Spiking was carried out using the same method as the ones used in the previous heavy metal spiking. EDXRF analysis on the spiked soils was carried out immediately after spiking to confirm the added Hg concentration in the soil. The Hg spiked PVC microcosms were stored in the same plant room as the other spiked soils.

4.3.1.3 Incubation and storage:

The unspiked control PVC microcosm, coal tar pitch and heavy metal spiked PVC microcosms were stored in an aerated and moisture regulated plastic box that was incubated at a controlled temperature between 18 °C- 20 °C (plant room) with a diurnal light cycle. The top lid of the plastic box was perforated to allow for easy air circulation and two beakers of water with a wet paper towel were used to regulate the loss of moisture in the box. The water content of the soil was maintained at 70 % WHC at 7 days intervals by checking the weight difference of the PVC microcosms. PVC microcosms for each treatment with its replicates (3 replicates) were harvested for analysis at 0, 1, 2, 3, 5, 7, 9, 12, 15, 20, 25, 30, 35 and 40 weeks after the spiking set up. Figure 4.2 is a picture of the storage box used to house the microcosms.

Figure 4.2: PVC microcosm storage box used during the main study of PAH bioremediation in soil

4.4 Experimental procedure for heavy metal analysis:

Spiked soil heavy metal concentrations for Pb, Cd and Hg were determined in two stages; total heavy metal content and bioavailable heavy metal concentration. The total heavy metal concentration was determined using energy dispersive X-ray fluorescence (EDXRF). The soil bioavailable Pb and Cd heavy metal content were determined by ethylene-diamine-tetra-acetic acid (EDTA) extraction method. The concentration of the extracted soluble metal quantified using flame atomic absorption spectrometer. The bioavailable fraction was only measured for the initial day of spiking and the final day of the experiment.

4.4.1 Total heavy metal concentration soil sample preparation for EDXRF

The soils total heavy metal concentration was determined using the same EDXRF protocol that was used in section 3.3.10.1.

4.4.2 EDTA extraction of metal from soil (bioavailability):

0.05 M EDTA was prepared as ammonium salt solution by adding in a beaker 14.61 g EDTA to 80 mL deionised water. The dissolution was aided by gradually adding 13 mL of ammonia solution (25 %). The solution was then transferred to a 1litre polyethylene container and approximately 900 mL of deionised water was added. The pH of the solution was adjusted to 7.0 by adding a few drops of HCl as appropriate and the solution was made up to 1 litre with deionised water.

2 g of representative soil samples from all the microcosms were each weighed into 50 mL centrifuge tubes and 20 mL of 0.05 M EDTA (pH 7.0) was added. The mixture was shaken in an end over end shaker at 30 rpm for 1hr at room temperature. The supernatant from the mixture was filtered through using a Whatman filter paper. The resulting extract was analysed for lead (Pb) and cadmium (Cd) concentrations using flame atomic absorption spectroscopy (FAAS). The FAAS was first calibrated using separate Pb and Cd standard concentrations of 0 mg L^{-1}, 2 mg L^{-1}, 4 mg L^{-1}, 6 mg L^{-1}, 8 mg L^{-1} and 10 mg L^{-1}. The FAAS value of the blank EDTA solution was also subtracted from the extract FAAS values in order to account for any error due to background solution. The Pb and Cd concentrations in soil extracts were obtained from the standard calibration graph that was plotted using the prepared Pb and Cd standard solution FAAS values.

4.5 Results and discussions

4.5.1. Characterisation of soil sample:

Soil chemical and physical properties were evaluated in order to determine the quality of the soil sample. The table 4.2 and 4.3 shows the obtained values for each soil chemical and physical properties evaluated:

4.5.2 Soil texture values:

Table 4.2: The percentage composition of the various soil fractions that make up the soil texture

Clay %	Fine Silt %	Medium silt %	Coarse Silt%	Coarse Sand%	Medium Sand%	Fine Sand%
2.59	4.47	11.66	12.32	4.97	14.79	48.66

4.5.3 Soil properties values:

Table 4.3: Measured soil sample properties**.**

PARAMETER	RESULTS
% Maximum Water holding Capacity	42.23 %
75 % Water holding Capacity	31.67 %
% Nitrogen	0.37 %
%Organic matter by Loss on Ignition	26.56 %
%Organic Carbon by Elemental Analyser Instrument	11.37 %
Soil pH	6.02

The soil texture values show that the soil has sandy loam characteristic 68.42 % sand, 28.45 % silt and 2.59 % clay. The soil also has an appreciable amount of organic carbon (11.37 %). The organic carbon also serves as nutrient for microbial community because carbon is one of the essential element for microbial growth [94]

Table 4.4: EDXRF analysis on untreated soil sample

Measured soil potentially toxic elements (mg kg^{-1} ± 1 sd)	
As	16 ± 3
Cd	<1
Cr	228 ± 45
Cu	66 ± 6
Hg	<1
Ni	30 ± 2
Pb	201 ± 7
Sb	11 ± 1
Se	<1
Sn	16 ± 1
Zn	147 ± 8

4.5.4: Soil heavy metal analysis

Table 4.5 shows the spiked metal concentrations for lead and cadmium at various stages during the 40 week study for both PAH amended and non-amended soils. The aim was to achieve approximate amendment levels of 100 mg kg^{-1} (low), 250 mg kg^{-1} (medium) and 500 mg kg^{-1} (high) for lead and cadmium. The actual initial concentrations are higher for both metals, though in the case of lead, we must also take into account the high background concentration. The concentrations of metals in

the soil generally decrease during the course of the study due to leaching following regular water addition to ensure constant soil moisture content. For the sterile microcosms, mercury chloride was added, initially at a concentration of about 500 mg kg^{-1} Hg, with a further amendment required after 7 weeks, taking the total to approximately 1,100 mg kg^{-1}. The repeated amendment was required because soil respiration analysis indicated some recovery in microbial activity at this point.

Table 4.5: Concentrations of lead and cadmium over the duration of the study in metal amended soils

			Cadmium or lead bioavailable concentration in soil (mg kg^{-1} ± 1 sd)			
PAH amendment status	Metal	Metal amendment level	0 wk	5 wk	20 wk	40 wk
Non amended	Cd	None	<1	-	-	-
		Low	148.9 ± 6.1	122.8 ± 9.8	127.2 ± 9.1	111.7 ± 11.4
		Medium	319.1 ± 11.2	267.9 ± 10.5	262 ± 9.1	260.9 ± 23.4
		High	567 ± 47.9	547.4 ± 21.3	535.1 ± 26.3	549.1 ± 16.2
PAH amended	Cd	None	<1	-	-	-
		Low	133.8 ± 21.2	117.5 ± 9	126.3 ± 10.1	115.5 ± 8.7
		Medium	301.5 ± 13	279.9 ± 11.4	273.6 ± 13.2	254.1 ± 9.5
		High	619.8 ± 68.5	502.7 ± 45.1	469.6 ± 82.7	465 ± 28.7
Non amended	Pb	None	206.6 ± 8.5			
		Low	340.4 ± 7.6	321.6 ± 14.3	323.1 ± 26.8	327.3 ± 17.3
		Medium	565.7 ± 43.3	514.7 ± 10.5	461.7 ± 9.1	444.1 ± 23.4
		High	816.7 ± 15.7	739.3 ± 57.4	684.2 ± 19.2	640.2 ± 23.5
PAH amended	Pb	None	210.1 ± 11.8	-	-	-
		Low	339.4 ± 36.2	305.6 ± 22.1	292 ± 23.7	291 ± 38.5
		Medium	522.9 ± 3	436.4 ± 14.7	415.7 ± 15.7	422 ± 30
		High	781.8 ± 44.5	684 ± 11.1	639.4 ± 64.4	637.1 ± 55.4
Non amended	Hg	n/a	643.4 ± 26.7	510.1 ± 48.1	1142.3 ± 14.7	939.1 ± 108.5
PAH amended	Hg	n/a	627.2 ± 19.7	538 ± 28.2	1115.1 ± 89.2	1061.1 ± 78.1

4.6.4.1 Soil bioavailable heavy metal

Calibration curves for Pb and Cd based on a standard concentration range of 0 - 10 mg L^{-1} with 5 calibration data points were obtained using Flame Atomic Absorption Spectroscopy (FAAS) and the regression coefficients (R2) obtained for both Pb and Cd calibration curves were 0.998 which shows the reliability of the standard solutions for the quantification of the metals concentrations in the soil solution.

Table 4.6 shows the bioavailable concentrations of Pb and Cd in the soil solution at 0 week and 40 weeks sampling periods for the PAH amended soils and the non-PAH spiked amended soils. The bioavailable concentrations of the metals show the concentration level of Pb and Cd that may be readily available to the microbial community during the course of the study. The total metal concentrations as shown in table 4.5 are not always available in soil solution [86, 110] due to the soil physicochemical properties such as the pH and soil organic matter content [90]. These

physicochemical properties of the soil influence chemical processes such as precipitation, dissolution, adsorption, desorption and chelation which takes place in the soil. These processes control the bioavailability of metals in soil [86]. The bioavailable metal concentration in the soil influences the soil microorganisms [110] which can be in a positive (stimulation) or negative (toxic) way. The stimulation on heavy metal on microbial activities has been observed at low concentrations of heavy metal by Bååth *et al.* [93] but the toxicity of the metals increases as the concentration of the metal increases. The bioavailable concentration of Cd at low level of amendment for the periods measured was < 100 mg kg^{-1} but the bioavailable Pb at the same level of amendment was > 100 mg kg^{-1} because of the high background concentration of Pb in the soil. The bioavailable concentrations at the initial period decreased at the latter period as seen in table 4.6 which can be due to leaching following regular water addition to ensure constant soil moisture content as already mentioned earlier. The soil chemical processes such as adsorption and precipitation can also reduce the bioavailable concentrations of heavy metals in soil

Table 4.6: EDTA Bioavailable concentrations of Pb and Cd in PAH amended and non-PAH spiked amended soil

			Cadmium or lead bioavailable concentration in soil (mg kg^{-1} ± 1 sd)	
PAH amendment status	Metal amendment	Metal amendment level	0 wk	40 wk
Non amended	Cadmium	None	<1	-
		Low	47.6 ± 0.6	45.3 ± 0.6
		Medium	235 ± 0.7	224.5 ± 7.4
		High	366 ± 33.9	313.7 ± 9.7
PAH amended	Cadmium	None	<1	-
		Low	52.9 ± 2.3	45 ± 0.6
		Medium	214.7 ± 7.1	192 ± 4.4
		High	337.6 ± 12.9	295.4 ± 5.6
Non amended	Lead	None	134.6 ± 5	137.5 ± 8.6
		Low	266.4 ± 8.6	254.9 ± 21.6
		Medium	426.8 ± 13.9	363.8 ± 5
		High	461.2 ± 37.8	521.4 ± 62.2
PAH amended	Lead	None	143.5 ± 5	120.3 ± 8.6
		Low	249.2 ± 29.8	223.4 ± 8.6
		Medium	340.9 ± 5	263.5 ± 17.9
		High	647.4 ± 13.1	478.4 ± 17.9

4.6.5: PAH biodegradation

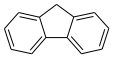
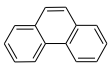
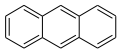
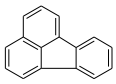
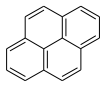
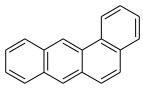
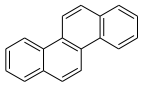
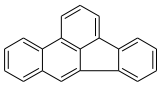
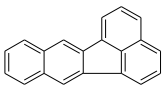
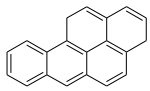
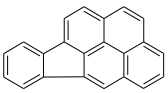
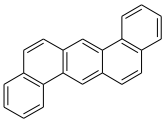
As discussed in the introduction, biodegradation of organic substances in soil involves a complex community of bacteria and fungi with multiple enzymic pathways, together with synergistic and inhibitory effects. In addition, the PAHs themselves partition between the aqueous phase and multiple soil phases, each possessing a different

degree of bioaccessibility, and therefore affecting the bioavailable fraction. Nevertheless, most literature PAH biodegradation studies in soil report that the rate of PAH loss is described by either a single [269-272] or double [272-274] exponential equation. In the present study we found that for microcosms without added metals, the rate of loss of extractable PAHs fits reasonably well to a double exponential: it seems that the bulk effect of these complex processes over significant time periods approximates to a sequential two-step reaction, as described by Eq. 4.1. Here, PAH_{TE} is the concentration of the total extractable PAH, k_a and k_b are the rates for the two stepwise processes, and Φ_a and Φ_b are the corresponding fractions of the reaction occurring via each process. All curve fitting analysis was carried out using the non-linear least squares method of Grafit version 7 [275].

$$[PAH_{TE}] = [PAH_{TE}] \cdot \Phi_a \cdot e^{-k_a \cdot t} + [PAH_{TE}] \cdot \Phi_b \cdot e^{-k_b \cdot t} \qquad \text{Eq. 4.1}$$

Table 4.7 summarises the rates for these two processes, together with the percentage (Φ_a x 100) of the reaction proceeding via the first step. The corresponding kinetic profiles are shown in Figure 4.3 and it is interesting to note the range of different behaviours: acenaphthene, fluorene and phenanthrene, for example, degrade rapidly, dropping to below 10 % of the starting concentration within 100 days, whereas naphthalene and acenaphthylene drop to about 35 % of the starting concentration in the same period before entering a slower degradation phase. There are two points to note here: firstly that the lower molecular weight PAHs, comprising, two and three benzene rings generally degrade at a faster rate than four- five and six-membered rings; this is also a common finding in the literature, and is most likely related to the restricted range of bacteria that can metabolise higher molecular weight PAHs [276, 277]. Nevertheless, whilst in general, the percentage of PAHs that are degraded in the first step appears to be correlated to k_a, there are exceptions, for example, acenaphthylene, which degrades rapidly in the first step, yet reaches only about 35 % of the starting concentration by 100 days; similarly, anthracene degrades relatively slowly, yet reaches less than 15 % of the starting concentration by 100 days. The second point is that the k_b values, with perhaps the exception of fluoranthene, appear remarkably similar. These points will be elaborated upon later in the discussion; however it is important first to try to ascribe a plausible mechanistic model to the observed biphasic kinetics.

Table 4.7: Initial concentrations and kinetic parameters for soil microcosm biodegradation studies shown in Figure 4.3.

PAH	Structure	Initial concentration (mgkg-1)	$k_a/10^{-2}$ d^{-1}	$k_b/10^{-2}$ d^{-1}	% of reaction proceeding via k_a (Φ_a x100)
Naphthalene		3.28±0.44	6.92±1.3	0.29±0.04	54.7±3.1
Acenaphthylene		1.44±0.02	19.74±1.84	0.26±0.02	58.1±0.9
Acenaphthene		7.75±0.40	27.2±2.75	0.32±0.05	90.1±0.7
Fluorene		9.53±0.13	24.39±3.39	0.46±0.05	85.7±1.1
Phenanthrene		93.43±6.53	12.06±0.84	0.38±0.06	94.8±0.5
Anthracene		27.56±2.02	4.87±0.48	0.46±0.07	82.4±2.6
Fluoranthene		217.6±13.5	19.02±8.75	0.94±0.04	40.9±3.4
Pyrene		187.8±4.8	3.68±0.35	0.47±0.08	80.7±3.2
Benzo[a]anthracene		167.9±16.0	3.93±0.73	0.34±0.06	61.1±5.1
Chrysene		196.1±31.7	14.75±3.71	0.34±0.03	51.3±2.4
Benzo[b]fluoranthene		324.7±14.2	4.81±0.5	0.22±0.02	42.3±1.8
Benzo[k]fluoranthene		138.1±7.6	6.05±2.62	0.34±0.04	31.1±4.9
Benzo[a]pyrene		254.9±12.6	5.75±1.37	0.34±0.02	32.6±2.9
Indeno[123-cd]pyrene		246.31±11.4	4.3±1.63	0.35±0.05	42±6.2
Dibenzo[a,h]anthracene		105.4±5.4	4.72±1.13	0.29±0.03	34.7±4.2
Benzo[g,h,i]perylene		184.5±4.4	5.43±1.79	0.38±0.04	41.1±4.2
Total	n/a	2166	8.58±0.79	0.43±0.01	40.6±0.8

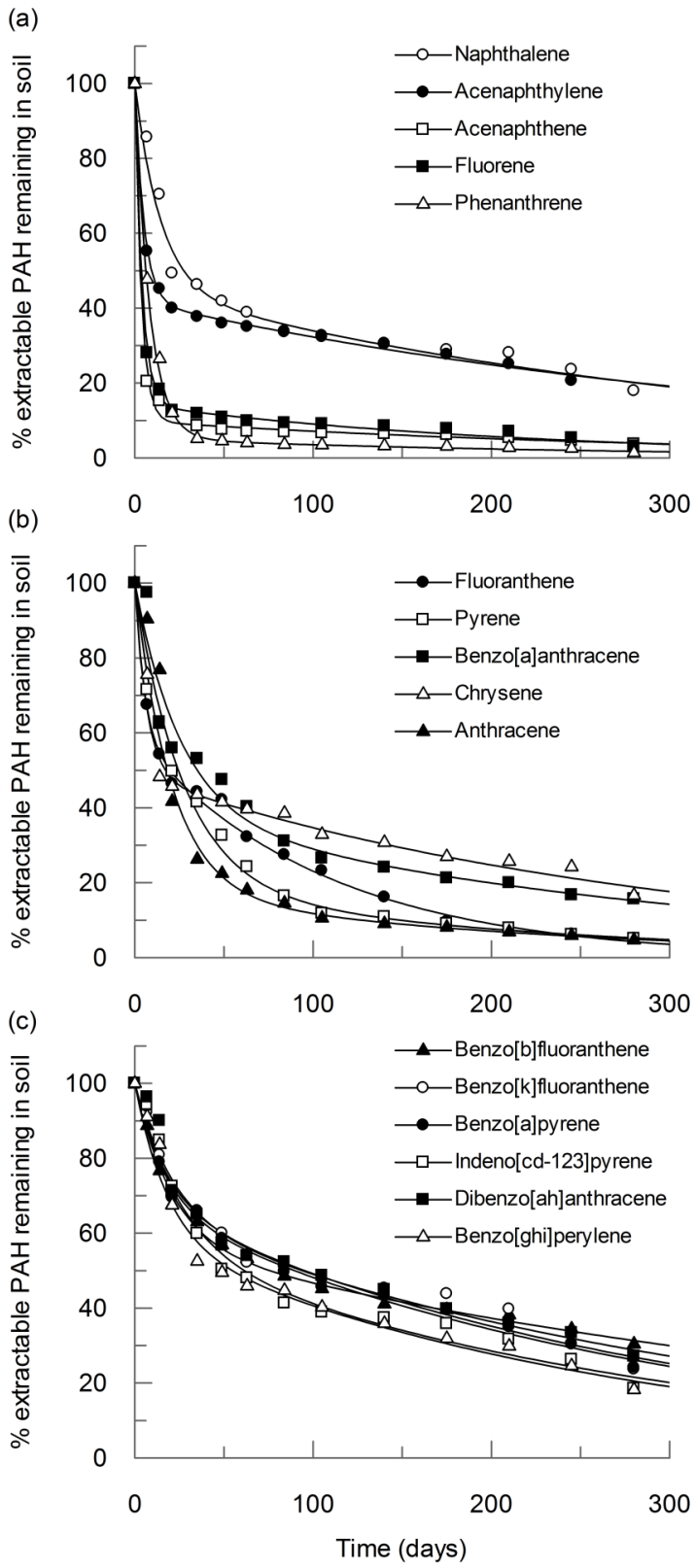
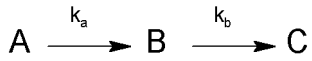


Figure 4.3: Degradation of 16 PAHs in soil microcosms over a period of 280 days without added metals. The curves are the best fit to a double exponential (Eq. 4.1) using the parameters listed in Table 4.7. All points are based on the average concentrations in three separate microcosms

4.6.6 Kinetic model

The approach used in the development of the kinetic model described in this section is based on the work of Deary [278]

Kinetics that conform to a double exponential equation can have an underlying reaction scheme that is as simple as the two-step process shown in Scheme 4.1, but can also be much more complex if the participating species are involved in equilibria and side reactions, as is likely to be the case for soil biodegradation processes.



Scheme 4.1: Sequential two-step reaction.

In finding a plausible underlying reaction scheme to explain the biphasic kinetic data it is important to be clear about what exactly is being measured during the biodegradation experiments. As discussed in the introduction, the interaction of PAHs and other organics with a soil matrix is a complex dynamic process. There will be an equilibrium between the adsorbed phase and the soil solution, but the adsorbed phase itself is complex, with multiple phases where the PAHs can adsorb, both to organic matter and inorganic components, though the former is the more important. Many studies have demonstrated that PAHs adsorbed onto organic matter sequentially transfer to phases that are less bioaccessible, and ultimately to phases within humin that are both non-bioavailable and non-extractable, even by robust extraction methods such as soxhlet and ASE, as used in this study (Dean *et al* have demonstrated that Soxhlet and ASE produce comparable extraction performance for hexaconazole spiked onto soils for 0, 1 and 52 weeks [279]). Thus, even without biodegradation we should expect to see that as a result of soil ageing, the concentration of the PAHs decline with time. This is amply demonstrated by the degradation profiles shown in Figure 4.4, where soil sterilised by the addition of mercury chloride still shows a significant decrease in extractable PAHs over the course of 280 days. Some caution should be exercised because soil respiration studies (reported in Chapter 5) did demonstrate a gradual partial recovery of the microbial community after mercury dosing and so some of the degradation could be due to microbial processes (the soil microcosms were re-sterilised with mercury at various intervals according to the observed respiration rates of the samples).

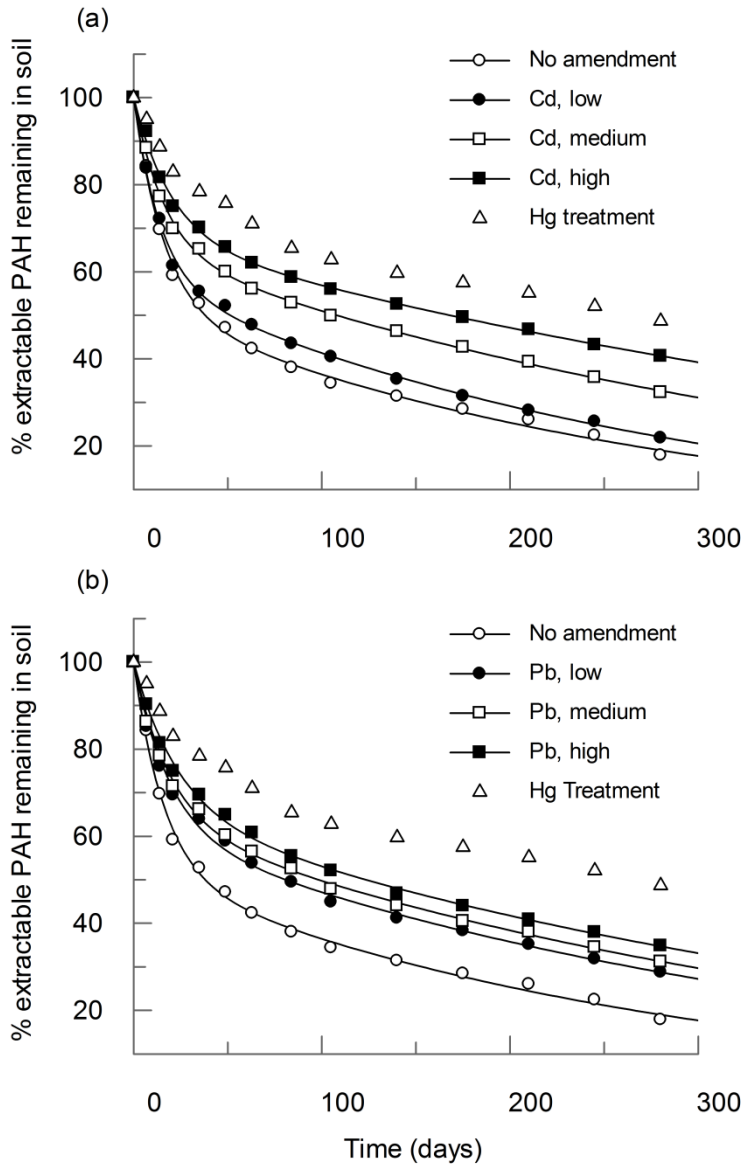
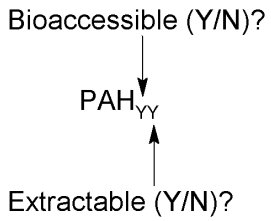


Figure 4.4: Influence of (a) cadmium and (b) lead on the degradation of the total PAH concentration in soil microcosms. The curves are the best fit to a double exponential (Eq. 4.1) using the parameters listed in Table 4.8. The points for the mercury treatment are also shown for comparison (see text for explanation). All points are based on the average concentrations in three separate microcosms

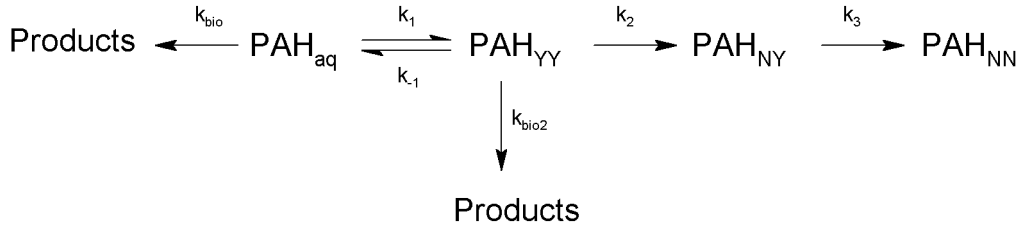
From a consideration of the above, there is likelihood the system could be simplified to a solution phase, a bioaccessible / extractable soil phase, a non-bioaccessible / extractable soil phase and a non-bioaccessible / non-extractable soil phase. The aqueous phase can be denoted as PAH_{aq}, whilst the soil phases are denoted using the convention of subscript notations illustrated in Scheme 4.2. In this example, PAH_{YY} is both bioaccessible and extractable.



Scheme 4.2: Convention for PAH subscript notation

There will be sub-phases within these overall soil phases, but it is, nevertheless, a reasonable simplification to make and is shown in Scheme 4.3. The scheme assumes that the concentration of the enzymes responsible for the biotransformations (k_{bio} and k_{bio2}) are constant.

An analytical solution to the reactions in Scheme 4.3 will yield a double exponential of the form shown in Eq. 4.1, since PAH_{YY} and its associated equilibrium species, equate to species A in Scheme 4.1, with PAH_{NY} and PAH_{NN} equating to species B and C respectively.



Scheme 4.3: Proposed scheme for transfer and degradation of PAHs in the soil environment

In Scheme 4.3, we consider that biodegradation may occur in both the solution phase (k_{bio}) and the solid phase (k_{bio2}), though the latter is likely to be the most important because of the low concentration of PAHs in the solution phase. As we have already discussed, fungi can directly metabolise soil bound organics, as can bacteria if biofilms are formed.

In finding an analytical rate equation for Scheme 4.3, we need to make a number of assumptions and simplifications. Firstly, it is likely that the forward and reverse reactions for the adsorption of PAH onto the soil matrix are rapid compared to the other processes and so we can write this as an equilibrium with the constant K, as in Eq. 4.2.

$$K = \frac{k_1}{k_{-1}} = \frac{[PAH_{YY}]}{[PAH_{aq}]} \qquad \text{Eq. 4.2}$$

From Scheme 4.3 we can write separate rate laws for the loss of the solution phase PAH (PAH_{aq}), and the bioaccessible soil PAH (PAH_{YY}) shown in Eqs. 4.3 and 4.4 respectively.

$$\frac{d[PAH_{aq}]}{dt} = -k_{bio} \cdot [PAH_{aq}] \qquad \text{Eq. 4.3}$$

$$\frac{d[PAH_{YY}]}{dt} = -k_{2} \cdot [PAH_{YY}] - k_{bio2} \cdot [PAH_{YY}] \qquad \text{Eq. 4.4}$$

We also need to define a mass balance for the total bioaccessible PAH (PAH_{TB}), i.e. in the soil and aqueous phases; this is shown in Eq. 4.5.

$$[PAH_{TB}] = [PAH_{aq}] + [PAH_{YY}] \qquad \text{Eq. 4.5}$$

Making substitutions from Eq. 4.2 and Eq 4.5 we can combine and re-write Eq. 4.3 and Eq. 4.4 in terms of $[PAH_{TB}]$, as shown in Eq. 4.5.

$$\frac{d[PAH_{TB}]}{dt} = -k_{bio} \cdot \left(\frac{[PAH_{TB}]}{K+1}\right) - k_{2} \cdot \left(\frac{[PAH_{TB}] \cdot K}{K+1}\right) - k_{bio2} \cdot \left(\frac{[PAH_{TB}] \cdot K}{K+1}\right) \qquad \text{Eq. 4.5}$$

Furthermore, because the value of K is typically much greater than 1[1] we can make the additional assumption that $K+1 \approx K$, thus allowing us to simplify Eq. 4.5 to Eq. 4.6, i.e. an assumption is made that the PAH is overwhelmingly in the bound form.

$$\frac{d[PAH_{TB}]}{dt} = -k_{bio} \cdot \left(\frac{[PAH_{TB}]}{K}\right) - k_{2} \cdot [PAH_{TB}] - k_{bio2} \cdot [PAH_{TB}] \qquad \text{Eq. 4.6}$$

Having defined the rate law for the bioaccessible fraction of the PAH, we now need to do the same for the non- bioaccessible, but extractable, PAH fraction (PAH_{NY}), which is formed from PAH_{TB} and lost as the PAH subsequently migrates deeper into the humin

[1] For pyrene, *K* ranges from 71 to 1155 for soils ranging between 0.11 and 2.28% OC respectively 265. J.C. Means, S.G. Wood, J.J. Hassett, and W.L. Banwart, *Sorption of Polynuclear Aromatic Hydrocarbons by Sediments and Soils.* Environmental Science Technology, 1980. **14**(12): p. 1524-1528.So is likely to be considerably higher with the high OC content of the soil used in this study.

pores to form the non-extractable phase (PAH_{NN}). This is described by Eq. 4.7, having again made the simplifying assumption that K+1 ≈ K.

$$\frac{d[PAH_{NY}]}{dt} = k_2 \cdot [PAH_{TB}] - k_3 \cdot [PAH_{NY}] \qquad \text{Eq. 4.7}$$

Finally, we need to consider the mass balance for the total extractable PAH (PAH_{TE}), Eq. 4.8

$$[PAH_{TE}] = [PAH_{TB}] + [PAH_{NY}] \qquad \text{Eq. 4.8}$$

An integrated rate equation can be obtained from Eq. 4.6 and Eq. 4.7 by carrying out a Laplace transformation using Mathcad 15 and the approach of Korobov and Ochkov [280]. The procedure, involves: (a) taking the Laplace transforms of Eq. 4.6 and Eq. 4.7, (b) adding together the two transformed equations (thus giving the total extractable PAHs, PAH_{TE}) and finally, (c) taking the inverse transform of the simplified form of the combined equation and rearranging to give the double exponential form shown in Eq. 4.9.

$$[PAH_{TE}] = [PAH_{TB}]_0 \cdot \frac{k_{bio} - K \cdot k_3 + K \cdot k_{bio2}}{k_{bio} + K \cdot k_2 - K \cdot k_3 + K \cdot k_{bio2}} \cdot e^{-\frac{(k_{bio} + K \cdot k_2 + K \cdot k_{bio2})}{K} \cdot t} + [PAH_{TB}]_0 \cdot \frac{K \cdot k_2}{k_{bio} + K \cdot k_2 - K \cdot k_3 + K \cdot k_{bio2}} \cdot e^{-k_3 \cdot t}$$

Eq. 4.9

This is the same form of equation as Eq. 4.1, with the pre-exponential terms equivalent to and Φ_a and Φ_b. We can also see that k_a and k_b in Eq. 4.1 have equivalences shown in Eq. 4.10 and 4.11 below.

$$k_a = \frac{(k_{bio} + K \cdot k_2 + K \cdot k_{bio2})}{K} \qquad \text{Eq. 4.10}$$

$$k_b = k_3 \qquad \text{Eq. 4.11}$$

Thus, k_a in Eq. 4.10 is a complex rate constant reflecting the equilibrium and various parallel reactions occurring for the bound and solution phase PAHs. k_b is simply equivalent to k_3, which is the rate for the abiotic transformation of the non bioaccessible / extractable PAH (PAH_{NY}) to the non-extractable phase (PAH_{NN}). Moreover, if we assume that because of limited solubility k_{bio} is small compared to k_{bio2} and k_2 then Eq.

9 equation reduces to Eq. 4.12 and the corresponding pre-exponential factor, Φ_a, reduces to Eq. 4.13 and Φ_b, reduces to Eq. 4.14

$$k_a = k_2 + k_{bio2} \qquad \text{Eq. 4.12}$$

$$\Phi_a = 1 - \frac{k_2}{k_2 - k_3 + k_{bio2}} \qquad \text{Eq. 4.13}$$

$$\Phi_b = \frac{k_2}{k_2 - k_3 + k_{bio2}} \qquad \text{Eq. 4.14}$$

We are now in a position to explain the range of kinetic profiles observed in Figure 4.3. For the first step, k_a, the key relationship is that between k_{bio2}, the rate at which the PAHs are biodegraded, and k_2, the rate at which the bioaccessible PAH, PAH_{YY}, migrates to the inaccessible yet extractable phase, PAH_{NY}. If $k_{bio2} >> k_2$; then Φ_a, the fraction of the overall reaction proceeding via k_a, will approach 1, i.e. 100%. This is the case for acenaphthene, fluorene and phenanthrene, where k_a is large, but also for anthracene and pyrene, where k_a is relatively small. However, in cases where k_{bio2} is equal to or lower than k_2 then Φ_a, will be 0.5 or lower, as we see for the majority of the high molecular weight PAHs, where k_a is relatively low, but also for acenaphthylene, where k_a is large.

In terms of how these rates relate to literature values, the rates for fluorene, anthracene and pyrene fall within the ranges listed in Maliszewska-Kordybach's review of such rates [269], though the value for chrysene observed in our study (14.75×10^{-2} d^{-1}) is significantly higher than the fastest rate listed (1.4×10^{-2} d^{-1})

For the second step, k_b, we have shown that this is likely to equate to k_3, the migration of the bio-inaccessible but extractable PAH, PAH_{NY} to the non-extractable fraction, PAH_{NN}. Our values are consistent with those reported for the same process in sterile soil for pyrene and benzo[a]pyrene [271].

4.6.7 Effect of heavy metals on PAH degradation

Figure 4.2 shows the effect of heavy metal addition on total PAH degradation; Table 4.8 shows the corresponding kinetic parameters. The net effect is that there is a significant reduction in the extent of overall PAH removal over the 280 day period of the study at increasing concentrations of lead and cadmium. From Table 4 we can see that overall, higher metal levels cause k_a to be depressed, though this is more obvious for

lead. Indeed for cadmium, there is the suggestion of slight stimulation at a lower concentration. Table 4.8 also shows that Φ_a x 100 is progressively reduced as the concentration of added metal is increased; this is the main determining factor in the overall reductions on extent of PAH removal. As has already been mentioned, sterilisation by mercury addition was used to create an abiotic control and this data is also included in Figure 4.4.

A summary of the effects of heavy metal addition on the percentage degradation of individual PAHs at 280 days is shown in Figures 4.5 and 4.6 for cadmium and lead respectively. In contrast to the results for total PAH concentration, many of the lower molecular weight PAHs show only small reductions in overall degradation, even at the highest metal concentrations. In contrast, higher molecular weight PAHs show marked reductions in overall degradation with increasing metal concentration.

The reason for the apparent lack of sensitivity of certain low molecular weight PAHs to metal addition is explained by equations 4.12 and 4.13. For PAHs where $k_{bio2} >> k_2$, such as acenaphthene, whilst increasing metal concentrations will cause a decrease k_{bio2}, the ratio of k_{bio2}:k_2 may still be large, meaning that Φ_a still remains close to 1. This is nicely demonstrated by the kinetic profiles shown in Figure 4.7 and the corresponding parameters in Table 4.8. Here, whilst k_a decreases from 27.23 10^{-2} d^{-1} to 8.89 x 10^{-2} d^{-1} Φ_a x100 remains high and so overall loss in extractable PAH falls below 10 % over the course of the study, with only very small differences observed, even at the highest cadmium concentration. On the other hand, for PAHs where $k_{bio2} \approx k_2$, such as dibenzo[ah]anthracene (Figure 4.8 and Table 4.8), reductions in k_{bio2} will have a marked effect on the ratio of k_{bio2}:k_2 causing a significant reduction in Φ_a x100; this is apparent from Table 4.8, at the highest cadmium concentration. Interestingly for this PAH, some stimulation of degradation is observed at the lowest cadmium concentration, as shown in both the kinetic profiles in Figure 4.6 for dibenzo[ah]anthracene and the parameters in Table 4.8. Similar behaviour has been observed in the literature at low concentrations of added metals [281-283].

It should also be noted that increasing metal concentrations did seem to have an inhibitory effect on k_b for both acenaphthene and dibenzo[ah]anthracene. In our kinetic model, it is assumed that k_b (= k_3) is an entirely abiotic process that should not be influenced by metal addition. Either there is some residual biological activity that is occurring parallel to the abiotic process, and is thus affected by the metal addition, or adsorption of metals to the organic component of the soil is affecting migration rates of PAHs from the non-bioaccessible phase to the non-extractable phase.

Table 4.8: Fitting parameters for soil microcosm biodegradation studies shown in Figures 4.2, 4.7 and 4.8.

PAH	Added metal	Amendment level	k_a / 10^{-2} d^{-1}	k_b / 10^{-2} d^{-1}	% of reaction proceeding via k_a (Φ_a x100)
Total PAHs	Cd	No amendment	6.15±0.67	0.36±0.02	47.9±1.8
		Low	7.09±0.5	0.35±0.01	58.5±1
		Medium	6.15±0.39	0.25±0.01	34.8±0.8
		High	5.01±0.34	0.18±0.01	31.8±0.9
	Pb	No amendment	6.15±0.67	0.36±0.02	47.9±1.8
		Low	5.3±0.48	0.27±0.01	38.4±1.4
		Medium	5.23±0.42	0.26±0.01	35.9±1.1
		High	4.28±0.36	0.23±0.01	33.7±1.3
Acenaphthene	Cd	No amendment	27.23±2.78	0.32±0.05	90.1±0.7
		Low	14.14±1.1	0.28±0.04	89±0.8
		Medium	15.54±2.18	0.37±0.08	88.6±1.4
		High	8.89±0.7	0.18±0.05	90.6±0.9
Dibenzo[ah]anthracene	Cd	No amendment	4.65±1.17	0.29±0.03	35.0±4.1
		Low	9.09±0.73	0.18±0.01	47.9±1.0
		Medium	5.92±1.15	0.16±0.02	30.4±2.1
		High	2.78±1.35	0.12±0.02	8.9±3.3

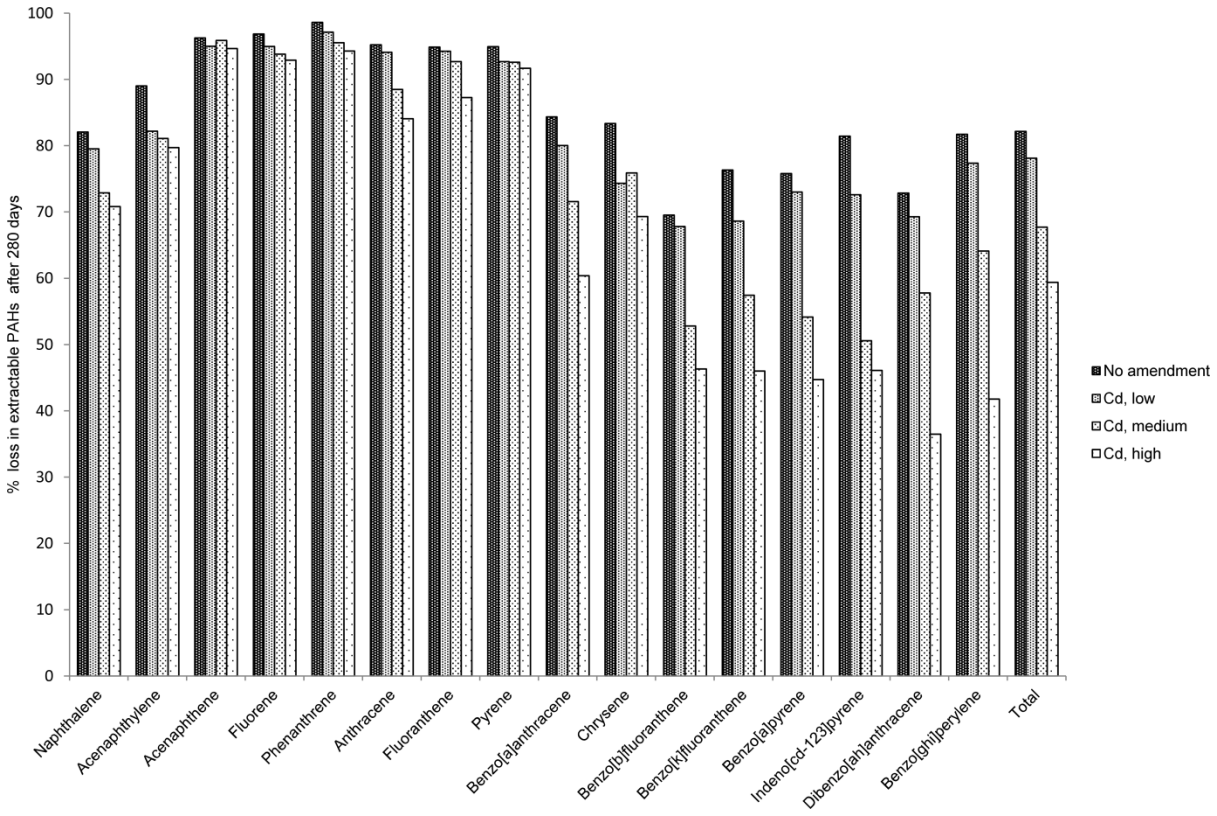


Figure 4.5: Biodegradation of the 16 USEPA priority PAHs after 280 days in the presence of added cadmium

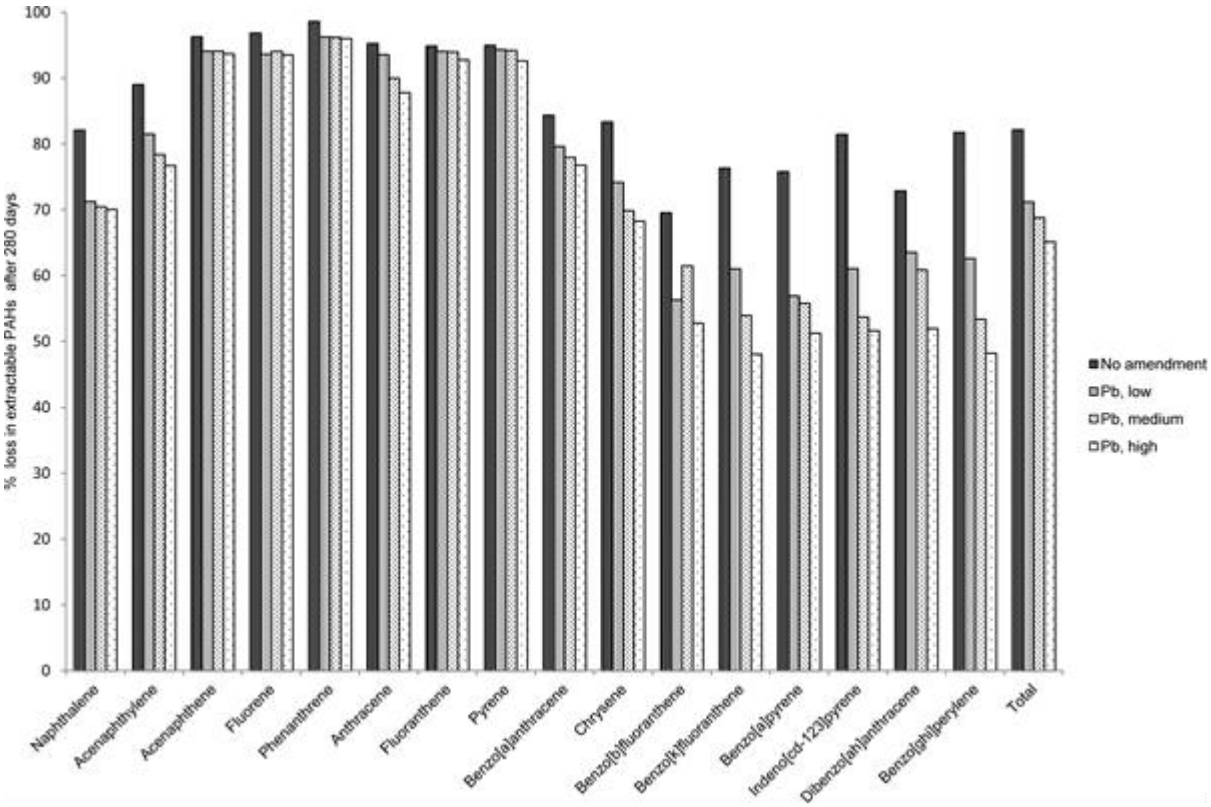


Figure 4.6: Biodegradation of the 16 USEPA priority PAHs after 280 days in the presence of added lead.

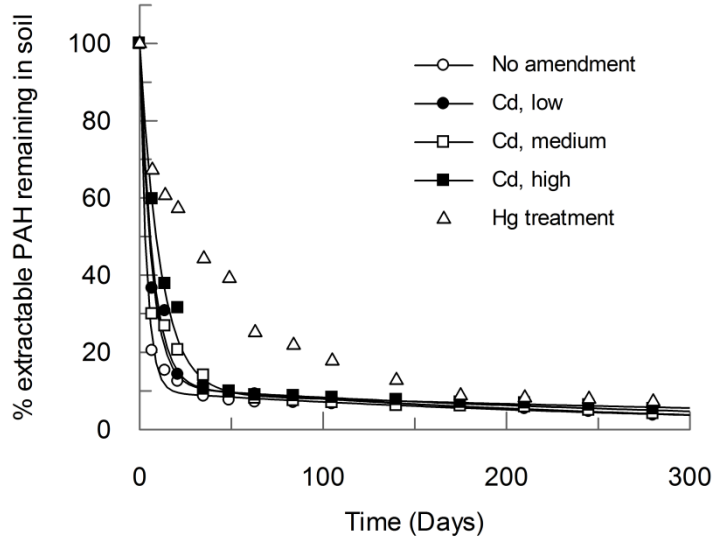


Figure 4.7: Influence of increasing concentrations of cadmium on the degradation of acenaphthene in soil microcosms. The Curves are the best fit to a double exponential (Eq. 4.1) using the parameters listed in Table 4.6. The points for the mercury treatment are also shown for comparison. All points are based on the average concentrations in three separate microcosms

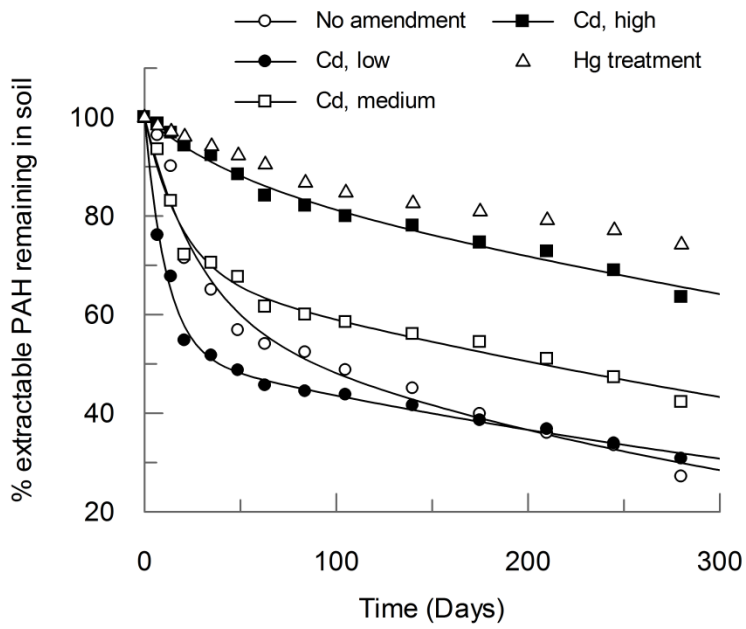


Figure 4.8: Influence of increasing concentrations of cadmium on the degradation of dibenzo[ah]anthracene in soil microcosms. The Curves are the best fit to a double exponential (Eq.4.1) using the parameters listed in Table 4.6. The points for the mercury treatment are also shown for comparison. All points are based on the average concentrations in three separate microcosms.

4.6.8 Application of the kinetic model to biodegradation data for all 16 US EPA priority list PAHs

We have shown in this Chapter that PAH degradation over a period of 40 weeks approximates to a double exponential. Moreover, a novel kinetic model has been developed to explain the various degradation profiles. This kinetic model is also consistent with the effects of heavy metal co-contaminants on PAH biodegradation.

Thus far, biodegradation data has been fitted to a simple bi-exponential decay, as described by Eq. 4.1. However, given that the developed kinetic model seems to describe the data well for both metal amended and non-amended soils, a more advanced approach to modelling can now be adopted. In Section 4.6.6 a simplification of Eq. 4.9 was described. The full form of the simplified equation is shown in Eq. 4.15.

$$[PAH_{TE}] = [PAH_{TB}]_0 \cdot 1 - \frac{k_2}{k_2 - k_3 + k_{bio2}} \cdot e^{-(k_2 + k_{bio2}) \cdot t} + [PAH_{TB}]_0 \cdot \frac{k_2}{k_2 - k_3 + k_{bio2}} \cdot e^{-k_3 \cdot t}$$

Eq. 4.14

This equation can be used directly to fit the kinetic data, thus generating values of the individual rate constants k_2, k_3 and k_{bio2}, rather than the complex rate constant, k_a. Thus, the biotic degradation rate, k_{bio2}, can be separated from the abiotic rates, k_2 and

k_3, thus providing much more insight into the biodegradation process and the effect that cadmium and lead co-contaminants have on it.

For a given individual PAH, we have degradation data for seven different conditions: unamended soil and spiked soil at three different concentrations for both lead and cadmium. Of the three rate constants, only k_{bio2} is likely to be significantly affected by the presence of metal co-contaminants; the other two rate constants, k_2 and k_3, relate to physical processes that should be unaffected by these changed conditions, even though there is evidence from Table 4.8 that k_3 shows some decrease in value at higher metal concentrations. Nevertheless, in these modelling studies, we have assumed that k_2 and k_3 are constant for all seven conditions for each PAH, with only k_{bio2} varying. This is accomplished in Grafit 7 using a global fit approach (all seven kinetic profiles are fitted simultaneously, using proportional error weighting).

The parameters obtained from the modelling are shown in Table 4.9 and the individual fits are shown in Figures 4.9 to 4.24. Generally, the fits are very good and so the kinetic model, as applied in this way to the data appears to hold.

From Table 4.9 we can make a number of observations. Firstly k_3 values are quite consistent as has been previously noted, though two, fluoranthene and pyrene, both compact 4-ring PAHs, have higher values than the others (there is some suggestion of a Gaussian distribution of k_3 with respect to molecular weight). For the other abiotic process, k_2, again the rates are fairly consistent across the 16 PAHs, with the exception of acenaphthylene, fluoranthene and chrysene which all have rate constants some two to three times larger than the average for the others. There is no obvious structural relationship that is common to these PAHs (structural features are likely to play a significant role in the physical processes).

As for the k_{bio2} values, these are consistent with the conditions to which the soils were subjected; generally, there is a reduction in k_{bio2} as the metal amendment level increases. Lead marginally seems to the more toxic metal, though it should be remembered that there was already a significant background lead concentration present in the soil before any spiking. There is evidence that for some PAHs there is slight stimulation in rate at low metal concentrations compared to the control. Also shown in Table 4.9 is the value for Φ_a, which is calculated from the individual rate constants according to Eq. 4.13. The variation in value for this parameter reflects the changes in the ratio of k_{bio2} to k_2, as previously discussed.

Table 4.9: Fitting parameters for soil microcosm biodegradation studies shown in Figures 4.9 to 4.24

PAH	Added metal	Added metal Conc.	k_a / 10^{-2} d^{-1}	Φ_a x100	k_{bio2} / 10^{-2} d^{-1}	k_2 / 10^{-2} d^{-1}	k_3 / 10^{-2} d^{-1}
Naphthalene	None	-	6.35±0.41	59.93±0.5	3.89±0.3	2.46±0.28	0.21±0.02
	Cd	Low	5.25±0.36	51.19±0.45	2.79±0.22	"	"
		Medium	5.71±0.38	55.27±0.47	3.25±0.25	"	"
		High	5.11±0.35	49.8±0.45	2.65±0.21	"	"
	Pb	Low	4.92±0.34	47.77±0.44	2.46±0.19	"	"
		Medium	4.97±0.34	48.32±0.44	2.51±0.2	"	"
		High	5.11±0.35	49.8±0.45	2.65±0.21	"	"
Acenaphthylene	None	-	17.77±0.71	59.85±0.83	10.72±0.57	7.05±0.43	0.21±0.01
	Cd	Low	20.75±0.84	65.68±0.94	13.7±0.72	"	"
		Medium	19.75±0.8	63.92±0.9	12.7±0.67	"	"
		High	20.55±0.82	65.34±0.93	13.5±0.7	"	"
	Pb	Low	17.64±0.71	59.55±0.83	10.59±0.57	"	"
		Medium	19.75±0.8	63.92±0.91	12.7±0.68	"	"
		High	17.01±0.69	58.04±0.81	9.96±0.54	"	"
Acenaphthene	None	-	18.26±0.99	91.87±1	16.8±0.98	1.46±0.12	0.3±0.04
	Cd	Low	13.61±0.78	89.03±0.79	12.15±0.77	"	"
		Medium	14.47±0.86	89.7±0.87	13.01±0.85	"	"
		High	12.36±0.77	87.89±0.78	10.9±0.76	"	"
	Pb	Low	11.96±0.7	87.48±0.71	10.5±0.69	"	"
		Medium	11.08±0.68	86.46±0.69	9.62±0.67	"	"
		High	12.25±0.72	87.78±0.73	10.79±0.71	"	"
Fluorene	None	-	18.55±0.77	87.82±0.78	16.33±0.76	2.22±0.13	0.32±0.03
	Cd	Low	18.32±0.79	87.67±0.8	16.1±0.78	"	"
		Medium	15.79±0.7	85.65±0.71	13.57±0.69	"	"
		High	13.68±0.62	83.38±0.64	11.46±0.61	"	"
	Pb	Low	16.45±0.71	86.24±0.72	14.23±0.7	"	"
		Medium	14.79±0.66	84.66±0.68	12.57±0.65	"	"
		High	17.84±0.8	87.33±0.81	15.62±0.79	"	"
Phenanthrene	None	-	11.46±0.57	94.73±0.58	10.88±0.57	0.58±0.07	0.45±0.06
	Cd	Low	9.6±0.48	93.66±0.48	9.02±0.47	"	"
		Medium	5.09±0.25	87.5±0.27	4.51±0.24	"	"
		High	3.53±0.17	81.17±0.19	2.95±0.15	"	"
	Pb	Low	4.86±0.26	86.85±0.28	4.28±0.25	"	"
		Medium	3.88±0.19	83.09±0.21	3.3±0.18	"	"
		High	4.88±0.26	86.91±0.28	4.3±0.25	"	"
Anthracene	None	-	7.84±0.58	78.37±0.63	6.25±0.53	1.59±0.24	0.49±0.05
	Cd	Low	6.72±0.46	74.48±0.52	5.13±0.39	"	"
		Medium	3.49±0.29	47±0.38	1.9±0.16	"	"
		High	3.21±0.28	41.54±0.37	1.62±0.14	"	"
	Pb	Low	5.98±0.42	71.04±0.48	4.39±0.34	"	"
		Medium	4.97±0.35	64.51±0.43	3.38±0.26	"	"
		High	4.63±0.33	61.59±0.41	3.04±0.23	"	"
Fluoranthene	None	-	13.63±1.59	43.23±2.08	6.42±0.85	7.21±1.34	0.93±0.02
	Cd	Low	14.44±1.67	46.63±2.14	7.23±0.99	"	"
		Medium	10.94±1.42	27.97±1.95	3.73±0.47	"	"
		High	9.23±1.36	13.13±1.91	2.02±0.26	"	"
	Pb	Low	12.24±1.49	36.25±2	5.03±0.65	"	"
		Medium	10.5±1.4	24.66±1.94	3.29±0.4	"	"
		High	10.86±1.42	27.39±1.95	3.65±0.46	"	"
Pyrene	None	-	5.79±0.43	68.76±0.51	4.2±0.34	1.59±0.27	0.7±0.05
	Cd	Low	4.07±0.31	52.82±0.42	2.48±0.16	"	"
		Medium	3.92±0.31	50.62±0.41	2.33±0.15	"	"
		High	3.83±0.3	49.2±0.41	2.24±0.14	"	"
	Pb	Low	4.45±0.34	57.6±0.43	2.86±0.2	"	"
		Medium	3.91±0.31	50.47±0.41	2.32±0.15	"	"
		High	3.74±0.3	47.7±0.41	2.15±0.14	"	"

Table 4.9 continued

PAH	Added metal	Added metal Conc.	k_a / 10^{-2} d^{-1}	Φ_a x100	k_{bio2} / 10^{-2} d^{-1}	k_2 / 10^{-2} d^{-1}	k_3 / 10^{-2} d^{-1}
Benzo[a]anthracene	None	-	4.64±0.43	60.42±0.53	2.93±0.28	1.71±0.32	0.32±0.03
	Cd	Low	3.53±0.35	46.73±0.48	1.82±0.15	"	"
		Medium	2.42±0.33	18.57±0.46	0.71±0.07	"	"
		High	2.62±0.33	25.65±0.46	0.91±0.08	"	"
	Pb	Low	3.01±0.34	36.43±0.46	1.3±0.1	"	"
		Medium	2.93±0.34	34.48±0.46	1.22±0.1	"	"
		High	2.69±0.33	27.85±0.46	0.98±0.08	"	"
Chrysene	None	-	15.15±0.88	53.46±1.08	8.23±0.61	6.92±0.63	0.28±0.01
	Cd	Low	11.55±0.72	38.6±0.95	4.63±0.34	"	"
		Medium	11.27±0.71	37.03±0.95	4.35±0.32	"	"
		High	10.29±0.68	30.87±0.93	3.37±0.25	"	"
	Pb	Low	11.04±0.7	35.69±0.94	4.12±0.3	"	"
		Medium	11.27±0.71	37.03±0.95	4.35±0.32	"	"
		High	8.95±0.65	20.18±0.91	2.03±0.17	"	"
Benzo[b]fluoranthene	None	-	5.6±0.35	46.15±0.46	2.66±0.18	2.94±0.3	0.14±0.02
	Cd	Low	5.26±0.34	42.58±0.45	2.32±0.15	"	"
		Medium	4.3±0.31	29.33±0.43	1.36±0.09	"	"
		High	3.93±0.31	22.43±0.43	0.99±0.07	"	"
	Pb	Low	4.47±0.32	32.1±0.44	1.53±0.1	"	"
		Medium	5.21±0.34	42.01±0.45	2.27±0.15	"	"
		High	4.42±0.32	31.31±0.44	1.48±0.1	"	"
Benzo[k]fluoranthene	None	-	4.44±0.28	42.62±0.37	1.99±0.12	2.45±0.25	0.17±0.01
	Cd	Low	4.08±0.27	37.34±0.36	1.63±0.09	"	"
		Medium	3.52±0.26	26.87±0.36	1.07±0.06	"	"
		High	3.09±0.25	16.1±0.36	0.64±0.04	"	"
	Pb	Low	3.77±0.26	31.94±0.36	1.32±0.07	"	"
		Medium	3.33±0.25	22.47±0.36	0.88±0.05	"	"
		High	3.12±0.25	16.95±0.36	0.67±0.04	"	"
Benzo[a]pyrene	None	-	4.01±0.39	44.91±0.52	1.9±0.17	2.11±0.35	0.18±0.02
	Cd	Low	3.7±0.38	40.06±0.51	1.59±0.14	"	"
		Medium	3.21±0.36	30.36±0.5	1.1±0.09	"	"
		High	2.8±0.36	19.47±0.5	0.69±0.06	"	"
	Pb	Low	3.25±0.36	31.27±0.5	1.14±0.09	"	"
		Medium	3.09±0.36	27.49±0.5	0.98±0.08	"	"
		High	2.83±0.36	20.38±0.5	0.72±0.06	"	"
Indeno[123-cd]pyrene	None	-	2.78±0.17	64.13±0.22	1.79±0.09	0.99±0.14	0.02±0.04
	Cd	Low	2.4±0.16	58.4±0.21	1.41±0.07	"	"
		Medium	1.84±0.15	45.6±0.21	0.85±0.04	"	"
		High	1.72±0.14	41.76±0.2	0.73±0.03	"	"
	Pb	Low	2.04±0.15	50.99±0.21	1.05±0.05	"	"
		Medium	1.78±0.14	43.75±0.2	0.79±0.03	"	"
		High	1.97±0.15	49.23±0.21	0.98±0.04	"	"
Dibenzo[ah]anthracene	None	-	5.34±0.35	42.17±0.47	2.35±0.16	2.99±0.31	0.17±0.02
	Cd	Low	6.16±0.38	50.08±0.49	3.17±0.22	"	"
		Medium	4.49±0.33	30.79±0.45	1.5±0.1	"	"
		High	3.24±0.32	2.61±0.44	0.25±0.06	"	"
	Pb	Low	5.08±0.34	39.1±0.46	2.09±0.14	"	"
		Medium	4.44±0.33	29.98±0.45	1.45±0.1	"	"
		High	4.22±0.32	26.17±0.45	1.23±0.09	"	"
Benzo[g,h,i]perylene	None	-	5.11±0.32	53.33±0.41	2.8±0.19	2.31±0.26	0.16±0.02
	Cd	Low	5.26±0.33	54.71±0.42	2.95±0.2	"	"
		Medium	3.82±0.28	36.89±0.38	1.51±0.1	"	"
		High	2.91±0.27	16±0.37	0.6±0.06	"	"
	Pb	Low	3.85±0.28	37.4±0.38	1.54±0.1	"	"
		Medium	3.27±0.27	25.72±0.37	0.96±0.07	"	"
		High	3.11±0.27	21.69±0.37	0.8±0.06	"	"

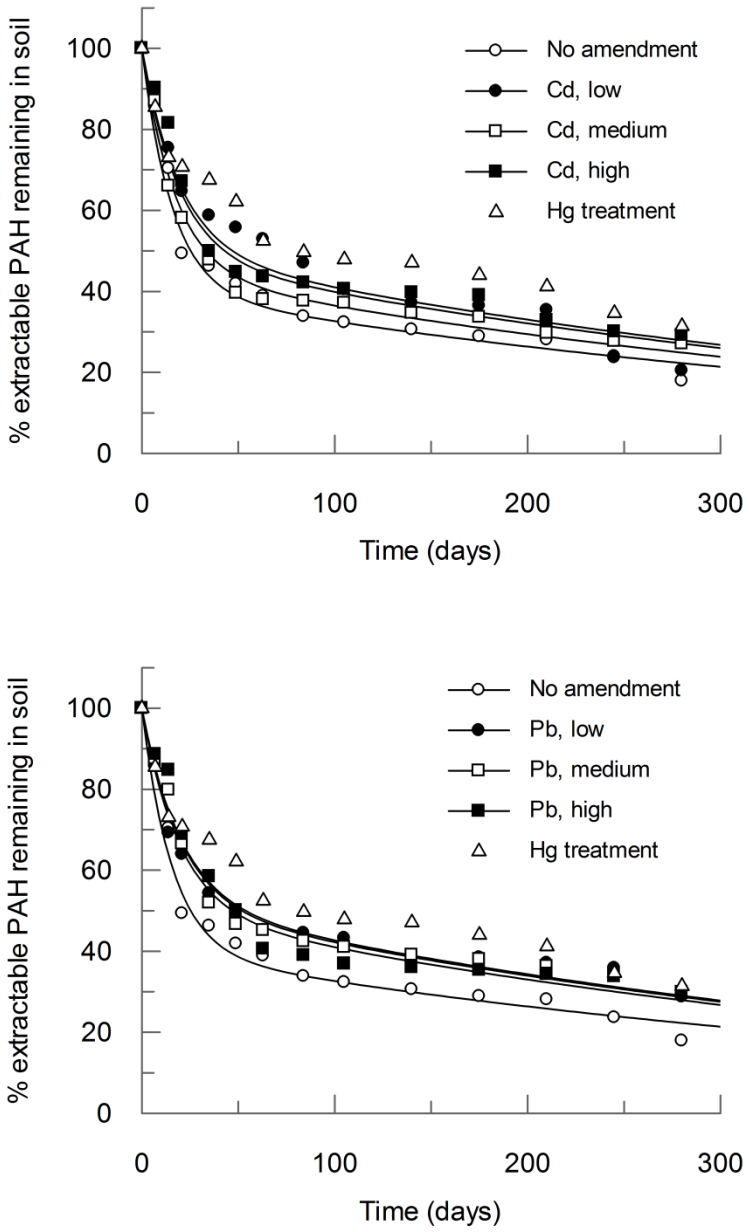


Figure 4.9: Influence of increasing concentrations of cadmium (upper plot) and lead (lower plot) on the degradation of naphthalene in soil microcosms. The curves are the best fit to a double exponential (Eq.4.15) using the parameters listed in Table 4.9. The points for the mercury treatment are also shown for comparison. All points are based on the average concentrations in three separate microcosms.

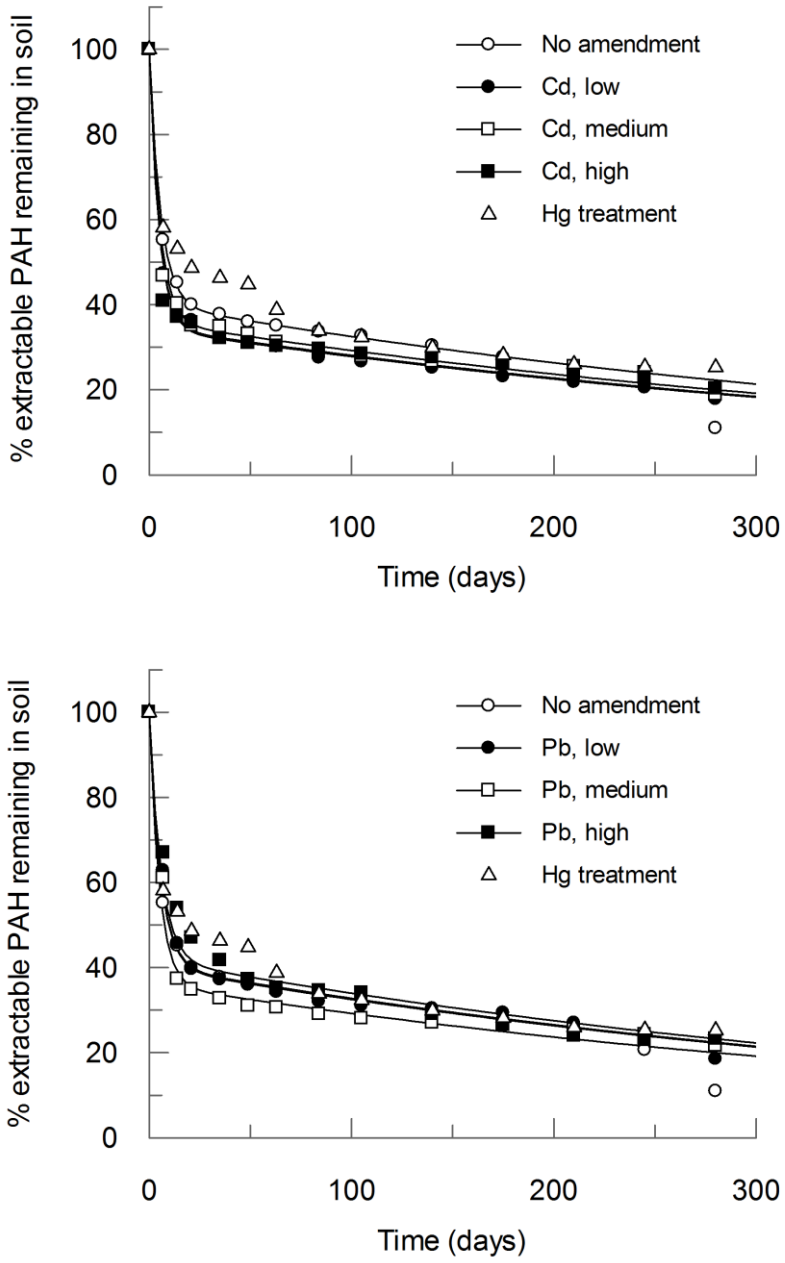


Figure 4.10: Influence of increasing concentrations of cadmium (upper plot) and lead (lower plot) on the degradation of acenaphthylene in soil microcosms. The curves are the best fit to a double exponential (Eq.4.15) using the parameters listed in Table 4.9. The points for the mercury treatment are also shown for comparison. All points are based on the average concentrations in three separate microcosms.

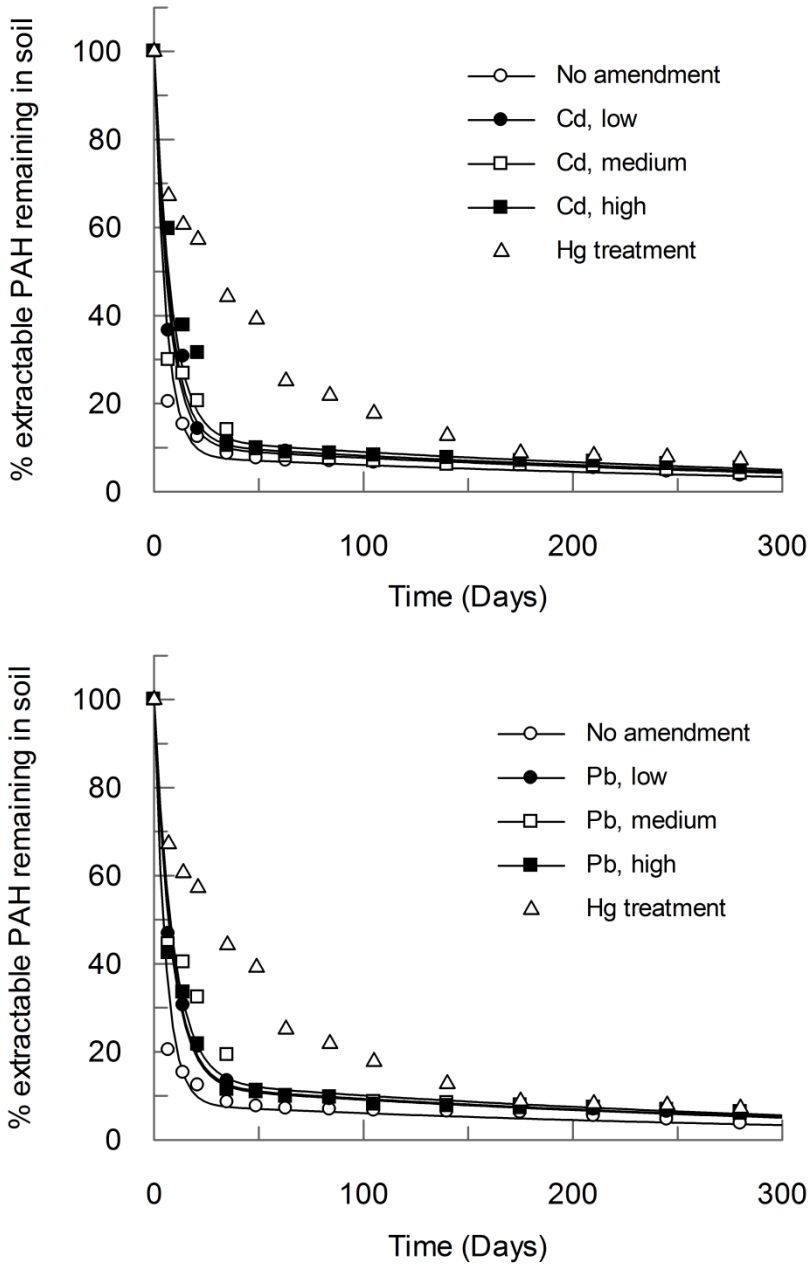


Figure 4.11: Influence of increasing concentrations of cadmium (upper plot) and lead (lower plot) on the degradation of acenaphthene in soil microcosms. The curves are the best fit to a double exponential (Eq.4.15) using the parameters listed in Table 4.9. The points for the mercury treatment are also shown for comparison. All points are based on the average concentrations in three separate microcosms.

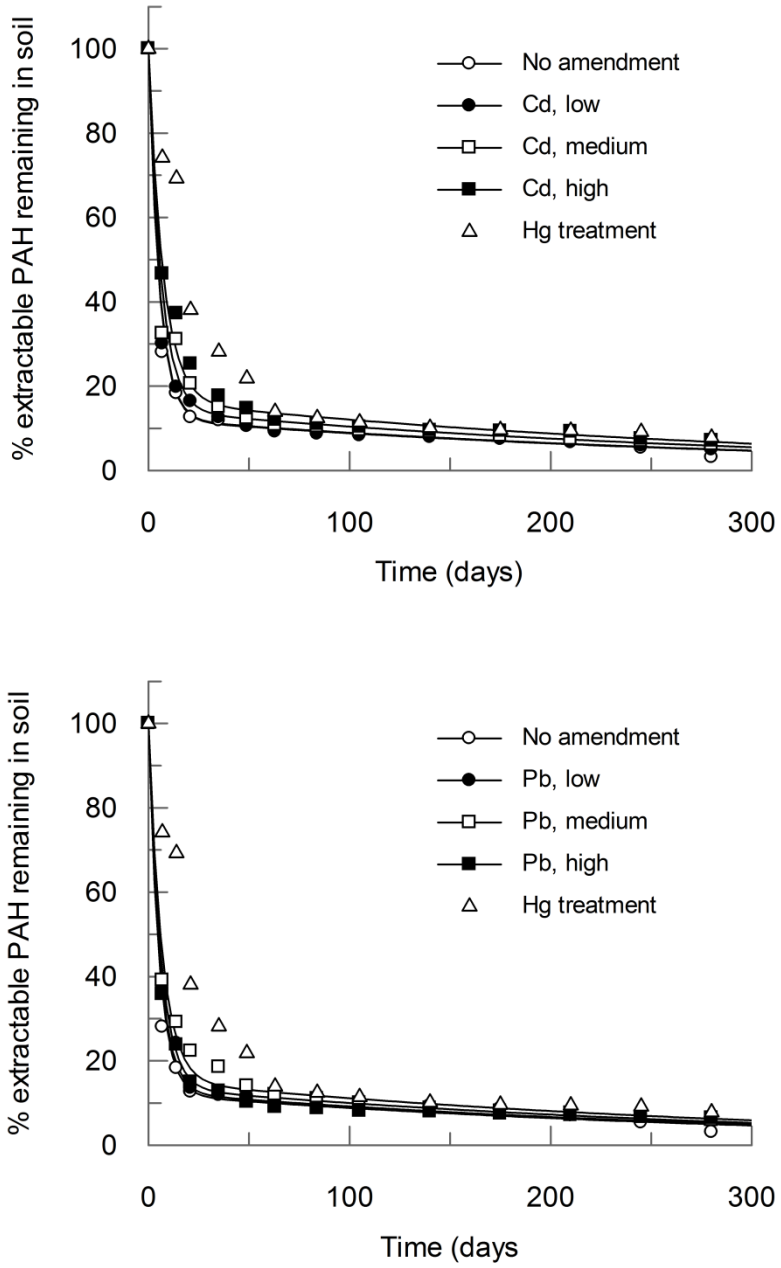


Figure 4.12: Influence of increasing concentrations of cadmium (upper plot) and lead (lower plot) on the degradation of fluorene in soil microcosms. The curves are the best fit to a double exponential (Eq.4.15) using the parameters listed in Table 4.9. The points for mercury treatment are also shown for comparison. All points are based on the average concentrations in three separate microcosms.

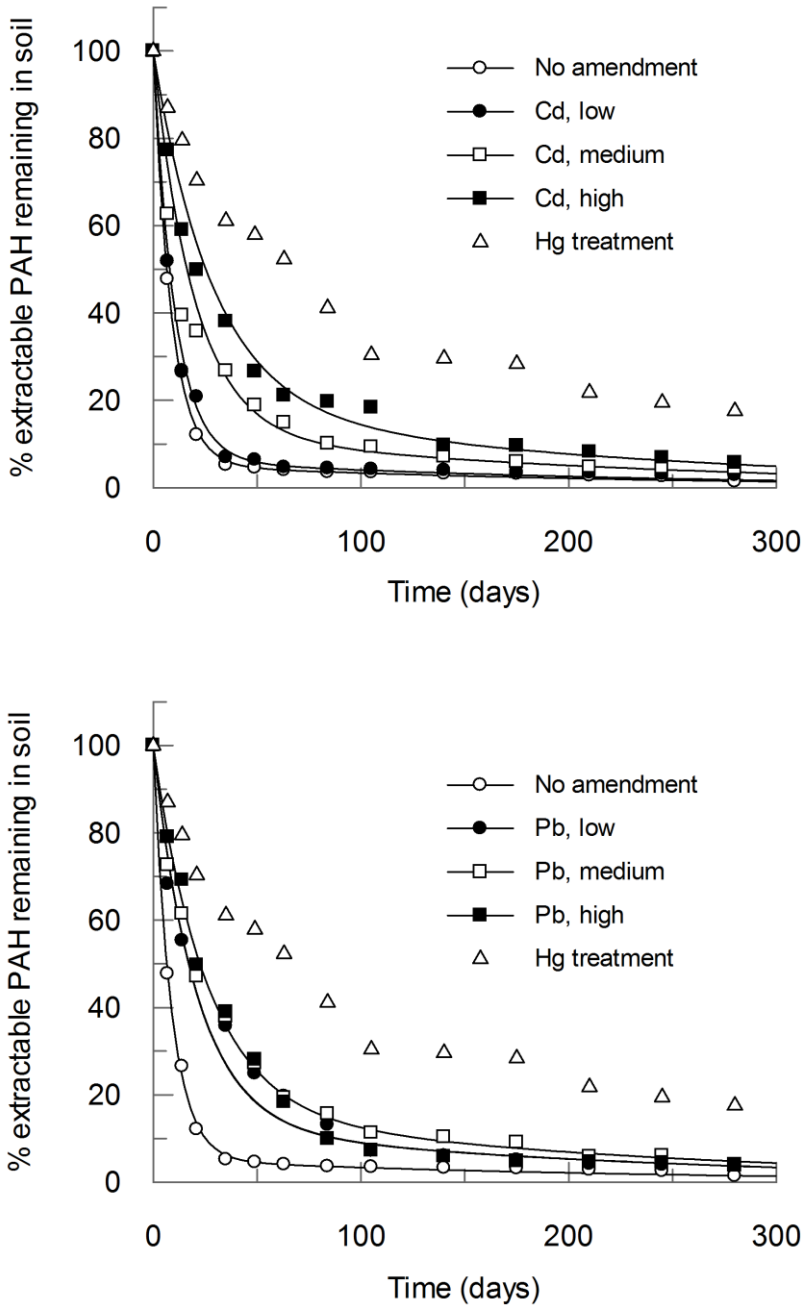


Figure 4.13: Influence of increasing concentrations of cadmium (upper plot) and lead (lower plot) on the degradation of phenanthrene in soil microcosms. The curves are the best fit to a double exponential (Eq.4.15) using the parameters listed in Table 4.9. The points for the mercury treatment are also shown for comparison. All points are based on the average concentrations in three separate microcosms.

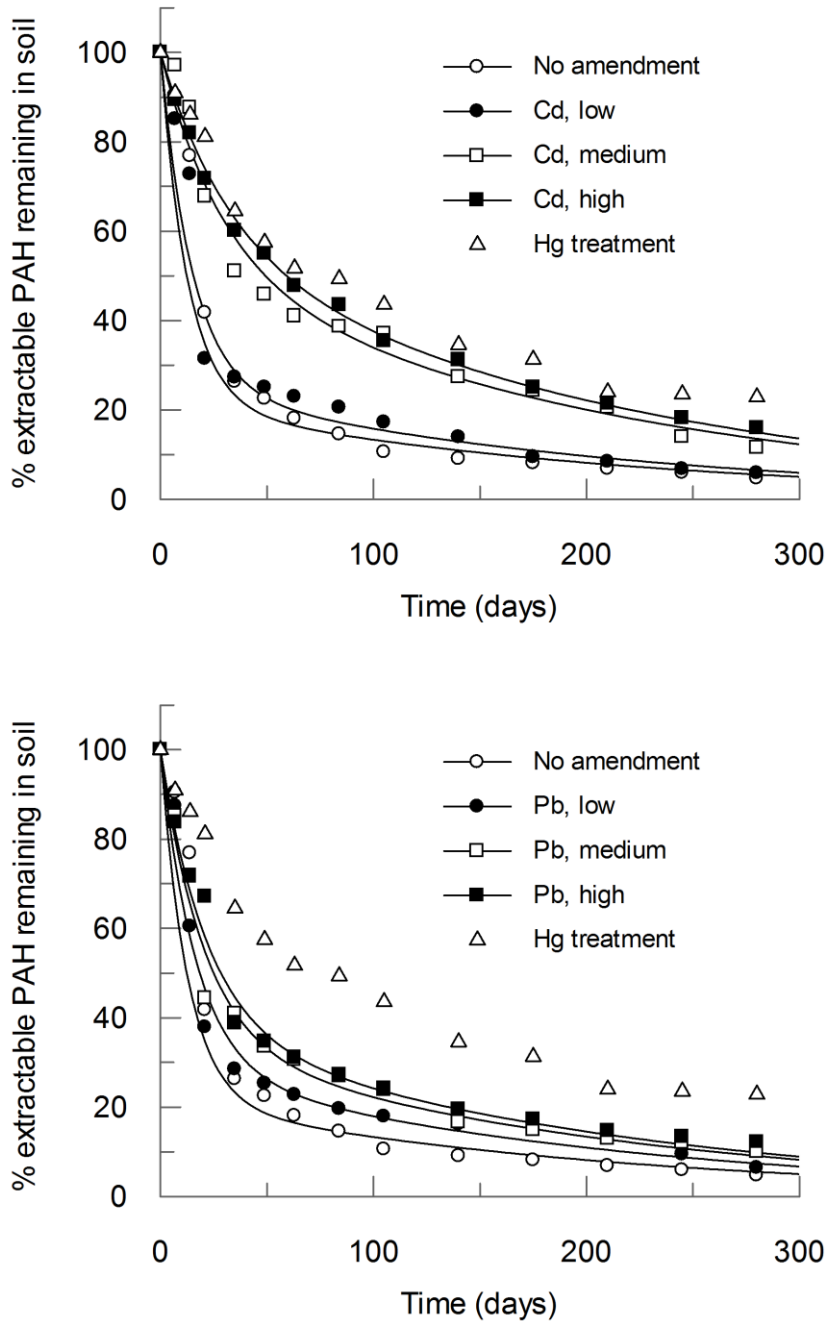


Figure 4.14: Influence of increasing concentrations of cadmium (upper plot) and lead (lower plot) on the degradation of anthracene in soil microcosms. The curves are the best fit to a double exponential (Eq.4.15) using the parameters listed in Table 4.9. The points for the mercury treatment are also shown for comparison. All points are based on the average concentrations in three separate microcosms.

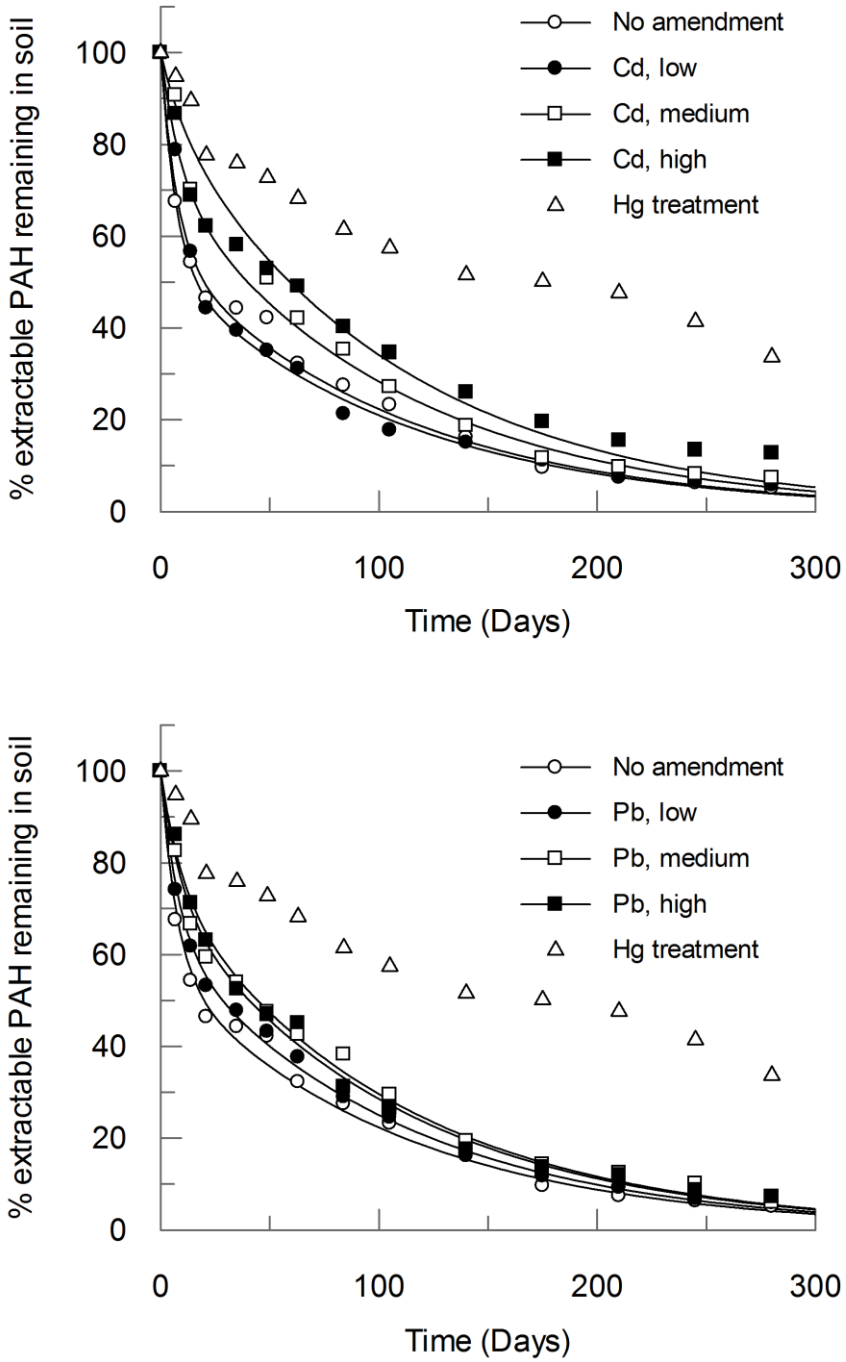


Figure 4.15: Influence of increasing concentrations of cadmium (upper plot) and lead (lower plot) on the degradation of fluoranthene in soil microcosms. The curves are the best fit to a double exponential (Eq.4.15) using the parameters listed in Table 4.9. The points for the mercury treatment are also shown for comparison. All points are based on the average concentrations in three separate microcosms.

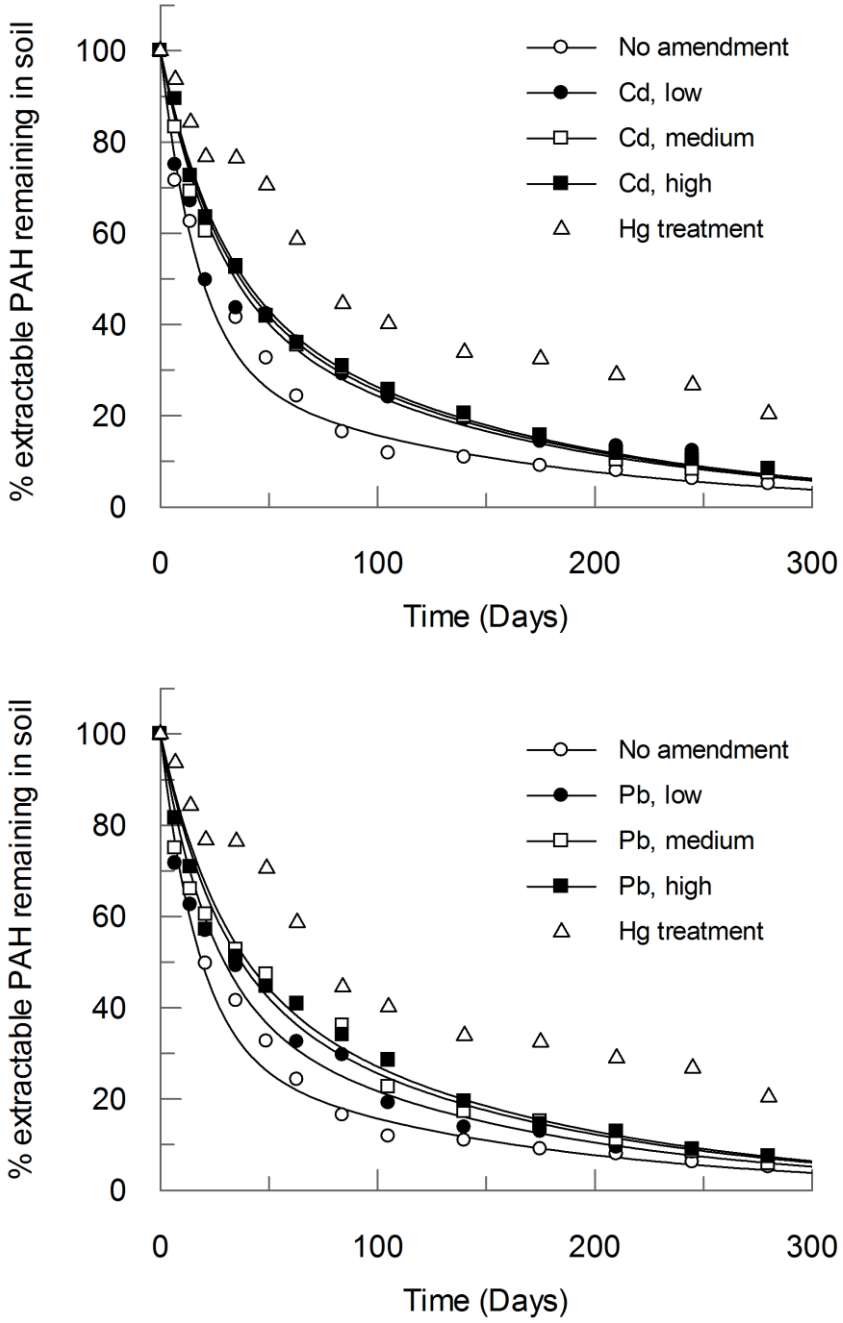


Figure 4.16: Influence of increasing concentrations of cadmium (upper plot) and lead (lower plot) on the degradation of pyrene in soil microcosms. The curves are the best fit to a double exponential (Eq.4.15) using the parameters listed in Table 4.9. The points for the mercury treatment are also shown for comparison. All points are based on the average concentrations in three separate microcosms.

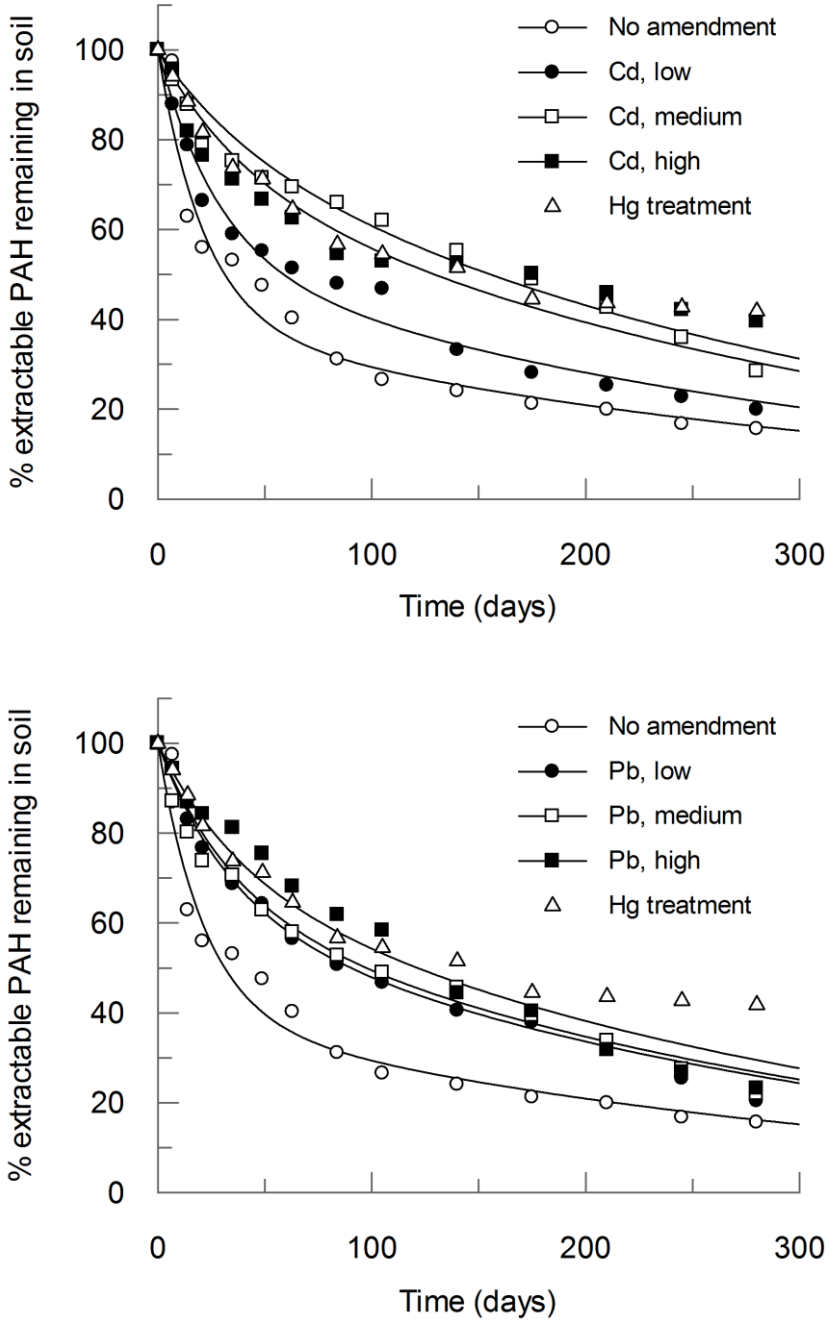


Figure 4.17: Influence of increasing concentrations of cadmium (upper plot) and lead (lower plot) on the degradation of benzo[a]anthracene in soil microcosms. The curves are the best fit to a double exponential (Eq.4.15) using the parameters listed in Table 4.9. The points for the mercury treatment are also shown for comparison. All points are based on the average concentrations in three separate microcosms.

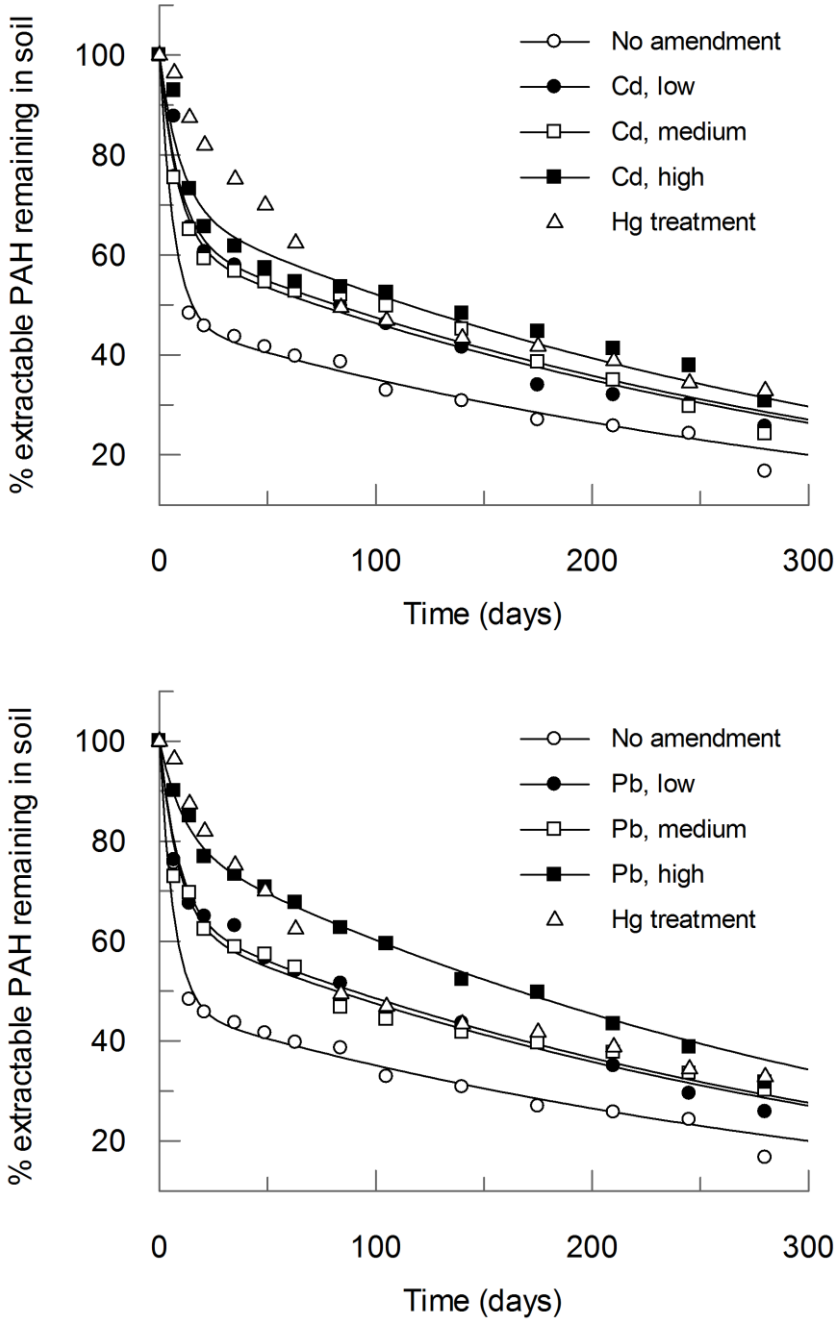


Figure 4.18: Influence of increasing concentrations of cadmium (upper plot) and lead (lower plot) on the degradation of chrysene in soil microcosms. The curves are the best fit to a double exponential (Eq.4.15) using the parameters listed in Table 4.9. The points for the mercury treatment are also shown for comparison. All points are based on the average concentrations in three separate microcosms**.**

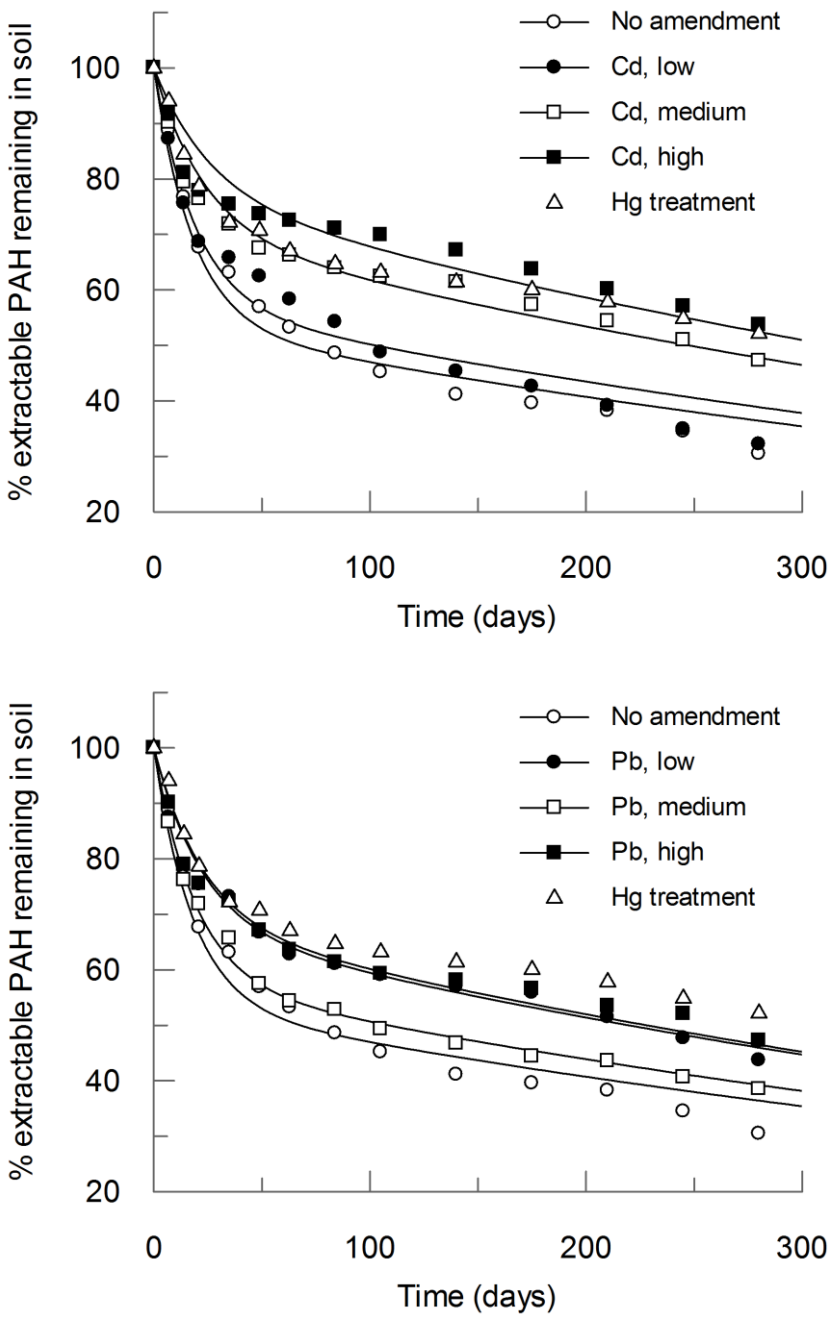


Figure 4.19: Influence of increasing concentrations of cadmium (upper plot) and lead (lower plot) on the degradation of benzo[b]fluoranthene in soil microcosms. The curves are the best fit to a double exponential (Eq.4.15) using the parameters listed in Table 4.9. The points for the mercury treatment are also shown for comparison. All points are based on the average concentrations in three separate microcosms.

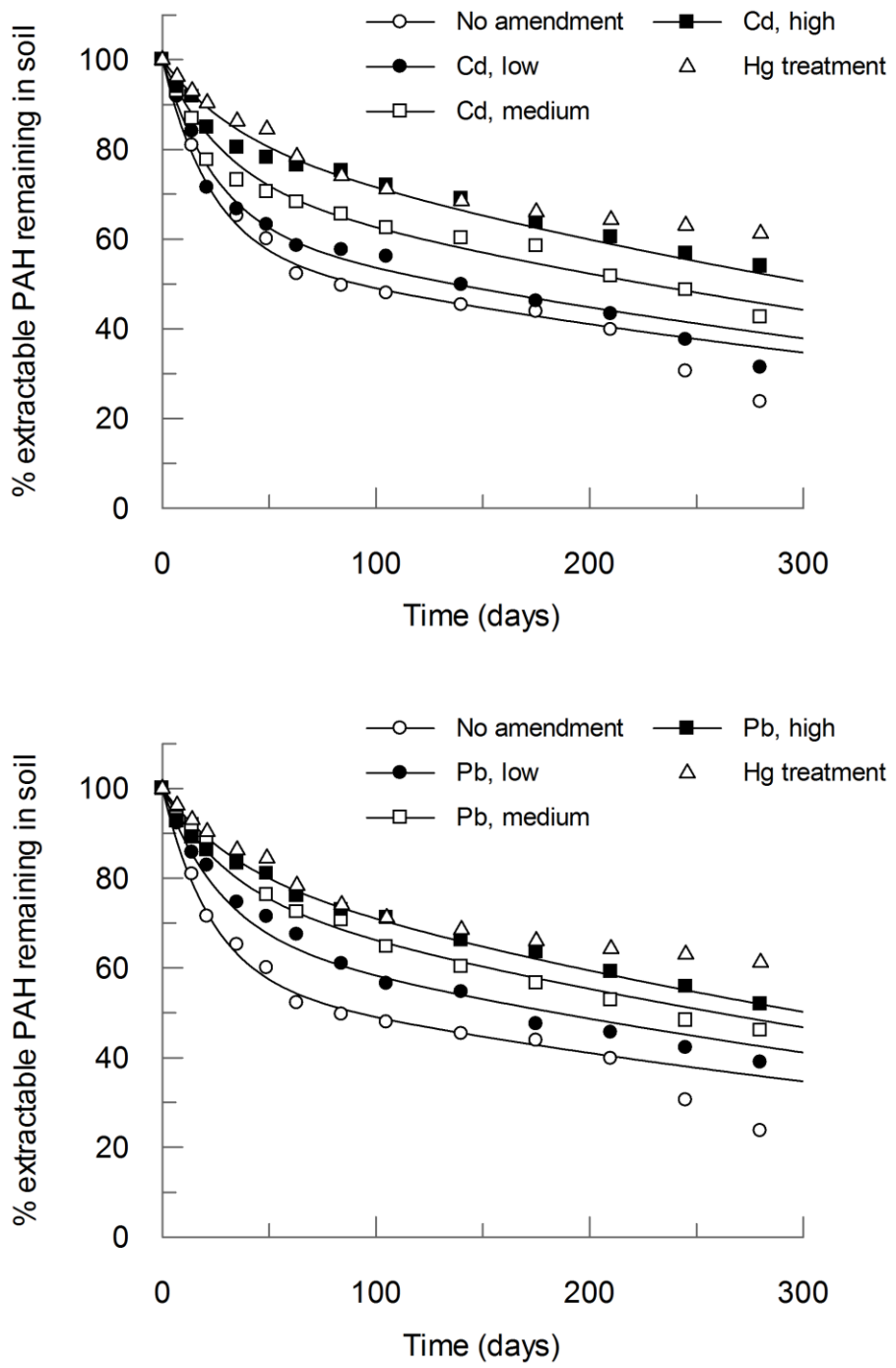


Figure 4.20: Influence of increasing concentrations of cadmium (upper plot) and lead (lower plot) on the degradation of benzo[k]fluoranthene in soil microcosms. The curves are the best fit to a double exponential (Eq.4.15) using the parameters listed in Table 4.9. The points for mercury treatment are also shown for comparison. All points are based on the average concentrations in three separate microcosms.

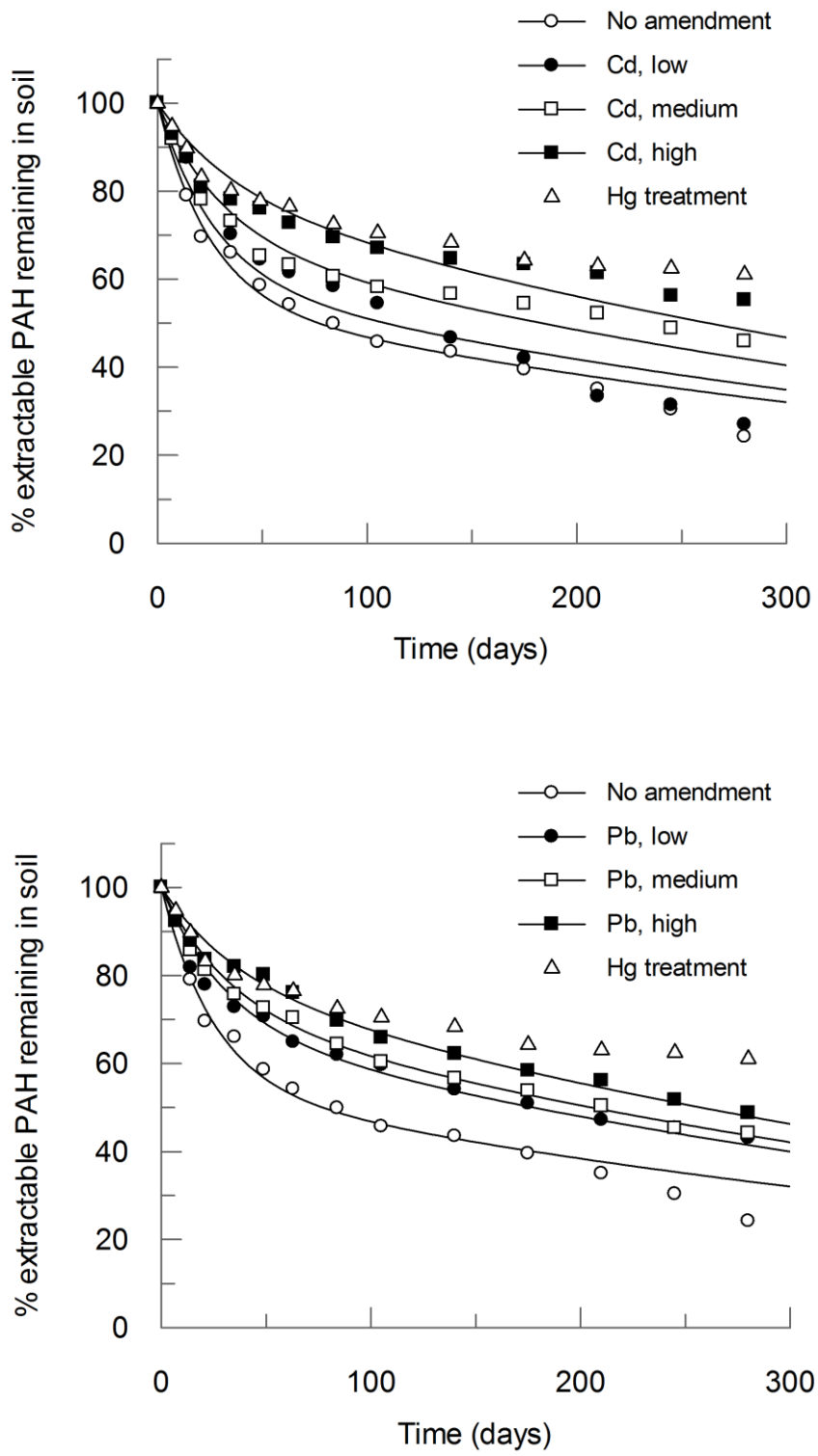


Figure 4.21: Influence of increasing concentrations of cadmium (upper plot) and lead (lower plot) on the degradation of benzo[a]pyrene in soil microcosms. The curves are the best fit to a double exponential (Eq.4.15) using the parameters listed in Table 4.9. The points for the mercury treatment are also shown for comparison. All points are based on the average concentrations of three separate microcosms.

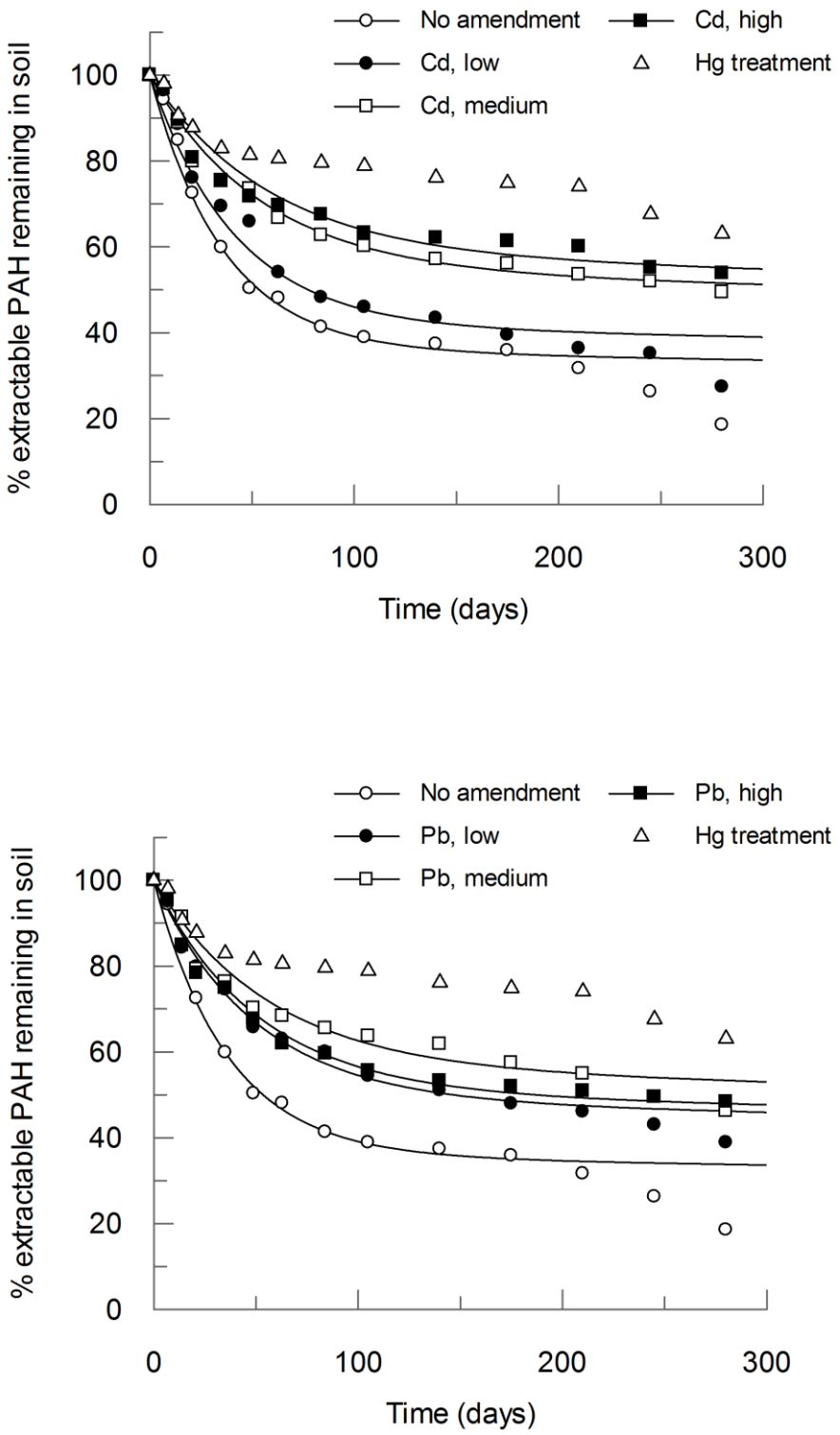


Figure 4.22: Influence of increasing concentrations of cadmium (upper plot) and lead (lower plot) on the degradation of indeno[123-cd]pyrene in soil microcosms. The curves are the best fit to a double exponential (Eq.4.15) using the parameters listed in Table 4.9. The points for the mercury treatment are also shown for comparison. All points are based on the average concentrations in three separate microcosms.

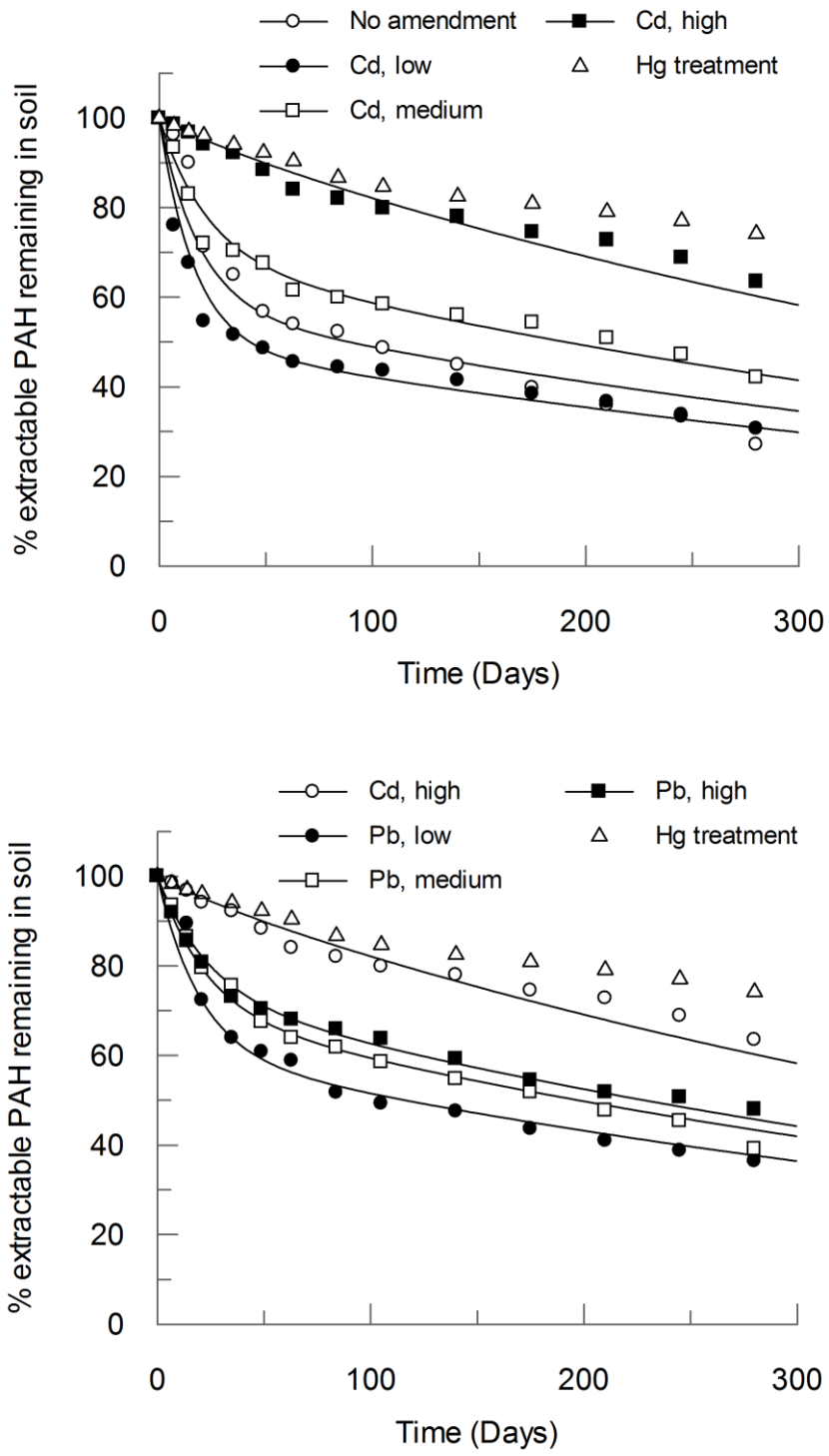


Figure 4.23: Influence of increasing concentrations of cadmium (upper plot) and lead (lower plot) on the degradation of dibenzo[a,h]anthracene in soil microcosms. The curves are the best fit to a double exponential (Eq.4.15) using the parameters listed in Table 4.9. The points for the mercury treatment are also shown for comparison. All points are based on the average concentrations in three separate microcosms.

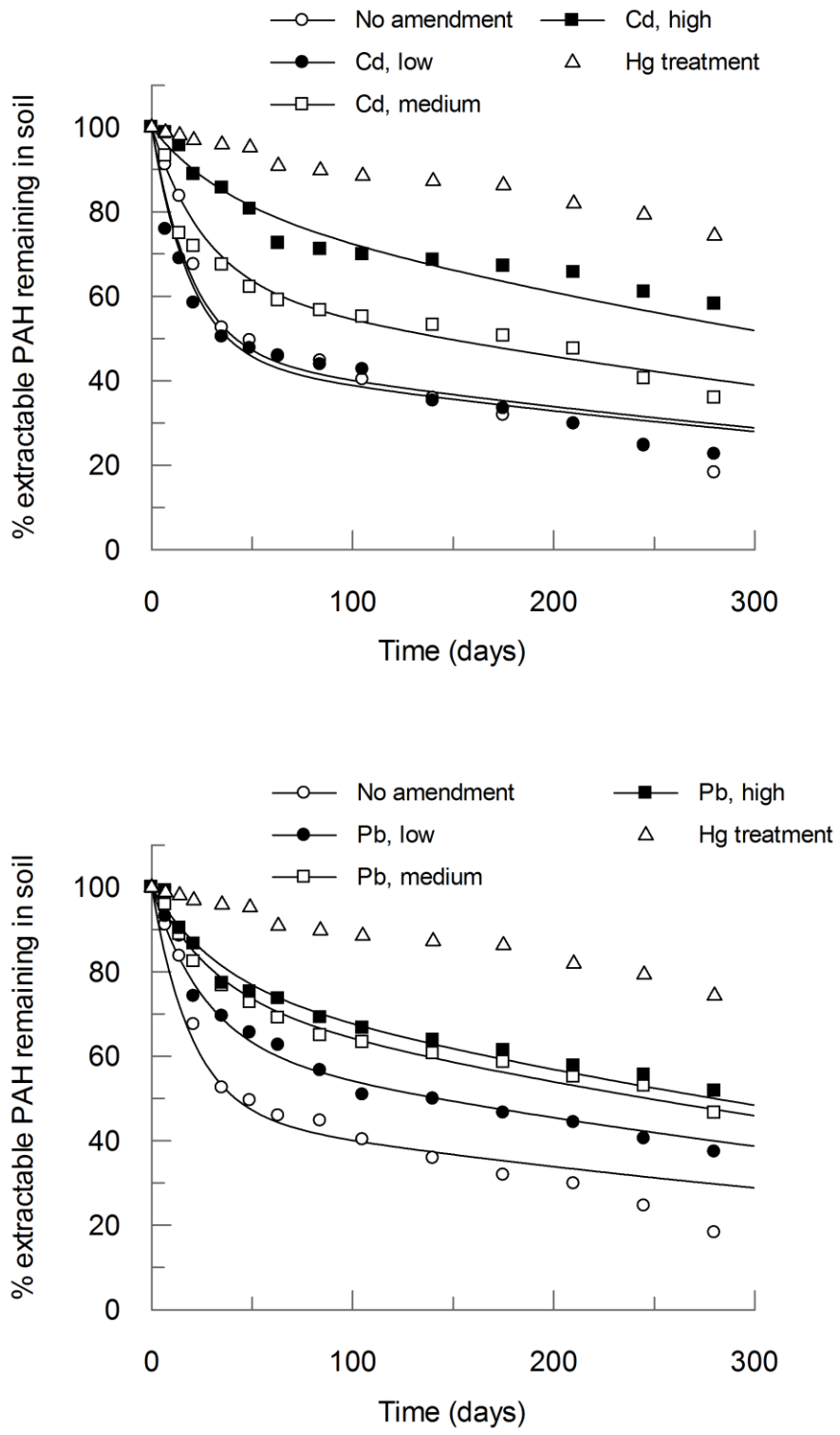


Figure 4.24: Influence of increasing concentrations of cadmium (upper plot) and lead (lower plot) on the degradation of benzo[g,h,i]perylene in soil microcosms. The curves are the best fit to a double exponential (Eq.4.15) using the parameters listed in Table 4.9. The points for the mercury treatment are also shown for comparison. All points are based on the average concentrations in three separate microcosms.

4.7 Conclusion

A novel kinetic approach has been developed and applied to the biexponential dependence of PAH concentration dependence on time. The model assumes that PAHs are distributed across a number of defined solution and soil phases, with the latter represented by phases displaying differing bioaccessibility and extraction properties. The key determining factor in PAH distribution and movement is the soil organic matter content with which the PAHs are likely to be mainly associated. The results of our model suggest that overall biodegradation is dependent on the respective rates at which the PAHs are (a) biodegraded by soil microorganisms and (b) migrate from a bioaccessible soil phase to a non bioaccessible phase. In addition, migration of PAHs to a non-bioaccessible and non-extractable phase associated with the humin pores gives rise to an apparent PAH removal process. Our model suggests that generally, two to four-ring PAHs biodegrade at a much faster rate than they are transferred to the bioaccessible phase, whereas for five and six membered rings the migration rate to the non-bioaccessible phase is more significant, leading to reduced overall degradation.

The presence of co-contaminants has the potential to inhibit the biotic process, but not the migration to non-bioaccessible phases, thus altering the balance of the respective rates, and, therefore, potentially the overall biodegradation after 40 weeks; overall in the presence of Cd and Pb this is relatively unaffected for two to four-ring PAHs, but shows significant impairment for five and six-ring PAHs.

The effect of metal co-contaminants on PAH degradation rates is consistent with our proposed kinetic model: this was demonstrated for the effect of cadmium on acenaphthene and dibenzo[ah]anthracene: the profiles and rates obtained from the bi-exponential decay were consistent with the proposed derivation for the complex rate, k_a.

This then allowed the modelling of biodegradation data for 16 USEPA priority list PAHs at each metal amended concentration (seven conditions for each PAH). This approach allows the individual rate constants to be quantified, rather than obtaining only a single complex rate constant. Thus, rates were obtained for the two abiotic processes, k_2 (transfer of soil-bound PAH from a bioaccessible soil phase to an inaccessible but extractable soil phase) and k_3 (transfer from bio-inaccessible but extractable soil phase to and non-extractable phase). These two rates were set as common to all seven conditions (for each PAH) in the multiple non-linear least squares analysis (proportional

error weighting) employed. The values were similar for most PAHs, though several were significantly higher than the average.

The value for k_{bio2}, the rate for degradation by the soil microbial community was independently determined for each of the seven soil conditions for each PAH. This value, showed the expected variation with amended metal concentration, i.e. a general decrease in rate as the metal concentration was increased, though for some PAHs there was evidence of stimulation in the rate at low applied metal concentrations.

Chapter 5: Effect of PAH and heavy metal co-contaminant on soil microbial community

5.1 Introduction

The soil microbial community plays an important role for example nutrient transformations and litter decomposition as well as having an influence on soil structural and hydrological properties [149, 181]. The high sensitivity of the community to temporal or spatial change represents a powerful tool for understanding community dynamics in the ecosystem and also on how community process affects ecosystem processes [177]. The soil microbial community also has a role in maintaining energy flow, element cycling, humification processes, plant pathology or plant growth promotion and degradation of pollutants [110, 172, 177]. The community might contain a significant part of the mobile pool of nutrients serving as a nutrient source or sink in tellurian ecosystems [177]. Microbial communities in soil differ to a great extent, for example, bacteria species present per gram of soil are estimated to be as many as 13,000 different species [133]. The microbial community constituent parts, which are a complex phenomenon, can be classified in different ways: the taxonomical composition of the particular biomass component, its metabolic abilities, and its biochemical and genetic characteristics.

These microbial classifications (size, diversity and activity), have often been used to characterise the effect of pollution on the soil ecosystem. Several studies have used soil microbial biomass [110, 113, 114], soil respiration [95], metabolic diversity [185] and enzyme activity [118] to measure the effect of pollution on soil. Pollutants such as PAHs can serve as an organic substrate for microorganisms thereby stimulating growth and they can also inhibit microbial enzyme activity when they occur as a mixture of compounds in the environment [92]. Heavy metals (Cd and Pb at high concentrations) are also toxic to microorganisms; they can inhibit microbial growth and denature microbial cell protein [83]. Microorganisms can also develop tolerance towards heavy metals by developing adaptive features as has been mentioned in Section 1.6.3.

Soil microorganisms in the presence of high concentrations of heavy metals can take up heavy metals from the environment. This is because of the negatively charged surface of microbial cells which causes the cell surfaces to have a high affinity for metal cations [16]. The heavy metals can then be sorbed (bio-sorption) by the microbial cell or can accumulate (bio-accumulate) on the cell surface. Some of the microbial cells are able to respond to this high heavy metal concentration by pumping out the metals from their cells or chemically modifying the metals [17, 18] thereby preventing them from accumulating and denaturing the cell's proteins. The ability of microorganisms

(bacteria) to resist heavy metal toxicity is because of genes carried on specific plasmids which are able to encode enzymes capable of detoxifying or pumping out metals [6]. Microorganisms that have resistance to high concentrations of several metals often carry multiple plasmids encoding the resistance to those metals. The tolerance of microorganisms to high heavy metal concentrations can enable them to carry out other activities such as the biodegradation of organic compounds (PAH) though the community as a whole can lose certain ecological function through the loss of metal-sensitive species that carry out those functions [84].

The occurrence of PAHs and heavy metals together, as can be seen in the most polluted soil environments, can both have a positive synergistic effect or a negative synergistic effect as discussed in Section 1.7.

Therefore, this chapter aims to evaluate the effect of 16 US EPA PAHs on soil microbial processes such as soil respiration and metabolic diversity. There is still a paucity of studies on the effects of these PAHs on microbial processes. Most studies have used five or less than 5 PAH compounds to examine the effect of PAH on microbial communities in order to minimise the number of variables, but PAH contamination, as stated earlier, occurs as a mixture of many different compounds.

This chapter also aims to evaluate the impact of heavy metals on microbial respiration, microbial biomass and metabolic diversity. There have been various studies [95, 141, 142] which seem to claim that microbial biomass and soil respiration are not affected by heavy metals because of the development of metal tolerance by microorganisms. This chapter will examine this effect and also examine if there is any correlation between soil respiration, microbial biomass and metabolic diversity in the presence of increasing concentrations of heavy metal.

The chapter will also evaluate any synergistic effect of PAH and heavy metal on microbial community under natural attenuation.

5.2. Experimental methodology

5.2.1. Overview

The experimental analysis for this chapter was carried out over a 40 week period. The experimental design used in chapter 4 covers the experimental protocol for this chapter. The unspiked microcosms and coal tar pitch (PAH spiked soil) spiked microcosms that were set up in Chapter 4 were harvested in accordance with the experimental plan for microbial characteristics analysis.

In addition to the PAH analysis reported on in Chapter 4, harvested soil samples were analysed for soil respiration, soil microbial biomass carbon and microbial metabolic diversity. The microbial metabolic diversity study was carried out at 0, 1, 2, 3, 5, 7, 9, 12, 15, 20, 25, 30, 35 and 40 weeks. The soil respiration study was carried out for the same number of weeks, except at 15 weeks where technical issues prevented analysis. The microbial biomass study was carried out on weeks 0, 1 and 40. Soil respiration measurement was carried out using the OxiTop manometric system; microbial biomass carbon measurement was determined using the total organic carbon method and soil microbial metabolic diversity was carried out using the Biolog Ecoplate community physiological profile.

5.2.2 Materials and reagents:

The ultra-pure water of conductivity 18.2 MΩ-cm was produced by a direct QTM Millipore system (Molsheim, France). Alcohol-free chloroform solvent ($CHCl_3$) and potassium sulphate (K_2SO_4) were Sigma-Aldrich reagents. Whatman No.1 filter paper (Sigma-Aldrich Ltd, Dorset, UK) was used to filter the extracted samples. Total Organic Carbon (TOC) was analysed using a direct method mid-range Test 'N' Tube which comprised: acid digestion solution vials mid-range TOC tubes, pH 2.0 buffer solution, sulphate funnel, micro indicator ampule for mid-range TOC, TOC persulfate powder pillows, pH paper and TOC 1000 mg L^{-1} C standard solution. The test kit was purchased from Hach-Lange LTD Salford, U.K. Sodium hydroxide pellets (Sigma-Aldrich, Dorset) was used to absorb carbon dioxide (CO_2) during the soil respiration study. BIOLOG Ecoplates (Techno-path distribution Ltd, Ballina, Tipperary, Ireland), were used for community physiology profile analysis. NaCl laboratory reagent from Fisher Scientific UK was used for extraction of microorganism from the soil.

5.2.3 Instrumentation and laboratory equipment

The details of the microplate absorbance reader used for the BIOLOG Ecoplate and the OxiTop respirometry system used for soil respiration can be found in Sections 2.5.4.1 and 2.5.4.2 respectively.

5.2.4 Experimental procedures:

5.2.4.1 Soil respiration experimental procedure:

Three replicate PVC microcosms for each treatment were harvested at each period of analysis for soil respiration measurements. The replicates were homogenised and a representative sample of 100 g was used to carry out soil respiration measurements. The respiration measurement from the three replicates represents the average respiration measurement for each treatment category.

The soil respiration experimental procedure used for this present study was the same procedure used in Section 3.3.7.2

5.2.4.2 Soil microbial biomass determination

The details on the procedure used for the microbial biomass determination can be found in Section 3.3.14.2.

5.2.4.3 Community level physiological profile using BIOLOG Ecoplate

The details on the procedure for the BIOLOG Ecoplate and its data analysis can be found in sections 3.3.8.5 and 3.3.8.6 respectively.

5.2.4.4 Soil microbial metabolic quotient (qCO_2)

The details on how the microbial metabolic quotient was determined can be found in section 3.3.15

5.3 Results and discussion:

5.3.1 Soil respiration:

Soil respiration rate was used to represent microbial activity during the duration of this present study and the effects of heavy metals, PAHs and the co-contamination of PAH and heavy metals. As shown in Figure 5.1 the soil respiration rate for the non-PAH spiked soils showed a steady decline over the 40 week period but the decline for the soil with the highest concentrations of heavy metal (500 mg kg^{-1} Pb and 500 mg kg^{-1} Cd) was more than the decline for the lower heavy metal (100 mg kg^{-1} Pb and 100 mg kg^{-1} Cd) concentrations. The soils amended with low concentrations (for both Pb and

Cd) had a steady decline after the first three weeks but on the fifth week, there was a sudden increase in their soil respiration value as shown in Figure 5.1. Heavy metals are known to inhibit growth and biochemical processes in microorganisms [82] which seems to show the reason for the decline in soil respiration values but soil microorganisms have also shown to be resilient towards heavy metal contamination in some studies [149] and may recover after the initial inhibition caused by heavy metals.

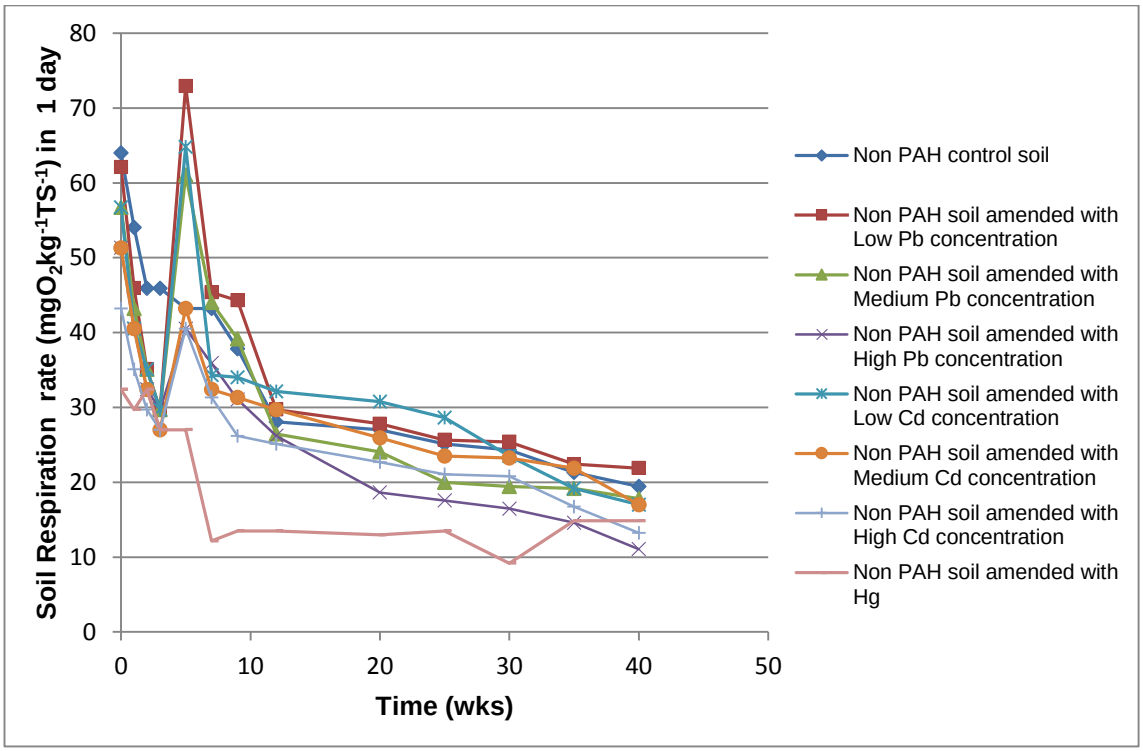


Figure 5.1: Soil respiration for non-PAH spiked soil comprising of non-PAH spiked control soil, and the three different concentrations of Cd and Pb-spiked non-PAH spiked soils.

Fungi have been shown in some studies to be more resilient to heavy metals than bacteria [95] and one of the reasons for the increase observed in Figure 5.1 for the soils amended with low concentrations of Pb and Cd could be the fungi utilising the organic carbon released by dead metal sensitive bacteria cells. The extra carbon may have enabled the Fungi to overcome the negative effect of the heavy metal contamination. Bacteria can also develop tolerance to heavy metals by binding the metal ions, using the efflux mechanism or making their cell wall impermeable to the metals [92]. Di´az-Ravina *et al.* [284] using thymidine incorporation to indicate soil bacteria activity in soil contaminated with heavy metal observed an initial reduction in bacterial activity after the first day of metal contamination and a subsequent recovery of bacterial activity on the second day in all the soil metal treatments (concentration range of 131 – 2,092 mg kg^{-1} of Zn). The recovery later stabilized at different sampling times for different metal concentrations. For example, soil amended with 523 mg kg^{-1} of Zn stabilised at a level similar to the control after 4 days of metal addition while soil

polluted with 1,046 mg kg^{-1} of Zn which had a delayed recovery (after 8 days of metal addition) stabilized after 16 days of metal addition. In another study Di´az-Ravina *et al.* [285] also using thymidine and leucine incorporation to indicate bacterial activity reported a large decrease in bacterial activity after 10 days of amending soils artificially with five different metals (Cu, Cd, Zn, Ni and Pb) using two concentrations of each metal (1,046 mg kg^{-1} and 2,092 mg kg^{-1}) except Pb and Ni concentration which was only 2,092 mg kg^{-1}. The decrease in activity was correlated to the level of metal amendment for soils amended with Cd, Cu and Zn though for soils amended with Pb and Ni there was no activity after the metal addition. They later observed a recovery for all the metal amended soil (except Pb and Ni) after 1 month. The recovery of bacterial activity for the soils treated with Pb and Ni was only observed 2 months after metal addition [285].

In the present study the soil sample used already had a background concentration of Pb (200 mg kg^{-1}), Zn (156 mg kg^{-1}) and Cr (180 mg kg^{-1}) because of the historic industrial activities (lead works and lead paint industry) that took place in the neighbourhood [227]. This may suggest that microbial community already had some degree of tolerance for the above-listed metals; however, the increase in metal concentration can still increase the stress burden on the community and there is also a question over the respective bioavailabilities of the historic and spiked metals. Some studies [84, 149, 284] have reported that microbial communities from polluted sites are usually more tolerant to increasing concentrations of heavy metals (especially metals found in the polluting site) than those from unpolluted sites. Witter *et al*. [84] observed that the microbial community from a metal amended sewage sludge site developed metal tolerance as a result of the background concentrations of the polluting metal (Zn concentrations ranged from 56 to 359 mg kg^{-1}). This might also explain the reason in the present study why the soil amended with a low concentration of Pb (100 mg kg^{-1} Pb) showed the highest activity in week 5 and also soils amended with medium Pb concentrations (250 mg kg^{-1} Pb) had a high respiration activity compared with other soils amended with medium Cd concentrations (250 mg kg^{-1} Cd) as seen in Figure 5.1. There has also been evidence of co-tolerance development by microbial communities on metals that were not previously exposed to them [84, 286] but may have a similar mode of action to the metals that are familiar to them [149]. Diaz-Ravina *et al*. [286] observed in their study that microorganisms from Zn contaminated sites exhibited resistance towards Zn and Cd amendment in the soil which suggests that there was a common resistance mechanism for both metals. They reported that resistance to both metals was found in the same plasmid of the tolerant microorganism [286]. The result also supports reports from other studies [95, 141, 142] which state that the increase in

basal respiration under environmental stress or pollution could be as a result of the increase in the more tolerant microbial community species when the most sensitive species have died because of the contaminants or environmental stress in the soil.

The soils amended with a higher concentration of Pb and Cd (500 mg kg^{-1}), though they showed some sign of recovery on week 5 of sampling, their respiration activities were much lower than the activities of the soils amended with low heavy metal concentration (100 mg kg^{-1}) and the non-PAH spiked control as seen in Figure 5.1. The non-PAH spiked control soil had a steady decline from the beginning of incubation which can be attributed to organic carbon limiting factor. Soil microorganisms (bacteria and Fungi) are usually carbon limited [95, 285] and because there was no addition of extra nutrient or a high number of bacteria dead cells that can serve as a source of readily available organic carbon, the respiration activity then started to decline as seen in Figure 5.1. The non-PAH spiked soil amended with Hg, which served as a sterile control, also showed signs of recovery after week 3 of sampling but the respiration activity was still very low compared to the other metal amended soils because of the mortality caused by the presence of Hg metal. The concentration of Hg was also increased (1000 mg kg^{-1}) on week 7 of sampling to maintain sterility but there was still some level of respiration activity observed after the additional Hg metal amendment to show that some microorganisms can withstand extreme heavy metal pollution. An example of this is *Pseudomonas aeruginosa* which is able to biotransform toxic mercury (Hg^{2+}) to atmospheric form (Hg^{0}) through the activity of its mercuric reductase enzyme [287]. Skohn *et al.* [106] observed the degradation of phenanthrene at a toxic Cu concentration of 7000 mgL^{-1} to imply that highly adapted Cu-resistant microorganisms were still performing the degradation of the parent phenanthrene.

However, the increase in soil respiration activity observed for all the non-PAH spiked metal amended soils on week 5 (Figure 5.1) might also be attributed to other factors such as pH, temperature and soil water content because these abiotic factors can influence microbial activity [149, 284]. In the present study, these factors apart from pH were kept constant. From the observations in Figure 5.1, it can be seen that soil respiration activity declined according to the increasing concentrations of the amended heavy metals, which shows the abiotic stress and mortality caused by the heavy metals. Similar trends are observed for the PAH heavy metal amended soils shown in Figure 5.2 which were kept under the same conditions as those in Figure 5.1.

The PAH heavy metal amended soils used in Figure 5.2 had a high respiration rate at the initial period of sampling but their respiration rate declined at the later periods of sampling. The interaction of both contaminants (PAH and heavy metal) with the soil microbial community seemed to have restricted their microbial respiration activity. PAHs though can serve as a readily available carbon source for microbial community which would increase soil microbial activity [23] but its interaction with heavy metals also can reduce soil microbial activity. Silva *et al*. [88] reported that metal toxicity is generally exhibited at the cellular level as a nonspecific binding with reactive macromolecules that interfere with physiological processes such as respiration and oxidative phosphorylation while PAHs disrupt membrane function due to nonpolar narcosis, and many are effective mutagens, cancer-causing agents and teratogens [88]. It has also been suggested that PAHs can enhance the easy penetration of heavy metals into microbial cells where the metals would then disrupt the cells functions [79, 103, 104]. These might be the reason that microbial respiration recovery as seen in Figure 5.1 was either in non-existent or very low in Figure 5.2.

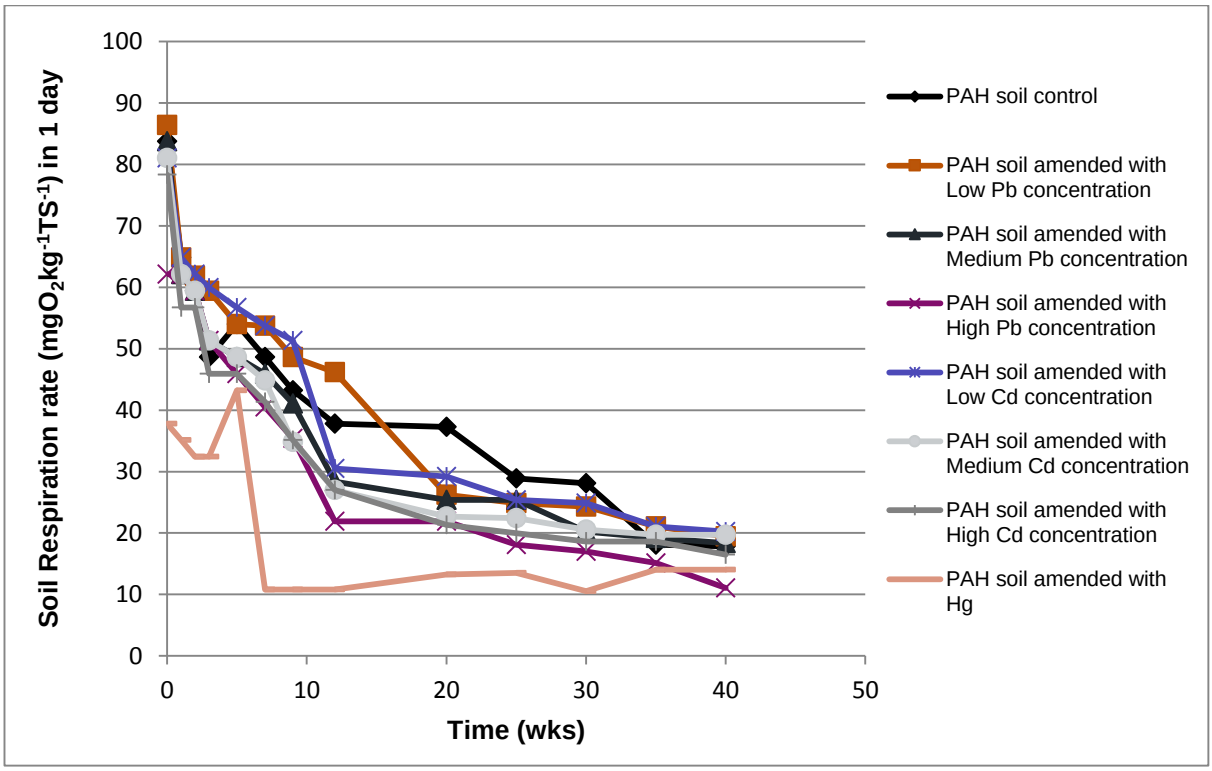


Figure 5.2: Soil respiration for PAH spiked soil comprising of PAH spiked soil control, and the three different concentrations of Cd and Pb-spiked PAH soils.

Soils amended with only PAH (no heavy metal amendment) had a high respiration activity at the initial stage because of the readily available carbon the PAHs provided [23] but it then declined (Figure 5.2).

The toxic effect of the high concentrations of some of the PAH mixture of compounds (e.g. benzo[b]fluoranthene 324.7 ± 14.2 mg kg^{-1}; benzo[a]pyrene 254.9 ± 12.6 mg kg^{-1}) can also be the reason behind the respiration activity decline during the period of the present study. Lu *et al.* [91] in their study observed that the incorporation of pyrene alone into soil had stimulating effect on the respiration rate which became higher than that of soil without pyrene. They reported that pyrene at a lower concentration in the soil could serve as a substrate for microflora, thus spurring microbial activity while microbial activity could also be suppressed by high concentrations of pyrene [91]. However, it has also been reported that PAHs can inhibit microbial enzyme activity when they occur as a mixture of compounds in the soil environment [92]. Gogolev *et al.* [105] observed that the growth of *A.oligospora* was reduced by 14 % at a concentration of 1 mg kg^{-1} of fluoranthene, with 10 and 100 mg kg^{-1} fluoranthene being more toxic but the level of toxicity between 10 mg kg^{-1} and 100 mg kg^{-1} fluoranthene was not different because of the low solubility of fluoranthene.

The reduction of soil respiration activity observed for the PAH soils at later periods can also be as a result of the low bioavailability of PAH compounds which reduced the amount of carbon substrates available for microbial utilization. The soils used in the present study had a high concentration of organic matter 26.56 % which can sequester PAHs and reduce their bioavailability [36]. Maliszewska-Kordybach *et al.* [89] in their study observed that increased organic substances and acidity of the soil reduced the toxic effect of PAH amendment in the soil as evidenced by the intensity of respiration that had no change compared to the control. The PAH soils amended with Hg also had a very soil low respiration activity because of the mortality caused by the high concentration (1000 mg kg^{-1}). The Hg amended soil started to show signs of higher respiration activity towards the end of the experimental period (40 weeks) which could mean that the soil was being recolonized by some highly Hg adaptive microorganisms.

The combined soil respiration results for both the PAH spiked soil and non-PAH spiked soil is shown in Figure 5.3. This shows that the PAH soils (metal amended and non-metal amended) had an initial higher respiration activity than the non-PAH spiked soils (metal amended and non-metal amended). The extra carbon source provided by PAHs as mentioned earlier enabled the microbial community to overcome the initial stress exerted by the heavy metals but subsequently the toxicity of both the PAH and heavy metal impacted on the microbial community which caused the decline in their respiration activity. Bååth [27] in his review on heavy metal stated that no effect of heavy metal was observed during the first week of degradation of different litter fraction in a litter decomposition study because of the easily degradable substrate that was still

available to the microorganisms. However, a decrease in degradation rate due to heavy metal pollution was observed with time.

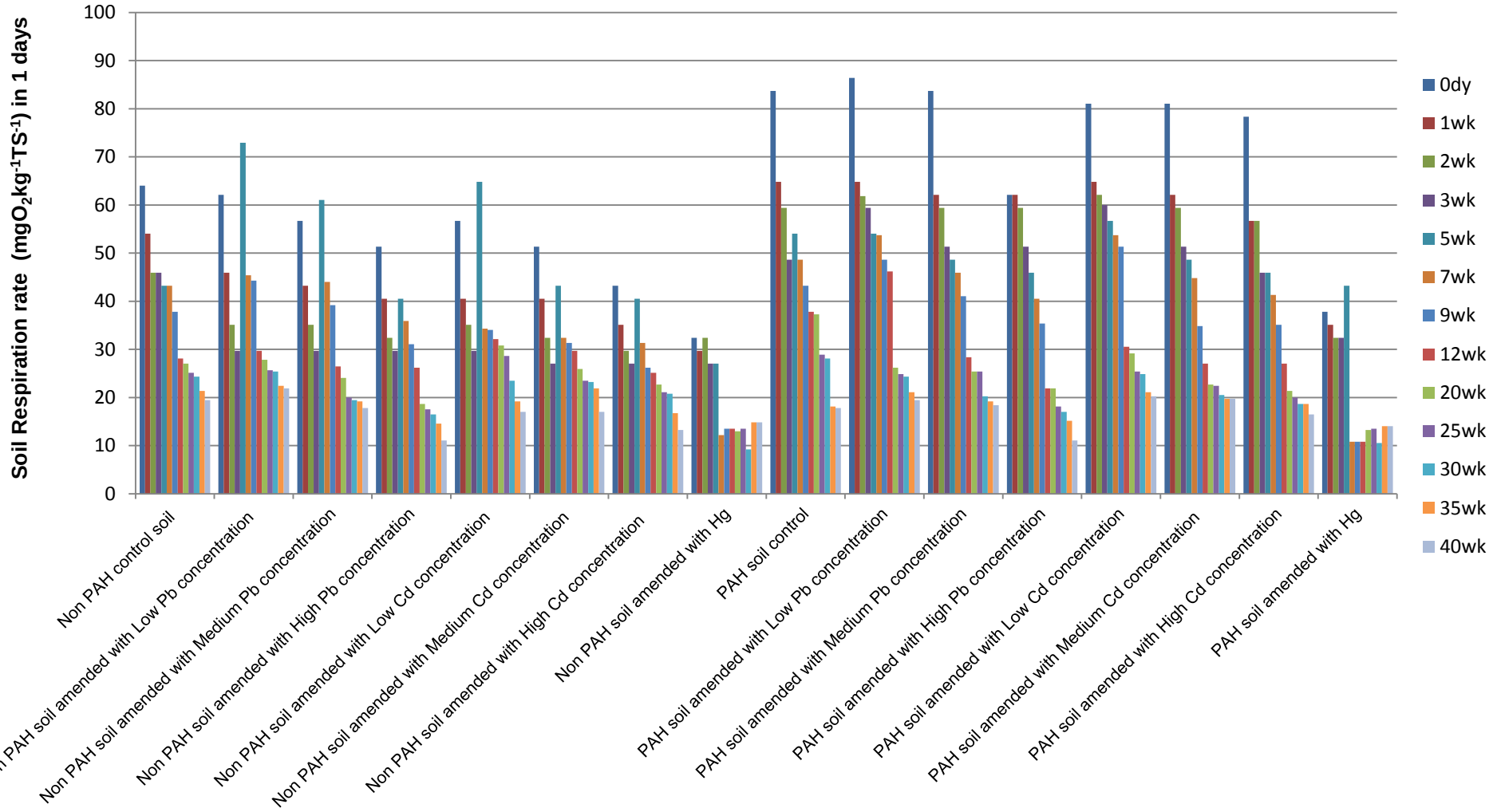


Figure 5.3: Soil respiration of both the non-PAH spiked soil and PAH spiked soil with their respective Cd and Pb heavy metal spiking over a 40 weeks period of sampling

The initial high respiration rate observed for the PAH soils (both for the PAH control and metal amended PAH soils) other than being because of extra carbon can also be a sign of metabolic stress exerted on the microbial community by both the PAHs and heavy metals. The high respiration rate observed for the non-PAH spiked metal amended soils can also be attributed to stress exerted on the microbial community by the heavy metals. The development of tolerance towards pollutants by microbial community can be due to the adverse effect exerted on the microbial community by the pollutant [84]. Fang et al. [26] observed an increase in the soil respiration rate with increasing heavy metal content which they attributed to stress on soil microbial community. Lu *et al.* [91] reported that the increase in production of CO_2 in the presence of PAH may be because of increased metabolic activity in reaction to metabolic stress. They also observed a stimulation effect of Cd on the basal respiration compared to the unspiked control but this stimulation effect decreased with increasing concentrations of Cd from 2.5 to 10 mg kg^{-1} [91]. These observations are similar to those made in the present study where the non-PAH spiked metal amended soils stimulated microbial basal respiration on the fifth week of sampling and stimulation also declined with increasing metal concentrations.

There have been several other explanations for observed changes in the soil basal respiration of contaminated soils: (i) microorganisms in less polluted soil consume a higher amount of the accessible carbon for assimilation and a smaller part of it is released as CO_2 in the dissimilation processes; (ii) microorganisms in highly contaminated soils required more energy to survive under unfriendly conditions, in this manner a higher amount of the carbon expended was lost as CO_2 and a smaller portion was ingested; (iii) under heavy metal adverse conditions, the energy utilization competence of microbial metabolic processes was decreased, which then required greater measures of carbon for maintenance and eventually increased respiration [91, 110]. Among the PAH soils the decline in the Pb amended soils was less than the decline in the Cd amended soils except for the PAH spiked soil amended with 500 mg kg^{-1} Pb which was lower towards the end of the experiment compared to the 500 mg kg^{-1} Cd at a similar period.

5.3.2 Soil microbial biomass:

Microbial biomass, which expresses the living constituent of the soil organic matter, [162, 163] was used to measure the effect of both heavy metals and PAH on the soil microbial community. The microbial biomass measurements were only carried out on soil samples harvested at the beginning and at the end of the experimental periods. As

shown in Figure 5.4 the soils amended with PAHs had a higher soil microbial biomass carbon concentration initially as expected because of the added PAH organic compounds which stimulated the increase in biomass. The PAH control soil had the highest biomass carbon concentration for the measured sampling periods but also showed signs of biomass decline after the initial period which can be partly due to the toxicity of some of the PAH compounds and non-bioavailability of PAH compounds at the later stages of the present study. The non-PAH spiked and PAH soils amended with low concentrations of heavy metals (100 mg kg^{-1} Cd and Pb) had higher microbial biomass than soils (PAH and non-PAH spiked) amended with high concentrations of heavy metals (500 mg kg^{-1}) which can be due to the mortality caused by the increase in metal concentrations. The high mortality rate observed for the soils with high metal concentrations could be as a result of the greater concentration of metals that were bioavailable to the microbial community, thereby exposing the microbial community to the toxic nature of the heavy metals. The distribution of heavy metals over the soil phases is significant to their interaction with soil microbial activity [101] since it is just the metal concentration in the liquid phase is viewed accessible to them.

Witter *et al.* [84] observed an increase in the amount of biomass for soils with a low rate of application of both sewage sludge and with metal amended sewage sludge. The opposite was observed for soils with a high application of sewage sludge and the lowest value of biomass was observed for soils with a high rate of metal amended sewage sludge [14]. Lu *et al.* [91] also observed that microbial biomass increased in the presence of pyrene alone but decreased in the presence of Cd alone and with pyrene and Cd combination as the concentration of Cd increased. Thavamani *et al.* [79] in their study also observed the decline in microbial biomass in soils contaminated with both heavy metal and PAHs.

An important observation in this present study was that the soils amended with Cd had a higher microbial biomass carbon than the soils amended with Pb. Diaz-Ravina et al. [286] observed a stimulatory effect of added Cd on bacteria extracted from soils polluted with the highest concentrations of Zn and Cd (32 mmol kg^{-1} and 16 mmol kg^{-1} respectively) and they also observed an increased level of tolerance to Zn and Cd for bacterial communities extracted from Pb, Zn and Cd-polluted soils. The level of tolerance towards Pb was to a less extent compared to Cd and Zn [286]. In another study [285] they observed that tolerance to Pb occurred two months after the Pb metal amendment which was late compared to tolerance towards Cd and Zn metal amendments. One explanation could be that tolerance to different metals may emerge at different times [286]. Witter *et al*. [84] in their study also observed an increase in

tolerance towards Zn and Cd at lower levels of metal contamination for soils that had a high rate of Cu, Zn and Cd metal amended sewage sludge.

However, the non-PAH spiked soil amended with Cd had a higher microbial biomass carbon at later sampling periods than the PAH soils amended with Cd (Figure 5.4) which shows that combination of PAH and heavy metals affected the microbial community. The same effect was also observed for the non-PAH spiked Pb amended soil and the PAH Pb amended soil.

The microbial biomass carbon for the PAH and non-PAH spiked soil after the first period of sampling was higher than the biomass carbon for all the heavy metal amended soils. The microbial biomass for the PAH amended soils was higher than that for the non-PAH spiked soil after the first two periods of sampling (0 day and 1 week) but became slightly lower than that for the non-PAH spiked soil at the end of the sampling period. As mentioned earlier, this can be due to the reduced bioavailability and the toxicity of the PAH compounds to microbial community. The microbial biomass carbon for the Hg amended soils was the lowest compared to all the soil treatments which show a high reduction of microorganism caused by the Hg metal. The biomass for the non-PAH spiked Hg amended soil was higher than the biomass for the PAH Hg amended soil (Figure 5.4) which is a further evidence of the negative synergistic effects PAH and heavy metal can have on soil microbial community.

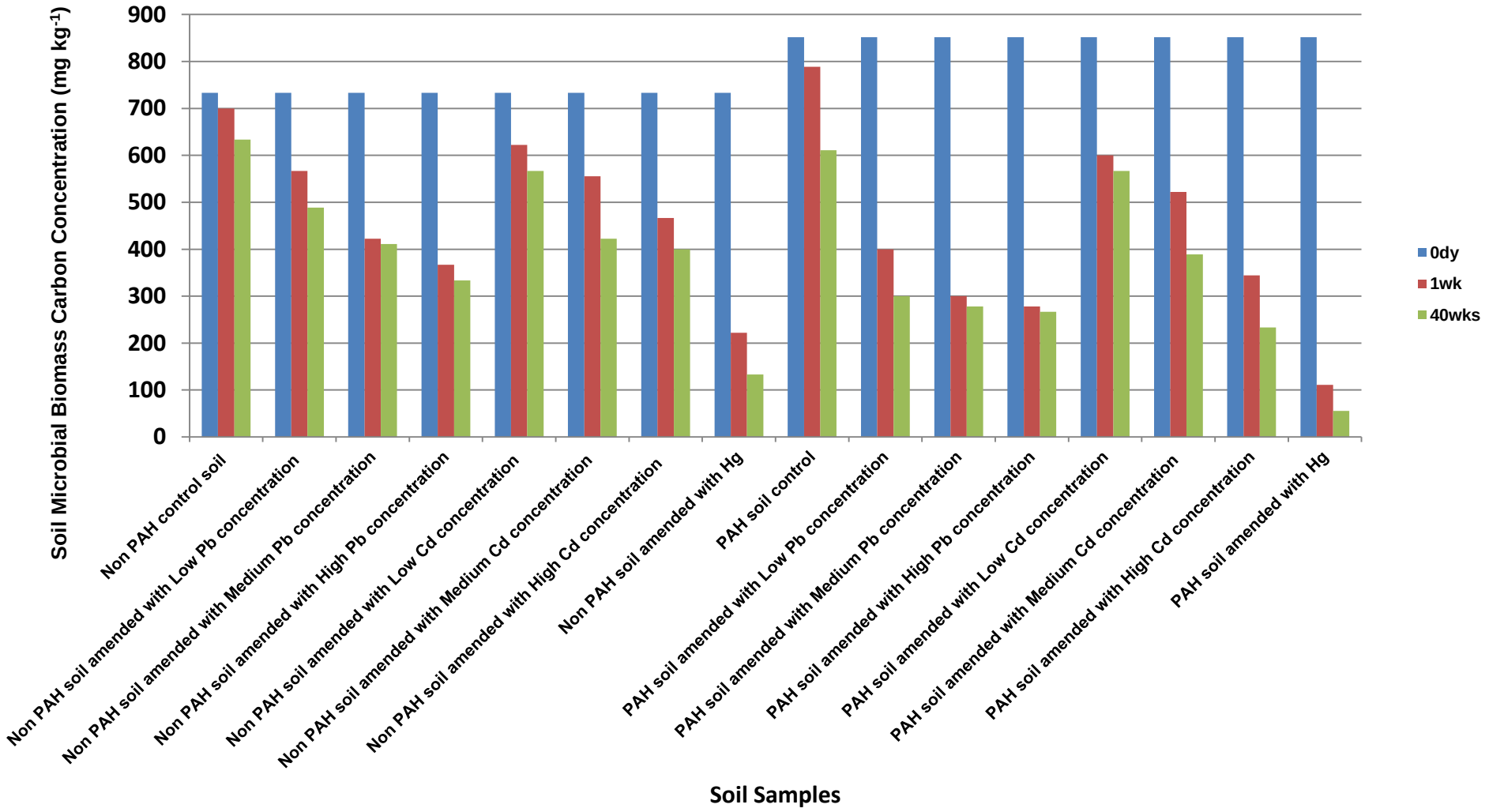


Figure 5.4: Microbial biomass carbon concentrations for non-PAH spiked soils; non-PAH spiked metal amended soil; PAH soils and PAH metal amended soils

5.3.3: Soil metabolic quotient:

The soil metabolic quotient which is the ratio of the soil respiration values to the microbial biomass carbon values (soil respiration: soil microbial biomass carbon) was used to indicate the stress on the microbial community by pollutants. The metabolic quotient is conversely identified with the efficacy with which microbial biomass utilises indigenous substrates [144]. Increase in metabolic quotient (qCO_2) is generally an indication of stress on microorganisms caused by contamination and ecosystem disturbances [267]. The result in Figure 5.5 shows that the soils with heavy metal amendment had the highest metabolic quotient indicating heavy metal stress on the microbial community. Zhang *et al.* [81] reported that heavy metal polluted soils had significantly higher qCO2, which indicates a greater energy demand for maintenance, a decrease in substrate quality and ultimately a decline in microbial metabolic efficiency. The high qCO_2 value of microorganisms in the presence of heavy metals and other contaminants could be best explained as the diversion of energy into physiological adaptations necessary to tolerate heavy metals. The consumed carbon substrate under this condition is then released as CO_2 with less being built into organic components or used for growth [81, 142, 145]. The PAH soils amended with heavy metal had a higher metabolic quotient than the non-PAH spiked soil metal amendment which confirms the additional stress exerted on microbial community by the interactions of PAH and heavy metals. The PAH soils amended with Pb metal had a higher metabolic quotient when compared with the PAH soils amended with Cd, likewise, the non-PAH spiked Pb soils and non-PAH spiked Cd soils, which shows that the Cd amended soils had seemed to have developed an increased tolerance to Cd metal than the Pb amended soils seemed to have done towards Pb. The metabolic quotient for the PAH control was the lowest at the sampling period measured which seem to show that the readily available carbon compounds provided by the PAHs reduced the stress on microbial community. This is in contrast to the non-PAH spiked soil which had a higher metabolic quotient than the PAH control which indicates that poor substrate quality can also exert stress on microbial community.

The soils amended with Hg also showed a high metabolic quotient especially the PAH spiked soil Hg amended soil which had the highest of all the other treatments which indicate that Hg amendment had the greatest effect on the microbial community. The metabolic quotient for all the treatments seemed to decline towards the end on the experiment as also observed by Zhang *et al.* [81] after 7 days in their study on the effect of heavy metals on microorganisms. This seems to indicate that more tolerant microbial community became more adapted to their environment.

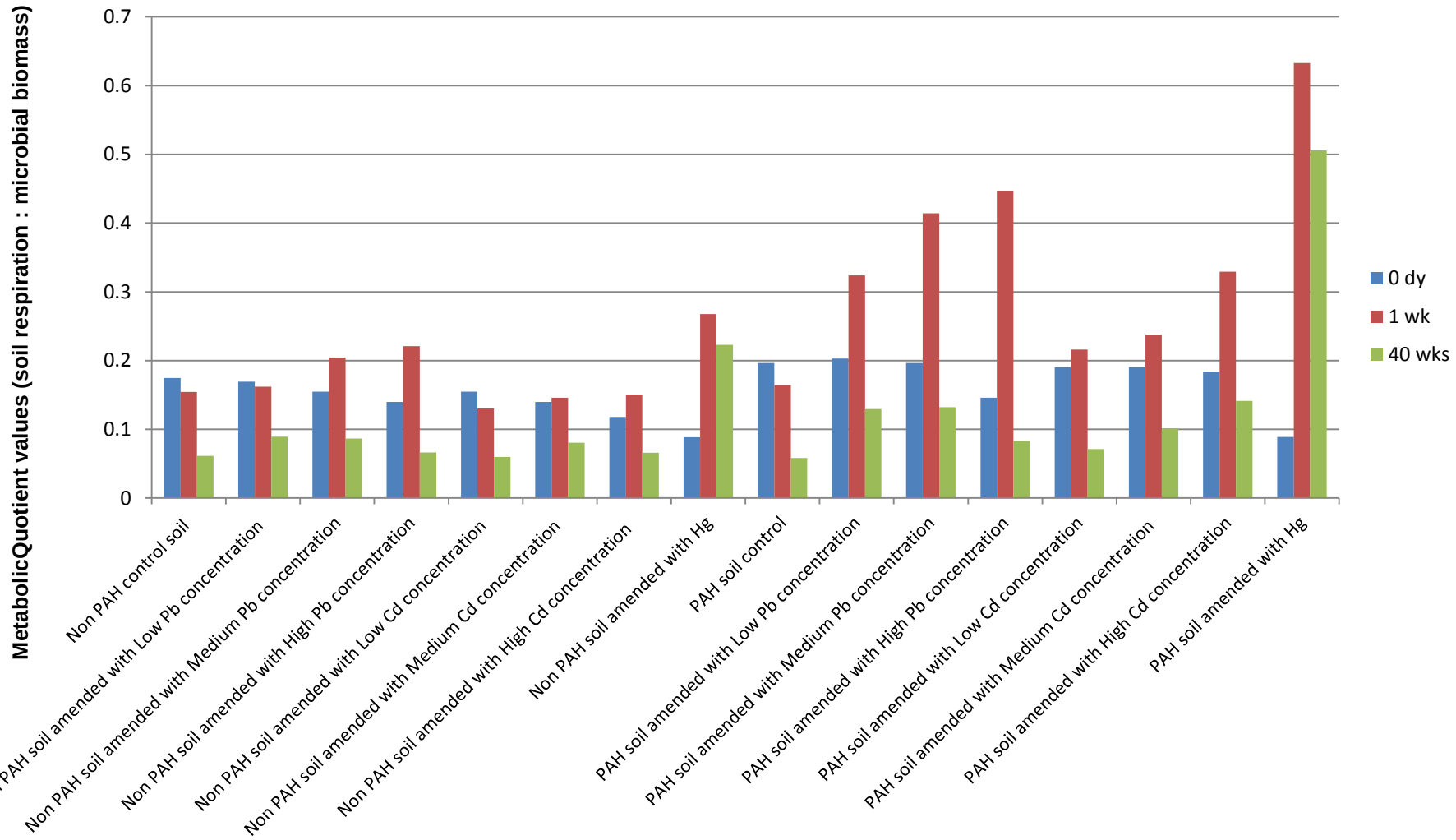


Figure 5.5: Metabolic quotient values for non-PAH spiked soil control; PAH spiked soil control; non-PAH spiked Pb amended soils; non-PAH spiked Cd amended soils; PAH Pb amended soil and PAH Cd amended soils

5.3.4: Community level physiological profile:

The community level physiological profile (CLPP) for soil microbial community from all the soil treatments was assessed using BIOLOG Ecoplates. The CLPP was used to characterize the soil microbial community function based on their catabolic diversity (sole carbon source utilization patterns). The BIOLOG Ecoplates used for this assessment provide a complete assessment of the microbial community structure because of the number, type and the diversity of substrates they contain.

The average well colour development values as seen in Figure 5.6 seem to reflect species metabolic activity and the ability of the microbial community to respond to substrates [82]. The decline of the metabolic activity as shown in Figure 5.6 can be due to a lower utilisation of the available carbon substrate in the BIOLOG Ecoplate and this can also be attributed to reduced species diversity in the microbial community. The presence of PAHs in the soil which can stimulate microbial activity can also lead to a change in the soil microbial catabolic diversity seen in Figure 5.6. The PAH control soil had more microbial metabolic activity than the non-PAH spiked control soil throughout the soil sampling and evaluation periods, which could be because of the extra carbon source provided by the added PAH compounds. The metabolic activity for non-PAH spiked soil was higher than the metabolic activity in all the metal amended soils except in the non-PAH spiked low concentration Pb soil which had higher metabolic activity at the initial periods (0 – 12 weeks) but became lower at the later stages of the evaluation (15 – 40 weeks). This observation for the non-PAH spiked soil with low Pb concentration could be that the Pb tolerant microbial species which were already in the soil (soil background Pb concentration: 206.6 ± 8.5 mg kg^{-1}) was not affected by the low additional Pb concentration at the initial stage but at the later stages the heavy metal effect caused its metabolic activity to decline. The decline was still not as much as the decline observed for soils with a low Cd concentration and higher concentrations of Pb and Cd

The PAH soils amended with heavy metals also had a higher metabolic activity than the non-PAH spiked metal amended soil at the initial period (0 day to 3 weeks) of sampling. The non-PAH spiked soil with Pb amendment had a higher metabolic activity than the PAH spiked soil amended with Pb after 3 weeks of sampling and evaluation which shows the negative synergistic effect of the PAH and Pb on the microbial community. The microbial community recovery for the non-PAH spiked metal amended soils which were observed in the soil respiration activity (Figure 5.1) on the fifth week of sampling was also observed in the average well colour development of the same soil treatments at the same period of sampling.

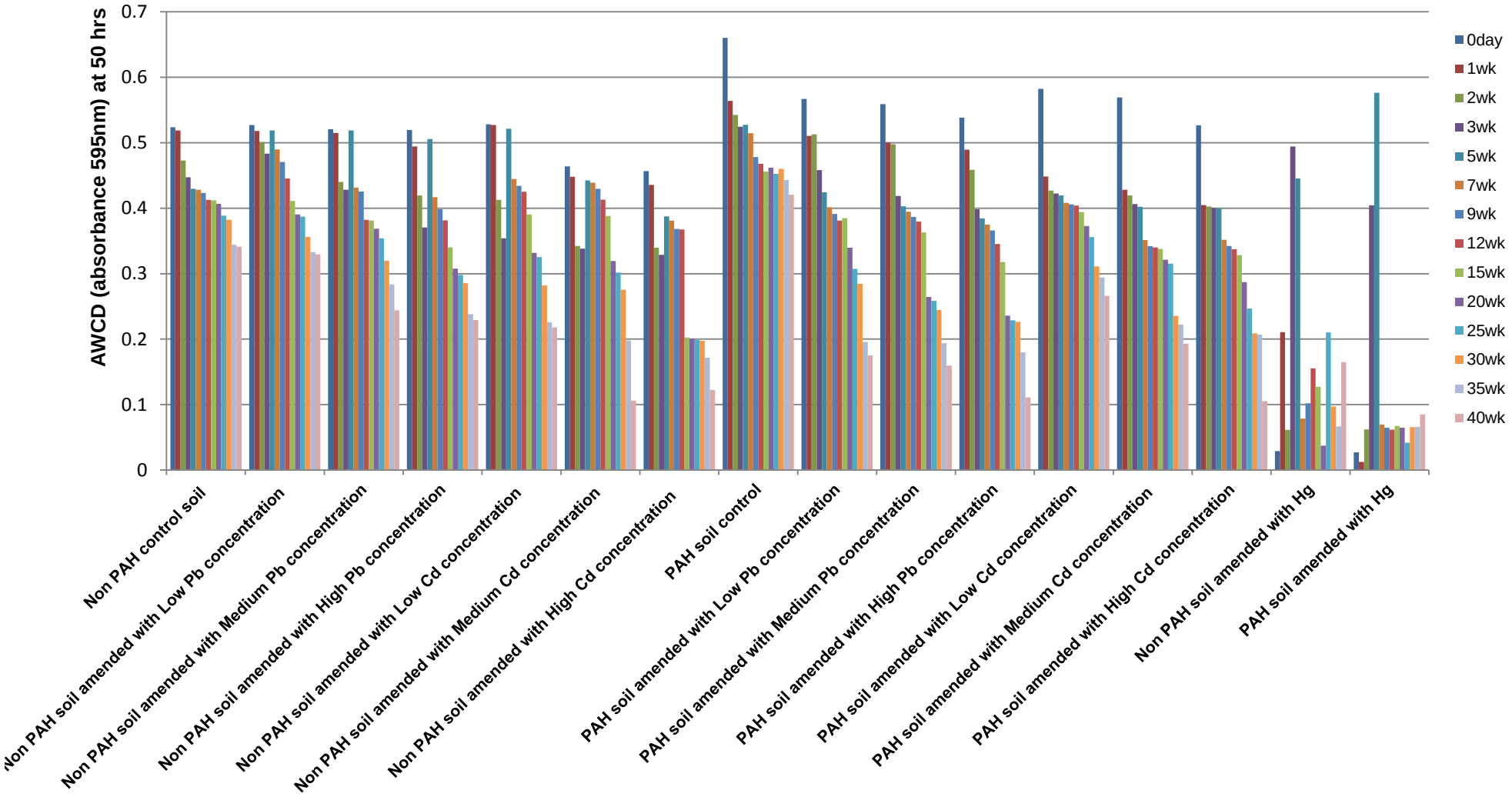


Figure 5.6: The average well colour development of the BIOLOG Ecoplate for non-PAH spiked soils control soils; PAH control soils; non-PAH spiked metal amended soils and PAH metal amended soil.

The high value observed in the AWCD for the non-PAH spiked metal treated soils on the fifth week of sampling could be because of a shift in microbial catabolic diversity whereby certain species of the community tend to metabolise more of a particular carbon substrate. The AWCD values for the non-PAH spiked soils amended with Pb metal were higher than those amended with Cd for most of the sampling weeks (especially 0 – 5 weeks) when comparing similar concentrations. This could be that the development of tolerance towards Cd by soil microbial community caused a change in their metabolic potential. This could imply that the metabolism of some of the Ecoplate carbon substrate for the non-PAH spiked Cd microbial community was lower than that of the microbial community from the non-PAH spiked Pb amended soils. The same observation can be seen for the PAH soils amended with Pb and those amended with Cd but in this observation, the metabolic activity for PAH spiked soil with Pb was only higher in the initial stages of the investigation (0 – 3 weeks). The AWCD for the PAH soils amended with Pb was lower than that amended with Cd at the later stages of the investigation. This could also imply that the negative synergistic effect of PAH and Pb on the microbial community metabolic activity was more than that for PAH and Cd community metabolic activity.

It is known that the tolerance of a community for a contaminant is a strong indicator for the presence of that contaminant at the level of truly adverse concentrations [84]. The development of tolerance to a contaminant by microbial community reduces the survival rate and the growth rate of the most sensitive species within the microbial community. The increased tolerance towards Cd and Pb by the microbial community as seen in Figure 5.3 and 5.4 might have caused a shift in the community composition whereby fewer carbon substrates were used more in the heavy metal amended soils than in the control soils. Jiang *et al.* [94] using an Ecoplate average well colour development (AWCD) observed that phenanthrene and Cd had a negative effect on the AWCD. It delayed the onset of colour development, reduced the rate of AWCD and decreased the maximum AWCD compared to the control. They reported that the presence of Cd and phenanthrene had a significant negative effect on microbial metabolism of L-serine, glycogen, and D-cellobiose, L-threonine, D-galacturonic acid, L-asparagine and itaconic acid in the Ecoplate well [94].

These effects on the metabolic activities of the microbial community were brought about by the different treatments used for this study. Haack *et al.* [182] in their study reported that when samples of similar inoculum density exhibit different patterns of positive and negative substrates responses, it probably reflects real differences in community composition. Massieux et al. [184] in their study, used Ecoplates to observe the differences in metabolic capabilities of the microbial community treated

with different concentrations of copper which confirmed the differences in the bacteria community structure exposed to copper treatment [184]. In this present study similar sample dilution was used for all the soil treatment which has already being observed in Section 3.4.10.1 as a method of standardizing the inoculum density of the microbial community. Therefore, the differences in patterns of Ecoplate substrate utilization observed for all the soil treatment would probably signify the differences in community composition.

5.3.4.1 Metabolic diversity:

The Shannon diversity index (H) is always used to track and understand shifts in communities over space and time. It was used in this study to provide information on the diversity of metabolised carbon substrates through the generated BIOLOG Ecoplate absorbance data.

Using the carbon substrate utilization profile (CSUP) gathered from a single BIOLOG Ecoplate at 50 hrs absorbance measurement, the substrate diversity (H) was calculated as in Eq. 5.1:

$$H = -\sum p_i \ln(p_i) \qquad (5.1)\ [232, 234]$$

Where:

H - Substrate diversity

p_i - Ratio of the activity of a particular substrate to the sums of activities of all substrates

The Shannon diversity index values seen in Table 5.1 shows that the PAH control soil had a more consistent and higher diversity value (between 3 – 3.2) than the non-PAH spiked control soil. The metabolic diversity values for the PAH Pb amended soils were much lower (2.9 – 2.6) than the control soils between the 25 week and 40 weeks of evaluation. The same observation can be seen for the PAH Cd amended soils that had values of 3 – 2.5 between 25 and 40 weeks of investigation. The metabolic diversity values for the non-PAH spiked low Cd amended soil ranged was between the range of 2.7 – 2.8 from 25 weeks to 35 weeks of evaluation; non-PAH spiked medium Cd soil had a low values: 2.9 between 7 and 12 weeks; 2.7 – 2.8 between 25 and 30 weeks; and a value of 2.5 at week 40. The non-PAH spiked high Cd also had lower values of 2.6 – 2.9 between 7 weeks and 12 weeks and 2.8 – 2.7 between 15 weeks and 25 weeks. These metabolic diversity values for the non-PAH spiked Cd soils were lower

when compared to the non-PAH spiked Pb soils which had values of 2.7 – 3.1 for most of the evaluation period.

Table 5.1: Metabolic diversity (H) values for the non-PAH spiked control, PAH control, non-PAH spiked Pb amended soil, PAH Pb amended soil, non-PAH spiked Cd amended soil and PAH Cd amended soil.

Substrate Diversity (H)																
PAH amendment status	Metal amendment	Metal amendment level	0 wk	1 wk	2 wk	3 wk	5 wk	7 wk	9 wk	12 wk	15 wk	20 wk	25 wk	30 wk	35 wk	40 wk
Non amended	Control	None	3.1±0.02	3±0.01	3±0.07	3.1±0.02	2.7±0.06	3.1±0.04	2.9±0.1	2.9±0.05	3±0.08	3±0.13	3±0.07	3±0.05	3±0.08	3.1±0.06
PAH amended	Control	None	3.1±0.04	3±0.06	3.1±0.04	3.1±0.06	3±0.02	3±0.02	3±0.02	2.8±0.04	3±0.06	3±0.02	3±0.04	3±0.02	3.2±0.03	3.2±0.03
Non amended	Cadmium	Low	3.1±0.02	3.1±0.04	3±0.05	3±0.03	3.1±0.01	3±0.05	3±0.03	2.9±0.06	3.1±0.02	3±0.08	2.8±0.04	2.8±0.19	2.7±0.05	3.1±0.04
		Medium	3.2±0.05	3±0.05	3±0.08	3±0.08	3±0.06	2.9±0.07	2.9±0.05	2.9±0.05	3.1±0.08	3.1±0.03	2.8±0.1	2.7±0.02	3.2±0.05	2.5±0.23
		High	3.2±0.04	3±0.06	3±0.07	2.9±0.15	3.0±0.07	2.6±0.04	2.7±0.01	2.9±0.08	2.8±0.1	2.6±0.08	2.7±0.02	2.7±0.14	3±0.15	3±0.03
PAH amended	Cadmium	Low	3.2±0.04	2.9±0.02	2.9±0.04	3±0.06	3±0.09	2.9±0.09	2.9±0.08	2.8±0.06	3±0.03	3.1±0.01	2.7±0.07	2.9±0.03	2.7±0.03	2.7±0.07
		Medium	3.3±0.03	2.8±0.04	3±0.001	2.9±0.03	2.8±0.06	3±0.04	3±0.08	2.8±0.07	3±0.05	3±0.02	2.8±0.06	2.8±0.03	3±0.01	2.5±0.18
		High	3.2±0.01	2.9±0.06	3±0.05	3±0.07	3.1±0.07	3±0.06	2.9±0.07	3.1±0.06	3±0.12	3±0.1	2.6±0.04	2.6±0.03	2.8±0.06	2.7±0.08
Non amended	Lead	Low	3±0.03	2.9±0.02	2.9±0.03	3±0.07	3.1±0.03	2.9±0.03	2.9±0.03	2.8±0.04	3±0.05	2.8±0.02	2.8±0.02	2.8±0.08	2.8±0.03	3.1±0.06
		Medium	2.9±0.05	3±0.08	2.8±0.04	2.8±0.08	3.1±0.03	2.9±0.04	2.8±0.01	2.8±0.09	2.8±0.04	2.7±0.02	2.7±0.05	2.7±0.06	2.8±0.11	2.7±0.03
		High	3±0.03	3±0.03	3±0.02	3.1±0.14	3±0.04	3.1±0.06	2.7±0.04	3.1±0.03	3±0.07	3.1±0.08	3±0.07	2.9±0.09	3±0.1	2.8±0.2
PAH amended	Lead	Low	3.2±0.03	3±0.03	2.8±0.04	3.1±0.02	2.9±0.06	2.8±0.02	2.7±0.04	2.7±0.04	2.8±0.03	3.1±0.02	2.9±0.1	2.9±0.12	2.7±0.11	2.6±0.07
		Medium	3.1±0.05	2.9±0.02	3±0.04	3±0.03	2.9±0.04	3.2±0.03	2.9±0.07	2.9±0.04	3±0.04	3.1±0.03	2.7±0.12	2.8±0.07	2.6±0.16	2.7±0.13
		High	3.2±0.03	3±0.04	2.9±0.03	3.1±0.05	3±0.02	2.9±0.07	3.1±0.02	2.8±0.04	3.1±0.12	2.9±0.03	2.8±0.1	2.8±0.11	2.7±0.04	2.6±0.07
Non amended	Mercury	n/a	1.7±0.23	2.4±0.49	2.4±0.45	2.9±0.1	2.9±0.15	3±0.22	2.5±0.41	2.6±0.2	2.6±0.21	2.5±0.06	2.8±0.2	2.3±0.58	2.3±0.12	2.9±0.14
PAH amended	Mercury	n/a	2.1±0.52	1.6±0.55	1.9±0.61	2.8±0.03	2.9±0.07	3±0.25	2.4±0.03	2.4±0.2	2.7±0.07	2.5±0.23	2.3±0.3	2.5±0.14	2.8±0.11	2.7±0.18

These reduced values show that less diverse BIOLOG Ecoplate carbon substrates were metabolised at those evaluation periods.

Substrate richness seen in Table 5.2 is a measure of the number of different substrates metabolised by the microbial community and was calculated as the number of wells with a corrected absorbance greater than 0.25 [180]. These substrate richness values show that there were a greater number of substrates metabolised by the PAH control soil than the non-PAH spiked control soil for most of the evaluation periods. It also shows that the PAH control soil had a greater number of metabolised substrates than the all the metal amended soils. The non-PAH spiked control soil had an equivalent number of metabolised substrates with the non-PAH spiked low Pb and Cd soils except at week 5 and week 25 where the metal amended soils had higher numbers of metabolised substrate than the non-PAH spiked control soil. The non-PAH spiked Pb amended soils had more metabolised substrate than the PAH Pb amended soil between 25 weeks and 40 weeks. The values also show that the PAH low Cd amended soil had more metabolised substrate than the non-PAH spiked low Cd amended soil between 25 weeks and 40 weeks.

Substrate evenness which is defined as the equitability of activities across all metabolised substrates was calculated using Equation 5.2.

$$E = H / H_{\max} = H / \log S \qquad (5.2)[120]$$

Where;

H = Shannon diversity indices value for each Ecoplate well

And S = Substrate richness value

The evenness values in Table 5.3 shows that there was greater substrate evenness in the PAH spiked soil control than in the non-PAH spiked soil control. The substrate evenness for the control soils (PAH spiked soil and non-PAH spiked soils) were more than that for all the metal amended soils. The substrate metabolism for the non-PAH spiked Pb amended soils were more even than that of the PAH Pb amended soil especially between the non-PAH spiked low Pb and the PAH low Pb which had values of 2.8 and 3.5 at week 35 (Table 5.3). The substrate evenness for the PAH Cd amended soils were slightly better than that of the non-PAH spiked Cd amended soils, especially at week 40 where the evenness value for the non-PAH spiked high Cd was 6.5 and the evenness for PAH high Cd was 5.3. The non-PAH spiked and PAH soils amended with Pb had more even substrate metabolism than the Cd amended soils for the majority of the evaluation period (for example at week 40 period).

It is likely that the PAH control soil had the better substrate metabolic diversity, richness and evenness than the non-PAH spiked control soil because of the added carbon substrate provided by the PAH compound to the soil microbial community. This also confirms the result obtained by Gomez et al. [266] in their study which showed that the application of organic amendment (compost) to the soil stimulated microbial population and it significantly increased their AWCD, metabolic diversity and substrate richness. The decline in substrate diversity, richness and evenness for the non-PAH spiked control could be that the poor organic carbon quality [149, 237] in the soil at the latter stages of evaluation affected the microbial community because there was no fresh nutrient addition to the soil during the period of this study. The combination of poor organic carbon quality and heavy metal could have also affected the metabolic diversity, richness, and evenness of the non-PAH spiked soils amended with heavy metals (especially non-PAH spiked low Pb and Cd soils) after the first week. Bååth [93] in his review on heavy metal stated that no effect of heavy metal was observed during the first week of degradation of different litter fraction in a litter decomposition study because of the easily degradable substrate that was still available to the microorganisms. However, a decrease in degradation rate due to heavy metal pollution was observed with time. The negative synergistic effect of PAH and heavy metals (Pb and Cd) reduced the metabolic diversity, richness and evenness of the PAH Pb and Cd soils. These effects were observed to be more than the effects observed for the non-PAH spiked Pb and Cd soils in most cases. For example, the evenness and substrate values at week 40 for non-PAH spiked Pb soils were much better than that for the PAH Pb soils.

Table 5.2: Substrate richness values for the non-PAH spiked control, PAH control, non-PAH spiked Pb amended soil, PAH Pb amended soil, non-PAH spiked Cd amended soil and PAH Cd amended soil.

Substrate Richness (S)															
PAH amendment status	Metal amendment	Metal amendment level	0 wk	1 wk	2 wk	3 wk	5 wk	7 wk	9 wk	12 wk	15 wk	20 wk	25 wk	30 wk	35 wk	40 wk
Non amended	Control	None	20.3±0.6	18.3±0.58	18±1	20±1	14.7±0.6	22	16.7±0.6	16±1.7	18	17	14±1.7	15±0.6	18±2	13±0.6
PAH amended	Control	None	22.3±1.2	19.3±0.6	22	19.7±1.2	21.3±0.6	19.7±0.6	18.3±0.6	16.3±0.6	17	21±1.2	19.3±0.6	17	19±2	19.7±0.6
Non amended	Cadmium	Low	20.3±0.6	21.3±0.6	17.7±1.5	16	22±1.5	18.7±0.6	19.7±0.6	16.7±0.6	19.3±1.2	14.7±0.6	16.3±1.5	12	10±1	10
		Medium	24.7±2.1	19.3±1.2	17±1.7	16.7±0.6	19.3±0.6	17±1	16.3±0.6	19±1	20	15.3±0.6	13.3±1.2	14	10±1.2	3
		High	19.7±0.6	18±1.7	15.3±1.2	14±1	16.3±1.2	10.7±1.5	12.7±1.2	18.3±0.6	8.7±1.5	8	11±1.7	12±0.6	12±1.7	4±2.6
PAH amended	Cadmium	Low	21.3±1.5	17±1.7	16.7±1.5	16	20	13.7±2.1	16.7±1.5	15.7±0.6	17.7±1.5	19	15±1	13	11±0.6	13.3±1.5
		Medium	26	16±1	20±1	16±1	17	14.7±1.2	16±1	14.7±0.6	17.7±1.2	15.7±1.2	15±1	11	10	8.3±0.6
		High	23	14.7±0.6	18.3±1.2	18.7±1.5	18±1	15±1	16.7±1.5	17.3±0.6	13.7±1.2	14.7±1.2	11.7±0.6	10	11.7±2	3.3±0.6
Non amended	Lead	Low	21	18±0.6	17.7±0.6	18.7±0.6	20.7±2.5	18.7±0.6	19.3±0.6	16.7±0.6	16±1	16	15±1	15±0.6	10±0.6	17.3±0.6
		Medium	18.7±0.7	22.7±2.3	16±0.6	15±1	21.7±0.6	15±1.2	16	14	14	11.3±0.6	13.3±1.5	13	12±0.6	10
		High	20.3±1.2	18.7±1.5	19.3±0.6	15.7±1.2	20±2	19.3±1.2	15±0.04	17.7±0.6	10.7±0.6	13.3±0.6	14	15±1.7	15±1.7	10.3±1.5
PAH amended	Lead	Low	22.3±0.6	20	18±0.6	19	16±1	11.7±0.6	12.3±0.6	15±1.2	13.7±1.2	18.3±0.6	10.7±1.2	15±0.6	6	8.3±0.6
		Medium	20.3±0.6	19.7±0.6	19	16	16.7±0.6	16.7±0.6	17.3±0.6	17.6±0.6	17±1	19±1.7	11.7±0.6	12±1	10±2.1	10±1
		High	23	18±0.6	18.3±0.6	19±0.6	18.3±1.2	15.3±0.6	20±1	13	19±1.8	14.3±1.5	11.3±1.5	11±1.2	10±0.6	4
Non amended	Mercury	n/a	2	8±8.7	1.7±2.1	15.7±2.5	15.3±1.5	0	4±1.7	7.7±0.6	5.3±1.5	0	5.7±0.6	4.7±3.8	1.3±0.6	9.3±1.5
PAH amended	Mercury	n/a	0	0	3	15.3±0.6	18.7±1.2	0	3±1	1	1.3±0.6	2±1	2	2±1	1±1	2.7±1.5

Table 5.3: Substrate evenness values for the non-PAH spiked control, PAH control, non-PAH spiked Pb amended soil, PAH Pb amended soil, non-PAH spiked Cd amended soil and PAH Cd amended soil.

Substrate Evenness (E)																
PAH amendment status	Metal amendment	Metal amendment level	0 wk	1 wk	2 wk	3 wk	5 wk	7 wk	9 wk	12 wk	15 wk	20 wk	25 wk	30 wk	35 wk	40 wk
Non amended	Control	None	2.3±0.02	2.4±0.03	2.4±0.06	2.4±0.03	2.3±0.02	2.3±0.03	2.4±0.1	2.4±0.06	2.4±0.1	2.5±0.1	2.6±0.1	2.5±0.1	2.5±0.1	2.7±0.06
PAH amended	Control	None	2.3±0.1	2.3±0.04	2.3±0.03	2.4±0.03	2.3±0.01	2.3±0.02	2.4±0.01	2.3±0.02	2.5±0.1	2.3±0.04	2.3±0.01	2.5±0.1	2.5±0.1	2.5±0.03
Non amended	Cadmium	Low	2.3±0.02	2.3±0.02	2.4±0.05	2.5±0.03	2.3±0.05	2.4±0.01	2.3±0.02	2.4±0.08	2.4±0.04	2.6±0.03	2.3±0.07	2.6±0.2	2.7±0.1	3.1±0.04
		Medium	2.3±0.03	2.3±0.01	2.4±0.03	2.5±0.1	2.3±0.05	2.4±0.03	2.3±0.03	2.3±0.07	2.4±0.06	2.6±0.03	2.5±0.03	2.4±0.1	3.1±0.2	5.3±0.5
		High	2.4±0.06	2.4±0.08	2.6±0.06	2.5±0.1	2.4±0.03	2.5±0.2	2.5±0.09	2.3±0.04	3±0.2	2.9±0.09	2.6±0.2	2.5±0.1	2.8±0.2	6.5±3.1
PAH amended	Cadmium	Low	2.4±0.03	2.3±0.08	2.4±0.08	2.5±0.05	2.3±0.07	2.5±0.2	2.4±0.07	2.4±0.08	2.4±0.06	2.4±0.01	2.3±0.1	2.6±0.1	2.6±0.1	2.4±0.1
		Medium	2.3±0.02	2.4±0.03	2.3±0.04	2.5±0.07	2.3±0.05	2.6±0.09	2.5±0.05	2.4±0.09	2.4±0.09	2.5±0.07	2.4±0.09	2.7±0.1	3±0.01	2.7±0.3
		High	2.3±0.01	2.5±0.09	2.4±0.05	2.4±0.03	2.5±0.02	2.6±0.06	2.4±0.04	2.5±0.04	2.6±0.02	2.6±0.02	2.5±0.07	2.6±0.1	2.7±0.2	5.3±0.8
Non amended	Lead	Low	2.3±0.02	2.3±0.02	2.3±0.04	2.3±0.04	2.3±0.07	2.3±0.04	2.3±0.03	2.3±0.01	2.5±0.1	2.3±0.02	2.4±0.04	2.4±0.1	2.8±0.1	2.5±0.08
		Medium	2.3±0.2	2.2±0.02	2.3±0.03	2.4±0.1	2.3±0.02	2.4±0.07	2.3±0.01	2.4±0.08	2.5±0.04	2.6±0.04	2.4±0.2	2.4±0.1	2.6±0.1	2.7±0.03
		High	2.3±0.02	2.3±0.06	2.3±0.04	2.6±0.06	2.3±0.05	2.4±0.08	2.3±0.04	2.5±0.05	2.9±0.1	2.8±0.1	2.6±0.06	2.5±0.2	2.6±0.1	2.8±0.4
PAH amended	Lead	Low	2.4±0.01	2.3±0.02	2.3±0.02	2.4±0.02	2.4±0.003	2.6±0.07	2.5±0.04	2.3±0.03	2.5±0.06	2.5±0.04	2.9±0.1	2.4±0.1	3.5±0.1	2.8±0.1
		Medium	2.4±0.03	2.3±0.02	2.3±0.03	2.5±0.02	2.4±0.01	2.6±0.04	2.3±0.04	2.3±0.04	2.5±0.07	2.5±0.06	2.6±0.2	2.6±0.1	2.6±0.1	2.8±0.2
		High	2.3±0.02	2.4±0.04	2.3±0.01	2.4±0.06	2.4±0.06	2.4±0.03	2.4±0.05	2.5±0.04	2.4±0.02	2.5±0.1	2.6±0.2	2.7±0.1	2.6±0.1	4.3±0.1
Non amended	Mercury	n/a	5.2±14.9	3.7±1.2	3.4±15.7	2.4±0.07	2.5±0.04	0	5.1±2.6	2.9±0.3	3.7±0.6	0	3.8±0.5	4.9±2.7	7.9±13	3±0.23
PAH amended	Mercury	n/a	0	0	4±1	2.4±0.02	2.3±0.02	0	5.7	0	8.8	6.3±1.5	6.7±14.1	6.6±2	9.3	5±1

5.4 Conclusion:

It has been observed in this present study that though PAHs can stimulate microbial activity by serving as an easily available carbon substrate, towards the end of the sampling period microbial activity for the PAH soils declined when compared to the control as observed in both their respiration activity and microbial biomass carbon concentration. The decline may be due to the non-bioavailability of the PAH compounds in the soil with high organic carbon content. It can also be due to the chronic toxic effect of the high concentrations of some of the PAH compounds that were available to the microorganisms. The non-PAH soils amended with Pb and Cd metals both seemed to have a higher soil respiration than the control which can be as a result of the stress exerted by the heavy metal and can also be due to increase of heavy metal tolerant microbial community though their microbial biomass concentration was lower than the biomass concentration of the control soil.

It can also be said from this present study that though it has been reported that Cd is more toxic to microorganisms than Pb [93] the microbial community seems to develop tolerance toward Cd amendment in soil faster than it does towards Pb amendment. This is associated with the observed increase in microbial biomass concentrations and lower metabolic quotient for the Cd amended soil than the Pb amended soil. The development of tolerance to Cd and Pb by soil microbial community seemed to have reduced their metabolic activity as observed in the AWCD and their metabolic diversity as observed in Figure 5.8 and Table 5.1 for the soils amended the metals (Pb and Cd). The study has also shown that PAHs though can stimulate microbial activity but its combination with heavy metal can have more adverse effect on the microbial activity.

Chapter 6: Biodiversity of soil microflora

6.1 Introduction

In the previous two chapters, it was seen that compared to the corresponding control treatment, the presence of heavy metal co-contaminants has a significant effect on both the degradation of individual PAHs (degradation rate and overall degradation) and on measures related to soil microbial activity, including soil respiration rate and microbial biomass concentration. For the latter two measures, whilst an overall decline does occur in the absence of added metals for both PAH-spiked and unspiked soils, it is more pronounced in the presence of different concentrations of lead and cadmium. In addition, analysis of overall results from Biolog Ecoplate analysis showed that the average well colour development (AWCD) at 50 hours is similarly affected, indicating that the viable inoculum concentration extracted from treated soils declines with time, but more so in the presence of added cadmium and lead. The decline in AWCD observed in Chapter 5 is consistent with results for PAH degradation, biomass and soil respiration. However, the true benefit of community level physiological profiling (CLPP) analysis, such as that obtained from Ecoplates, is the ability to determine changes in the diversity of the soil microbial population as a result of different treatments, as previously discussed. Thus, the question of whether, in addition to the overall decline in inoculum size, the diversity of microorganisms has also been affected, can be explored. There are many different approaches to analysing CLPP data, several of which will be explored in this Chapter.

6.2 Data normalisation and visualisation

To recap, Biolog Ecoplates, contain 32 wells per test as seen in Table 6.1, one of which is blank and the other 31 are different environmentally applicable carbon substrates of which at least nine are considered as constituents of plant root exudates [171]. The substrates are based on the ecological functions the microbial community perform within the ecosystem [171, 181]. The 32 wells are duplicated three times on one plate so as to allow triplicate determinations.

Table 6.1: ECO plates substrates, their corresponding well numbers and associated guild grouping [181]

Substrate	**Plate Code**
Control:	
Water	A1
Amino Acids (n=6):	
L-Arginine	A4
L-Asparagine	B4
Glycly-L-Glutamic Acid	F4
L-Phenylalanine	C4
L-Serine	D4
L-Threonine	E4
Carbohydrates (n=10)	
D-Cellobiose	G1
i-Erthritol	C2
D-Galactonic acid y-lactone	A3
N-Acetyl-D-glucosamine	E2
Glucose -1-phosphate	G2
β-Methyl-D-glucoside	A2
D,L-α-Glycerol phosphate	H2
A-D-Lactose	H1
D-Mannitol	D2
D-Xylose	B2
Carboxylic acids (n=9)	
y-Hydroxybutyric acid	E3
α-Ketobutyric acid	G3
D-Galacturonic acid	B3
D-Glucosamine acid	F2
Itaconic acid	F3
D-Malic acid	H3
Pyruvic acid methyl ester	B1
2-Hydroxybenzoic acid	C3
4-Hydroxybenzoic acid	D3
Amines (n=2)	
Phenylethylamine	G4
Putrescine	H4
Polymers (n=4)	
α-Cyclodextrin	E1
Glycogen	F1
Tween 40	C1
Tween 80	D1

In order to obtain an overall ‘feel’ for the CLPP data, 3-D bar charts were constructed that allowed the determination of trends and patterns for each well over the course of the 40 week study. Looking at overall trends in these figures, Figure 6.1, shows the effect of mercury treatment on both PAH-spiked and non-spiked soil. For the PAH

spiked control, 6.1(a), the majority of wells show significant absorbance development, and in most, but not all, cases there is a gradual decline in absorbance with time, mirroring the overall AWCD reported in Chapter 5. After treatment with mercury, 6.1(b), there is an almost complete cessation of activity across all wells, though this quickly re-establishes by week 5, as also evident from the AWCD and the soil respiration data. After week 5 it was necessary to apply a second mercury treatment, as previously noted, which results in a sustained reduction of activity over the remaining period of the study, thus giving an abiotic control to which the other treatments could be compared. For the corresponding results for non-PAH spiked soils, 6.1(c) and 6.1(d), similar trends are observed, though there does appear to be more activity across the wells for the mercury treatment, even after the second treatment: this may indicate a more resilient microbial community in the absence of PAH spiking. Figures 6.2 to 6.5 show similar plots for cadmium and lead spiked soils (with PAHs present and absent). There are discernible differences between the plots, with lower overall absorbances and a reduction in the number of wells that are positive when heavy metal co-contaminants are present. Some of these differences have been discussed above.

Some comment can also be made about trends involving specific substrates. It is apparent that the number and type of carbon substrate metabolised by the PAH control soil was more than that metabolised by the non-PAH spiked-amended soil as seen in Figure 6.1 (a) and (c). For example, the metabolism of D-cellobiose (G1) and β-methyl-D-glucoside (A2) was greater in the PAH control soil than in the non-PAH spiked control soil. The metabolism of D-xylose (B2) was lower in all the metal treated soils than in the control soils as seen in Figures 6.1 – 6.5. The metabolism of N-acetyl-D-glucosamine (E2) was also affected in all the metal treated soils except in the non-PAH spiked low concentration Pb-treated soil which was not affected. The metabolism of L-serine (D4) and D-mannitol (D2) was greater in the non-PAH spiked Pb amended soil than in the non-PAH spiked Cd amended soils (Figure 6.2 and 6.3). The metabolism of D-malic acid (H3) was more in the PAH Pb amended soils than in the PAH Cd amended soils (Figure 6.4 and 6.5) but β-methyl-D-glucoside (A2) was metabolised to a greater extent in the PAH Cd soils than in the PAH Pb soils.

Whilst visual differences are apparent from the plots, it is only through statistical analyses that a quantifiable estimation of the significance of the difference between the different treatments can be obtained. It is common practice to normalise absorbance data prior to statistical analyses [120, 177, 178, 234, 264]. This is done at each time point by dividing the absorbance of individual wells by the AWCD: the results of this normalisation are shown in Figures 6.6 to 6.10.

6.3 Ordination analysis techniques

Simple ordination analysis techniques such as Principal Component Analysis work by taking the overall variance of the sample set, which may arise as a result of many different environmental factors, and reducing this down to a smaller number of artificial variables (Eigenfactors), whilst seeking to retain as much of the variance as possible within these pseudo variables. PCA is often referred to as a dimension reduction technique. Usually, sample values for the first two of the pseudo-variables are plotted on a scatter plot, whereby differently treated groups of samples may separate out on the two axes. Subsequently, the diagram may be interpreted in terms of correlations between the pseudo-variables and environmental variables which can be plotted on the same graph.

For the analysis of Biolog Ecoplate data, PCA has often been used, as has Correspondence Analysis (CA) and Detrended Correspondence Analysis (DCA). One disadvantage of using PCA for ecological samples is that the technique assumes a linear dependence of species response (well colour development in our case) to one or more environmental variables, whereas in reality the species may have an optimal, or unimodal, relationship with an environmental variable. Correspondence analysis, on the other hand, is a unimodal method, though not itself without problems, often giving rise to distorted patterns (arch effect) that arises from the co-relation between the pseudo-variables. For this reason, a variant of CA called DCA is often used for ecological datasets: this is based on work by Hill *et al*. [288] in which a 'detrending' algorithm is applied, based on splitting the ordination plot into segments and centering each on zero, so as to remove any distortions. Garland [179] has compared both PCA and DCA for soil microorganism samples obtained from roots of different crops and confirms that DCA is marginally superior though both techniques accomplished good separation of the groups. DCA was used in the present study.

6.3.1 Detrended correspondence analysis (DCA)

Canoco 4.5 was used to carry out the DCA analysis. The software requires two files to be prepared: one containing the 'species' data for each of the samples, which in this study equates to the normalised absorbance data for each of the 31 wells, and an environmental variables data file that contains the values of the time (0 to 40 weeks), and the toxic metal and PAH spiking status. Files were converted to Canoco 4.5 format using the WincanoImp utility program.

The results of the first run are shown in Figure 6.11. Here, a global analysis of all the data was carried out, i.e. using all of the sample data (triplicates averaged) and using all of the environmental variables to interpret the observed separation along the two

axes. The samples appear fairly clustered, with little separation of groups, for example, PAH-spiked and non-spiked (non-filled and filled circles respectively), which appear intermingled. The red vectors on the graph indicate the respective magnitudes of these environmental variables in describing the position of the samples on the scatter plot, with time being by far the major determinant (time was included as a scalar variable, whereas PAH, Pb and Cd are nominal variables, either present or absent, i.e. there is no scalar concentration variable). It is important to emphasise that the results of the DCA analysis are in no way influenced by the choice of environmental variables: the latter are simply used to correlate with the two ordination axes of the DCA analysis, providing some level of interpretation of the ordination plot.

On the basis of the results of the global analysis, it was decided to group further DCA analyses into individual time periods, thus removing the main determinant from the plot, hopefully allowing other factors to emerge more clearly. For these analyses, each of the triplicate set of samples for each treatment is included separately rather than averaging: this allows the 'within sample' variation to be compared to that observed for the different treatments. The results of analyses at 0, 1, 5, 12, 25, 35 and 40 weeks are shown in Figures 6.12 to 6.25 respectively. In the Figures, the individual samples (in triplicate) are labelled using the convention indicated in the caption to Figure 6.12. The overall trends are complex to interpret and it is clear that the degree and nature of the separation of the samples along the two ordination axes vary significantly with time, most probably representing complexes changes in the soil microbial ecology, reflecting both the influence of toxic metals and the absence or presence of PAHs, which themselves may serve to stress the microbial system.

There are, however, a number of common themes, in particular, the reasonably clear separation of the PAH-spiked and non-spiked samples, and the general closeness of the triplicates for each treatment. In addition, and generally to a lesser extent than the influence of PAH-spiking, the presence or absence of individual metals causes specific groupings to emerge. The general trend with the separation of groups of samples appears to be for greater separation to be observed as the length of time increases. At 40 weeks for example, there are a clear series of groups located within the overall separation of PAH-spiked and non-spiked samples. Metal spiked samples of different concentrations appear distinct from the non-spiked samples. Garland has observed similar complex changes emerge for a time series study carried out for soil inocula extracted from different crop types [179].

It is clear that Biolog Ecoplates are effective at separating out the effects of different treatments, indicating that the composition of the microflora is changing with time and

treatment. Nevertheless, the total variation described by the DCA analysis is quite small, as indicated by Table 6.2 which shows the cumulative percentage variance for the first four Eigenvalues generated by the DCA analysis; the first two of these form the basis of the ordination plots shown in Figures 6.11 to 6.18. The maximum variance explained by the first two Eigenvalues (37.1 %) is obtained for the DCA analysis carried out for the week 5 data, where the greatest separation between PAH-spiked and non-spiked was seen. For week 25 the value is 35 % and for the remainder, the values are approximately 30 %. These are quite low compared to similar studies, for example, Garland's study where 50 % of the variance was described by the first two Eigenvalues [179].

6.3.1.1 Derivation of eigenvalues in DCA

The Eigenvalues were derived through the non-linear rescaling of the ordination axis generated by CA. This is designed to eliminate the arch effect generated by CA. The rescaling thus attempts to balance the within site variance at all points along the ordination axis by partitioning the axis into small groups, broadening the groups with sites with small within-site variance as well as contracting the groups with sites with huge within site variance. The site scores are subsequently calculated as weighted averages of the species scores, additionally, the scores are standardized such that the within-site variance is on par with 1. The CA Eigen scores were calculated by using the reciprocal averaging algorithm on the weighted average regression (WA) values of the species data and environmental variable data [289].

6.3.2 ANOVA applied to Shannon Diversity indices

Whilst the DCA analysis did manage to separate out groups of samples based on treatment, thus indicating that the diversity of the microflora is affected by both treatment and time, the analysis did not yield a definitive assessment of significance. An alternative and more intuitive approach is to analyse the Shannon Diversity indices (SDI) for each treatment and time period. Canonica 4.5 calculates this value automatically from the normalised well absorbance data. In Figures 6.26 to 6.30, scatterplots are presented of SDI as a function of time, with each figure displaying a series of related plots. The size of the circle is proportional to the SDI, scaled according to the range of values for each set of plots.

It is immediately apparent from these plots that the diversity of soil microflora, as determined by Ecoplate analysis, appears to decrease with exposure to heavy metal

contaminants of the soil. The pattern is complex, with no apparent relationship between metal concentration and reduction in SDI. The reduction in SDI becomes more pronounced with the length of time, as might be expected with long-term stress on an ecological system. The corresponding SDI for the samples that were not spiked with metals remains relatively constant throughout the course of the study. Figure 6.30 shows a comparison plot of PAH-spiked with non-spiked soil without added metals present.

From the overall SDI plot, it does seem that the non-PAH spiked heavy metal soil samples retain more diverse communities over time compared to the PAH spiked heavy metal soil samples, consistent with the PAHs and heavy metals placing extra stress on the microbial community, even though the same PAHs are providing an additional carbon source, causing the respiration rate to increase, at least initially. It has been reported that PAHs disrupt membrane function due to nonpolar narcosis [88] and enhances the easy penetration of heavy metals into microbial cells where the metals would then disrupt the cells functions [79, 103, 104]. This could be the reason for the negative synergistic effect (low microbial diversity) of PAHs and heavy metals on soil microbial community over time.

An ANOVA test was carried out on data from each of the plots presented in Figures 6.26 to 6.30 and the results of these analyses are shown in Table 6.3. The Table shows the p-values for each of the sets of data, together with the results of Tukey's post-hoc analysis of individual relationships (comparison of each of the metal treatments with the control). Significant p-values (P<=0.05) are indicated in red. In addition, the Levene parameter is shown: this is based on the null hypotheses that the variances within the samples are equally distributed: a significant value (p<=0.05) for this parameter indicates that the null hypothesis is rejected and that the variances are not equal. For these results, the Levene test indicates that the null hypotheses can be accepted for the majority of samples.

In Table 6.3 the majority of the ANOVA p-values are significant for all four groups of treatments, though this gives no indication of which tests differ. The Tukey's post-hoc tests for comparisons to the control confirm the trends indicated by the graphs, i.e. the metal treatments result in statistically lower SD indices, particularly at later weeks in the study. The tests also support the notion that PAH-spiked soils in the presence of metal co-contaminants have less diverse microflora at later weeks.

Table 6.2: Cumulative percentage variance of well data for the first four Eigenvalues derived from DCA analysis

Week number	Eigenvalue number and cumulative percentage			
	1	2	3	4
0	18.6	29.2	37.1	41.3
1	17.3	29.2	37.0	41.3
2	20.8	31.6	39.4	43.6
3	27.0	39.8	48.2	51.6
5	29.1	37.1	42.3	45.1
7	26.8	37.7	42.6	46.1
9	23.1	34.7	40.3	43.9
12	20.6	31.0	37.7	42.6
15	21.3	35.1	41.0	44.7
20	21.4	32.0	37.3	40.8
25	20.6	35.3	41.3	45.2
30	23.9	37.9	43.8	46.7
35	17.4	29.3	37.0	41.4
40	20.5	30.4	35.1	38.8

Table 6.3: Results of ANOVA and Tukey's post-hoc tests for Shannon Diversity indices obtained from Biolog Ecoplates for four different groups of soil treatments. Significant p values (<=0.05) are indicated in red.

Experiment	Statistic	Week number													
		0	1	2	3	5	7	9	12	15	20	25	30	35	40
No PAH, Cd	ANOVA	**0.003**	0.104	0.514	0.126	**0.000**	**0.000**	**0.002**	0.997	**0.003**	**0.001**	**0.000**	0.189	**0.001**	**0.002**
	Post-hoc tests:														
	Low Cd v No Cd	0.786	0.075	0.918	0.436	**0.000**	0.082	0.078	0.997	0.164	0.998	**0.010**	0.939	**0.002**	0.999
	Med Cd v No Cd	**0.003**	0.610	0.887	0.643	**0.001**	**0.006**	0.993	1.000	0.277	0.727	**0.002**	0.290	1.000	**0.003**
	High Cd v No Cd	**0.041**	0.598	0.918	0.094	**0.001**	**0.000**	**0.039**	1.000	0.115	**0.001**	**0.000**	0.254	0.150	0.659
	Levene	0.465	0.205	0.752	0.203	0.304	0.484	**0.021**	0.709	0.223	0.112	0.147	0.064	0.093	**0.007**
No PAH, Pb	ANOVA	0.058	0.171	**0.001**	**0.001**	**0.000**	**0.000**	**0.006**	**0.002**	**0.010**	**0.000**	**0.000**	**0.011**	**0.006**	**0.003**
	Post-hoc tests:														
	Low Pb v No Pb	0.554	0.352	**0.022**	0.057	**0.000**	**0.000**	0.749	0.236	0.640	**0.025**	**0.006**	0.683	**0.010**	0.959
	Med Pb v No Pb	**0.041**	0.988	**0.002**	**0.002**	**0.000**	**0.000**	0.211	0.058	0.102	**0.003**	0.294	**0.019**	**0.010**	**0.011**
	High Pb v No Pb	0.631	0.960	1.000	0.850	**0.000**	0.573	**0.024**	0.063	0.725	0.429	**0.001**	0.989	0.191	0.063
	Levene	0.541	**0.009**	0.243	0.063	0.732	0.420	**0.024**	**0.044**	0.568	**0.015**	0.149	0.664	0.145	**0.047**
PAH, Cd	ANOVA	**0.006**	**0.020**	**0.001**	0.179	**0.003**	**0.022**	0.395	**0.003**	0.864	0.077	**0.000**	**0.000**	**0.000**	**0.001**
	Post-hoc tests:														
	Low Cd v No Cd	0.420	**0.044**	**0.000**	0.629	0.912	**0.041**	0.937	0.995	0.985	0.344	**0.001**	**0.007**	**0.000**	**0.005**
	Med Cd v No Cd	**0.004**	**0.021**	0.285	0.145	**0.009**	0.785	0.998	0.907	0.830	0.969	**0.029**	**0.000**	**0.003**	**0.000**
	High Cd v No Cd	0.174	0.377	**0.028**	0.868	0.860	0.974	0.395	0.100	0.972	0.604	**0.000**	**0.000**	**0.000**	**0.005**
	Levene	0.166	0.144	0.081	0.439	0.139	0.218	0.274	0.615	0.064	**0.005**	0.651	0.842	0.083	**0.050**
PAH, Pb	ANOVA	0.158	0.642	**0.000**	0.055	**0.011**	**0.000**	**0.000**	**0.001**	**0.021**	**0.000**	**0.026**	**0.017**	**0.001**	**0.000**
	Post-hoc tests:														
	Low Pb v No Pb	0.193	0.976	**0.000**	0.966	**0.014**	**0.001**	**0.000**	**0.004**	0.068	**0.009**	0.820	0.104	**0.004**	**0.000**
	Med Pb v No Pb	0.992	0.630	**0.003**	0.133	0.249	**0.016**	0.146	0.824	0.996	**0.009**	**0.039**	**0.020**	**0.001**	**0.001**
	High Pb v No Pb	0.437	0.761	**0.002**	0.988	0.993	**0.018**	**0.006**	0.451	0.837	**0.001**	0.068	**0.028**	**0.001**	**0.000**
	Levene	0.695	0.193	0.921	0.274	0.315	0.110	0.051	0.987	**0.043**	0.788	0.541	0.139	0.098	0.132

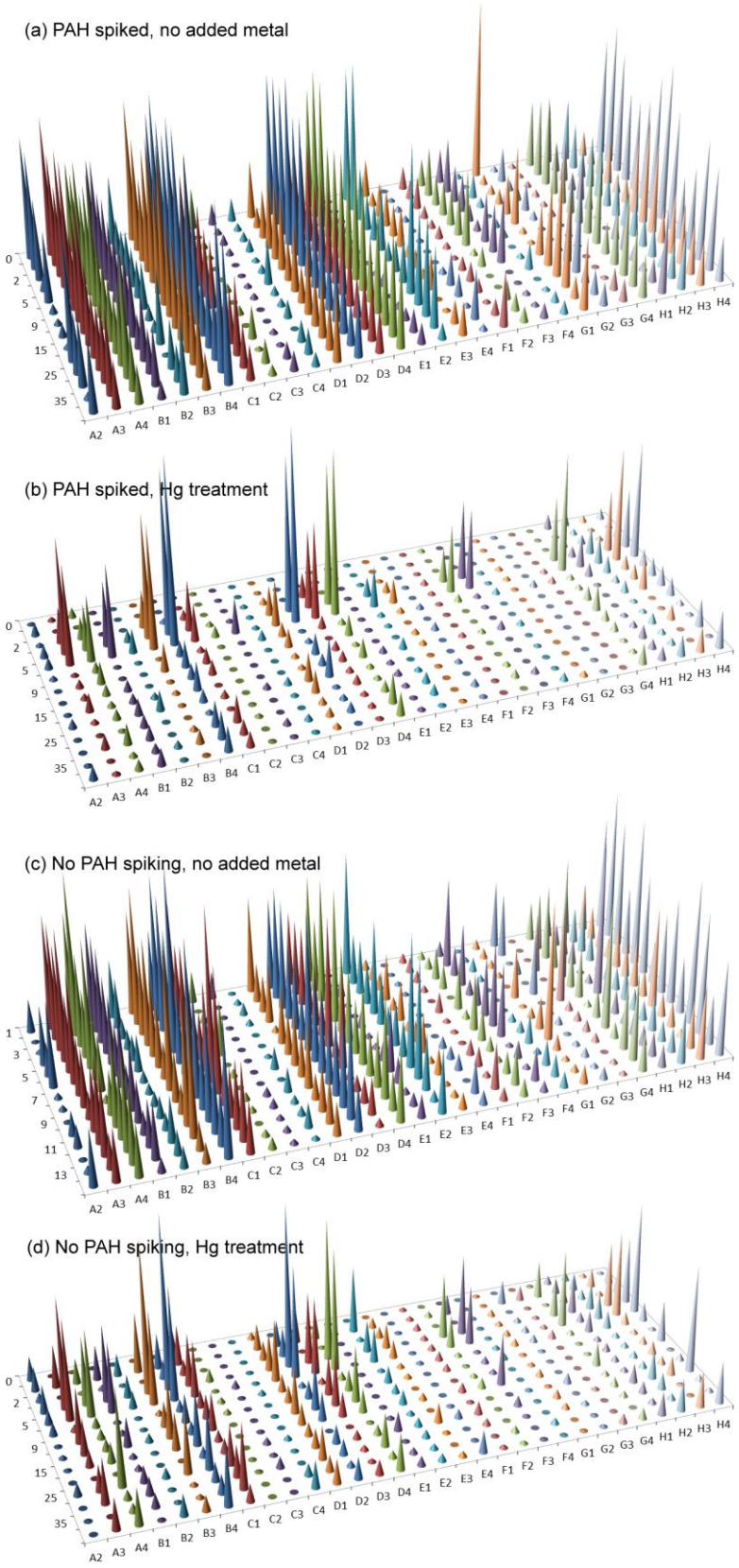


Figure 6.1: Impact of mercury addition to PAH-spiked and non-spiked soils, as determined by the variation in well absorbance values for all 31 substrates of a Biolog Ecoplate (average of three replicates) over a 40 week period. The time point and well number are shown on the x- and y- axes respectively. A common absorbance scale (z-axis) is applied to all 4 plots (0 to 2.5 absorbance units)**.**

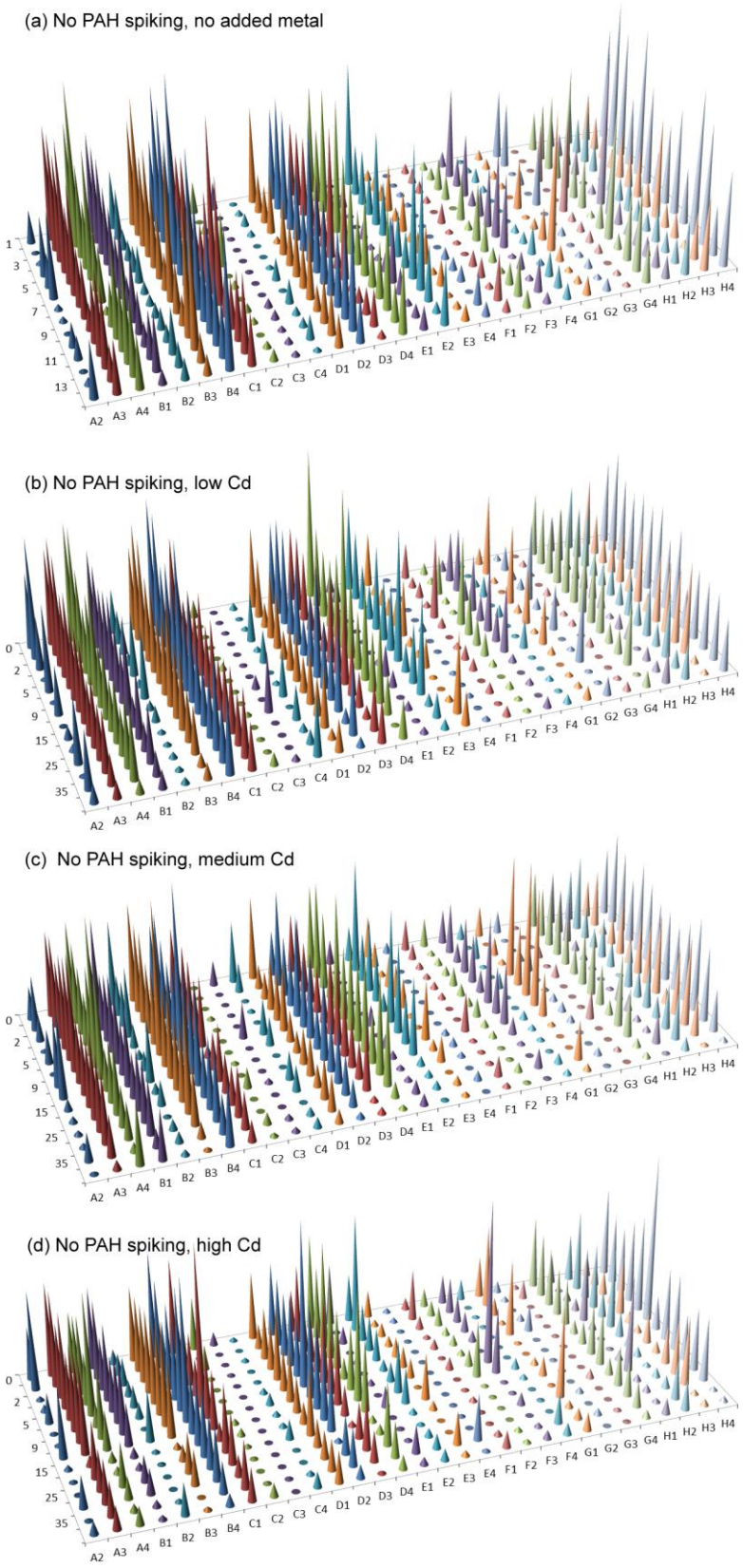


Figure 6.2: Impact of cadmium addition to non-PAH spiked soil samples, as determined by the variation in well absorbance values for all 31 substrates of a Biolog Ecoplate (average of three replicates) over a 40 week period. The time point and well number are shown on the x- and y-axes respectively. A common absorbance scale (z-axis) is applied to all 4 plots (0 to 2.5 absorbance units).

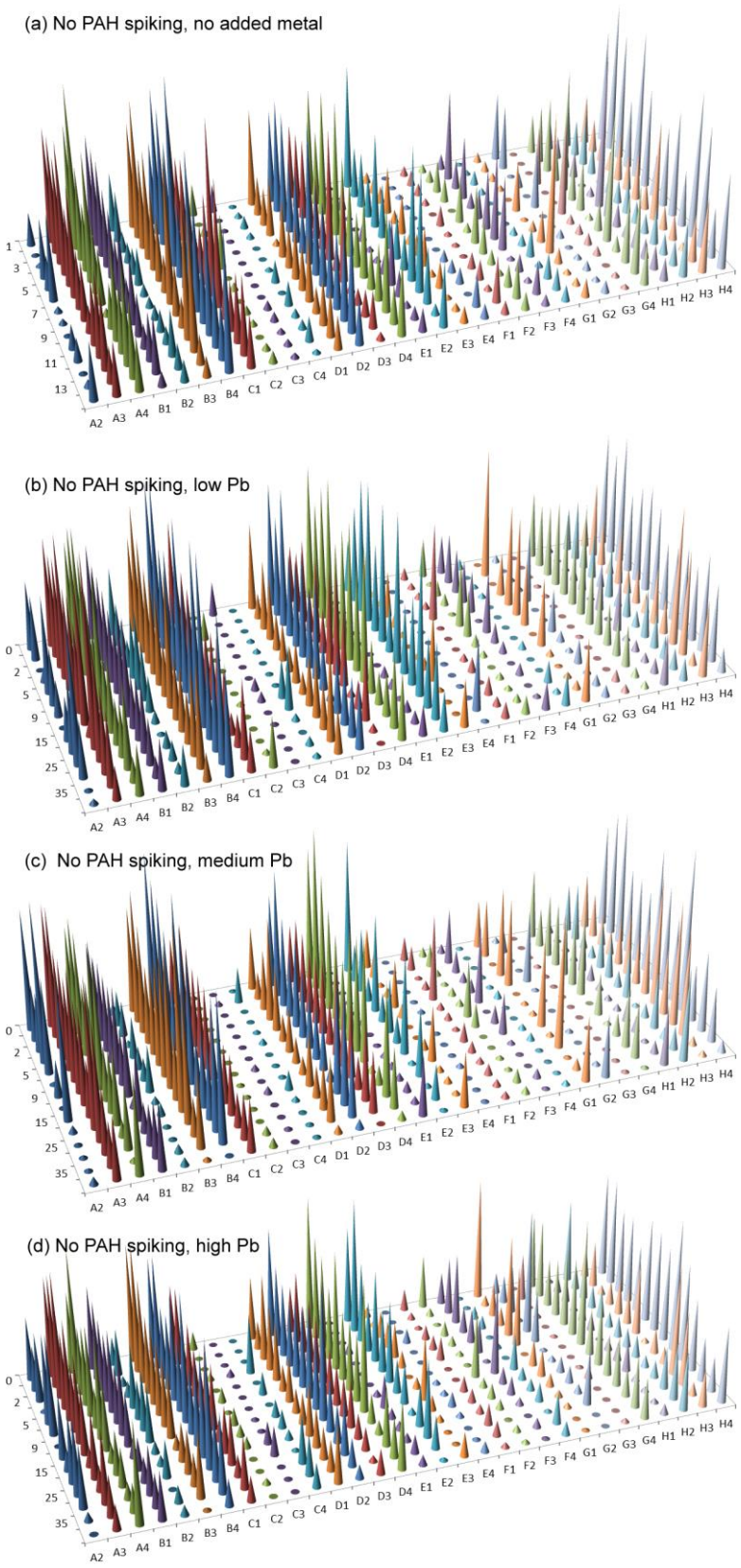


Figure 6.3: Impact of lead addition to non-PAH spiked soil samples, as determined by the variation in well absorbance values for all 31 substrates of a Biolog Ecoplate (average of three replicates) over a 40 week period. The time point and well number are shown on the x- and y-axes respectively. A common absorbance scale (z-axis) is applied to all 4 plots (0 to 2.5 absorbance units).

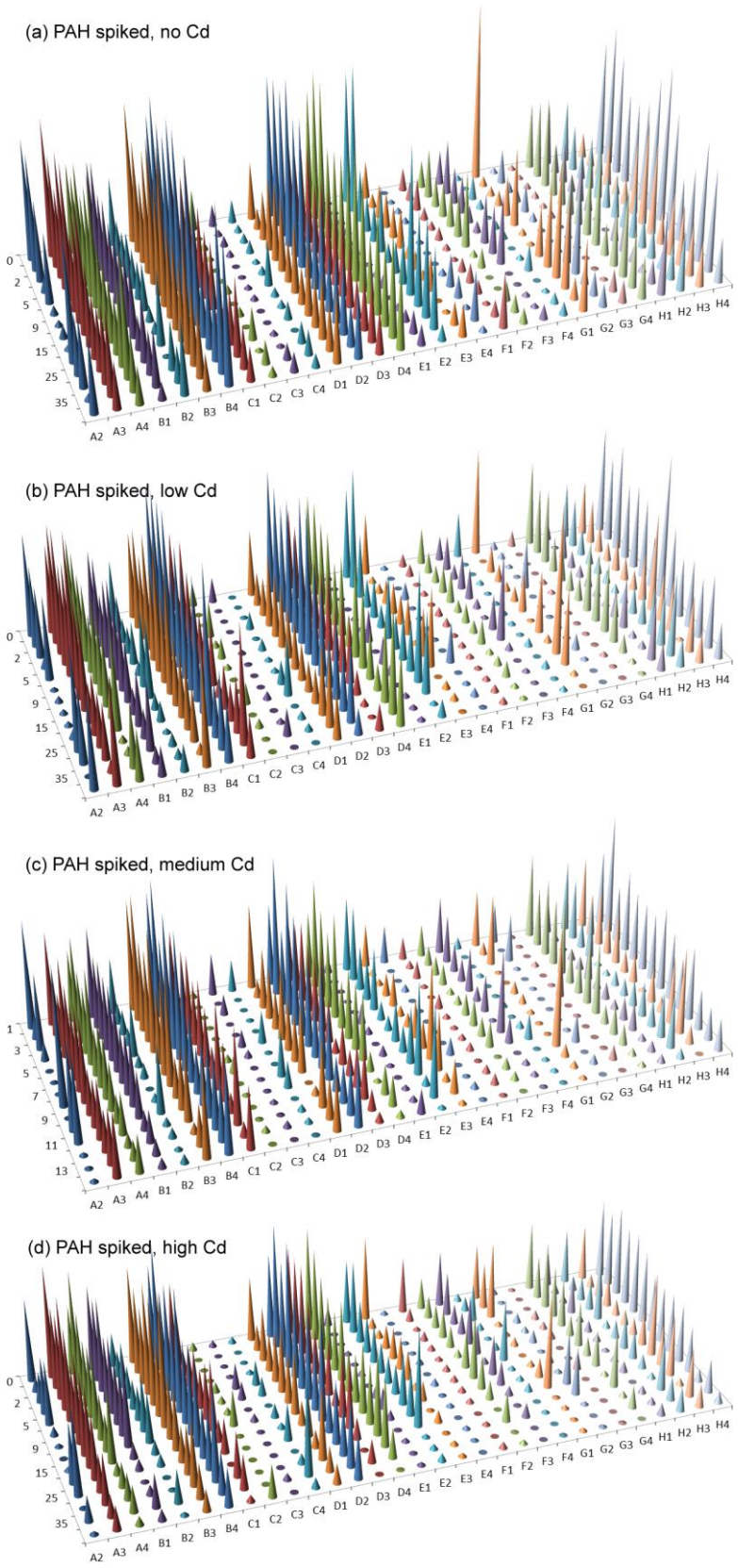


Figure 6.4: Impact of cadmium addition to PAH spiked soil samples, as determined by the variation in well absorbance values for all 31 substrates of a Biolog Ecoplate (average of three replicates) over a 40 week period. The time point and well number are shown on the x- and y-axes respectively. A common absorbance scale (z-axis) is applied to all 4 plots (0 to 2.5 absorbance units).

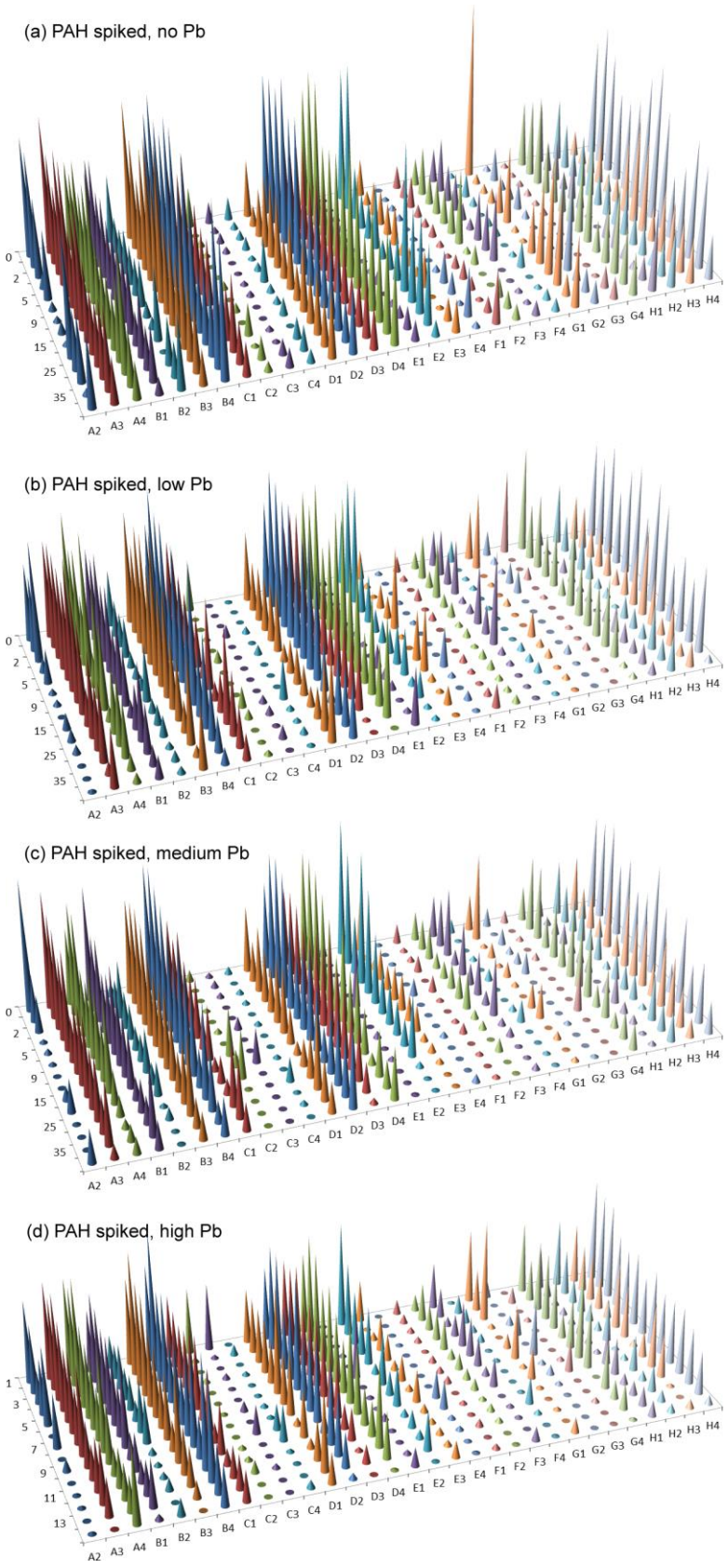


Figure 6.5: Impact of lead addition to PAH spiked soil samples, as determined by the variation in well absorbance values for all 31 substrates of a Biolog Ecoplate (average of three replicates) over a 40 week period. The time point and well number are shown on the x- and y- axes respectively. A common absorbance scale (z-axis) is applied to all 4 plots (0 to 2.5 absorbance units).

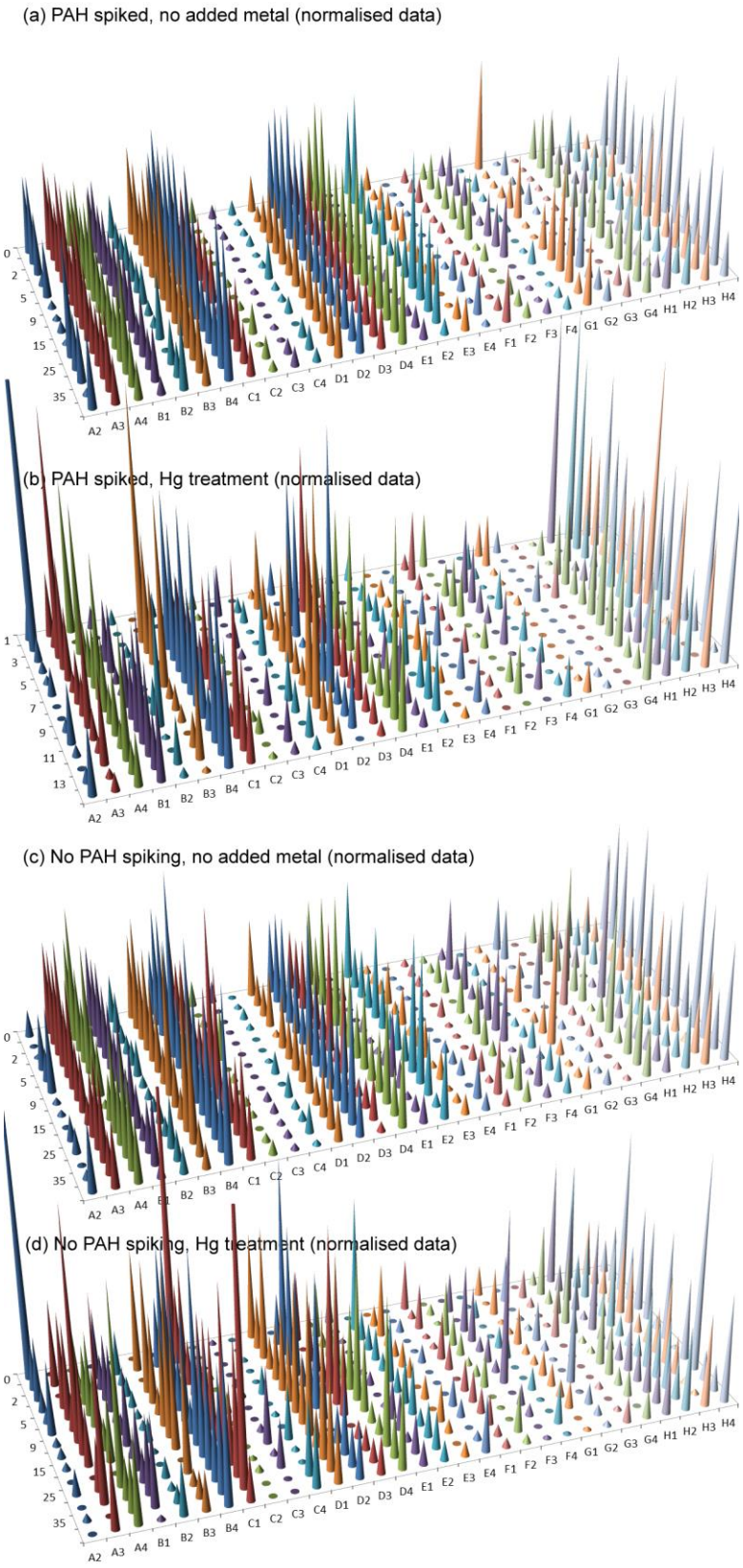


Figure 6.6: Impact of mercury addition to PAH-spiked and non-spiked soils, as determined by the variation in normalised well absorbance values for all 31 substrates of a Biolog Ecoplate (average of three replicates) over a 40 week period. The time point and well number are shown on the x- and y- axes respectively. A common absorbance scale (z-axis) is applied to all 4 plots (0 to 6 units).

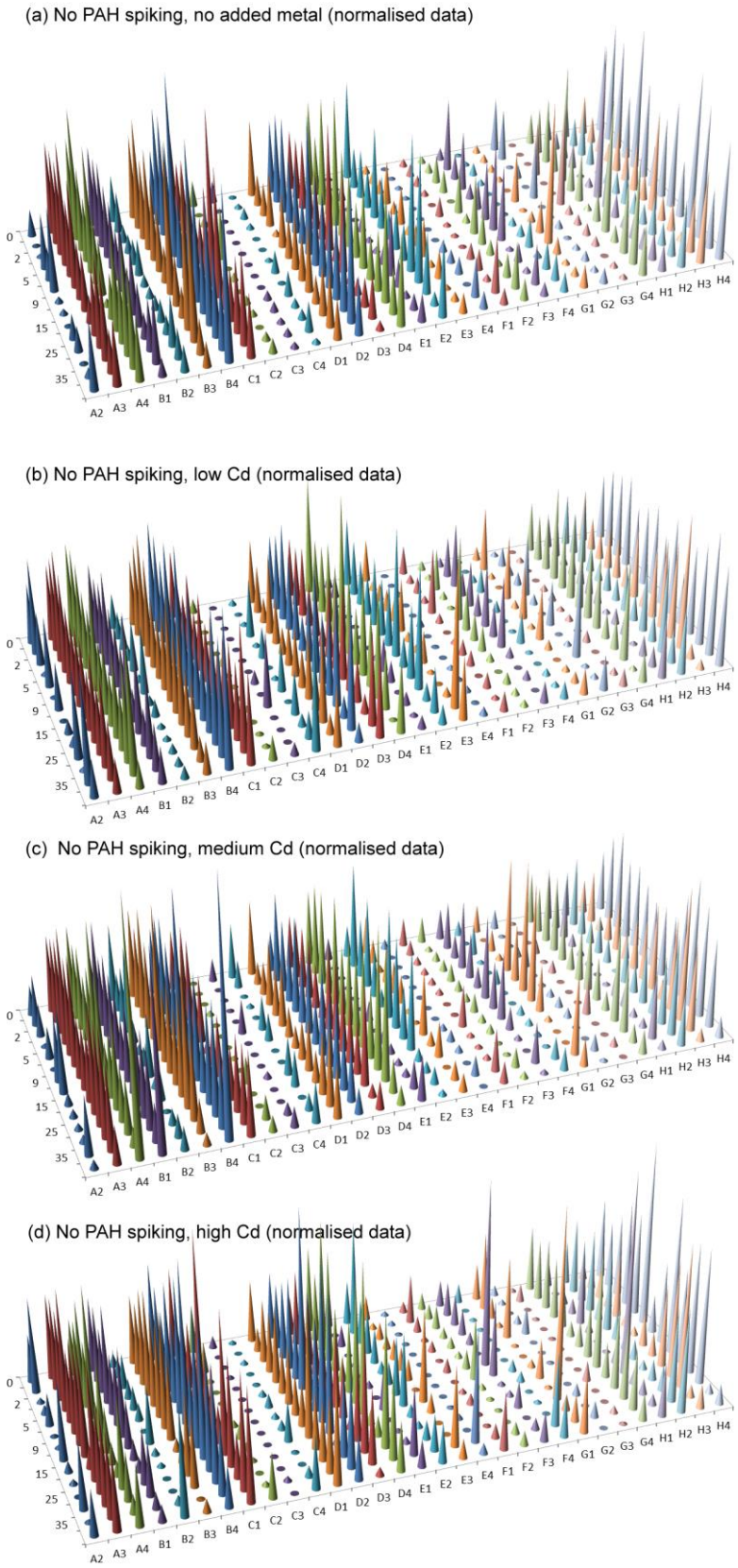


Figure 6.7: Impact of cadmium addition to non-PAH spiked soil samples, as determined by the variation in normalised well absorbance values for all 31 substrates of a Biolog Ecoplate (average of three replicates) over a 40 week period. The time point and well number are shown on the x- and y- axes respectively. A common absorbance scale (z-axis) is applied to all 4 plots (0 to 6 units).

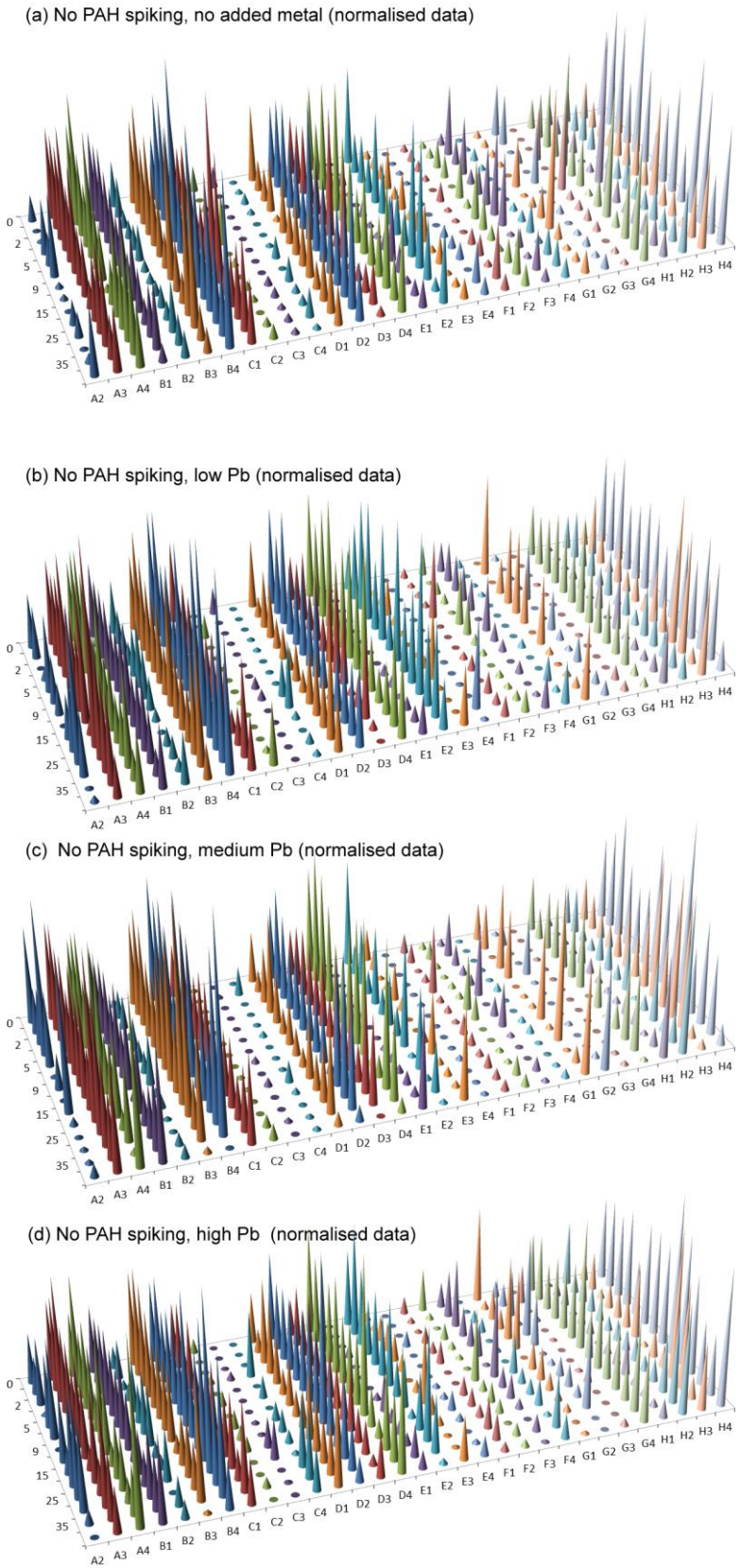


Figure 6.8: Impact of lead addition to non-PAH spiked soil samples, as determined by the variation in normalised well absorbance values for all 31 substrates of a Biolog Ecoplate (average of three replicates) over a 40 week period. The time point and well number are shown on the x- and y- axes respectively. A common absorbance scale (z-axis) is applied to all 4 plots (0 to 6 units).

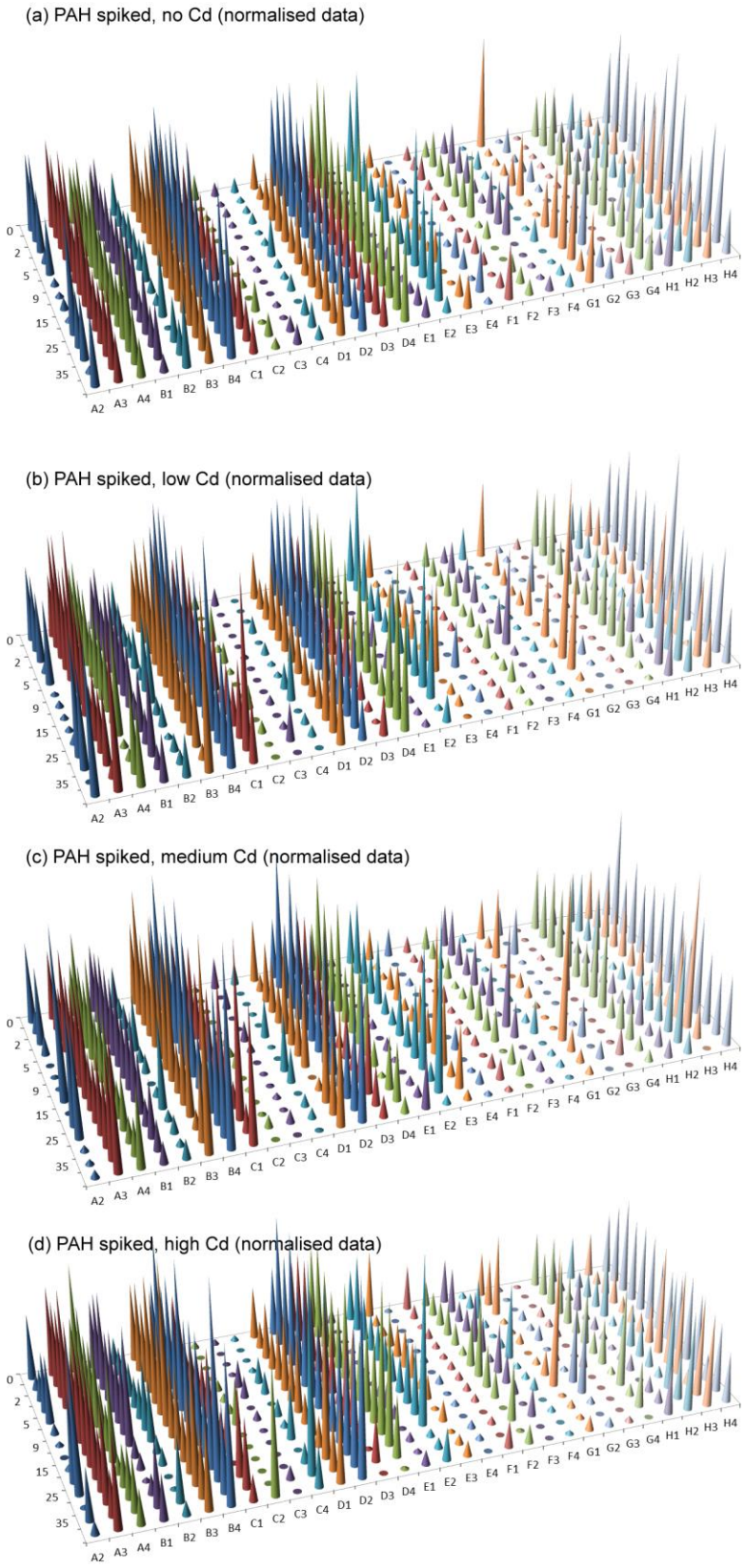


Figure 6.9: Impact of cadmium addition to PAH spiked soil samples, as determined by the variation in normalised well absorbance values for all 31 substrates of a Biolog Ecoplate (average of three replicates) over a 40 week period. The time point and well number are shown on the x- and y- axes respectively. A common absorbance scale (z-axis) is applied to all 4 plots (0 to 6 units).

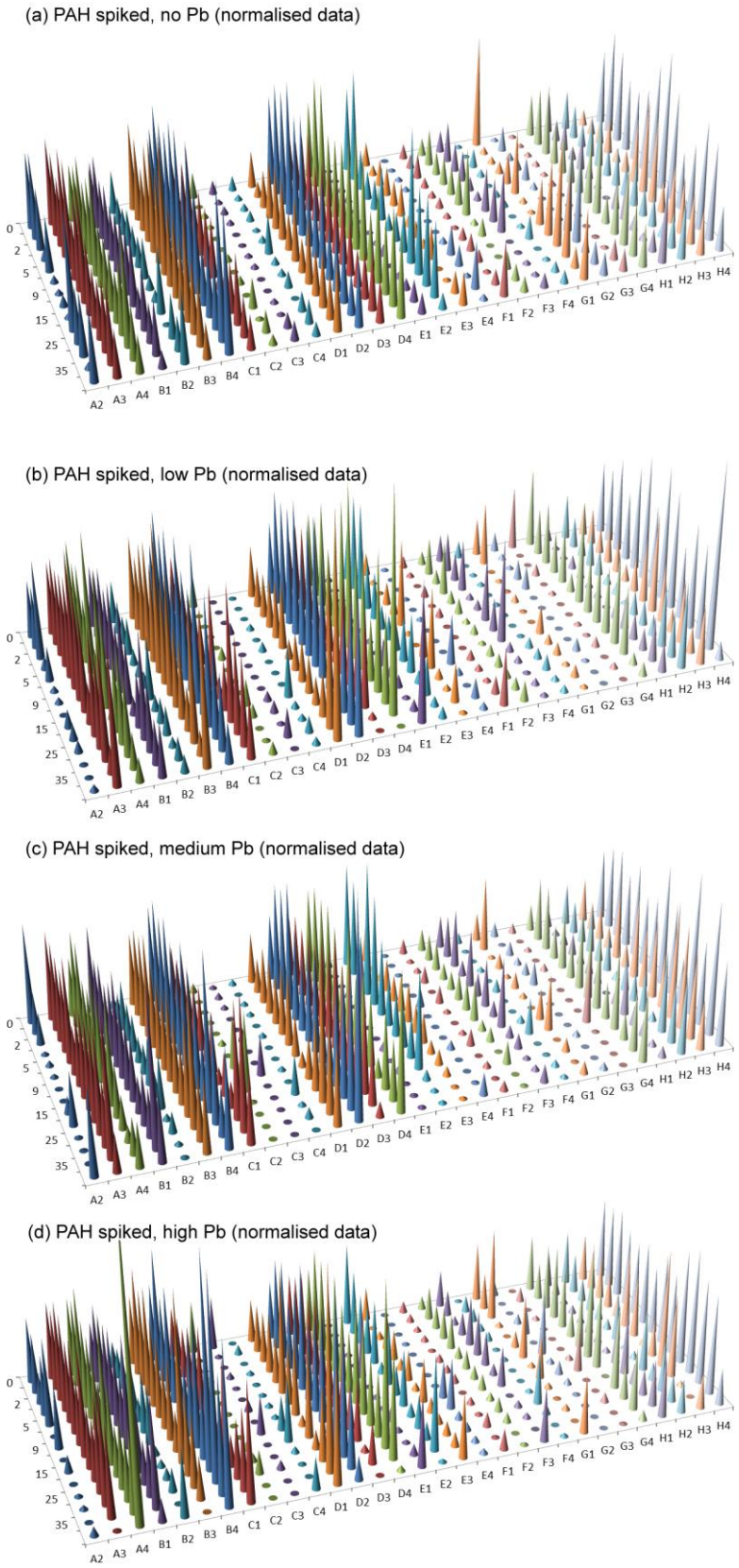


Figure 6.10: Impact of lead addition to PAH spiked soil samples, as determined by the variation in normalised well absorbance values for all 31 substrates of a Biolog Ecoplate (average of three replicates) over a 40 week period. The time point and well number are shown on the x- and y- axes respectively. A common absorbance scale (z-axis) is applied to all 4 plots (0 to 6 units).

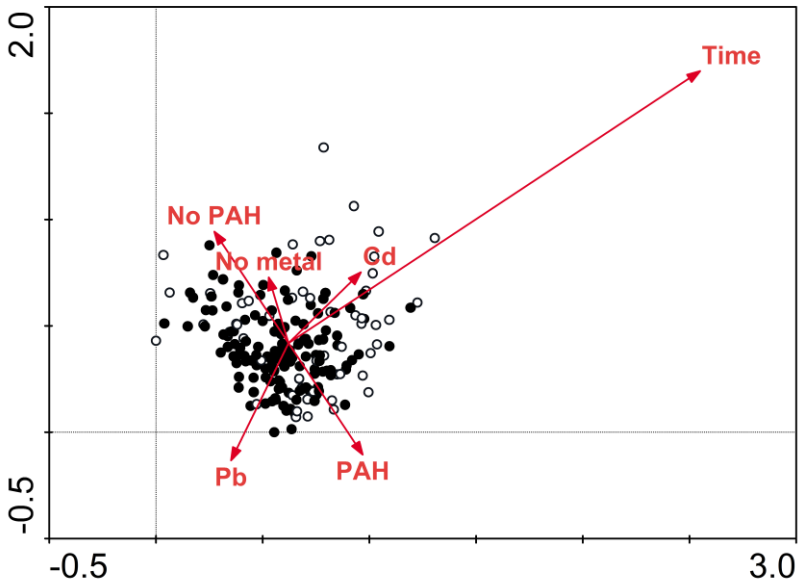


Figure 6.11: Ordination diagram for DCA analysis of Biolog Ecoplate analyses for all soil samples, i.e. all treatments and all time periods. The well absorbances used in the DCA analysis were normalised by dividing by the AWCD for the plate; each point (sample) is based on the average of three replicates. Open circles represent the PAH spiked soils and filled circles the non-spiked soils. The vectors shown in red are respective magnitudes and directions of the environmental factors that are responsible for the separation of the sample values in the DCA plot**.**

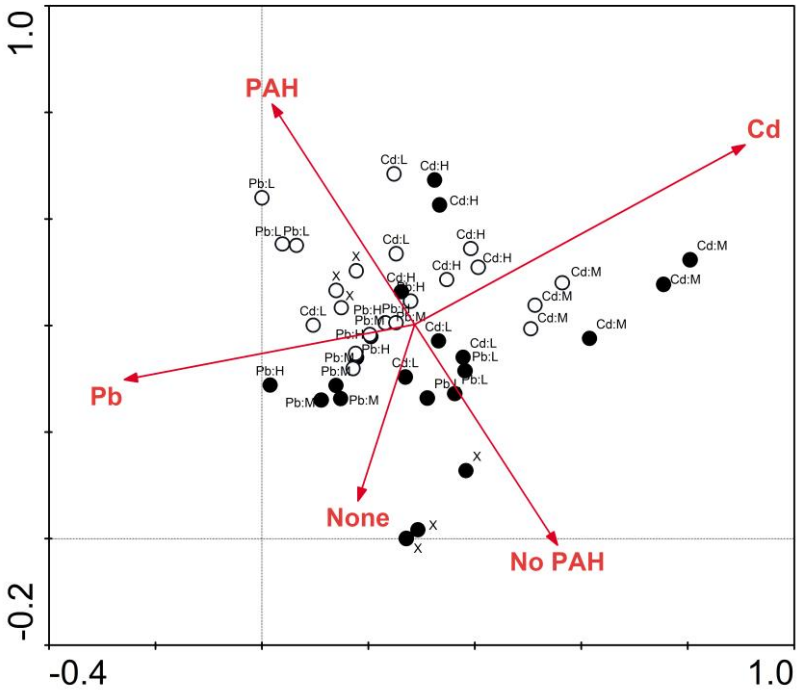


Figure 6.12: Ordination diagram for DCA analysis of Biolog Ecoplate analyses for soil samples at 0 week. The well absorbances used in the DCA analysis were normalised by dividing by the AWCD for the plate; each replicate is shown. Open circles represent the PAH spiked soils and filled circles the non-spiked soils. In the notation adjacent to the symbols, L, M and H denote low, medium and high metal concentration respectively and X indicates no metal treatment. The vectors shown in red are respective magnitudes and directions of the environmental factors that are responsible for the separation of the sample values in the DCA plot.

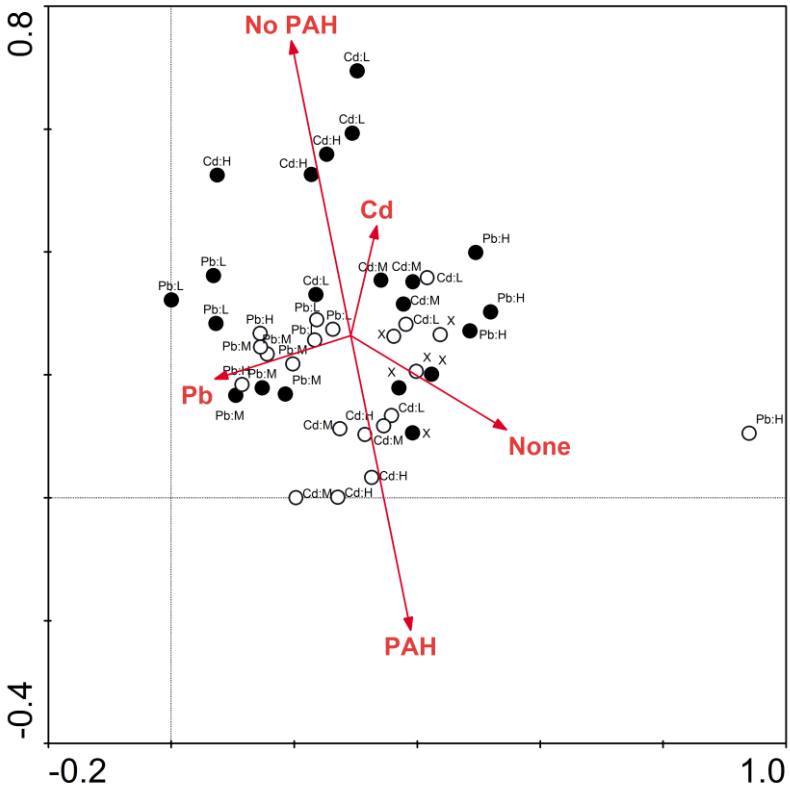


Figure 6.13: Ordination diagram for DCA analysis of Biolog Ecoplate analyses for soil samples at 1 week. Symbol and vector notation as described in Figure 6.12.

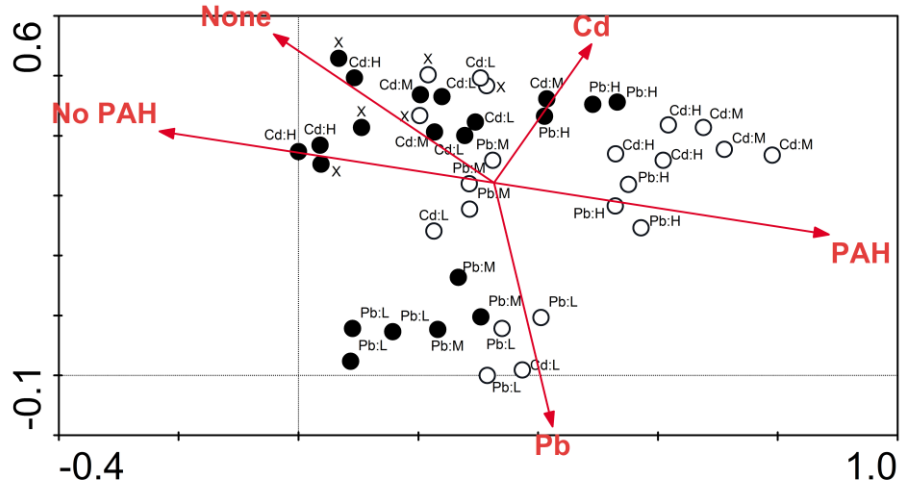


Figure 6.14: Ordination diagram for DCA analysis of Biolog Ecoplate analyses for soil samples at 2 weeks. Symbol and vector notation as described in Figure 6.12.

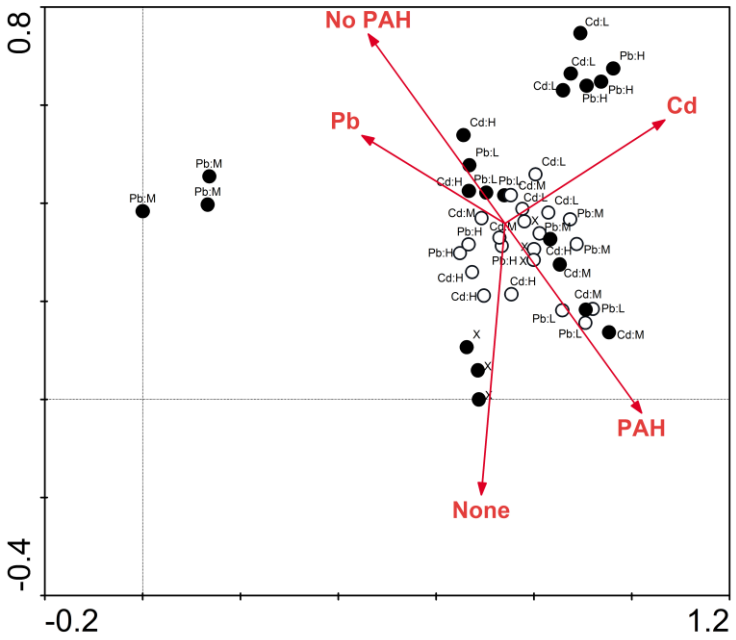


Figure 6.15: Ordination diagram for DCA analysis of Biolog Ecoplate analyses for soil samples at 3 weeks. Symbol and vector notation as described in Figure 6.12.

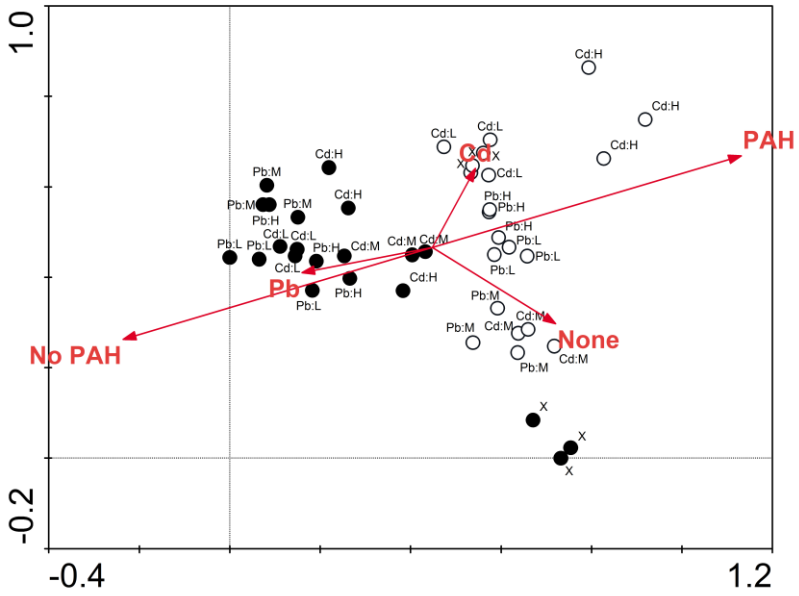


Figure 6.16: Ordination diagram for DCA analysis of Biolog Ecoplate analyses for soil samples at 5 weeks. Symbol and vector notation as described in Figure 6.12.

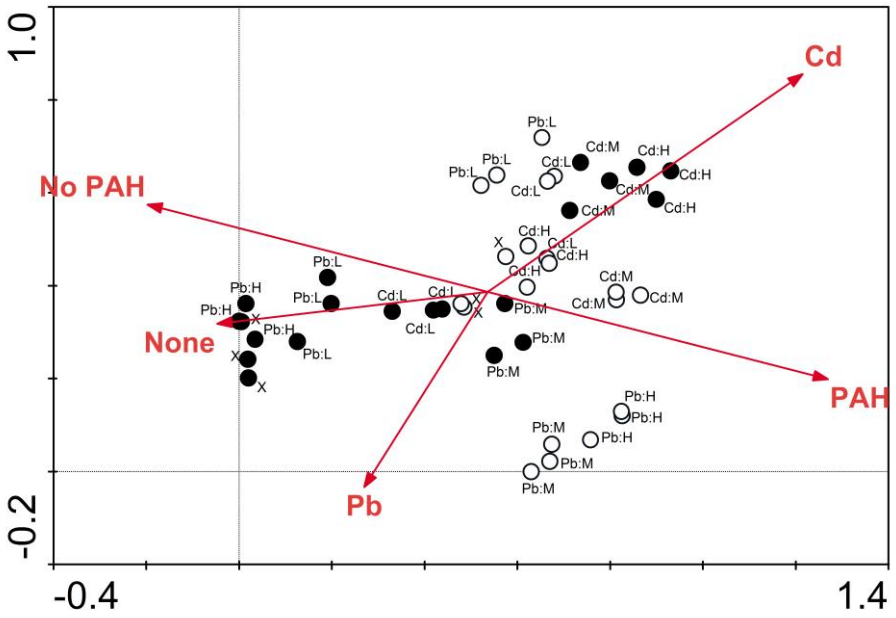


Figure 6.17: Ordination diagram for DCA analysis of Biolog Ecoplate analyses for soil samples at 7 weeks. Symbol and vector notation as described in Figure 6.12.

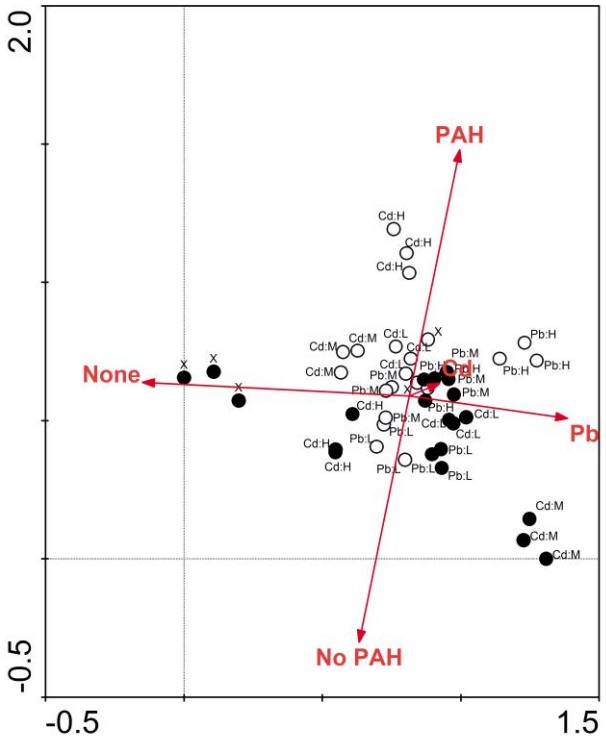


Figure 6.18: Ordination diagram for DCA analysis of Biolog Ecoplate analyses for soil samples at 9 weeks. Symbol and vector notation as described in Figure 6.12.

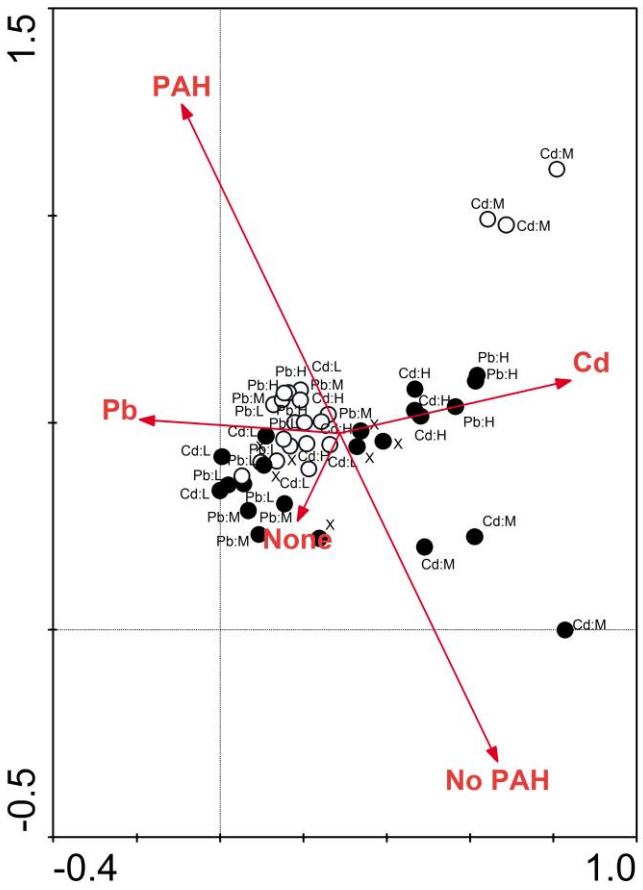


Figure 6.19: Ordination diagram for DCA analysis of Biolog Ecoplate analyses for soil samples at 12 weeks. Symbol and vector notation as described in Figure 6.12.

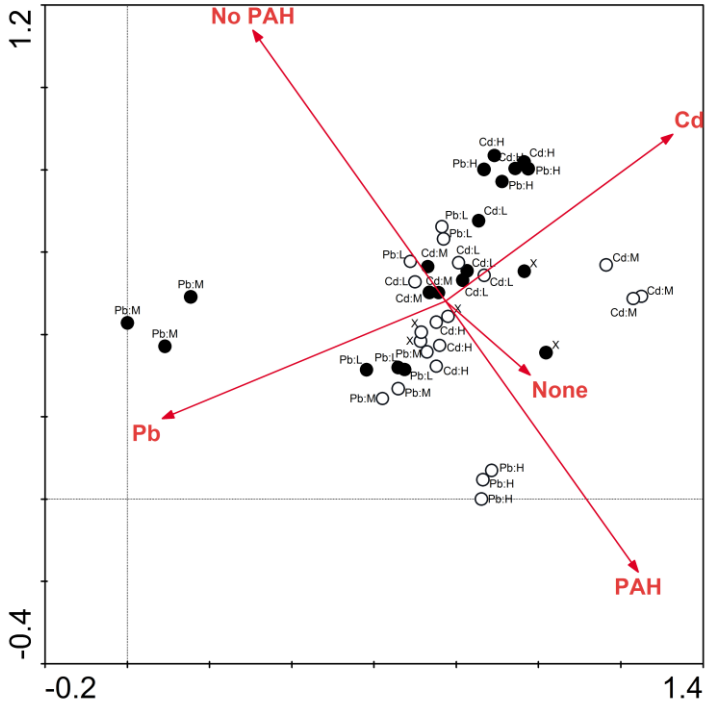


Figure 6.20: Ordination diagram for DCA analysis of Biolog Ecoplate analyses for soil samples at 15 weeks. Symbol and vector notation as described in Figure 6.12.

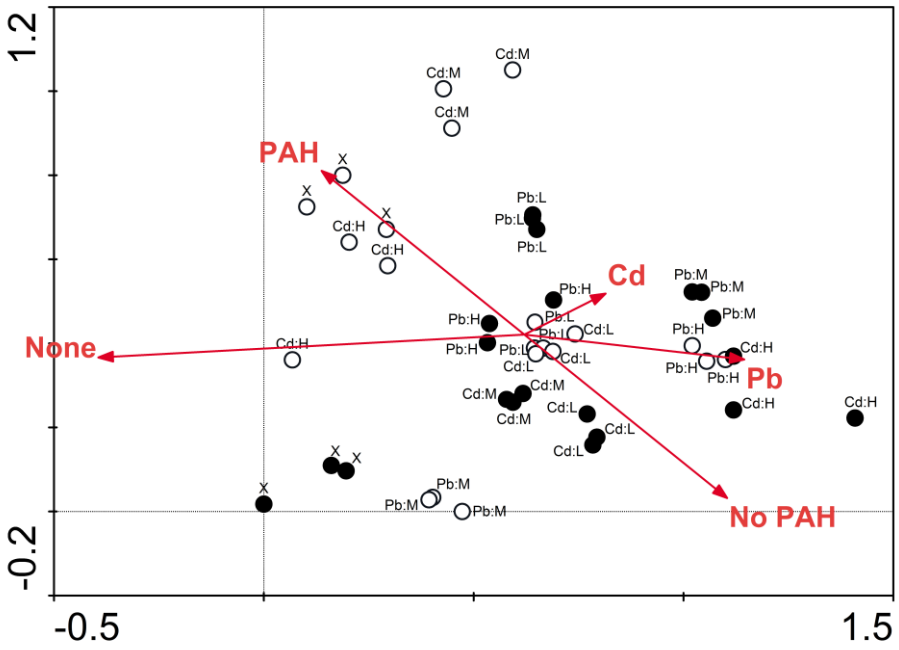


Figure 6.21: Ordination diagram for DCA analysis of Biolog Ecoplate analyses for soil samples at 20 weeks. Symbol and vector notation as described in Figure 6.12.

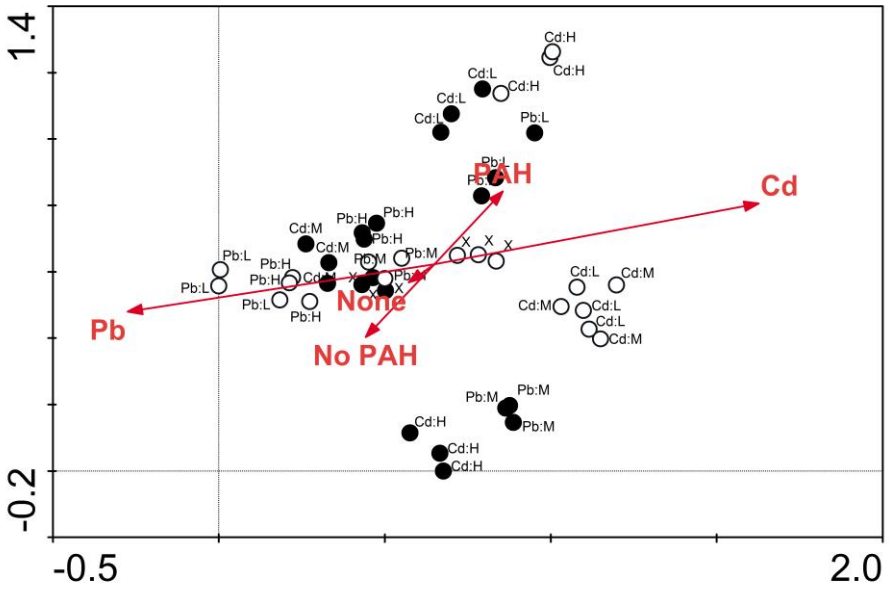


Figure 6.22: Ordination diagram for DCA analysis of Biolog Ecoplate analyses for soil samples at 25 weeks. Symbol and vector notation as described in Figure 6.12.

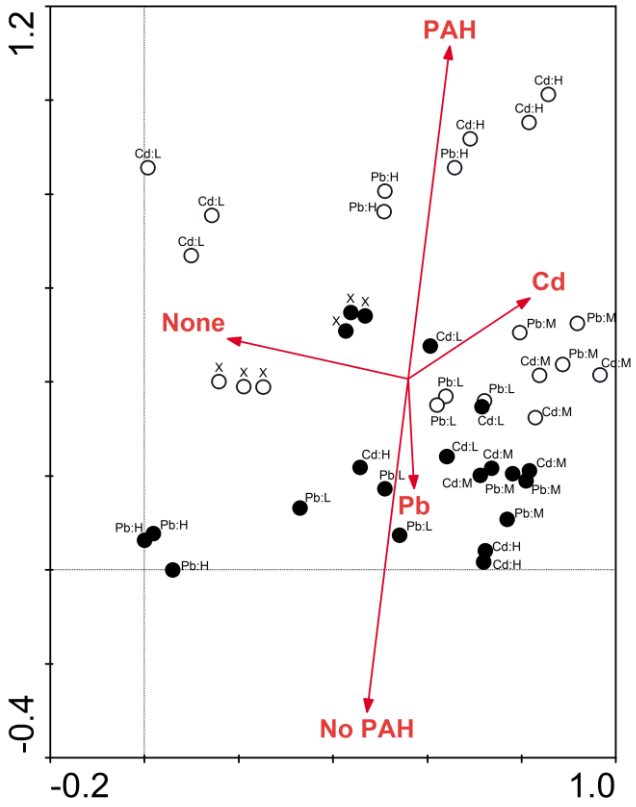


Figure 6.23: Ordination diagram for DCA analysis of Biolog Ecoplate analyses for soil samples at 25 weeks. Symbol and vector notation as described in Figure 6.12.

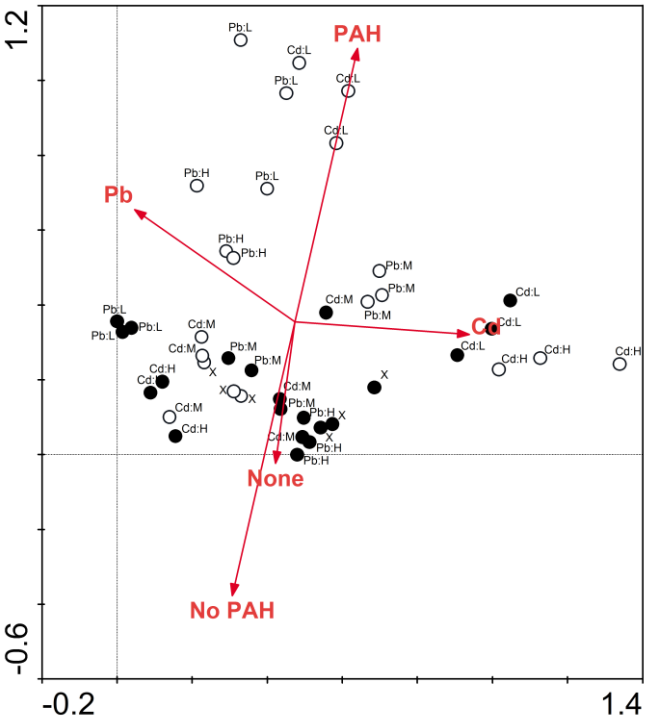


Figure 6.24: Ordination diagram for DCA analysis of Biolog Ecoplate analyses for soil samples at 35 weeks. Symbol and vector notation as described in Figure 6.12**.**

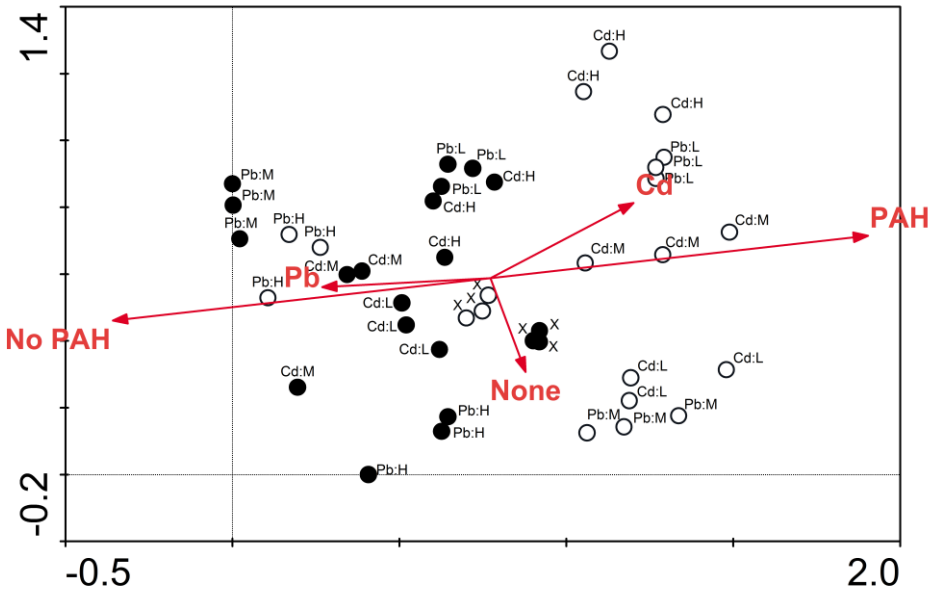


Figure 6.25: Ordination diagram for DCA analysis of Biolog Ecoplate analyses for soil samples at 40 weeks. Symbol and vector notation as described in Figure 6.12.

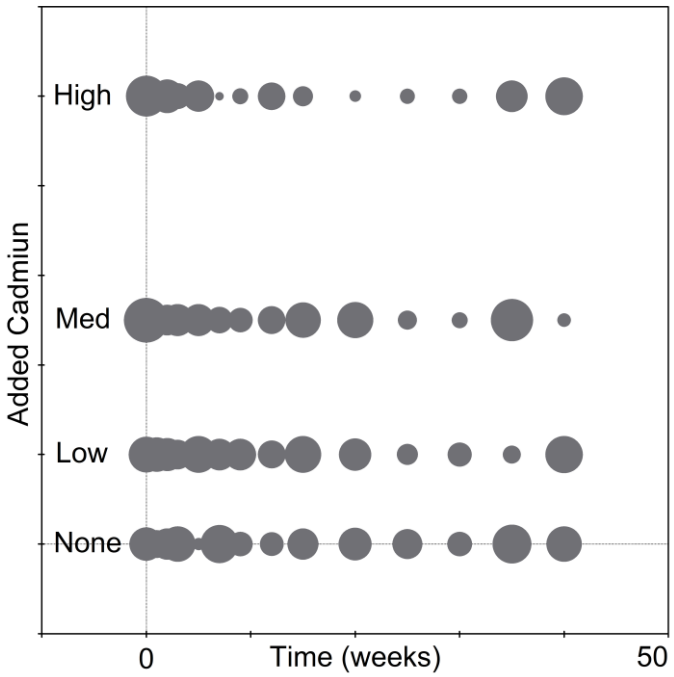


Figure 6.26: Relative magnitudes (circle size) of the Shannon Diversity Index for Biolog Ecoplate analyses carried out in triplicate for non-PAH spiked soil in the presence of different cadmium amendment levels.

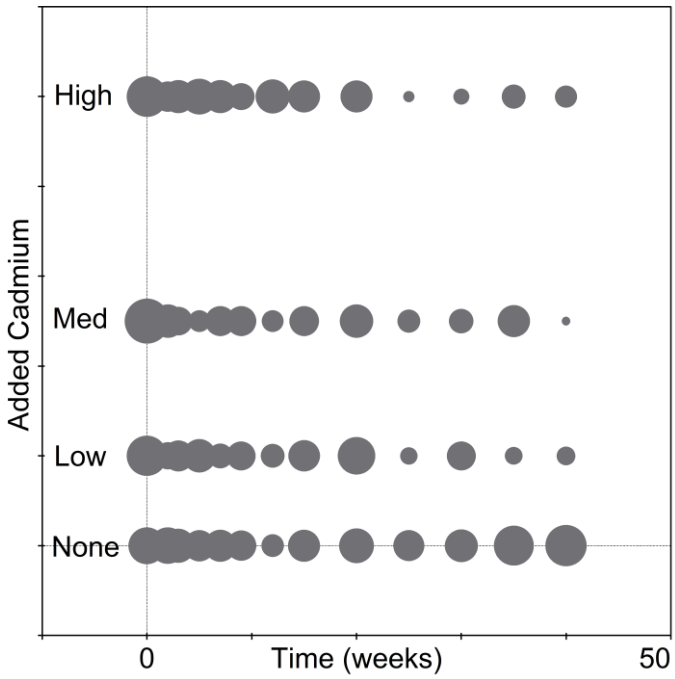


Figure 6.27: Relative magnitudes (circle size) of the Shannon Diversity Index for Biolog Ecoplate analyses carried out in triplicate for PAH-spiked soil in the presence of different cadmium amendment levels.

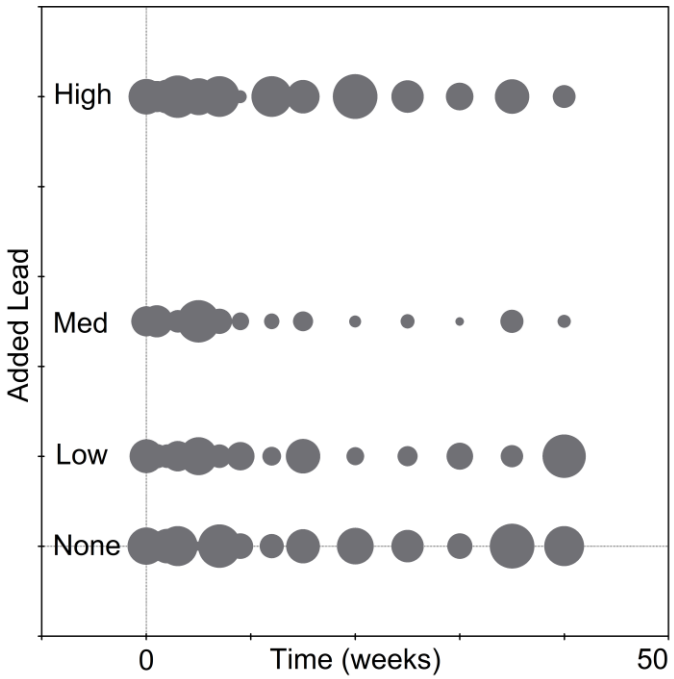


Figure 6.28: Relative magnitudes (circle size) of the Shannon Diversity Index for Biolog Ecoplate analyses carried out in triplicate for non-PAH spiked soil in the presence of different lead amendment levels.

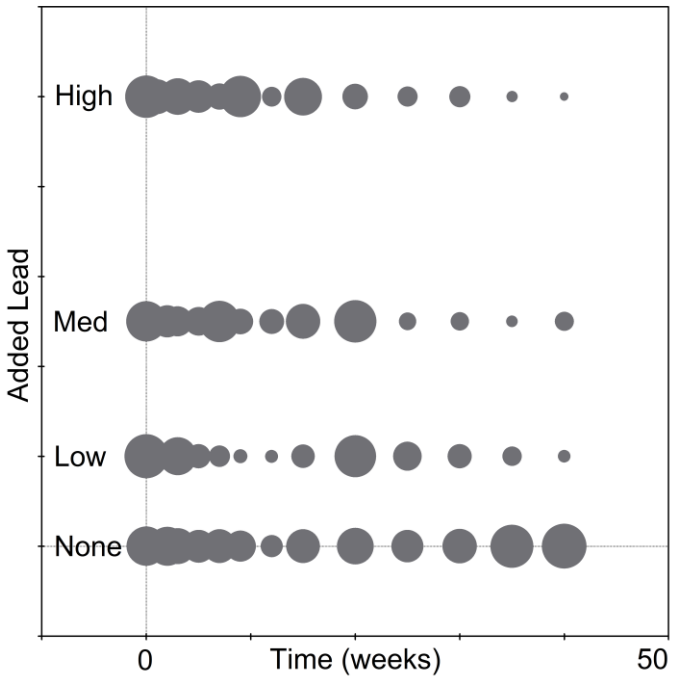


Figure 6.29: Relative magnitudes (circle size) of the Shannon Diversity Index for Biolog Ecoplate analyses carried out in triplicate for PAH-spiked soil in the presence of different lead amendment levels.

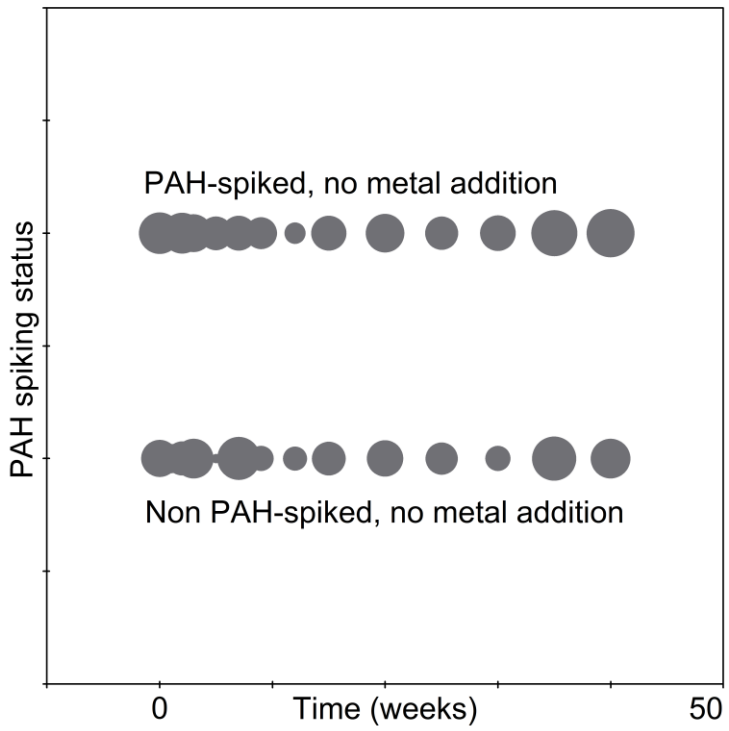


Figure 6.30: Comparison of relative magnitudes (circle size) of the Shannon Diversity Index for Biolog Ecoplate analyses carried out in triplicate for both PAH-spiked and non-spiked soil without any metal amendment**.**

6.4 Conclusion

Detrended correspondence analysis (DCA) was applied to the microbial community data set generated using the BIOLOG Ecoplate and it separated the soil microbial community metabolic activities according to the different treatments (non-PAH spiked; PAH; Pb PAH; Cd PAH; non-PAH spiked Pb; non-PAH spiked Cd) that were given to the soils. ANOVA was also applied on the data to test the statistical significance of the observed separation and it showed that the heavy metal treatment caused a significant shift in the diversity of the microbial metabolic activity. This significant change in microbial diversity was observed when a Shannon diversity indices score plot was made.

Chapter 7: Conclusions and future work

7.1 Conclusion

The studies described in this thesis centred on the biodegradation of polycyclic aromatic hydrocarbons (PAHs) in a situation akin to monitored natural attenuation (MNA), which is often the only economic and practical way of remediating large areas of land contaminated with organic substances. The biodegradation of PAH under laboratory conditions posed some difficult challenges such as determining the suitable temperature and moisture conditions, soil handling techniques and sustaining soil microbial activity for the duration of the study. It was these challenges that necessitated the need for preliminary studies to be carried out. The preliminary studies were first carried out to determine appropriate soil handling techniques and storage conditions so as to have as small an impact on the soil biology as possible. Some of the results from this first preliminary study showed that the common practise of drying out soil prior to sieving introduced artefacts in the results because of the sudden increase in soil microbial activity upon re-wetting. This sudden increase was caused by the increase in the amount of microbial carbon and nitrogen once the soil has been remoistened. The increase in the amount of carbon and nitrogen can be attributed to the nutrients released from decomposed soil microorganisms killed by drying and subsequently mineralised by the few living microorganisms. This microbial activity increase can easily be misjudged as the effect of amended contaminant if this study was not carried out. Drying and rewetting the soil also changes the distribution of soil organic matter (SOM), by increasing the amount of dissolved organic matter which alters the soil's original properties. The laboratory storage conditions can also affect the experimental results if the right amount of moisture content (< 50 %) and adequate temperature conditions is not maintained. If the storage vessel lacks a proper air circulation system, microbial activities can also be affected. These preliminary findings demonstrated the importance of the study and led to the introduction of better soil handling techniques and better soil storage environment.

Some of the introduced changes were: minimal soil handling and manipulation techniques whereby; soil samples were only air dried for 24 hours to remove excess moisture and sieved using a 2 mm sieve as recommended by the American Society for Testing and Materials (ASTM); the soils were also stored in vessels with proper ventilations and the temperature was maintained at 18 – 20 ^{o}C; the soil moisture content was also maintained at 75 % of its water holding capacity by regularly adding distilled water to soil when it was required. The temperature and moisture conditions for

the soils were kept constant in order to eliminate the variability effects of these conditions and maximise the effects of the spiked pollutants on the soil microbial community.

There were also preliminary studies centred on the optimisation of analytical procedures and instruments for the PAH biodegradation study. The optimisation enabled the adoption of an efficient, precise and accurate analytical procedures used during the USEPA 16 priority PAH biodegradation study. Some of the optimised analytical procedures were: the use of 2 g of alumina sorbent inside ASE's sample cell for in-situ clean up; calibration of GC-MS instrument for an efficient 16 PAH data quantitation; selecting an appropriate time for BIOLOG Ecoplate analysis; determining the suitable PAH spiked soil spiking ratio that would neutralise the effect of acetone on the soil microbial community; determining the spiking technique that enhance the homogeneity of the spiked contaminants and evaluating a suitable sample dilution factor that would eliminate the effect of inoculum density during community physiological profile analysis using BIOLOG Ecoplate. The test on the reproducibility of BIOLOG Ecoplate replicate which was also carried out showed that the BIOLOG Ecoplate was highly reproducible when the appropriate dilution factor was applied. The low relative standard deviation of 17 % after the first day of soil sampling which later reduced to 7.82 % after 14 days of sampling using inoculums from three soil sample replicates on one Ecoplate showed a good reproducibility value for BIOLOG Ecoplate analysis.

The derived analytical procedures obtained from this second preliminary study was applied to the biodegradation of 16 US EPA PAHs in a soil containing high organic carbon content (11.37 %). The study was carried out in PVC tube soil microcosms comprising approximately 250 g of soil over a 40 week period and the effect of heavy metal co-contamination was also investigated. PAHs were applied to the soil using a coal tar pitch source dissolved in acetone, giving a total PAH concentration in the spiked soil of 2166 mg kg^{-1}. Three different Cd and Pb amendments of low, medium and high metal concentration were carried out on the PAH soil. The respective resulting concentrations for Cd and Pb ranged from 133 to 620 mg kg^{-1} and 340 to 817 mg kg^{-1}. Mercury amendment was also made to give an abiotic control. The biodegradation results in the absence of metal amendment showed a variety of behaviours for individual PAHs: acenaphthene, fluorene and phenanthrene, for example, degrade rapidly, dropping to below 10 % of the starting concentration within 100 days, whereas naphthalene and acenaphthylene drop to about 3 5 % of the starting concentration in the same period before entering a slower degradation phase. A novel kinetic model was developed to describe the different degradation profiles: these were nearly all

biphasic in nature, but the extent of the different phases seemed to be adequately explained by a model in which the main biological degradation phase takes place in the soil rather than solution phase. In addition, the model is consistent with the migration of PAHs adsorbed onto the soil to increasingly less bioavailable and ultimately non-extractable soil phases associated with the humin pores. In general, lower molecular weight PAHs, comprising, two and three benzene rings generally degrade at a faster rate than four- five and six-membered rings. The results in the presence of heavy metal co-contaminants showed that Cd and Pb have the potential to inhibit the biotic process, but biodegradation was relatively unaffected for two to four-ring PAHs in the presence of these metals. There was also a significant impairment for the biodegradation five and six-ring PAHs. The effect of metals on PAH degradation rates is varied though generally there was an inhibitory effect that was dependent on concentration. Nevertheless, some stimulation of degradation was observed at low cadmium amendments for some PAHs, including for dibenzo[ah]anthracene. The effects of the metals were consistent with the developed kinetic model.

The effects of different combinations of PAHs, Pb and Cd on soil microbial community activity were investigated using soil respiration, soil microbial biomass carbon, metabolic quotient and the community level physiological profile. For the soil respiration measurement, the Oxi-Top manometric technique was used to evaluate the activity of the entire soil microbial community. The measured soil respiration rate values showed that the presence of PAHs stimulated the activities of the soil microbial community by acting as an additional carbon source to the microorganisms, this effect later declined towards the end of the experimental period because of the increasing non-bioavailability of the PAH compounds (migration towards non-bioavailable phases) and also because of the chronic toxic effect of the high concentrations of some of the PAH compounds (e.g. 217.6 ± 13.5 mg kg^{-1} of fluoranthene). The soils with Cd and Pb also had a reduced soil respiration values because of the effects of heavy metal on soil microorganisms such denaturing of microbial cell proteins. The PAH spiked soils amended with Cd and Pb had an initial high soil respiration rate value compared to the non-PAH spiked soils but the negative synergistic effect of PAH and heavy metal caused a greater decline in their respiration rate values towards the end of the experimental period (from week 30) especially for the PAH soils amended with Cd.

The microbial biomass carbon also showed the effect of heavy metal on the soil microbial communities. The decline in the biomass for the heavy metal amended soils was greater when compared to the control soils and there was a greater decline in the PAH soils amended with Pb and Cd compared to their respective corresponding non-PAH spiked soils. The soil metabolic quotient for the PAH soils amended with heavy

metals (Cd and Pb) also showed a higher metabolic stress on the community. There was evidence of a stimulation effect observed on the microbial activity (especially the microbial biomass values) of the Cd amended soils compared to the Pb amended soils.

The community level physiological profile which was evaluated using Shannon diversity indices values (H) derived from BIOLOG Ecoplate showed that the heavy metal amendment on both the non-PAH spiked and PAH soils caused a shift in the metabolic diversity of the microbial community when compared to their respective control soils. The substrate richness value for the PAH control soil was higher than the richness value for the non-PAH spiked control soil and the metal amended soils. The reason for this would be because of the added carbon source provided by the PAH compounds. The substrate richness for the non-PAH spiked control soil was higher than the richness values for the metal amended soils especially towards the end of the experimental period. The reason for this observation is also because of the effect of heavy metal on the soil community. The substrate evenness for the PAH control and the non-PAH spiked control was also better than the substrate evenness for the heavy metal amended soils. These values also confirm the toxic effect of heavy metals on the soil microbial community. Another observation was that the metabolic diversity and evenness for the Pb amended soils were better than the Cd amended soils which seem to show that the microbial community had more tolerance to the added Pb heavy metal than the Cd metal. The reason for this could be because the soil already had a background Pb concentration (206.6 ± 8.5 mg kg^{-1}) which would have made the microbial community to develop a better tolerance mechanism for Pb than for Cd metal.

Additionally, the AWCD values for the PAH control soil was higher compared to the non-PAH spiked control soil because of the extra carbon source provided by the PAH compounds. The PAH soils amended with heavy metal had a lower AWCD values compared to their PAH control soils and the non-PAH spiked soil amended heavy metals also had a lower AWCD compared to the non-PAH spiked control soil except the non-PAH spiked soil with low concentrations of Pb which had a higher value AWCD than the control at the initial stages (0 – 12 weeks) of sample evaluation but their AWCD value declined when compared to the control at the later stages of sample evaluation (15 – 40 weeks). This observation could be because the Pb tolerant microorganisms that were already present in the soil (background Pb concentration: 206 ± 8.5 mg kg^{-1}) were not affected by the added low Pb concentration but at the later stages poor organic substrate quality combined with the effect of Pb heavy metal caused their metabolic activity to decline.

The detrended correspondence analysis plots for selected weeks (0, 1, 5, 12, 25, 35 and 40) analysed shows the various separation of microbial community according to the soil treatments; the non-PAH spiked soils were separated from the PAH amended soils; the non-PAH spiked control soil were also separated from the non-PAH spiked soil with heavy metals; the PAH control soils were separated from the PAH spiked soil with heavy metals and the heavy metals soils were also separated according to the type and concentrations of the heavy metals. An ANOVA analysis on Shannon diversity indices values shows that the separation caused by the heavy metal soils and the PAHs soils were statistically significant.

7.1.2 The Novel aspect of this study

In this study the biodegradation of 16 US EPA priority PAH in the presence of three different concentrations (low, medium and high) of Cd and Pb was investigated. In contrast, the majority of the previous research on the effect of heavy metal on PAH biodegradation has only been performed on a limited number of PAH compounds in a simplified model system in order to minimize the number of variables. This study, using coal tar pitch spiking represents a more realistic scenario because PAHs are generally present as a mixture of compounds in a complex environmental condition.

A kinetic model was developed and applied to the biexponential dependence of PAH concentration time dependence in an attempt to explain the biphasic nature of these profiles. The model assumed the following phases in which the PAHs may be located: a solution phase in which metabolism of dissolved PAHs can take place; a bioaccessible soil phase where metabolism also takes place; a non-bioaccessible soil phase; and a phase associated with the humin pores that is non-extractable by soxhlet or accelerated solvent extraction. The latter phase is associated with removal of PAHs at a fairly constant rate that is also observed for the abiotic control.

This study also employed the use of a wide range of techniques to assess both the biological transformation of the PAHs but also the biological parameters associated with the soil microbial community. Oxi-Top soil respiration manometric measurement; soil microbial biomass carbon; and BIOLOG Ecoplate analysis were all used to evaluate the effect of PAH and heavy metal co-contamination of soil microbial community. The Oxi-Top system is a unique system that has been mostly used for measuring biological oxygen demand (BOD) but it was used for soil respiration measurement in this study.

Another novel aspect of this study was monitoring under natural attenuation the biodegradation of 16 US EPA PAHs in the presence of heavy metal co-contamination in a soil with high organic carbon content (11.37 %).

7.3 Future work

The migration of PAHs between the different organic phases in the soil would need to be investigated further because the organic matter content in the soil is the key determining factor in PAH distribution and movement and PAHs are likely to be mainly associated with it. There was a need to carry out a total extraction on PAHs in the soil in order to confirm that some of the PAHs have migrated into a phase that was not extractable by accelerated solvent extraction ASE. Further investigations can also be carried out to determine the bioavailable PAH fraction in the soil using cyclodextrin in order to have a better understanding of different phases of PAHs in the soil.

The kinetic model developed from this study was based on the interactions of PAHs and the high organic content of the soil. Therefore, it would be interesting to carry out further kinetic studies on soils with different organic content in order to understand the different associated PAH phases and their biodegradation rate for such soils and to determine whether the model holds for these different soil types.

There also a need to carry out further identification of the tolerant and stimulated microbial species that evident in the soil respiration and soil microbial biomass of the heavy metal amended soils (Pb and Cd). The community level physiological profile was only able to differentiate the catabolic diversity of these communities but further investigations using molecular techniques such as denaturing gradient gel electrophoresis would be useful additional techniques for the identification of the tolerant species which carried out the PAH biodegradation.

Additionally it would be interesting to carry out PAH biodegradation studies over a longer period of time (10 years) and under different natural plots and conditions such as arid, semi-arid woodland plots which are characterised with different soil microbial community (especially ligninolytic Fungi and bacteria) so as to better understand the biodegradation rates and mechanisms under such conditions. Moreover, soils taken from industrial, rather than Greenfield sites, as was the case for this study, would provide yet more insight into the biodegradation process.

Finally further investigations on the biodegradation of 16 US EPA PAHs in the presence of other organic compounds such PCBs and other heavy metals (e.g. Ni, Cu) can be carried out in order to understand the interactions of PAHs, PCBs and heavy metals with the soil organic matter content and soil microbial community.

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