ENHANCED VERMIREMEDIATION OF HYDROCARBON CONTAMINATED LAND USING BIOSURFACTANT: AN INTEGRATED APPROACH

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This thesis is submitted to Middlesex University in partial fulfilment of the requirement for the degree of Doctor of Philosophy

January 2019

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Dedication

I hereby dedicate this work to my parents Mr Ezekiel Olatunde Popoola and Mrs Rose-Anne Ajibola Popoola for the unconditional and unending love and support from childhood till date, you both encouraged me to strive for success in life.

Declaration of originality

I declare that the work submitted herein is my original work and has not been submitted in parts or full to any other university for any qualification. Where others' ideas, words, graphics, calculations are used, I have referenced the source in accordance with the university guidelines. Results reported in this thesis were generated from the research that I personally carried out under effective supervision. This thesis contains approximately 49,000 words including references, appendices and has approximately x figures and tables.

SOLOMON POPOOLA

January 2019

Signature

Date

Acknowledgment

I wish to express my profound gratitude to Almighty God for his grace and granting me the strength through this journey.

I would very much like to appreciate and express my gratitude to my supervisors Prof Diane Purchase and Dr Huw Jones, under your supervision I became a confident researcher in my field. I would like to express my gratitude for your constructive criticisms and suggestions throughout this journey, thank you for believing in me and supporting me through this journey, I am indeed grateful.

I would like to say a very big thank you to the technical team (Manika Choudry, Dr. Anneke Prins, Dr Leonardo Pantoja-Munoz and Alejandra Gonzalez Baez) for your constant support all through the journey I am indeed grateful.

I would also like to say a very big thank you to Dr Dirk Wildeboer of Middlesex University and Dr Rens De Groot of Imperial College London for their input in this work, this study could not have been possible without their input.

Also, to siblings Yetunde Popoola and Enitan Popoola Ikuoriah, friends and colleagues amongst whom are Anthony Futughe, Obed Amobghoa, Imeobong Antia, Cynthia Osemeke, I want to say a big thank you for your support in every way.

To others who I may have missed out, I do appreciate your support every step of the way, thank you.

Abstract

Polycyclic aromatic hydrocarbon (PAH) contamination in soil continues to be one of the biggest environmental challenges because of the persistence and carcinogenicities of the contaminants. There is a need to seek updated environmental friendly ways to remediate these pollutants and vermiremediation provides a promising solution.

Earthworms, because of their burrowing activity result in the accumulation of several lipophilic organic pollutants from their surrounding environment via the absorption of the contaminant through their body wall and also through intestinal uptake when soil passes through the gut. This makes them suitable model organisms in the remediation of PAHs from contaminated land. Biosurfactant produced by bacteria is known to aid mobility and bioavailability of contaminants which enhances bioremediation of hydrocarbons. The toxicity of these hydrocarbon compounds are yet to be fully understood. This research investigates the effect of biosurfactant on vermiremediation using anecic earthworm species (*Lumbricus terrestris)* and epigeic species (*Eisenia hortensis)* and studies the joint impact of PAHs and biosurfactant on the biochemical processes in both species by examining the monooxygenase activities (EROD and MROD activity of cytochrome P450 [CYP1A1 and CYP1A2]) and the antioxidase activity (GST activity). A 28-day experiment was conducted by exposing the two species of earthworms in soil spiked with selected 3, 4 and 5 ringed hydrocarbons [phenanthrene (PH), fluoranthene (FL) and benzo(a)pyrene (BAP) at 180 mg Kg^{-1} combined (60 mg Kg^{-1} each) and BAP alone at 60 mg Kg^{-1} under the same conditions. A time response relationship was established between the concentration of the three PAHs and length of exposure. In the presence of biosurfactant (0.1 g L^{-1}), *E. hortensis* removed on average (mean ± stdev) 91% ± 5.2% PH, 70% ± 8.4% FL and 27% ± 4% BAP after 7 days whereas *L. terrestris* removed approximately 89% ± 0.5%, 67% ± 6.4% FL and 30% ± 1.4% BAP. These values were significantly higher than the control without the addition of biosurfactant ($p = <0.05$), *E. hortensis* removed only 26% ± 1.7% PH, 17% ± 6.2% FL and 8.4% ± 0.7% and *L. terrestris* 25% ± 4.5% PH 24% ± 8.8% FL and 6.4% ± 3% BAP. A one-way ANOVA analysis showed that there was a significant difference in the degradation of FL and PH in control soils in the presence of biosurfactant compared to the control soils without biosurfactant. Also, a significant difference between degradation of PH, FL and BAP by *E. hortensis* in the presence of biosurfactant, compared to its control (absence of biosurfactant, degradation by *L. terrestris*

with biosurfactant was observed compared to the control (without biosurfactant). In the presence of biosurfactant (0.1 g L^{-1}), the degradation was similar in both types of hydrocarbons with R-sq values of 85.6%, 85.9% and 94.1%

for removal of PH, FL and BAP. This shows that the integration of vermiremediation and biosurfactant hold a promising approach to optimized and accelerated biodegradation of heavy molecular weight (HMW) PAHs in soil.

Further investigation of enzymatic activities in a time response relationship over a 28-day period was conducted. Ultra-performance liquid chromatography (UPLC) analysis showed that both EROD and MROD activities were induced between days 0 and 2 of exposure. The level of increase in enzymatic activities were similar in both genera (between 2 and 2.5-fold higher in EROD and between 2 and 2.5-fold increase in MROD activity). Enzymatic activities decreased by day 7, with complete mortality of *L. terrestris* by day 28, indicating the presence of monooxygenases in earthworms further indicating the potential in degrading PAHs.

Results obtained indicate that an epigeic species when used with biosurfactant performed slightly (≤5%) better because of their tolerance limits than anecic species which are more sensitive to several parameters such as temperature, moisture content, contaminants. Vermiremediation (using either species) was enhanced with biosurfactant. It is a promising, sustainable and quick technique to remediate PAH contaminants when compared to some of the other conventional approaches such as land filling, soil vitrification or incineration that could be expensive or pose some negative effects in the environment over a period of time.

Overall the integrated approach of vermiremediation with biosurfactant completely removed 3 and 4 ringed hydrocarbons and 80% of 5-ringed hydrocarbons as stated earlier, where the application of rhamnolipid biosurfactant (0.1 mg Kg^{-1}) posed no deleterious effect in either specie of earthworms, thus indicating that this could be a promising technique in remediating organic pollutants such as PAHs.

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List of abbreviations

B	Biosurfactant
BAP	Benzo(a)pyrene
BSA	Bovine serum albumin
CEC	Cation exchange capacity
CMC	Critical micelle concentration
CYP450	Cytochrome p450
D/W	Dry weight
E	*Eisenia hortensis*

EC_{50}	Effect concentration
EROD	Ethoxyresorufin *O*-deethylase
FL	Fluoranthene
GCFID	Gas chromatography flame ionization detector
GSH	Glutathione
GST	Glutathione *s*-transferase
ICP-OES	Inductive coupled plasma optical emission spectrometry
L	*Lumbricus terrestris*
MICROTOX	Microtoxicity
MROD	Methoxyresorufin *O*-deethylase
NADPH	Nicotinamide adenine dinucleotide phosphate
NIG	Nigeria
OECD	Organization for economic development and co-operation
P	Pollutant
PAH	Polycyclic aromatic hydrocarbon
PH	Phenanthrene
S	Soil
SOD	Superoxide dismutase
SOM	Soil organic matter
SMC	Soil moisture content
UPLC	Ultra-performance liquid chromatography

USEPA	United states environmental protection agency
W/W	Wet weight
WHC	Water holding capacity

CHAPTER 1: INTRODUCTION

1.1 ENVIRONMENTAL PROBLEMS OF POLLUTION

1.1.1 DEFINITION OF CONTAMINATION AND POLLUTION

The presence of chemical compounds beyond the limits of naturally present concentration in the environment is referred to as environmental contamination (Agnello et al., 2014). Generally, contaminants are undesired compounds in the environment, though their presence at certain concentrations does not necessarily mean they are harmful. However, pollution is the presence of compounds at concentrations far beyond naturally present concentration initiated generally by anthropogenic factors and causes detrimental facts to the environment as well as its inhabitants thus affecting biochemical processes in flora and/or fauna (Agnello., 2014). Hence all pollutants are contaminants, however not all contaminants are pollutants (Chapman, 2007). It is worth noting that despite the differences between both words, it is not uncommon to use both interchangeably as is done in several scientific communities as well as in this written thesis.

1.1.2 OVERVIEW OF SOIL POLLUTION

There are two main sources of soil contamination, and they are natural and anthropogenic in origin. The major sources of contamination are manmade. The majority of manmade pollution in Nigeria comes from industrial and commercial activities, waste disposal and mining of crude oil activities, accidental spillage of chemicals, application of pesticides to soil, improper waste disposal such as landfills and leaching are few examples of manmade activities that lead to introduction of both organic and inorganic pollutants to soil (Abioye et al., 2011; Okparanma et al., 2010; Okoro and Ikolo, 2007). Two main sources of environmental pollution are usually used; if there is a single source of pollution (known as point-source pollution) which would usually be at high concentrations, and if the pollution is spread in the environment and cannot be traced to single source is known as diffuse-pollution or non-point-source (Mirsal, 2004). The nature and extent of pollution varies widely depending on site of pollution, from long term exposure at low concentrations leading to associated risks, to immediate extermination of plant or animal species. Furthermore, polluted sites pose an imminent threat to ground

and surface water thus potentially contaminating drinking water. Overall, there has to be a pathway to link receptors to the source of pollution (directly or indirectly) to allow contact, otherwise there is no imminent risk posed (Wilson, 1991). When there is a pathway linking the source and receptor, it is essential to apply suitable risk assessment methodologies to identify possible associated issues which might be of concern, and highlight suitable actions to be implemented (BASOL, 2014). Presently, remediation technologies can be grouped into four major types; 1) physical and chemical methods, 2) biological methods, 3) thermal destruction methods and 4) fixation methods. The decision to choose specific methods is as a result of cost-benefit assessment which takes into consideration several factors such as cost, time of remediation, soil type, type/concentration of contaminant, post-remediation use of site etc. However, it Is beyond the scope of this present study to evaluate the distinctive features and properties of each remediation technology. This study focuses on a single bioremediation technology (vermiremediation) which is discussed solely.

1.1.3 OVERVIEW OF POLLUTED SITES IN NIGERIA

Inadequate regulations and law enforcements have resulted in large cases of contaminated land as well as potentially contaminated land across Nigeria (Abioye, 2011). This has led to several cases of contamination due to negligence.

Oil theft through pipeline vandalization, poorly maintained and ageing pipelines are major causes of oil spills. These spills led to pollution of land, air and water-ways, and in turn affected the livelihood of neighbouring villages by polluting and decreasing farm produce, fish production and clean water supplies (USEIA, 2016).

The average lifespan of a pipeline as stipulated by the gas and oil standards (GOST) is 33 years (Achebe et al., 2012), this led to a study to ascertain causal factors of oil pipeline failures NNPC: mechanical failure was responsible for 42%, third party activity such as vandalism responsible for 24%, 18% caused by corrosion of pipelines, 10% by man/operator error and 6% by natural occurrences such as floods, erosion, bush burning etc. (Figure 1.1). NNPC observed that the pipeline reliability was inversely proportional to its age, where reliability of pipeline aged around 20 years were at a capacity of 46% and that above 30 years at 25% or less. It was also reported that Rivers state showed the highest rates of oil spills at 32% while Cross-Rivers state showed the least only with 2% occurrence (Figure 1.2).

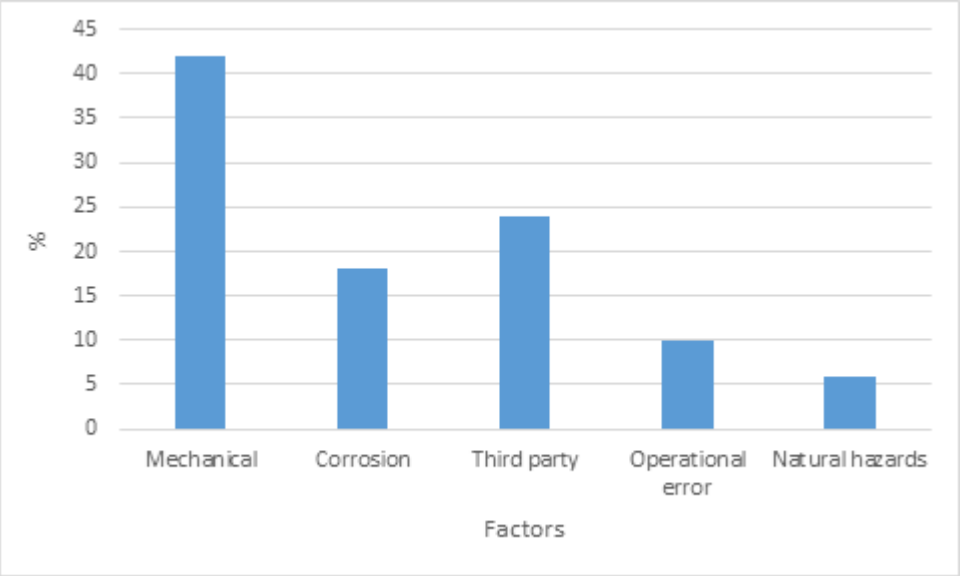


Figure 1. 1 Causing factors (in %) of pipeline failure (Source: Achebe et al., 2012).

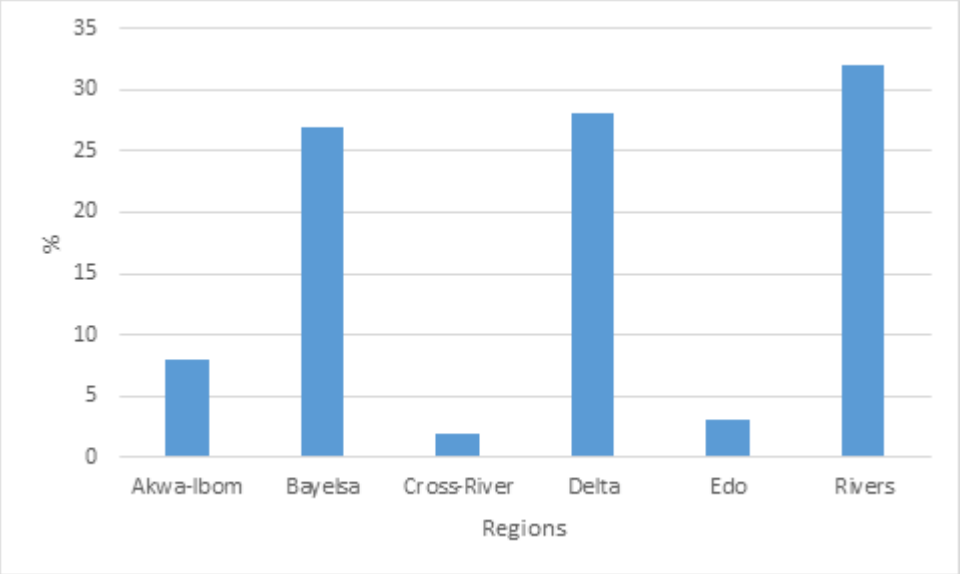


Figure 1. 2 Proportion of spill in the Niger-Delta between the periods of 1999-2005 (Source: Achebe et al., 2012).

There are over a thousand contaminated sites across the Niger Delta region, and with the trend in cases such as pipeline vandalism, this number has been estimated to rise by 30% by 2020 (Amnesty USA report, 2017). In addition to this trend, the response time has been reported to be slow, with expensive conventional techniques in remediation, with only an

estimate of under 10% of contaminated land in the last 10 years being remediated. Furthermore, it is worth noting that the number of potentially contaminated land are estimated to be as much as known contaminated land if not more (Abioye, 2011).

Polycyclic aromatic hydrocarbons (PAHs) are some of the several compounds in crude oil. They are globally ubiquitous, and hence a global problem (Kanalay and Harayama, 2000; Harmsen et al., 2007). According to the USEPA, PAHs represent an approximate of 13% of the total soil contaminants (Sayara et al., 2010). Following heavy metals and mineral oils, PAHs are the third largest constituents of oil pollutants. Other constituents include chlorinated hydrocarbons and phenols which are not so common (Schippers et al., 2014).

Between 1976 and 1996, there were a total of 6,647 oil spills in Nigeria that is an equivalent of 2.4 million barrels (bbl) (approximately 157 $L.bbl^{-1}$) released into surrounding environments, with 23.17% of the spill being recovered as reported by the Department of Petroleum Resources (DPR) (Egberongbe et al., 2007). Between 1970 and 2000 there were 7000 oil spills in Nigeria, as well as 2000 major spills and thousands of smaller spills yet to be attended to that date back decades (Vidal, 2014). Ordinioha and Brisibe (2013) suggested an estimate of 7000 spills should have a value of 13 million barrels, which is an average of 240,000 barrels yearly. In 2012, Amnesty International reported that thousands of indigenes of Bodo village in the Niger Delta of Nigeria, whose daily sustenance was solely dependent on fishing and farming were severely affected by two oil spills in 2008 with spills between 1440 and 4320 bbl/d, resulting in a total estimate between 103,000 and 311,000 barrels over the 72 days of spill.

There is very little information regarding the negative impacts caused by the activities of companies operating in these areas in the Niger-Delta regions of Nigeria (Vidal, 2014). This spill (Bodo community spill) has been reported to have had several adverse effect on the health of locals of this communities including high child mortality rate, increased malnutrition rates, low life expectancy, tiredness, itchy nose, red eyes, sore throat, diarrhoea, ear pain, as well as a risk of induced abortion due to the contaminated water consumed (Omorede, 2015; Sebastian and Hurting, 2004). Further to these adverse health effects, the economic reforms and crises in the country has led to an increase in the exploitation and impoverishment of locals in the community, with all complaints and grievances to democratic institutions not heard or investigated (Obi, 2009).

Soil contamination from hydrocarbons are mainly from petroleum products, which is becoming a global problem rapidly because of its toxicity (Abioye, 2011). These petroleum products are comprised of a range of saturated products such as methane (CH_4), ethane (C_2H_6) and propane (C_3H_8), right through the straight and branched chains up to $C_{76}H_{154}$ (Abioye, 2011).

According to Okparanma et al. (2010), there are potentially 5.7 contaminated land sites per every 10,000 inhabitants where PAHs are the second most abundant contaminant found in Nigerian soils (Figure 1.3).

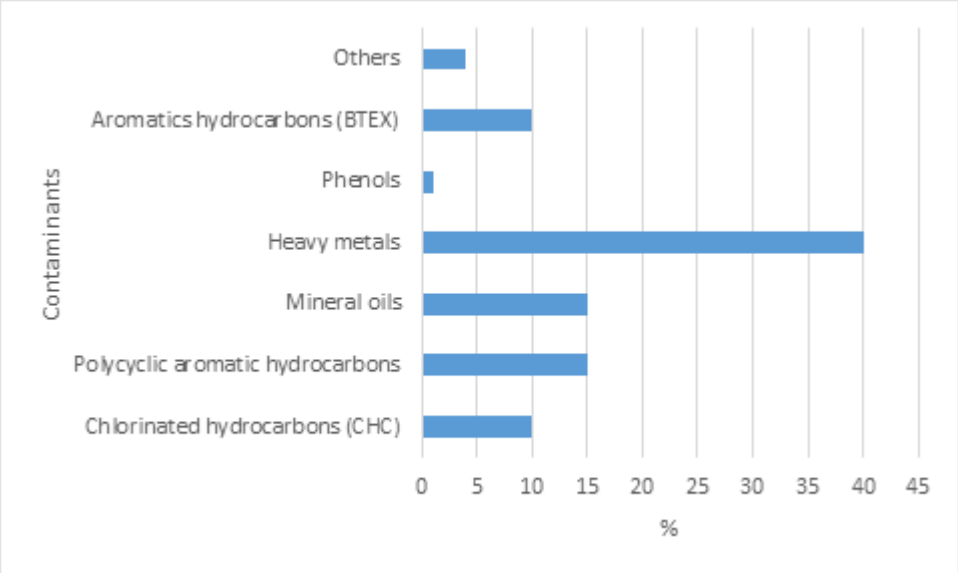


Figure 1. 3 Frequency of the most abundant contaminants present in Nigerian soils (Source Okparanma et al., 2010).

1.2 PAHs: CONTAMINANTS OF INTEREST

PAHs are made up of two or more benzene rings e.g. naphthalene (2-rings), phenanthrene (3-rings) having molecular weights of 128 and 178 g mol^{-1} respectively are referred to as low molecular weight (LMW) (Flores and Mestahoward, 2001). Those made up of 4 rings and above such as pyrenes (4 rings), fluoranthene (4 rings) and benzo(a)pyrene (5 rings) with molecular weight of 202, 202 and 252 g mol^{-1} respectively are referred to as high molecular weight (HMW) PAHs (Pineda-Flores and Mesta-Howard, 2001; Niederer et al., 1995; Kanaly and Harayama, 2000).

Pathway of PAH entry into the environment includes incomplete combustion of organic substance such as fossil fuels, hence the reason it is largely distributed in this vicinity. The listed EPA16 possess distinctive characteristics of high hydrophobicity that increases with

increase in molecular weight of the compound which also decreases the solubility in water. These features play an important role in the behavioural characteristics of the compound upon entry into soil matrix where they have a strong adsorption to soil matrices. It can be said that an increase in the molecular weight of PAHs determines how persistence they are in the environment (Mohan et al., 2006; Cerniglia, 1992; Gan et al., 2009).

1.3 VERMIREMEDIATION OVERVIEW

Two popular forms of remediation of contaminated sites are; 1) dig and dump- which involves soil excavation, transporting polluted soil offsite where they are buried in a landfill site and monitored. However, this method does not treat the polluted soil and poses the potential risk of leaching into groundwater systems and contaminating it, and 2) soil washing- which is designed to remove inorganic pollutants from polluted soil. However, this technology generates sludge as a secondary by-product that requires further treatment.

Recently, there has been a global realization that mechanical, chemical and thermal technologies of remediating polluted sites can be financially and environmentally unsustainable, hence the shift in focus to bioremediation technologies that can be both environmentally and financially sustainable.

Over the last few decades, the use of earthworms in biodegradation (vermiremediation) has been extensively studied and reported to proffer a cost effective and sustainable technology in remediating polluted lands globally. It has been reported that earthworms bioaccumulate these pollutants in their body tissue where they either biodegrade or biotransform with the aid of enzymes (Brown and Doube, 1994). Furthermore, the gut of an earthworm has been reported to be a microbial incubator hosting several microbial degrading species further enhancing degradation of pollutants (Ramteke, 1992; Brown and Doube, 1994).

Several species from all three groups of earthworms (epigeics, endogeics and anecics) such as *Eisenia fetida, E. hortensis, E. andrei, Lumbricus rubellus, Aporrectodea tuberculata, A. longa, L. terrestris, Dendrobaena rubida, Eudrilus eugeniae, Allophobora chlorotica* etc. have used and reported to remove significant amount of pollutant from soil under the right conditions (Rorat et al., 2017; Rodriguez-Campos et al., 2019; Chachina et al., 2016). Several studies have indicated that *Eisenia* species are most versatile and are capable of tolerating very high concentration of contaminants by bioaccumulating them in their body tissues (Hanna and Weaver, 2002; Contreras et al., 2005; Rorat et al., 2017). For the purpose of this

present study, commercially available species from the endogeic (*E. hortensis*) and anecic (*L. terrestris*) were used in conducting research.

The key limitation to bioremediation technologies is time of remediation and this is as a result of bioavailability of pollutants in the soil. Semple et al (2004) define bioavailability as *"the proportion of a chemical compound that is freely available to living organisms, thus able to cross the cellular membrane of the organism from the medium where the organism lives at the given time"*. Thus, the major focus of this research is exploring biological amendments to increase bioavailability of pollutants thus optimising the removal of pollutants from soil within shorter timeframe.

1.3.1 BIOSTIMULATION-ASSISTED VERMIREMEDIATION

Biostimulation is the addition of organic compounds with the aim of optimising the vermiremediation processes. The selected additive in this research was rhamnolipid biosurfactant. Surfactants are amphiphilic compounds that possess both hydrophilic and hydrophobic properties in their structure, and the main function is decreasing surface tension between compounds (Ma et al, 1995).

Rhamnolipid biosurfactant (biologically produced surfactants) is one of the most studied groups of surfactants because of its effectiveness in reducing the water surface tension and oil-water surface tension in addition to emulsifying oil thus increasing the mobility of recalcitrant compounds (Zhao et al., 2016; Gudina et al., 2015; Amani et al., 2013). Hence, the selected biosurfactant used to enhance vermiremediation in this study was rhamnolipid biosurfactant.

1.4 AIMS

This research aims to investigate an optimised and accelerated removal of polycyclic aromatic hydrocarbons (PAHs) by earthworms enhanced with biosurfactant and their combined effect on the biochemical processes in species of earthworms. The impact on biochemical processes in earthworms is very important because this combined bioremediation approach, to the best of our knowledge, has not been used before hence there is little or no knowledge what toxic effect the combination of PAHs and biosurfactant could pose at molecular levels in earthworms.

CHAPTER 2 – LITERATURE REVIEW

2.1 BACKGROUND

2.1.1 CONTAMINATED SITES IN NIGERIA

The presence of chemical compounds and elements beyond the limits of naturally present concentration in the environment is referred to as environmental contamination (Agnello et al., 2014). This is a cause for concern as it poses risks to the environment and human health. In contaminated land there is a cause (usually) to remediate the land of its present contaminants to restore the land for potential uses, while this does not apply to potentially contaminated sites (Van-Liedekerke et al., 2014).

Nigeria is known to be the largest producer of petroleum in Africa, with an estimate 192 trillion cubic feet (Tcf) natural gas reserves in June 2017 (DPR, 2017). In 2014, Nigeria produced 780, 000 mbbls and 1.55Tcf of natural oil and gas where a majority of this reserves are located in the Niger-Delta region of Nigeria (USEIA, 2016). The key factor that shapes the market value of crude oil is density of the oil, which further categorises them into light molecular weight (LMW) or heavy molecular weight (HMW), which are the key groups of organic compounds found in crude oil. Most hydrocarbons present are alkanes, cycloalkanes and polycyclic aromatic hydrocarbons PAHs Fig 2.1.

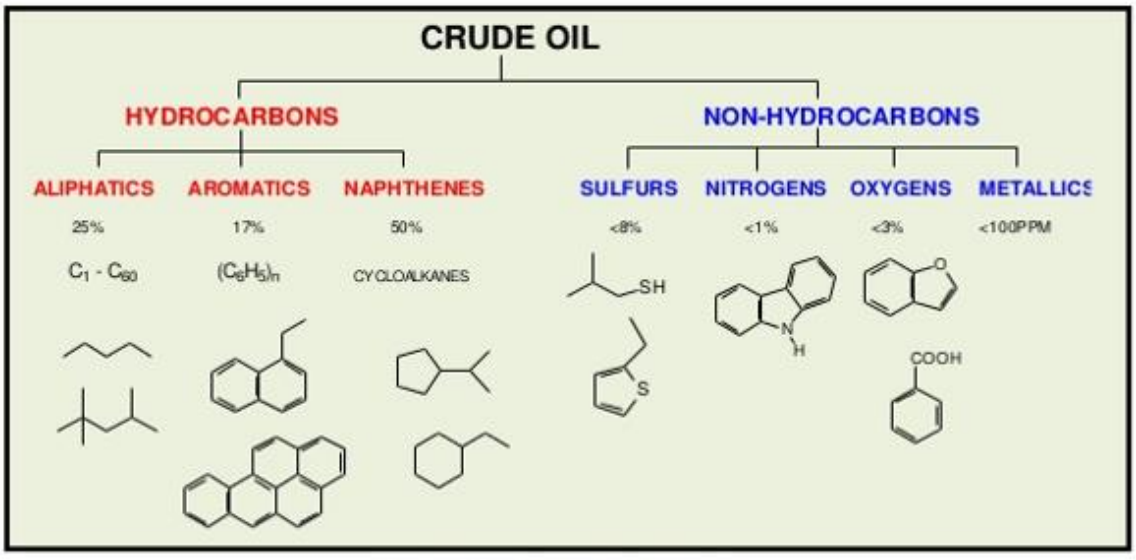


Figure 2. 1 Composition of crude oil (Source: Ogunkeyede, 2016)

Table 2. 1 Fractional composition and specific gravity of Nigerian crude oil

Crude oil fractions	Saturation (%)	Aromatics (%)	Asphaltenes (%)	Residue (%)	Specific gravity
Bonny light	81.11	7.20	2.48	0.21	0.84
Medium	64.90	13.37	13.37	8.36	0.98
Escravos light	69.74	22.05	2.56	5.65	0.78
Forcados blend	58.89	11.10	340	26.61	0.88

(Source: Ogunkeyede et al., 2015)

Production of crude oil in Nigeria plateaued in 2005 where it reached its peak of 2.44 million barrels per day (bbl/d) after which there was a sharp decline in production due to the infiltration of militant groups forcing the shutdown of production of big companies by any means possible (USEIA, 2016). The Nigerian National Petroleum Cooperation (NNPC) was set-up in 1977 to regulate the natural gas and oil industries, with the development of upstream and downstream development and regulations being their secondary responsibilities (USEIA, 2016). Subsequently, the Department of Petroleum Resources (DPR) was established in 1985 under the Ministry of Petroleum Resources with responsibilities to regulate leases and permits, environmental standards and general compliance for the key international oil companies such as Shell, Exxon Mobil, Elf/Total, Chevron, etc.). Though Nigeria as a country produces large volumes of oil, production is constantly cut off by instability and supply disturbances, which in turn leads to losses as high as 500,000 bbl/d (Khusanjanova, 2011). In 1971, Nigeria joined the Organization of Petroleum Exporting Countries (OPEC), twenty years after oil exploration in the oil rich Niger Delta state Bayelsa was first commenced in the 1950s (USEIA, 2016).

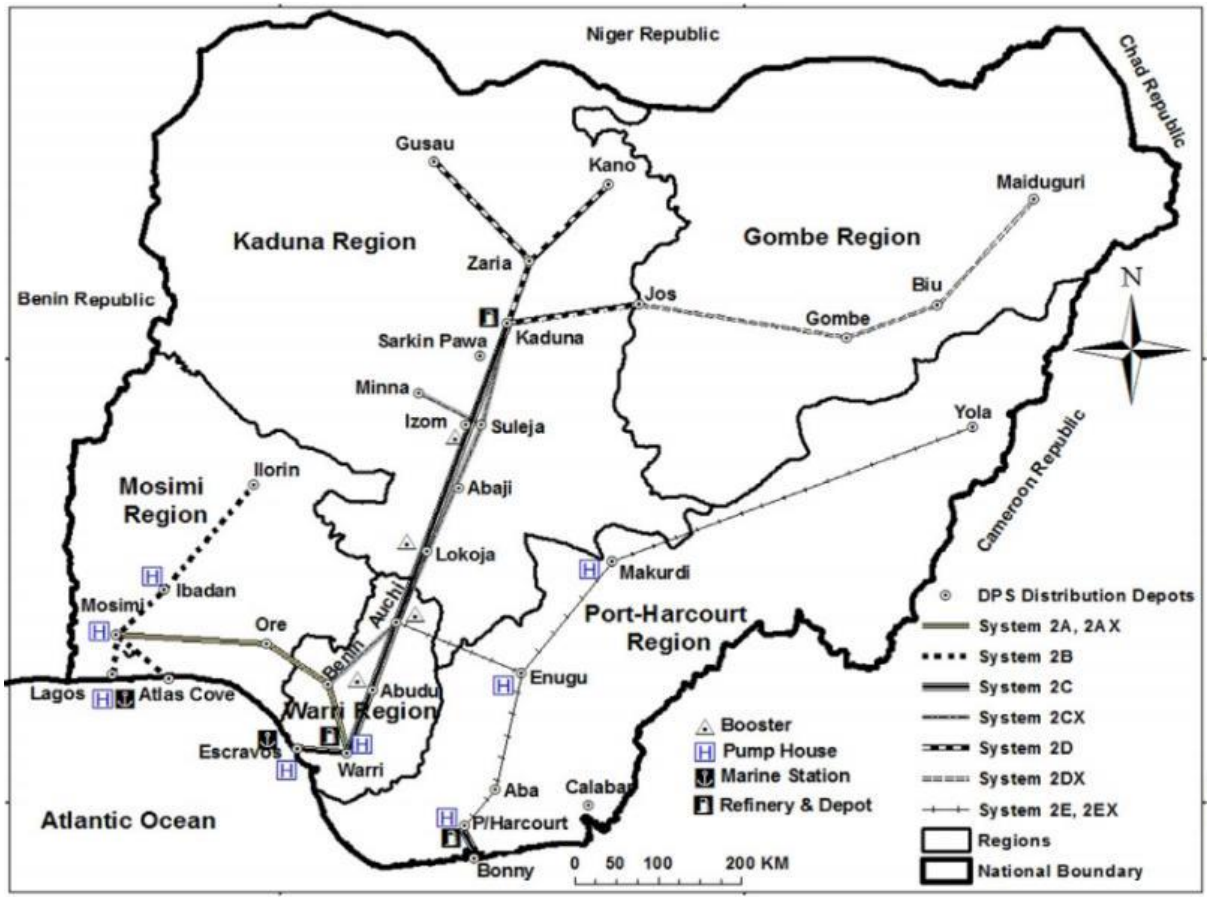


Figure 2. 2 Map of Nigeria showing the pipeline network and facilities (Source: Ambituuni et al., 2015).

There was a slight recovery of oil production between 2009 - 2010, however it was below its peak production as a result of the ongoing disruptions in supply. In 2013, there was further supply disruption due to reasons such as oil theft through vandalization of the oil pipelines which in turn led to the shutdown of Nembe Creek Trunkline in Warri and Trans Niger Pipeline and a huge loss on the shipment of multiple crude products (Fig 2.2). On average, production of crude oil between January to October 2013 was approximately 200 million bbl/d, this was similar to the levels in 2008/2009 where disruption was at its highest (USEIA, 2016).

2.2 THE CHEMICAL COMPOSITION OF CRUDE OIL

Crude oil hydrocarbons are generally categorised into four groups, saturates (cyclic, branched and unbranched alkanes), resins (oil surface structures that are polar and dissolved in saturates and aromatics), aromatics (ringed aromatic hydrocarbons that can either be monoaromatic hydrocarbons (MAHs) or polycyclic aromatic hydrocarbons (PAHs), and asphalthenes (brown solids dispersed in saturated and aromatics (Speight, 1999; Kanaly and

Harayama, 2000). Based on the four groups of crude oil hydrocarbons, saturates are found on the outermost layer and are mostly vaporized, while asphalthenes make up the innermost layer because of their huge molecular mass (Kanaly and Harayama, 2000).

Hamme et al. (2003) implied that the susceptibility of crude oil hydrocarbons to biodegradation is in the following order alkanes > light aromatics (MAHs) > heavy aromatics (PAHs) > asphalthenes. Resins are easily degraded because of their light molecules (Spiecker et al., 2003).

Other constituents of crude oil that are non-hydrocarbons with moieties including sulphur, nitrogen, oxygen and metallics as illustrated in fig 2.1, where crude oil sulfur content plays a role in the categorising of crude oil as sweet or sour, hence affecting the market value of crude oil product.

2.2.1. PAHs AS SOIL CONTAMINANTS AND THEIR TREATMENTS

MADEP (2002) defined natural background concentrations of contaminants in soil as that concentration of hazardous materials that can be attributed to ecological and geological conditions and not to manmade activities (Table 2.2).

Table 2. 2 Natural PAHs background concentrations in soil

PAHs	Background soil concentrations µg Kg^{-1}	Background soil	Concentrations	(µg Kg^{-1})
		Rural soil	Agricultural soil	Urban soil
Acenaphthene	500	1.7	6	-
Acenaphthylene	500	-	5	-
Anthracene	1000	-	11-13	-
Benzo(a)anthracene	2000	5-20	56-110	169-59,000
Benzo(a)pyrene	2000	2-1300	4.6-900	165-220
Benzo(b)fluoranthene	2000	20-30	58-220	15000-62000
Benzo(e)pyrene	-	-	53-130	60-14000
Benzo(g,h,i)pyrelene	1000	10-70	66	900-47000
Benzo(k)fluoranthene	1000	10-110	58-250	300-26000
Chrysene	2000	38.3	78-120	251-640
Dibenzo(a,h)anthracene	500	-	-	-
Fluoranthene	4000	0.3-40	120-210	200-166000
Fluorene	1000	-	9.7	-
Indo(1,2,3-cd)pyrene	1000	10-15	63-100	800-61000
Naphthalene	500	-	-	-
Phenanthrene	3000	30	48-140	-
Pyrene	4000	1-19.7	99-150	145-147000

(Adapted from MADEP, 2002; Mumtaz and George, 1995) high concentrations in urban soils in America was reported as a result of anthropogenic factors

Based on the total PAH concentration in soil, Malawaska and Wikomirski (2001) established a general soil assessment table based on the PAH content (Table 2.3) (Sayara et al., 2010).

Table 2. 3 PAH threshold limits in soil surface area (adapted from Sayara et al., 2010)

Total PAH content (µg Kg^{-1} dw)	Soil assessment
<200	Unpolluted (natural content)
200-600	Unpolluted (increased content)
600-1000	Slightly polluted
1000-5000	Polluted
5000-10000	Heavily polluted
>10000	Very heavily polluted

PAHs in the environment is a cause for concern in public, regulatory and scientific related matters, this is due to their toxicity, carcinogenicity, teratogenicity, mutagenicity and their ability to bioaccumulate and biomagnify along the food chain (Rorat et al., 2017). This has led to several investigations in understanding the bioremediation mechanisms associated with the remediation of PAHs in contaminated land over the recent several decades, leading to advanced developments and use of these technologies (Semple et al., 2001; Contreras-Ramos et al., 2008). Every contaminated site possess unique challenges, thus approaches in remediating contaminated sites vary from site to site. Several factors are considered when selecting a remediation approach for contaminated sites and some of these factors include, type of contaminant, threshold limits of contaminants based on legislation, soil type, time limits for remediation, geographical region of contaminated site and very importantly is cost. It is worth noting that treatments could be unique to contaminated site, thus not any treatment can be applied to a contaminated land hence a cost-benefit analysis is used in selecting treatment methods (Beskoski et al., 2012). Table 2.4 and 2.5 highlights an overview of treatment groups and examples of their application. However, biological treatments are the group of choice because they are eco-friendly and cost effective (Sayara et al., 2010; Gan et al., 2009; Hernandez et al., 2014).

Table 2. 4 Remediation technologies

Remediation treatment	Main features
Bioremediation	Organism and microbial activities that function in transforming or mineralizing contaminants to a less toxic form.
Chemical	Chemical reactions that function by neutralizing, fixing or destroying environmental contaminants.
Thermal	Use of heat to exterminate contaminants through methods like pyrolysis, incineration or gasification.
Physical	Includes the relocation of contaminated soils to landfill sites, or containment.
Solidification/vitrification	This involves encapsulating the contaminants within a monolithic structure that is with or without chemical fixation. This technology makes use of very high temperatures in fusing contaminated materials.
Integrated remediation techniques	This is a combination of different methods in remediation of cites, this approach could avoid the disadvantages posed by single methods hence leading to better results.

(adapted from Gan et al., 2009; Sayara et al., 2010; Conteras-ramos et al., 2005).

Table 2. 5 Potential of established and emerging technologies for treating PAHs contaminated field soils

Site	Technology description	Volume/area of treated soil	Initial PAH concentration (mg Kg-1)	Time (days)	PAHs	% PAH remediated	Study type	References
A. Thermal treatment - Incineration and thermal desorption [c]								
[b] Superfund, US	Incineration	142,000,000 kg	1000	480	Nap, Ace, Acy, Flu, Phe, Ant, Flt, Pyr, BaA, Chr, BbF, BAP	90	F	Acharya and Ives (1994)
[a] Former wood treatment, US	Thermal desorption	29,800,000 kg	30.6	130	BAP	99.9	F	Baker et al. (2007)
[a] Lampblack residuals, US	Soil venting thermal desorption	70 kg	1000	35	Nap, Phe, Flt, Pyr, BaA, Chr, BbF, BkF, BaP, BghiP, IcdP	90	B	Hosseini (2006)
B. Physical treatment - Soil washing/solvent extraction [c]								
[b] Bedford, UK	Soil washing with water and mixed organic solvents	1 g	11,600	1	Nap, Flu, Flt, Pyr, BbF, BkF, BaP, IcdP, BghiP, PHE, AnT, Pyr	96.3	B	Khodadoust et al. (2000)
[b] MGP, China	Extraction with sunflower oil	150 g	5453	7	Flu, Phe, Ant, Flt, Pyr, BaA, Chr, BbF, BkF,	81-100	B	Gong et al. (2005)

					BaP, DahA, BghiP, IcdP			
[b] 30-year-old MGP, France	Extraction with cyclodextrin	50 g	655	7	Phe, Ant, Pyr	99	B	Viglianti et al. (2006)
[b] Former chemical plant, Italy	Soil washing with humic acid	10 g	4560	1	Total PAHs (individuals not specified)	90	B	Conte et al. (2005)
C. Chemical treatment - Chemical oxidation [c]								
[b] MGP, US	Oxidation with Fenton reagent	9 kg	1164	40	Chr, BaA, BbF, BkF, BaP, IcdP, DahA	87-95	P	Pradhan et al. (1997)
[b] Former steel manufacture, France	Oxidation with KMnO4	10 g	1550	4	Nap, Ace, Acy, Flu, Phe, Ant, Flt, Pyr, BaA, Chr, IcdP, BbF, BkF, BaP, DahA, BghiP	70	B	Lemaire et al. (2013)
D. Biological treatment [c]								
[b] POPILE superfund, US	Open land farming units (tilling and nutrient addition)	0.46 m (depth) 1.22 m (width) 6.1 m (length)	13,000	730	Ace, Flu, Phe, Ant, Flt, Pyr, Chr, BaA, BbF, BbF, BaP	91-95	P	Hansen et al. (2004)
[b] Creosote treatment plant, US	Static pile compost system with poultry manure	350 kg	1086.9	570	Nap, Ant, Phe, Flu, Pyr,	98	B	Atagana (2004)

[a] Oil refinery, Serbia	Biopile	2.7 m3	3.6	500	Chr, Flt, BaP Nap, Ace, Acy, Flu, Phe, Flt, Pyr, BaA, Chr, BbF, BkF, BaP, DahA, IcdP, BghiP	77	F	Maletic et al. (2009)
[b] Agricultural land, China	Microbe assisted phytoremediation by alfalfa	1.5 kg	10.1	90	Nap, Ace, Acy, Flu, Phe, Flt, Pyr, BaA, Chr, BbF, BkF, BaP, DahA, IcdP, BghiP	51	G	Teng et al. (2011)
[b] Gas work, Australia	Vermiremediation	5 kg	11,820	84	BaA, Chr, BbF, BkF, BaP, DahP, BghiP	70-90	B	Sinha et al. (2008)
E. Integrated approaches								
[b] Oil treatment plant, Mexico	Enhanced system of biostimulation/bioaugmentation with filamentous fungi	10 g	7560	35	Nap, Ace, Phe, Ant, Flt, Pyr, Chr, BkF, BaP, IcdP	50-70	B	Mancera-Lopez et al. (2008)
[b] Former wood treatment facility, US	Landfarming with bioaugmentation and biostimulation	-	13,000	480	Ace, Flu, Phe, Ant, Flt, Pyr, BaA, Chr, BbF, BkF, BaP	87	B, P	Straube et al. (2003)

[b] Farm, Canada	Multiprocess phytoremediation system e land farming þ bioaugmentation þ phytoremediation	1 Kg	500-3000	120	Nap, Ace, Acy, Flu, Phe, Ant, Flt, Pyr, BaA, Chr, BbF, BkF, BaP, DahA, IcdP, BghiP	55-80	B	Huang et al. (2004a,b)

[a] In-situ, [b] Ex-situ, [c] Established technology, [d] Emerging technology; B - Bench-scale; P - Pilot-scale; G - Greenhouse study, F - Field-scale. (Adapted from Kuppusamy et al., 2017)

Juwarkar et al. (2010) highlighted the costs of several remediation technologies as seen in Table 2.6. The cost to run an *in-situ* remediation is far less than that of *ex-situ* remediation, and ranges from $30-$100 per cubic meter (Van Cauwenberghe and Roche, 1998). Based on the conditions at site of contamination, the remediation cost will vary. According to USEPA (1995) the cost of running a slurry phase remediation is $170 per ton, while solid phase bioremediation such as biopiling would cost approximately $260 per cubic meter, vermiremediation is relatively one of the most cost-effective technologies of bioremediation, costing between $20-$50 per ton contaminated soil (USEPA, 2018).

Contrary to conventional remediation technologies such as incineration that are expensive to run and less eco-friendly, bioremediation has been regarded as a safe method in remediating contaminants such as VOCs, PAHs, PCBs, pesticides as well as organic solvents. By transforming contaminants into less toxic forms, bioremediation remains the most sought eco-friendly remediation technologies, where they represent an average of 25% of technologies explored in the remediation of crude oil contaminated land (Beskoski et al., 2011; Semple et al., 2001; Othman et al., 2011; Mohan et al., 2006).

Table 2. 6 A comparison between remediation cost in soil

Treatment	Cost ($/ton of soil)
Biological	5-170
Chemical	12-600
Physical	20-170
Solidification	17-170
Thermal	30-750

(Source: Juwarkar et al., 2010).

Bioremediation involves soil organisms and microorganism activities. The success of these activities are based on several factors which includes the type and concentration of contaminant, availability of macro and micro nutrients in soil, toxicity of contaminant, bioavailability and bioaccesibility of contaminant as well as availability of activated enzymes (Ward et al., 2003). The outcome of all bioremediation technologies is based upon taking all

the site-specific parameters into account. Inadequate monitoring of bioremediation parameters however could lead to adverse effects such as more toxic intermediate products such as epoxides that could be more potent in the environment (Juwarkar et al., 2010).

Remediation standards are much more established in developed nations in regard to the quality of treated soils and methods of remediation. In addition, ecotoxicological tools are used in assessing the quality of methods that are explored in remediating soils. This tools not only take into account the toxicity of the parent compound, but also the intermediate products that are formed in the process, making the assessment very useful (Beskoski et al., 2012). Overall bioremediation has an advantage of remediating contaminants that are readily available into less toxic forms, unlike non-biological technologies that could possibly leave traces of minute but bioavailable concentrations of contaminants in the soil with potential of biomagnification in the food chain (Beskoski et al., 2012).

2.3 FEATURES OF PAHs

2.3.1 STRUCTURE, PHYSICAL AND CHEMICAL PARAMETERS OF PAHs

PAHs are compounds made up of two or more fused benzene rings that are either clustered or linear, containing hydrogen and carbon atoms (Gan et al., 2009). PAHs are comprised of several compounds, although the USEPA have highlighted a sub-grouping (EPA16) which represent the 16 top PAHs that have been categorized into having carcinogenic, mutagenic or teratogenic properties, and of the EPA16, 7 have been classified has being carcinogenic to human (Li et al., 2008).

PAHs containing 2-3 rings or LMW PAHs have been reported to have acute toxic effects to several organisms. However, their carcinogenicity has not been confirmed. In contrast, HMW PAHs containing 4-7 rings have been reported to be carcinogenic to organisms such as birds and mammals (Gan et al., 2009). The physical and chemical properties of the EPA16 priority pollutants are highlighted in Table 2.7. The most important parameter as highlighted by INCHEM (1998) is the water solubility of these contaminants which reduces availability of the contaminants in remediation, solubility of contaminants decreases as the PAH rings increase. Hence HMW PAHs are less readily available for degradation compared to LMW PAHs (Franco

et al., 2006; Cerniglia, 1992). The organic carbon partitioning co-efficient (log K_{oc}) is also used to indicate the bioavailability of PAHs for degradation and is directly proportional to the molecular weight of the PAH; as the PAH weight increases or decreases, the log K_{oc} also increases. Similarly, the octanol-water partition coefficient (log K_{ow}) is also directly proportional to the molecular weight of PAHs and being used to indicate the potentials of a compound adsorbing to the soil matrix resulting in bioaccumulation and biomagnification (Gonzalez, 1992; Juhasz and Naidu, 2000; Baumard et al., 1998).

The physico-chemical properties of PAHs affect their behavioural patterns even under the same conditions, and thus could impact on their transformation in bioremediation.

2.3.2 PAHs SOURCES

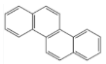
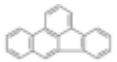
PAHs are ubiquitous in the environment being present in soils, air, water and groundwater (Bamforth and Singleton, 2005). Anthropogenic activities remain one of the leading causes of PAH entry into the environment, while some natural means of entry include volcanic eruptions or forest fires. The main activities leading to PAH entry are accidental oil-spills from mining, vehicular emissions, incomplete combustion of fossil fuels and incineration (Maila and Cloete, 2002; Kafilzadeh et al., 2000; Johnsen et al., 2005, Contreras-Ramos et al., 2005). Beskoski (2012) estimated that 0.1% of mined crude oil is accidentally discharged into its surrounding environment annually leading to contamination. Industrial activities that make use of fossil fuels generate high concentrations of PAHs which can be seen in their surrounding environments. Such industries include, petroleum refineries, thermal power generation, wood preservation and iron smelting companies (Zhang et al., 2011).

Table 2.8 highlights the data produced by Juhasz and Naidu (2000), showing the concentrations of PAHs as produced from various industrial activities and how it is reflected in their surrounding environmental soils. Wood preservation sites generates a high concentration of PAH as seen from their surrounding soils which is 3-4-fold higher than the soils from creosote production areas and as high as 20-fold compared to soils from gas manufacturing. Interestingly, concentrations of HMW PAH in the same soils from wood preservation sites though very high, in total PAHs are very low while those from gas manufacturing and creosote production are rather high. Thus, the source of contamination is

crucial in determining the concentration of the individual PAHs present in the contaminated site, and generally this needs to be taken into account when selecting a remediation technology to achieve a successful remediation (Antizar and Ladislao et al., 2004).

Table 2. 7 EPA16 physico-chemical parameters and structure (adapted from Antizar-Ladislao et al., 2004; INCHEM, 1998).

PAH	MP[a]	Formula [a]	Structure [a]	Melting point (° C) [a]	Boiling point (°C) [a]	Log K_{ow} [a]	Water solubility at 25°C (µg L^{-1})[b]	Vapour pressure (Pa at 25°C) [a]	Henry's law constant at 25°C (KPa) [b]
Naphthalene	128	$C_{10}H_8$		81	218	3.00-4.00	3.17×10^4	10.9	4.89×10^{-2}
Acenaphthylene	152	$C_{12}H_8$		95	270	3.70	-	5.96×10^{-1}	114×10^{-3}
Acenaphthene	154	$C_{12}H_{10}$		96.2	279	3.93-5.07	3.93×10^3	5.96×10^{-1}	1.48×10^{-2}
Fluorene	166	$C_{13}H_{10}$		115-116	294	4.18	1.98×10^3	8.86×10^{-2}	1.01×10^{-2}
Anthracene	178	$C_{14}H_{10}$		218	342	4.46-4.76	73	2.0×10^{-4}	7.3×10^{-2}
Phenanthrene	178	$C_{14}H_{10}$		100.5	338	4.45	1.29×10^3	1.8×10^{-2}	3.98×10^{-3}
Fluoranthene	202	$C_{16}H_{10}$		108.8	383	4.90	260	2.54×10^{-1}	6.5×10^{-4}
Pyrene	202	$C_{16}H_{10}$		150.4	393	4.90	135	8.86×10^{-4}	1.1×10^{-3}
Benzo(a)anthracene	228	$C_{18}H_{20}$		160.7	425	5.61-5.70	14	7.3×10^{-6}	1.2×10^{-6}

Chrysene	228	$C_{18}H_{20}$		253.8	431	5.61	2	5.7×10^{-7}	6.7×10^{-7}
Benzo(b)fluoranthene	252	$C_{20}H_{12}$		163.3	481	6.57	1.2^{11}(20 ° C)	-	5.1×10^{-5}
Benzo(k)fluoranthene	252	$C_{20}H_{12}$		215.7	480	6.84	0.76	-	4.4×10^{-5} (20 ° C)
Benzo(a)pyrene	252	$C_{20}H_{12}$		178.1	496	6.04	3.8	8.4×10^{-7}	3.4×10^{-5} (20 ° C)
Dibenzo(a,h)anthracene	278	$C_{22}H_{14}$		266.6	535	5.80-6.50	0.5 (27 ° C)	3.7×10^{-10}	7×10^{-6}
Indenol(1.2.3-cd)pyrene	276	$C_{22}H_{12}$		163	536	7.66	62	-	2.9×10^{-5} (20 ° C)
Benzo(g,h,i)pyrelene	276	$C_{22}H_{12}$		278.3	542	7.23	0.26	6×10^{-8}	2.7×10^{-5} (20 ° C)

Where [a] = Antizar-Ladislao et al., 2004, and [b] = INCHEM, 1998.

Table 2. 8 Concentrations of PAHs recovered from different activities (adapted from Juhasz and Naidu, 2000; Antiszar-Ladislao et al., 2004)

PAHs (mg Kg^{-1})	CP[a]	WP[a]	WP[b]	SFMn[a]	GW[a]	PC[b]	MGP[a]	MGP[b]	SFMs[a]	COGEMA[a]
Naphthalene	1131	500	3925	6494	-	186	10	97	673	-
Acenaphthylene	33	-	49	3651	-	-	6	28	79	28
Acenaphthene	-	7100	1368	21319	2	43	46	49	705	2
Fluorene	650	1900	1792	2497	225	87	16	14	32	4
Anthracene	1595	6400	4434	7902	379	156	84	26	266	51
Phenanthrene	334	2500	3307	1440	156	53	6	11	2	58
Fluoranthene	682	2200	1629	10053	2174	137	62	73	419	195
Pyrene	642	1000	1303	9481	491	99	51	47	-	173
Benzo(a)anthracene	-	300	171	1670	317	33	20	16	496	88
Chrysene	614	1000	481	2392	345	-	21	15	305	52
Benzo(b)fluoranthene	-	560	140	2271	498	-	48	21	513	99
Benzo(k)fluoranthene	-	-	-	-	-	-	-	-	-	-
Benzo(a)pyrene	-	60	82	536	92	15	10	14	224	106
Dibenzo(a,h)anthracene	-	-	23	120	207	-	21	7	64	46
Indenol(1.2.3-cd)pyrene	-	<30	-	192	2451	12	5	33	27	-
Benzo(g,h,i)pyrelene	-	<30	-	-	-	-	16	-	-	-
Total PAHs	5863	23600	18704	70633	7337	821	352	451	3815	974
% HMW PAHs	33	22	20	38	90	36	72	50	54	78

Note: CP- creosote production site, WP- wood preservation, SFMn- superfund site Minesota, GW, gas work, PC- petrochemical sites, MGP, manufacturing gas plant, SFSMs- superfund Mississippi, COGEMA- French MGP site. concentration of total PAHs does not determine the total PAHs present thus influencing choice of renediation. Source: [a] (Antizar-Ladislao et al., 2004) [b](Juhasz and Naidu, 2000)

2.3.3 BIOAVIALABILITY OF PAHs IN THE ENVIRONMENT

Once in the environment, PAHs pose toxic effects to human and animal health. This happens through inhalation, ingestion or dermal contact with the contaminant (Othman et al., 2011). The World Health Organization (WHO) has categorized some of these PAHs (4-7 rings) as having cancerous as well as several other adverse effects (Antizar-Ladislao et al., 2004).

PAHs adsorb on to solid matter with organic matter due to their hydrophobic nature where they then have the ability to bioaccumulate and biomagnify in sediments, soils and animals and become persistent in the environment (Ma et al., 2005; Cerniglia, 1992; Gan et al., 2009).

The time of contact between the PAH and the soil plays a crucial role in the bioavailability of the contaminant. The contaminant is transported to soil micro-pores where it is incorporated into the matrix and becomes stable in a solid phase thus reducing its availability for degradation, a process referred to as soil ageing. The mechanism of soil ageing that aids the locking of PAHs in soil is known as sequestration, which involves two stages, diffusion and sorption. This process limits the availability of PAHs to readily available organisms and microorganisms for remediation (Wick et al., 2011; Mohan et al., 2006; Semple et al., 2001).

PAHs' hydrophobic nature and low solubility makes them reliant on the sorption mechanism in the soil for transport, hence soils with high organic matter attract organic pollutant where the pollutant becomes locked and stable and less bioavailable (Bathi et al., 2007).

The adverse effects of PAHs as highlighted in Section 1.3 has led to several investigations into methods or technologies to explore in remediating contaminated land.

2.4 IMPACT OF CRUDE OIL ON HUMAN HEALTH AND ECOSYSTEMS

Crude oil can cause eye, nose, skin and lung irritation in humans, excessive exposure to crude oil on short term basis could also increase chances of drowsiness, nausea headaches, and rise in blood pressure, asphyxiation, or even lung damage (Agnello et al., 2014).

Due to the diversity of the compounds present in crude oil, with emphasis on PAHs, crude oil can be carcinogenic, mutagenic and teratogenic with respect to human health (Bamforth and Singleton 2005; Grant et al., 2005). Until recently, the carcinogenic impact of some crude oil components such as diesel on humans was not clearly known. However, the World Health

Organization have categorized crude oil as a 'major cancer risk' in the same categories as asbestos, arsenic and mustard seed (NTP, 2011).

The impact of oil spills in terrestrial and aquatic environments are felt at similar magnitudes in the environment, and this pose either direct, indirect or acute (short term) and chronic (long term) impacts on living organisms.

Effects of oil spills can persist in the environment long after spills, some lasting for up to 30 years (Peterson et al., 2003). This could can a shift in the population structure, species, diversity, and distribution of organisms, loss of habitat, and loss of prey species which could alter the food chain structure (Peterson et al., 2003). The impact of these spills is most felt in areas that are shielded away from weathering processes such as shorelines and soft surfaces.

Crude oil causes harm to wildlife through inhalation, ingestion and absorption, in aquatic environment, floating diesel may affect planktons which includes fish eggs, algae as well as eggs of other vertebrates. In turn fish that feeds on these organisms come in contact with ingested crude oil, and the contamination may result in increased concentration in higher trophic levels also known as biomagnification) (U.S fish and wildlife service, 2004).

It is of key importance to note that the quantity of an oil spill is not totally reflective of the impact it poses, since a great loss could come about from a small spill in ecologically sensitive areas (Freedman, 1989). With only a few tons of crude oil spill in the environment at certain seasons could result in a massive loss of species (Kingston, 2002). An example is the Exxon Valdez 1989 spill that spilled 245,000 barrels of oil and claimed the lives of 250,000 birds mostly murres (Piatt and Ford, 1996). Kornberg (1982) also reported a little operational spill oil bilge from a tanker that took the lives of 30,000 sea birds near Norway in 1981 at a time where they are seasonally present at this location. The Braer spill on the other hand that released 595,000 barrels of oil into the surrounding environment claimed only 1,500 bird lives despite its larger quantity (Heubeck, 1997). A possible explanation for the variation in response as reported by Kingston (2002) was attributed to the breeding seasons, which would either increase or reduce the impact of the oil spill. Hence with only a few thousands of barrels of oil released into the environment could cost the extinction of an entire species of organisms.

Birds (particularly diving ones) and fish happen to be two most susceptible species in aquatic oil spills (Al-Majed et al., 2012). Several authors have also reported the adverse effects most chemical dispersants used to control the spills have had on flora and fauna (e.g. Al-Majed et al., 2012). Environments surrounded by petroleum refineries as well as that surrounding oil tankers are well exposed to constant contamination from frequent oil spills or waste water discharge from oil operations (Freedman, 1989). Several cases of both carcinogenic and non-carcinogenic diseases have been reported in fish and shellfish (Fabacher et al., 1991).

Freedman (1989) observed in their study that vegetation with meristematic tissues were not completely damaged after exposure to oil spills, instead they had a substantial regeneration post exposure. On the other hand, lower plants such as bryophytes exposed alongside boreal forest vegetation were susceptible shortly after exposure and almost completely wiped out (Freedman, 1989). There are several cases where recovery of polluted sites and species prove to be almost impossible, such a case is the Amoco Cadiz spill that wiped an entire population of amphipod (sand hopper), which were the dominant species found in this area. Recovery in this site was well over 10 years, this is due to the highly sensitive nature of the sand hoppers (Kingston, 2002).

Impact of oil spills in the environment as well as on human lives are more keenly felt in third world countries than in developed countries. This is due to several factors such as response time to spills, insufficient infrastructures, technologies, or skills for mitigating oil spills. Other contributing factors include the lack of awareness in local population, as well as the lack of enforcement of environmental policies in the communities. One significant example is the SHELL oil spill in the Bodo community in Ogoni land Rivers state Nigeria in 2009, where millions of oil barrels was released into the environment contaminating lands, water and groundwater (Tregaskis, 2013). The spill not only hindered all farm production but also made its way into the rivers killing a great number of aquatic life, thereby disrupting fishing activities which is the major source of livelihood of the locals from this community.

Due to the magnitude of the spill, the water from the river (which is used by locals on a daily basis for all purposes including drinking) and agricultural lands are completely polluted, however due to the levels of poverty in this areas, some of the locals are still forced to use fetch water from this same river and use for same domestic purposes and harvest crops from

lands as before it was contaminated not fully aware of the deleterious impact it poses to their health (Aljazeera, 2018).

The aftermath effect of oil spills poses more severe effects if the clean-up response time is not immediate. Both Kingston (2002) and Ba-Akdah (1996) stated that there is a possibility of bioaccumulation of contaminants in the body tissue of exposed organisms which could pose somewhat of a hazardous effect in the environment. This in turn could result in several cases of biomagnification along the food chain in the environment (this comes about by feeding upon organisms that have bioaccumulated contaminants in their tissues, thus leading to the magnification of hazardous contaminants as further feeding occurs in the food chain), this is a product of a long-term exposure to crude oil (Samantha et al., 2002).

Asides from the toxicity that these pollutants pose to human health, they are also great threat of fire outbreak as their compounds are volatile and highly flammable (Volkering et al., 1998). Kvenvolden and Cooper (2003) reported that the estimate of crude oil spillage per year to be 600,000 metric tons with an uncertainty range of 200,000 metric tons per year. On entering the environment, be it by accident or through human activities, they lead to soil and water pollution (Holliger et al., 1997). Due to the regular occurrence of oil spillage, pollution due to the introduction of petroleum as well as its derivatives have been termed the most prevalent environmental problem which needs to be cleaned up (Pirollo et al., 2008).

Innovative approaches towards soil treatments both *in-situ* and *ex-situ* have globally been on the rise over the last few decades. The major advantages of bioremediation technologies the limited disturbance of site, affordable cost and eco-friendly approach as compared to its conventional rivals such as soil vapour extraction, furthermore, bioremediation approaches are coherent with the sustainable development strategy that is a part green engineering (Sayara et al., 2010).

Bioremediation has gained a lot of attention over several years because of its eco-friendly nature as well as capital costs. Some of these low-cost technologies include phytoremediation, bioslurping, land farming, composting, vermiremediation and many more. The major challenge in bioremediation of PAHs is the bioavailability of the hydrocarbons to the degrading organisms. Biosurfactant has been suggested as a dispersive agent to enhance

bioremediation (Cernilagia, 1997; Franco et al., 2006; Singh et al., 2007) biosurfactant is believed to be biodegradable, less toxic and low-cost to produce. However, there appears to be a gap in knowledge regarding the actual toxicity biosurfactant poses when combined with organic contaminants at the cellular level.

Several conventional techniques have been explored in remediating contaminated land. These techniques can be grouped into different categories such as thermal treatment, chemical treatment and biological treatment, with a combination of groups in some cases. This research intends to achieve an accelerated yet eco-friendly and efficient removal of PAHs in spiked samples that can be applicable on a larger scale of contaminated sites in Nigeria.

2.5 EARTHWORMS AND VERMIREMEDIATION

Over the last few decades, several bioremediation techniques have been developed and explored in mitigating oil spills and its damages. Some of these bioremediation techniques include bioslurry, land farming, composting, bioslurping and bioventing/biosparging. Table 2.9 and 2.10 highlights the general advantages and disadvantages of bioremediation.

Table 2. 9 Advantages and disadvantages of bioremediation techniques.

Advantages	Disadvantages
They are cost effective, costing less than conventional techniques such as incineration.	Contaminants could be unavailable for microbial degradation.
It is a natural process that occurs with the aid of indigenous microbes transforming contaminants into other forms.	Extrapolation of results from laboratory to field could be difficult.
Several bioremediation techniques are *in-situ*, thus reducing risk of transferring contaminants.	Process of bioremediation can be time consuming.
Bioremediation techniques are capable of transforming a wide range of contaminants into less toxic forms.	There have been concerns that by-products of bioremediation could be toxic.

Table 2. 10 Summarized advantages and disadvantages of bioremediation technologies

Technology	Examples	Pros	Cons	Factors to consider
In-situ	Bioventing Biosparging Bioslurping	Most cost effective Treats both soil and water Non invasive	Time of remediation Difficulties to monitor Environmental constraints	Presence of metals as well as other inorganic compounds Ability of indigenous microbes to degrade contaminants Environmental parameters Site-specific parameters Distribution of contaminants
Ex-situ	Landfarming Composting	Cost efficient, Procedure can be done on site	Bioavailability of contaminant Time of treatment Space for treatment	Same as above
Bioreactors	Slurry and aqueous reactors	Optimised degradation, Effective use of inoculants	Expensive to run Requires excavation of soil	Concentration of contaminants Bioaugmentation, Toxicity of amendments

Overall the major limitation of most bioremediation technology is time of remediation stemming from reduced bioavailability of contaminants. A channel of bioremediation which in the last few decades is the use of earthworms in remediation soil contamination (vermiremediation). These organisms have been reported as one of the most active organisms in the soil due to their burrowing activities and ubiquity in almost every soil type globally (Contreras et al., 2008; Hernandez-Castellanos et al., 2013, Dendooven et al., 2011).

Vermiremediation combines application that are environmentally sustainable, economically viable and socially acceptable. Earthworms are known to be environmental engineers because of their experience of living in soil alongside its associated physico-chemical/ biological parameters for over 600 million years (Contreras et al., 2005). Over the last few

decades, the role of earthworms as waste and soil engineers as well as promoters of plant growth has been and widely studied (Blouin et al., 2013). However, their roles in wastewater treatment and remediation of contaminated soils have not been fully explored. The applications of earthworm that includes vermicomposting, vermifilteration, vermi-agroproduction and vermiremediation are cost effective means to solve social economic environmental and health problems.

Earthworm bioengineering technologies are self-regulated, self-promoted and self-improved, with very little to no energy being required and a wormery is relatively easy to setup, operate and to maintain. They are reported to be more effective than other forms of bio-production, bio-conversion and bio-degradation technologies (Appelhof, 1997; Wang, 2000).

2.5.1 EXAMPLES OF VERMIREMEDIATION

Hartenstein et al. (1980) reported the ability of earthworms in bioaccumulating very high concentrations of cadmium (Cd), lead (Pb), mercury (Hg), calcium (Ca), manganese (Mn), zinc (Zn) and iron (Fe) in their body tissues. In particular up to 100 mg Kg^{-1} dry weight of cadmium has been reported in the body tissue of earthworms where it binds with the protein metallothionein. *L. terrestris* has been reported to bio-accumulate up to 90-180 mg/g lead*, L. rubellus* 2600 mg/g and *D. rubida* 7600 mg/g of dry weight.

Ma et al. (1995) studied the effect of *L. rubellus* on the remediation of soil containing two of USEPA16 phenanthrene and fluoranthene at 100 µg Kg^{-1} of soil. They observed a significant reduction of both hydrocarbons in experiments with earthworms in them that occurred at a faster rate compared to their controls without earthworms. Up to 86% of phenanthrene was removed after 8 weeks of exposure. Using different concentrations, Contreras-Ramos et al. (2006) studied the uptake and removal of three PAHs phenanthrene, anthracene and benzo(a)pyrene for 11 weeks using *E. fetida*. There was a 2-fold reduction in the concentration of anthracene on addition of earthworms with an overage of 51% while microbes alone were able to remove 23% within the same timeframe. There was a 43% reduction of BAP compared to the 13% reduction reported in the control and phenanthrene was completely removed in an experiment with earthworms and the controls removed 77% of phenanthrene within the same time.

Schaefer et al. (2005) reported that the increase in the catabolic activities of microorganisms in the gut of *E. fetida* aided the removal of 91% total hydrocarbon (from 1074 mg Kg^{-1} to 96 mg Kg^{-1} of soil) of crude oil in 56 days. Bolan and Baskaran (1996) studied the effects of earthworms vermicast on the removal of radiolabelled herbicide C^{14} – atrazine, C^{14} – metasulforon methyl, C^{14} – 2,4 dichlorophenoxyacetic acid in soil using *L rubellus* and *A. caliginosa*. They reported a greater absorption of the herbicides by the vermicast compared to the control.

Rodriguez-Campos et al. (2019) evaluated the removal of total petroleum hydrocarbons (TPHs), alkanes and PAHs using a combination of phytoremediation (*Panicum maximum*), bioaugmentation (encapsulated bacterial consortium) and vermiremediation (*Pontoscolex corethrurus*) for 112 days. They observed a significant increase in earthworm biomass (2 – 2.6-fold) in the presence of bioaugmented bacteria. Furthermore, they reported that most of the PAHs and alkanes were removed by day 28 of the treatment. They reported PAH removal of 54-62% (2 and 3 rings), 54-92% (4-rings), 80% (5-rings) and 70% (6-rings) using a combination of these three bioremediation technologies

Sinha et al. (2008) studied the effect of earthworms on PAHs on contaminated soils from former gas industry in Brisbane Australia where coal was used in the production of gas. Recorded concentration of PAH in the soil was 11,820 mg Kg^{-1} of soil. After 12 weeks they reported a 60% removal of PAHs compared to the 47% removed by the control.

2.5.2 BIOLOGY OF EARTHWORMS

As earthworms vary greatly in species, so does their environmental needs, their ecological niches, their behaviours, and life histories. Depending on soil moisture, temperature, food as well as other suitable environmental factors, earthworms could produce cocoons all year round, however the species and climate determines the number of cocoons produced per season. Earthworm bodies are made up of 65% proteins (of which 70-80% are high grade lysine rich proteins on dry weight basis), 14% carbohydrates, 14% fats and 3% ash. Depending on species and environmental factors, their lifespan could vary between 3-7 years. There is a direct correlation between the number of cocoons produced in all species and the extent to which earthworms are exposed to adverse environmental factors such as extreme

temperature, predators and desiccation. With burrowing species producing fewer number of cocoons and epigeics producing more cocoon (Satchell, 1967). The time of earthworm hatching, up until the time it reaches sexual maturity is also dependent on the species. Butt (1993) reported a period of 3 months to reach maturity in *L. terrestris* where temperature plays a part in the time it takes a cocoon to hatch (15 – 20 ^{o}C). Satchell (1967) also suggested that lumbricids in the field have a short life cycle that spans at most a few months based on the constant exposure to environmental factors as opposed to their normal longevity that could span between 4-8 years. Species such has *A. longa* and *L. terrestris* have been kept in captivity for as long as 10 and 6 years respectively. The earthworms' prominent clitellum is active during the breeding period which is only half the adult lifespan of an earthworm.

Earthworms have similar physical structures across a wide range of species (Sinha et al., 2002; Sherman, 2003). They belong to the Phylum Annelida that means ringed. The rings that can be seen all around the earthworms' body are called segments. *E. fetida* has about 95 segments while *L. terrestris* has about 150 segments. Their bodies have no protruding appendages or sense organs and they are streamlined, this supports their gliding movement in the soil. The circulatory, nervous, excretory, digestive, muscular and reproductive systems in earthworms are well developed. Earthworms' anterior end contains a prostomium, a lobe that is covering the mouth and aids burrowing of earthworms. Usually a pair of setae (bristles) is found on each segment of the earthworm which can be retracted or extended, which function in movement of the worms. The skin glands secrete lubricating mucus helping the gliding movements through the soil as well as stabilizing their casts. Earthworms breathe through the skin hence why the skin needs to remain moist to facilitate respiration. Due to their aquatic ancestry, earthworms can thrive in water for months and will only die if they dry out. Earthworms are photosensitive hence why a brief exposure to sunlight would paralyze them and prolonged exposure is fatal to them (Edwards and Bohlen, 1996).

2.5.3 DIGESTIVE SYSTEM

Earthworms are sometimes referred to as a giant gut (Edwards and Bohlen, 1996), because the gut extends throughout its body (Fig 2.3). Worms ingest soil and food from the soil surface, where it passes through the gut. Ingested matter is initially attacked by strong muscles where

it is mixed and moved through the tract while enzyme-filled fluids are being produced and infused with these materials. The digestive fluids then release a variety of microorganisms, amino acids and sugars in addition to decomposing plants and animal and soil that was ingested. The worms' intestinal membrane then absorbs the finer particles where it utilises it as energy source and for cell production (Liebeke et al., 2015).

Earthworms are known to have a rudimentary alimentary canal that extends all the way from its mouth to the anus (Edwards and Bohlen, 1996). Food is taken up from the mouth which is a tiny crescentic aperture that is located just below the prostomium in the first segment, where it is transferred into the pharynx (Liebeke et al., 2015). The pharynx which is shaped like a pear is a muscular chamber that extends through to the 4th segment. The salivary gland is located on the dorsal inner side of the pharynx. Pharynx aids the food being pumped into the mouth where it is mixed with saliva that is secreted in the salivary glands. Earthworm saliva is made up of mucin and proteolytic enzymes, where they both function by lubricating and digesting food respectively (Konig and Varma, 2006). Food is then transferred to the oesophagus (a narrow tube that is thin walled and extends to the 7th segment), then into the crop where the food is temporarily stored and moistened. As soon as food gets moistened, it is transferred to the gizzard that is oval in shape and thick walled and very muscular, this extends to the 9th segment of the body. In addition to having same function as the crop, the gizzard grinds the food into finer particles.

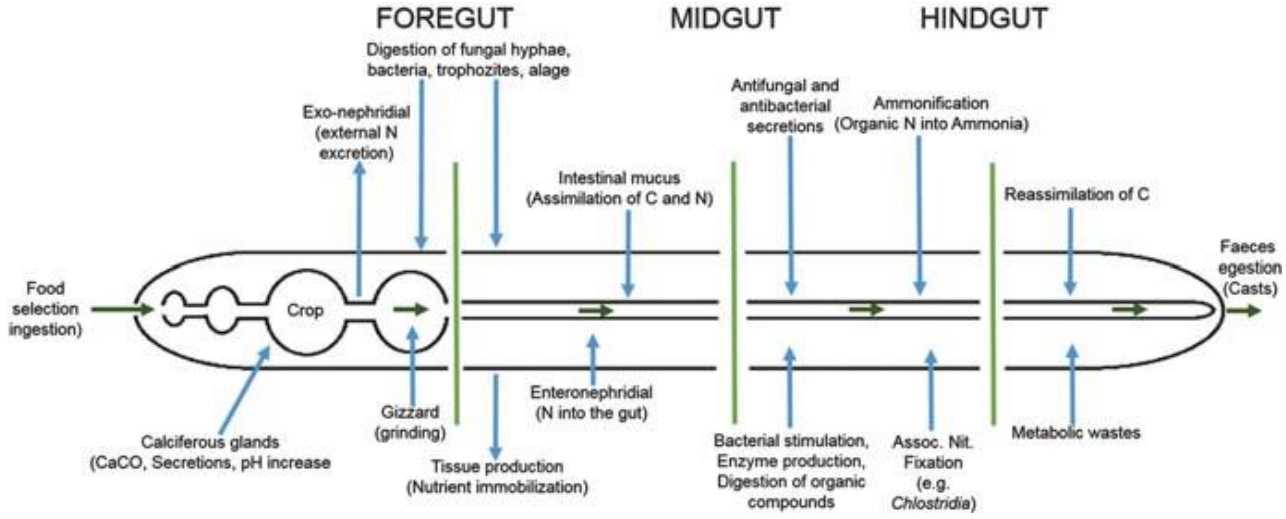


Figure 2. 3 Schematic diagram representing different internal component of the Drilosphere, from uptake to excretion in earthworms (reproduced from Brown et al., 2000).

Circular contraction of the gizzard muscles results in the physical digestion of the food, and the gizzard naturally contains grits or gravel to aid this task. Food then moves into the gut which as mentioned spans the majority of the body. The gut extends from the 15th segment, all the way to the anus. The intestinal caeca can be located on the 26th segment as a protrusion towards the 22nd segment. The internal intestine is folded, and these folds are referred to as villi (Hickman and Reid, 2005), and a typhlosole (large villus) is located on the mid dorsal side. A yellowish chloragogen tissue is found all around the intestine and dorsal vessels, this functions for fat and glycogen synthesis, similar to functions of liver cells in higher organisms. Chloragogen cells are released into the coelom when they are filled with fat where they are cells that freely float and called eleocytes that function in transporting materials to various body tissues. Earthworms have folding intestinal walls which allows for a larger surface area for soil and organic matter and nutrients. Soils and undigested products are excreted in the form of cast from the anus where it is acid neutralized, revitalised and rich in nutrients (Edwards and Bohlen, 1996).

2.5.4 MECHANISM OF DIGESTION

Nutrients are derived from the organic matter present in soil which could be in the form of compost, decomposing plants or food, rotifers, protozoa, bacteria, fungi, nematodes and other microorganisms. Some species are even particular about the types of decomposing food they eat and the conditions which they are in. Table 2.11 show the food and soil consumption rates of selected earthworms. The most popular species whose digestive process has been well studied is *E. fetida*. Segment 1-14 'the reception zone' secrets acid mucus which contains amylase, the oesophagus houses calciferous glands that secrets amorphous calcium carbonate particles. Segment 15-44 is the secretory zone; the gizzard is present in this zone where the soil or food is ground and passed to the intestine where it is absorbed into the bloodstream through intestinal epithelial cells and transported to various body tissues for use and storage. Segment 44 to the anus is the absorptive zone, here the undigested soil and food particles unused are wrapped up in peritrophic membrane and ready to be excreted as cast (Liebeke et al., 2015).

Earthworms are known to facilitate different processes, physically they are known as mixers, crushers and aerators, biologically they are known as incubators and stimulators in decomposition, and chemically they are known as degraders. The front Section of worms' function as grinders, alter the physical appearance of ingested soil or organic matter thus increasing chances of digestive enzymes for their functions. Soil or organic matter takes approximately 2.5-7 hours to pass from mouth to anus of *E. fetida* (Hartenstein et al., 1981). Earthworms and their gut microorganisms produce several essential enzymes that aid further digestion of ingested soil and organic matter. Excreted cast is very rich in nutrients and microbial population and further mineralization of the cast occurs by the aid of microorganisms. Generally, the bacteria that are present in the foregut aids digestion of organism matter, the actinomyces that are present in the mid gut through their antagonistic activities kills pathogens, and the fungi in the hind gut aids the binding of the waste as casts.

Table 2. 11 Earthworm substrate consumption

Earthworm species	Consumption rate (mg g^{-1})	Food substrate
Allolobophora longa	20	Soil
Eisenia fetida	10-5000	Activated sludge
Eisenia hortensis	200-20,000	Activated sludge with dead leaves
Eudrilus euginae	2000-5000	Activated sludge mixed along with sludge
Eudrilus euginae	3000-7000	Activated sludge
Lampito marutii	700-2800	soil
Lumbricus terrestris	27-80	Elm leaves
Lumbricus terrestris	10-30	soil
Octolasium sp.	29	Soil
Pheretima elongata	375-700	Potato peel
Eudrilus euginae	300-600	Potato peel
Eisenia fetida	300-600	Potato peel

Megascolex megascolex	715-1400	Press mud
Eudrilus euginae	625-1250	Press mud
Eisenia fetida	600-1200	Press mud
Megascolex megascolex	650-1300	Cow dung
Eudrilus euginae	1000-2000	Cow dung
Eisenia fetida	800-1650	Cow dung
Eisenia fetida	30-35	Garlic waste
Eudrilus euginae	30-35	Onion waste

(Adapted from Munnoli et al., 2010)

2.5.5 CLASSIFICATION

Earthworms have a broad diversity with different history, and inhabit different ecological niches, and also have been classified based on their feeding and burrowing activities into three distinct classes: epigeic, anecic and endogeic (Bouche, 1977) See table 2.12.

Table 2. 12 Ecological categories and niches of earthworms and their characteristic features and beneficial traits

Ecological category	Species	Ecological niche	Characteristic features	Beneficial traits
Epigeics	*E. fetida, E. hortensis, L. rubellus, L. castaneus, L. festivus,Eiseniella tetraeedra, Bismastus minusculus, B. eiseni, Dendrodilus rubidus, Dendrobaena veneta, D. octaedra*	Leaf litter compost, superficial soil layer	Small size. Uniform body pigments, active gizzard, high reproduction rate, short life-cycle, tolerant to xenobiotics, phytophagus	They are good biodegraders and nutrient releasers, they are efficient in producing compost, and they accelerate early decomposition of litter.
Endogeics	*Aporrectodea caliginosa, A. trapezoids, A. rosea, Millsonia anomala*	Top or sub-soil	Small-large in size, weak body pigmentation, medium length life cycle, moderate tolerance to xenobiotics, geophagus	Promotes changes in soil physical structure, utilizes energy from poor soils hence can be used for soil improvements
Polyhumic endogeic	*Octolasion cyaneum, O. lacteum*	Topsoil (A1)	Small size, unpigmented, burrows horizontally, rich soil feeder	
Mesohumic endogeic	*Pontscolex corethurus, Allolophobora chlorotica*	A and B horizon	Medium body size, unpigmented, burrows extensively horizontally, bulk soil feeder	
Oligohumic endogeic	*Aminthas sp.*	B and C horizon	Very large in size, unpigmented, burrows extensively horizontally, feeds on poor deep soil.	
Anecics	*L. terrestris, L. Polyphemus, A. longa*	Permanent deep soil burrows	Large in size, pigmented dorsally, burrows extensively horizontally, low reproduction rate, long life cycle, sensitive to xenobiotics, nocturnal, phytophagus	Improves relationship and movement of air and water from deep layers to the surface through the vertical burrows formed hence aids nutrient mixing.

(reproduced from Pathma and Sakthivel, 2012)

2.5.5.1 EPIGEICS

This type of earthworms are composting species capable of degrading organic waste rapidly breaking them down into finer particles by grinding them in their gizzard in their mouth which all earthworms possess. Epigeics dwell in the litter/top-sub soil surface, they dwell in organic rich soil horizons. They ingest the waste and soil and excrete holorganic faecal pellets. These are small sized earthworms that are well pigmented and also a very high metabolic and reproductive rate, which is representative of their adaptive qualities to variable environmental conditions. Other than increasing decomposition rate, based on these activities, this also affects the population of other surrounding inhabiting organisms (Dominguez et al., 2003). Microorganisms that grow in organic waste supplies the earthworm with nourishments and not the organic waste itself, and simultaneously they increase microbial activities in the waste in order to produce cast that are well fragmented and rich in active microbial population than the initial consumed waste. During this process, nutrients such as N, P and K are released and converted to readily available forms than the original waste. Gut retention time of waste in the earthworm is quite short, a few hours at most, and an average population of earthworms consume a huge quantity of organic matter at once. Epigeic earthworms represented largely by the *Eisenia* species do not build burrows and inhabit the organic matter rich segment of the soil. Since they do not burrow deep into soil, they are easy to culture and handle as well as they have high ability to adapt and withstand foreign parameters. However, this group though top soil dwelling groups could burrow between 0 - 20 cm of top soil which potentially makes them viable groups for degradation dependent on desired purpose of remediation sites.

2.5.5.2 ENDOGEICS

This group of earthworms inhabits deeper zones in the soil profile and they feed on soil and organic matter. With very little pigmentation, this species burrow extensively horizontally, creating burrows that are deep and branched and filled with cast materials. These species have the potential to burrow deep into soil (Satchell, 1983), with a long duration of time until they attain maturity and are more tolerant to starvation compared to epigeic species. Endogeic species are important for soil mixing, decomposition of roots and aeration of soil. Some of the species in this group include *A. caliginosa, A. rosea* and *Octolasion cyaneum*.

Species such as *Eudrilus euginae* construct deep burrows in which they remain the majority of the time where they feed on soil and the organic matter therein. Endogeics are the only group of earthworms that feed primarily on soil (geophagus), having burrowing activities that leaves the soil well mixed, aerated and structured.

2.5.5.3 ANECICS

These species of worms construct and inhabit temporary to permanent vertical burrows that could extend as deep as several metres down the soil profile. Permanent burrows have a micro-climate gradient, where the earthworms could inhabit the surface portions of the burrow or the deeper end of the burrow depending on the conditions of the soil. These species are nocturnal, tending to burrow to the surface of the soil at night to feed on surface litter or manure and to drag their food into their burrows. They also pass their cast at the surface of the soil. Some earthworm species have been seen to build their cast at the burrow openings which are called *middens*. These are made up of earthworm cast, soil as well as some parts of the surface litter. Some distinctive characteristics possessed by this species are their large matured body size, with darker colours at their anterior and dorsal ends, and they have a rather slow reproduction cycle. This species is very important in organic matter decomposition, nutrients cycling, and formation of soils and they optimise pedological soil processes globally. Examples of species belonging to this group are *L. terrestris, A. longa* and *A. trapezoids*. Some anecic species such as *L. terrestris* has been reported to aid degradation of organic pollutants in soil. This species construct burrows and feed on organic matter from soil surface which they convert into humus. If displaced from their burrows, this species cease growth and ultimately leads to mortality (Sherman, 2003).

2.6 MECHANISM OF VERMIREMEDIATION

Mining activities, use of land fill sites for disposal of toxic wastes, oil and gas drilling activities and continuous use of synthetic agro-chemicals on farmLands have led to the contamination of lands (some which are arable), groundwater and waterways.

A popular form of contaminated land remediation was soil excavation, which involves transporting polluted soil offsite where it is buried and monitored. However, this method does not treat the contamination and, there is the risk of leaching of contaminants from the

landfill to surrounding water or groundwater posing greater health risks. There are potential risks of landfill explosions from methane escape through crack openings in the site.

Earthworms have been used in different functions such as for land reclamation, land recovery and rehabilitation of soils with low levels of minerals, open cast mining soils, polder soils, cutover peats and closed landfills (Lowe and Butt, 2005). The earthworms' sphere of influence within the environment is termed the driliosphere. This includes the burrows, the above and below ground cast, the gut and the microbial community associated and all its processes, earthworm contact with the soil and the biological and physicochemical interactions associated with it (Brown et al., 2004).

Several researchers have investigated the roles potential roles earthworms play in environmental monitoring and sustainability as well as their positive roles in agro-ecosystems (Maenpaa et al., 2002; Dada et al., 2016). Earthworms have been reported to biodegrade municipal, industrial and agricultural waste (Fraser-Quick, 2002; Datar et al., 1997; Edwards, 1998), organochlorines and organophosphate pesticides(Gaveo et al., 2001; Haimi et al., 1992), heavy metals (Hartenstein et al., 1992; Contreras-Ramos et al., 2006; Dada et al., 2016), crude oil hydrocarbons (Martin-Gil et al., 2005; Njoku et al., 2016; Rodriguez-Campos et al., 2019), polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Ma et al., 1995; Singer et al., 2001; Sinha et al., 2008). Several earthworm species (especially *Eisenia* spp.) are resistant to several chemical contaminants including organic pollutants such as hydrocarbons and inorganics such as heavy metals in soil (Hernandez-Castellanos et al., 2013; Rorat et al., 2017). Azizi et al (2013) reported over 50% reduction in PAH concentration using *E. rubellus* exposed to PH, anthracene and BAP for 30 days. Azaripa et al (2013) observed the potential of *Eudrilus eugeniae* in reducing concentration of macro-elements such as magnesium and sodium ions, as well as salts like phosphates and nitrates in sheep manure. According to Dabke (2013), *E. fetida* seeded in soil reduced the chromium levels in soil with no mortality recorded. Furthermore, they reported the productions of cocoons after treatment which indicated improved soil conditions. Azaripa et al. (2013) reported a 50-80% reduction in concentration of trace elements and soluble salts in garden soil and sheep manure by *Eudrilus eugeniae*. Njoku et al. (2018) reported 50-80% reduction in concentration of dichlorvos pesticides using both *E. eugeniae* and *L. terrestris*. However, they further

reported the potentials of *E. eugeniae* performing better than *L. terrestris* species. It has been reported that earthworms bio-accumulate these contaminants in their body tissue and would either biodegrade or biotransform them into less toxic form with the aid of enzymes. Also, the gut of earthworms is said to be a microbial incubator hosting several microbial degrading species (Ramteke and Hans 1992; Brown et al., 2004).

Earthworms have the ability to bioaccumulate toxic chemicals in their body tissue. This can be illustrated using the 1976 Seveso plant explosion in Italy, with massive contamination of site with several contaminants including TCDD (2,3,7,8- tetrachlorodibenzo-p-dioxin) that wiped out several species of flora and fauna except earthworm species that survived and thrived the contamination. Earthworms in this area were seen to have bioaccumulated dioxin in their tissue and concentrated it over 14.5-fold above the environmental concentration (Satchell, 1983).

With the right conditions of moisture, temperature, organic food, earthworms can be seen to multiply and reproduce between 0.2 – 1 million organisms per hectare within a short time frame of about 3 months for vermiremediation (Bhawalkar, 1995).

Earthworms can uptake chemicals from the soil via two routes, either orally or via absorption through the moist body wall in the interstitial water. They either bio-accumulate the chemicals or biodegrade them into less toxic forms. Furthermore, they possess metallothioneins which are proteins that have a high capacity to bind heavy metals in earthworms. Earthworms also possess chloragogen cells that act by accumulating heavy metals absorbed by the gut, immobilizing them in the spheroidal chloragosomes and debris vesicle contained in the cell (Ireland, 1983).

2.6.1 MICROBIAL DEGRADATION MECHANISM

The earthworm drilosphere, which support remediation of contaminants is symbiotically synergistic with its gut and vermicast degrading activities. In microbial degradation, the first step of an intercellular attack is an oxidative process. The conversion of the organic pollutant in a step by step form to produce intermediates of the central intermediary metabolism

occurs via a peripheral degradation pathway (Fig 2.4), an example is the tricarboxylic acid cycle.

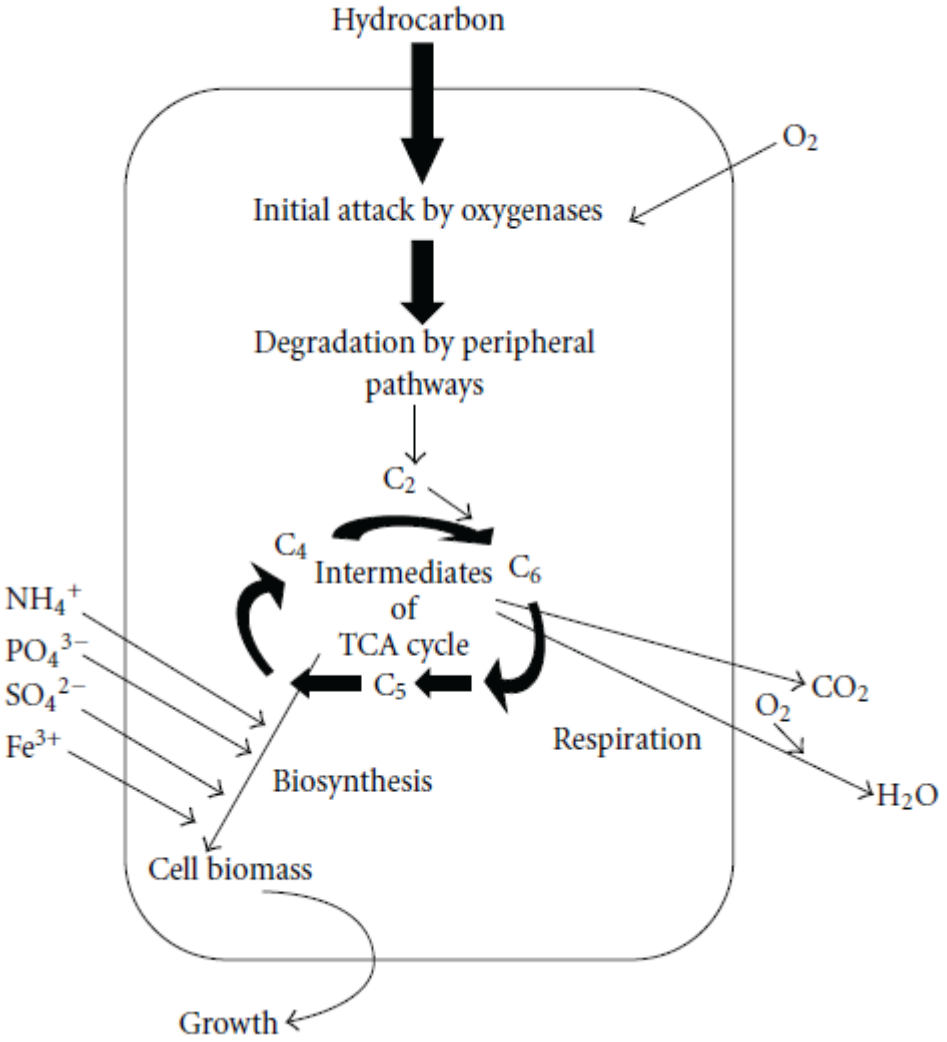


Figure 2. 4 Main principles of aerobic degradation of hydrocarbons by microorganisms (Adapted from Fritsche and Hofrichter, 2000).

Specific enzymes are responsible for the degradation of petroleum hydrocarbons. Initial attack on xenobiotics can be seen in Fig 2.5 (Fritsche and Hofrichter, 2000). There are other mechanisms in which microbes' aid degradation and they include

1. The attachment of microbial cells to the target substrate
2. Production of biosurfactant by microorganisms (Hommel, 1997).

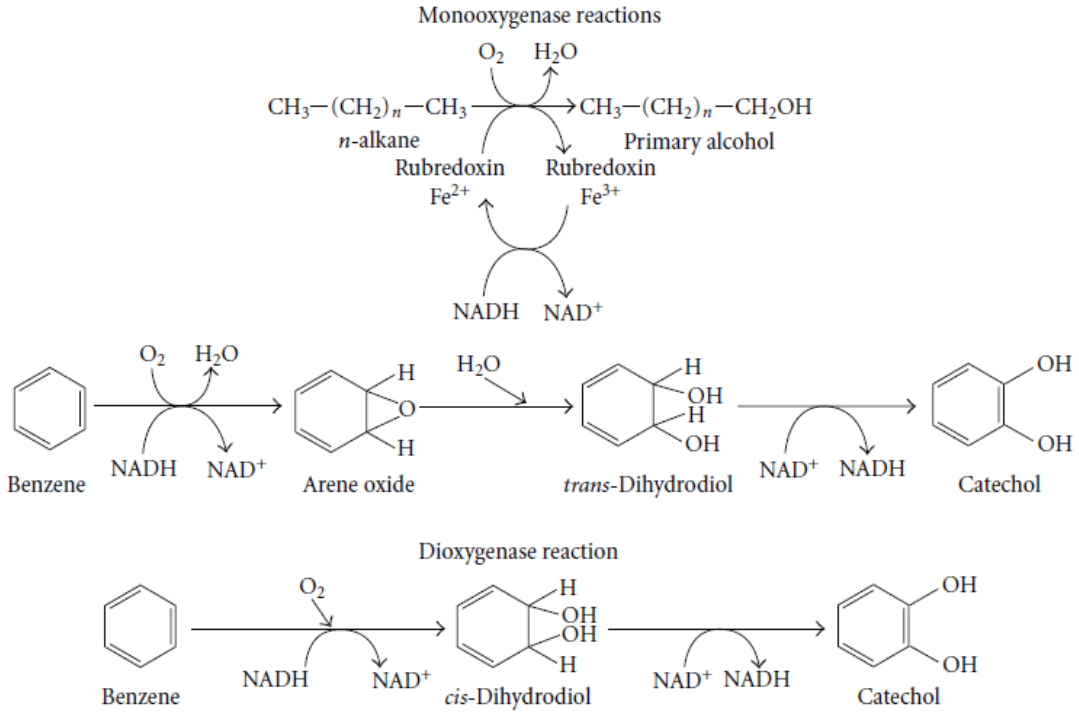


Figure 2. 5 Enzymatic reactions involved in the degradation processes of hydrocarbons (Adapted from Fritsche and Hofrichter, 2000).

2.6.2 METABOLIC PATHWAYS

Metabolic pathways are very important in environmental sciences, they provide insight into the degradation of xenobiotic substances by living organisms and microorganisms. Metabolism of xenobiotics occurs in three phases. In phase I there is an introduction of polar or reactive group into the xenobiotics by monooxygenase enzymes such as cytochrome P450 oxidases. This then leads on to phase II which is the conjugation of the polar compounds from phase I, catalysed by transferase enzymes like glutathione S-transferase. Phase III involves the conjugated compound being identified by efflux transporters and excreted out of the cell. However, prior to this, compounds may require further processing which would be done in phase III before excretion.

CytochromeP450 (CYP450) alkane hydroxylases is composed of a super family of Heme-thiolate monooxygenases that play a crucial role in the degradation of hydrocarbons, oil, fuel additives and in general, xenobiotics (Van Beilen and Funhoff, 2007). Generally, enzyme systems are setup to introduce oxygen in the substrate to initiate biodegradation, this is however dependent on the length of the chain of hydrocarbon. Larger eukaryotes have

several P450 families with several individual isoforms of P450 for metabolic transformation of different substrates. A few species of microorganisms have also been reported to have CYP450 and they are involved in the degradation of petroleum hydrocarbons (Zimmer et al., 1996).

2.6.2.1 PHASE I TRANSFORMATION

This phase involves the action of several enzymes to introduce polar or reactive groups into the substrate. Hydrolysis is one of the most common forms of modification that is catalysed by cytochrome P450 oxidases. The mode of action of this enzyme is the incorporation of an oxygen atom into the non-activated hydrocarbons, this could possibly result in either introduction of a hydroxyl group, or O-, N- and S- dealkylation of the substrate (Guengerich, 2001). Mechanism of action of CYP450 is by the reduction of cytochrome bound oxygen and the production very reactive oxyferryl species as illustrated below (Schlichting et al., 2000).

$$O_2 + NADPH + H^+ + RH \rightarrow NADP^+ + H_2O + ROH$$

2.6.2.2 PHASE II CONJUGATION

This phase involves the conjugation of active metabolites from phase I with compounds such as glutathione (GSH), glucuronic acid or glycine. Unlike phase I metabolite, the products from phase II conjugation have a higher molecular weight and are less active than their substrate. Introduction of a large anionic groups such as glutathione will function by detoxifying reactive electrophiles thus producing metabolites that are very polar and can easily be transported for excretion. A large group of transferases are responsible for catalysing these reactions, this groups are capable of metabolising almost any group of hydrophobic compounds containing nucleophile or electrophile groups (Jakoby and Ziegler, 1990).

There are a number of ways in which phase I reactions could occur namely; oxidation, reduction, hydrolysis, cyclization, decyclization as well as addition and removal of hydrogen, and they all occur through the functions of oxidases. If metabolites are polarized after this, they could be excreted from here. However, this is normally not the case, and metabolites usually require a combination with an exogenous substrate that forms a polar group. One of

the most common oxidation reactions of phase I is conversion of C-H bond to C-OH, by turning an inactive compound to an active one which could be less toxic or more potent (Akagah et al., 2008).

2.6.2.3 PHASE III MODIFICATION AND EXCRETION

Upon completion of the conjugation of the metabolites in phase II, conjugates may require further metabolising which occurs in this phase, where they then are excreted from the cell (Homoloya et al., 2003). Proteins supporting the excretion are members of ATP-binding cassette transporters with the ability to catalyse the transport of a large spectrum of hydrophobic anions by utilizing the energy of ATP binding as well as hydrolysis to transport metabolite and excrete from the cell (Homoloya et al., 2003).

2.6.3 MONOOXYGENASE ENZYMES: CYTOCHROME P450 (CYP450)

CYP450 proteins are hemoproteins, a superfamily that contains heme as a cofactor (Jakoby and Ziegler, 1990). Cytochrome enzymes make use of substrate ranging from small to large molecules in enzymatic reactions. In electron transfer chains, CYP450's are the terminal oxidase enzymes, hence generally called P450-containing systems. They derive their popular name from their wavelength of absorption on the spectrophotometer (450 nm) when they are in their reduced state and complexed with carbon monoxide (Omura and Sato, 1964).

They have been found to exist in almost every living organism, microorganism and viruses, ranging from animals, plants, bacteria, fungi. They are however absent in some microorganisms such as *Escherichia coli* (Achazi et al., 1998) and are therefore not omnipresent. Cytochrome enzymes deliver electrons to reduce the iron, requiring a protein partner. CYP450s can be classified into different groups based on the electron transfer of proteins: microsomal P450 systems, mitochondrial P450 systems, bacterial P450 systems, CYB5R/cyb5/P450 systems, FMN/Fd/P450 systems and P450 only systems (Akagah et al., 2008).

2.6.3.1 NOMENCLATURE AND GENE

Based on the naming nomenclature of cytochrome P450, cytochrome is denoted by an italicized *CYP* symbol, this is followed by a number which designated which family it belongs to, then a letter that indicates the subfamily followed by another number that indicates individual gene (e.g. *CYP*1A1). However, at protein or mRNA levels, it is recommended that CYP not be italicized.

2.6.3.2 CHARACTERIZATION OF CYP450

Several CYP450 enzymes and isoenzymes over the last few decades have been isolated and purified from both terrestrial and aquatic organisms (Stegeman and Kloepper-Sam, 1987; Buhler and Williams, 1988; Buhler and Williams, 1989; Stegeman, 1989; Stegeman et al., 1990). The most comprehensive studies have been done on fish species such as rainbow trout, as well as pigs and rats.

Several studies carried out on trout isolated and purified two forms of P450 from matured trout kidney and named P450 KM1 and KM2 with molecular weight of 54 and 52 kDa. With the use of specific polyclonal antibodies designed against P450 KM2 it was shown that it was completely absent in female and juvenile species hence only present in adult male specimens. (Andersson, 1991; Williams et al., 1983; Lorenzana et al., 1988; Celander et al., 1989a).

2.6.3.3 MOLECULAR PROPERTIES OF CYTOCHROME P450 SYSTEMS

All isoenzymes of CYP450 have a single polypeptide chain that has iron-protoporphyrin IX which is bound loosely by hydrophobic forces, covalent and electrostatic bonds. Isoenzymes of P450 have molecular weight range from 45-60 KDa. Contained in the planar porphyrin ring are four of the iron ligands, the fifth iron ligand is a thiolate group that is from a residue of cysteine found in the peptide backbone, and the 6^{th} and final ligand is the oxygen binding site during monooxygenase reaction (Fig 2.6).

CYP450 is located in the endoplasmic reticulum, alongside several P450 isoenzymes and several other enzymes. Electrons are transferred from the NADPH through flavoprotein and

NADPH-CYP450 reductase, P450 then introduces an oxygen atom into the substrate, reducing the second atom to form water.

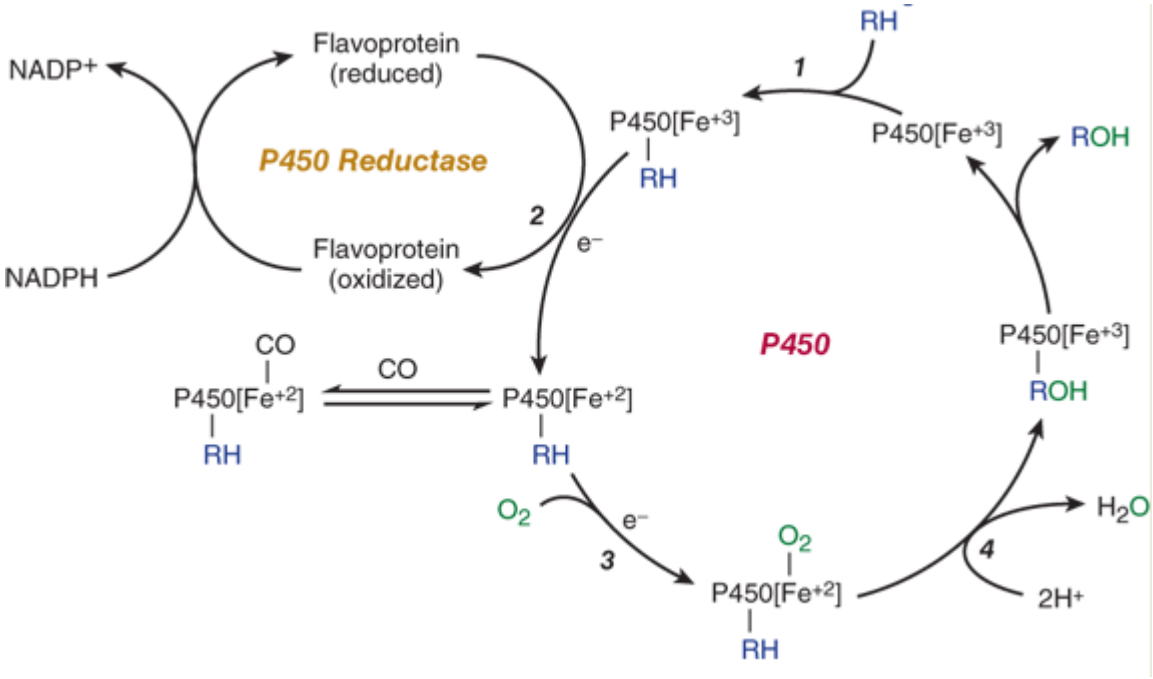


Figure 2. 6 CYP450 monooxygenase cycle showing the 6 iron ligand bonds (Source: De Montellano, 2005).

Although monooxygenase reactions in P450 mediated transformations have the same basal features, there are significant differences in the chemistry of the substrate and product ranging from complex steroids, polyunsaturated fatty acids right down to simple benzenes. Activities in substrate that undergo specific transformations have been studied and can be analytically measured. One of the major focus of these studies was to design a prototype substrate for measuring specific monooxygenase enzyme activities in samples from tissues of different organisms (Lubet et al., 1990). Since there are various isoenzymes of P450 in the tissue, the substrate should ideally illustrate only the activities of specific isoenzymes, this is because the isoenzymes should have a substrate specificity as a result of the difference in active site topography (Lewis et al., 1994). However, this only apply to some isoenzymatic reactions that are substrate specific in PAH in the subfamily CYP1A (e.g. hydroxylation of BAP, AHH; ethoxyresorufin O-deethylation). These catalytic tools are important in the study of

environmental pollutants on ecology. However, it should be noted that these *in vitro* catalytic activities are not always a reflection of P450 reactions *in vivo*.

2.6.4 CYP1A PROTEINS

These enzymes are monooxygenases involved in biotransformation of xenobiotics and endogenous compounds. The actions of CYP in the xenobiotic metabolism (phase I) makes xenobiotic compounds more hydrophilic hence they become more accessible for conjugation (phase II) and excretion. Some CYP1A enzymes are involved in this reactions with several other CYP enzymes. There are 2 types of CYP1A enzymes present in humans CYP1A1 and CYP1A2, which have been reported in some fish species such as rainbow trout. In other species like earthworms, they may have multiple CYP1A forms, but to date they are simply referred to as CYP1A enzymes (Brown and Doube, 2004; Rorat et al., 2017; Cao et al., 2012; Maity et al., 2008).

2.6.4.1 CYP1A INDUCTION

CYP1A genes are expressed continuously however they can also be induced upon exposure to an inducer. Induction of CYP1A enzymes upon exposure to xenobiotics is one of the key traits of this enzymes. Until the late 20th century it was believed that these genes were only expressed in vertebrates, but they have subsequently been found to be expressed in a few invertebrate species (Cao et al., 2012). CYP1A is generally induced through an aryl hydrocarbon receptor (Ah-receptor) pathway. The Ah-receptor can be referred to as a ligand-activated transcription factor that is found in the cell cytoplasm. Upon binding of the receptor to a ligand, it is translocated into the cell nucleus. The complex bind with the partner protein ARNT, then binds to DNA at specific points which in turn will result in the induction of target gene expressions (Pollenz, 2002). Example of target genes that are involved in the biotransformation of xenobiotics are CYP1A, CYP1B, glutathione S-transferase, NAD(P)H, UDP glucuronosyltransferase, aldehyde-dehydrogenase (Nebert and Gonzalez, 1987; Celander et al., 1993; Handley-Goldstone et al., 2005). Denison and Nagy (2003) however reported that a few CYP1A inducers are weak Ah-receptor ligands, which has led to other suggestions of CYP1A regulations such as the retinoic acid receptor signal transduction pathway and tyrosine

kinase activation pathway (Delescluse et al., 2000). In mammals, the stabilization of CYP1 mRNA has been since suggested as an induction mechanism (Kimura et al., 1986; Okey, 1990).

2.6.4.2 INDUCERS AND INHIBITORS OF CYP1A

The major pollutants that initiate the Ah-receptors are planar molecules and hydrophobic pollutants such as polyhalogenated aromatic hydrocarbons (PHAHs) and polyaromatic hydrocarbons (PAHs). This includes co-planar polychlorinated biphenyls (PCB's) polychlorinated naphthalenes (PCNs), polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDDs/Fs). The listed PHAHs, 12 out of the 209 PCBs, 7 out of the 75 PCDDs, and 10 of the 135 PCDFs have a high affinity of binding to the Ah-receptor with some of them having brominated analogues that are agonists (Van Den Berg et al., 1998; Behnish et al., 2003). One of the most potent inducers of CYP1A known to date is 2,3,7,8-tetrachloro—dibenzo-p-dioxin (TCDD) (Van Den Berg et al., 1998). The EPA16 with hydrocarbon rings of 4 or more are also major inducers of CYP1A because of their ability to bind to the Ah-receptors (Billiard et al., 2002; Behnish et al., 2003; Lee and Anderson, 2005). CYP1A has been reported to have the ability to metabolize some of this PAHs. Furthermore, BAP which is a CYP1A inducer has also been characterized as an inhibitor CYP1A activity in humans (Shimada and Guengerich, 2006).

Some phytochemicals such as flavones are synthetic Ah-receptors that are not environmental pollutants, an example of this is β-naphtoflavone (βNF) which are experimental inducers of CYP1A. Other ligands that can be found in the diet as natural substances as well as from endogenous metabolism, include carotinoids, flavonoids, indole-metabolites of arachidonic and tryptophan acid metabolites (Denison and Nagy, 2003). PHAHs and PAHs serve as substrate for CYP1A but can also serve as inhibitors of reactions CYP1A catalyse such as EROD (Doostdar et al., 2000; Denison and Nagy, 2003). Antifungal drugs such as ketoconazole, sulfamethoxazole (antibiotics), diclofenac (non-steroidal anti-inflammatory) have all shown the capacity to inhibit activities of CYP1A in fish (Hegelund et al., 2004; LaVille et al., 2004).

Ah-receptors are induced by a range of chemicals, with all attempts of an endogenous ligand for Ah- receptor abortive.

2.6.4.3 SOURCES OF CYP1A INDUCERS AND INHIBITORS

Though both PAHs and PCDDs/Fs have the ability to form naturally in the environments, most of their introduction into the environment is via anthropogenic sources such as mining and incomplete combustion of crude oil (Stieglitz and Vogg, 1987). Though PCBs are now banned from being produced, they were earlier produced in mass quantities and there is still a legacy of PCBs that escaped into or persisted in the environment via existing PCB containing materials such as electrical equipment with PCB insulators.

It has been seen from studies that polyaromatic hydrocarbons induces CYP1A as much as PCBs even when the areas have higher concentrations of PCBs contamination (Engwall et al., 1997; Sundberg et al., 2005). Mostly the source of PAH is incomplete combustion such as during crude oil fractioning and gas flaring, vehicle emission, wood burning, wear and tear of tyres and asphalt (Bostrom et al., 2002).

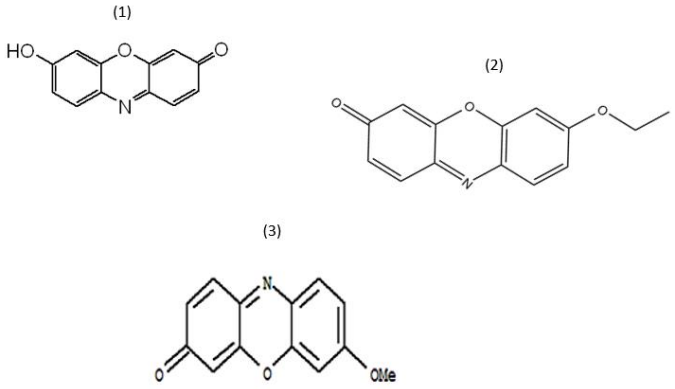


Figure 2. 7 Chemical structure of (1) resorufin, (2) 7-ethoxyresorufin and (3) 7-methoxyresorufin.

2.6.5 OXIDATIVE STRESS

Cell/tissue injury could be a result of oxidative stress that originates from an imbalance between reactive oxygen species (ROS) and antioxidants (Sies, 1991). Several anomalies in cell metabolism such as DNA strand break are as a result of oxidative stress (Halliwell and Aruoma, 1991). An increased in intracellular calcium Ca^{2+} resulted in damage of membrane ion transporters as well as other proteins as well as lipid peroxidation (Hyslopp et al., 1988; Gutterdidge and Halliwell, 1989: Halliwell and Aruoma, 1991). Damage caused could either be direct e.g. oxidation of hydrogen peroxide to thiol groups, or indirect. Hence an excessive

increase in free intracellular Ca^{2+} can lead to the activation of proteases and nucleases that can attack the cytoskeleton and DNA fragmentation (Sies, 1991). Thiol-containing proteins such as cell surface thioredoxin, protein kinases and cell surface receptors have similar trends in responding to oxidative stress in ways that offsets cell metabolism (Martin and Dean, 1991). There is little difference between the regulatory properties of ROS and its damaging properties dependent on the generation of ROS and aldehydes.

Oxidative stress is dependent on the degree of stress that is imposed, the mechanism by which it is imposed, length of stress and the nature, all these provide information on how oxidative stress results in cell damage of the targeted cells; e.g. increase in lipid peroxidation of damaged cells, or even cell death (Saran and Bors 1989; Laurindo et al., 1991). It has been reported that cell injury caused by the increase in free intracellular Ca^{2+} is associated more with protein and DNA damage rather than lipid peroxidation. Lipid peroxidation occurs at a later stage that occurs simultaneously with cell apoptosis (Coghlan, 1991; Halliwell and Gutterdidge, 1989; Raven, 1991).

2.6.6 ANTIOXIDANT DEFENCE

Antioxidants are compounds with the potentials of delaying or inhibiting oxidative damage to target cells. They are stable compounds that act by donating an electron to neutralize a free radical hence their importance. On reception of an electron from an antioxidant, the neutralized compound becomes polar and inactive breaking the chain of reaction of oxidation. On donating an electron, an antioxidant itself becomes a free radical, however they are not toxic compounds, this is due to the fact that they possess the ability to accommodate this change and remain stable (Niki et al., 2005).

Regulation of cellular aldehydes are carried out by enzymes in the antioxidase defence group such as glutathione S-transferases (GSTs) that conjugates GSH to aldehydes to detoxify it, and aldehyde dehydrogenase (ALDHs) which catalyses the oxidation toxic aldehydes into acids (Gondhowiardjo 1993). Further to conjugating aldehydes, GSTs also have the potentials to catalyse conjugation reaction of glutathione to xenobiotics, resulting in the polar electrophiles that are then excreted from the cells. Increasing ROS are jointly attacked by the spectrum of antioxidase defence enzymes which are, superoxide dismutase (SOD),

glutathione reductase (GR), glutathione peroxidase (GPx) and catalase (CAT). (Saint-Dennis et al., 1999).

2.6.6.1 GLUTATHIONE

Glutathione (GSH) are tripeptides that are produced in the cytosol from amino acids: glutamate, cysteine and glycine. Known to be one of the most important non-protein thiols in biological systems, GSH is an important modulator for cellular homeostasis serving other functions such as detoxification of xenobiotics (Meister and Anderson, 1983; Christie and Costa, 1984).

GSH functions by reducing the disulphide bonds formed within cytoplasmic proteins to cysteine mediated by glutathione peroxidase via a non-enzymatic pathway via the donation of an electron, which results in conversion of two molecules of GSH to an oxidized form glutathione disulphide (GSSG) (Van der Oost et al., 1998). GSH, described as the body's master antioxidant has the potential to be reduced back by glutathione reductase (GR) where NADPH serves as an electron donor (Flohe, 1998).

Other antioxidase enzymes are SODs that function by catalysing the conversion of toxic derivatives of O_2 into H_2O_2 (McCord and Fridovich, 1988), this is then converted by CAT and GPx into H_2O and O_2. Hence changes in this cellular pathway could possibly serve as indicators in living organisms (Espinosa-Reyes et al., 2010).

2.7 ENVIRONMENTAL FACTORS ASSOCIATED WITH VERMIREMEDIATION

Several factors affect the everyday functioning of an earthworm, such as favourable environmental conditions for feeding, growth, reproduction and survival. Due to the widespread application of vermitechnology, earthworms (epigeic and anecic groups) are cultured on a large scale, and the following conditions are vital in optimising their growth.

The distribution, diversity and population of earthworms is greatly pH-dependent (Staaf, 1987). Earthworms have an affinity for soils of neutral pH. Edwards et al. (1991) observed that the pH range of 5.0-9.0 is best for maximum productivity in vermiculture. Most of the epigeic species conform to this pH range, although Dominguez et al. (2011) observed that when left to roam freely, this species migrated towards pH of 5.0 (Dominguez et al., 2011).

Earthworm biomass is made of about 80-90% water, and they lose water through their skin hence moisture is very important for their growth and survival (Grant, 1955; Wever et al., 2001). The worms' body weight as well as preventing body water loss are key factors in determining the success of a vermiculture (Munnoli et al., 2010).

In drought, some groups of earthworms migrate towards more favourable conditions (moist soil) and in the absence of any, they curl up into a ball that is lined with mucus to keep themselves cool until favourable conditions are re-established. Excess water such as rainfall could also affect worms as this alters soil pH which is not favourable to several species (Gajalakshmi and Abbasi, 2003).

Temperature is by far the most important factor that determines the biological functions of an earthworm, from metabolism, respiration, growth and reproduction (Munnoli et al., 2010). Earthworms' response to temperature changes differs from specie to specie (Dominguez et al., 2011). Rutikar (1997) suggested temperature ranges of 20-30^{o}C for vermibeds, while Reinecke et al. (1992) observed that temperatures of 25^{o}C support cocoon incubation in epigeics and temperatures higher than 25^{o}C reduces the production of hatchlings.

Earthworms are photosensitive animals, migrating from anywhere there is light towards darker regions. When exposed to ultraviolet rays from sunlight, they become paralysed and continuous exposure could be fatal. Even though earthworms have an affinity for moist areas including water, they would migrate away from most or water areas with depleted oxygen or large build-up of carbon dioxide. This is because earthworms are very sensitive to anaerobic conditions. Earthworms respire by diffusing oxygen through their body walls because they do not have respiratory organs (Dominguez et al., 2011). Hence earthworms require a certain degree of oxygen concentration in soils or water for them to stay active otherwise it could be fatal.

Earthworms have an affinity for soils with high organic matter content (Edwards and Bohlen, 1996). In the absence of foods with organic matter content, earthworms turn to soil to derive organic matter (Munnoli et al., 2010). It has been reported by several authors that there is a strong correlation between biomass, population and soil organic matter content (Doube et al., 1997; Contreras-Ramos et al., 2006).

Several groups of earthworm are very selective of the soil type and texture in which they thrive, this is because texture of the soil has direct impact on few soil properties such as the water holding capacity, nutrient content as well as the cation exchange capacity (Lavelle and Martin, 1992). Light and loamy soil appeared to support more species of earthworms than heavy clay or very sandy soils (Shaw and Pawluk, 1986).

Overcrowding is detrimental to earthworms and would negatively impact on their biomass as they compete for space to thrive. Dominquez et al. (2001) suggested not more than 16 worms in 100g of vermibed for epigeic earthworm species.

2.8 LIMITATIONS ASSOCIATED WITH VERMIREMEDIATION

Physical, chemical and biological parameters affect the activity of earthworms in vermiremediating contaminated land, in addition the bioavailability of contaminants, as well as toxicity of the contaminant all play roles in limiting vermiremediation. These activities are further discussed below.

2.8.1 ASSOCIATED MICROBIAL POPULATION

A very important factor that may limit the activity of vermiremediation is the population of its associated gut and surrounding microorganisms. However, this can be controlled by optimizing soil conditions for optimal earthworm activity which in turn would should improve microbial growth because of the symbiotic relationship between the two (Binet et al., 1998).

2.8.2 CONCENTRATION OF CONTAMINANTS

PAHs could pose inhibitory effects on earthworms and associated microorganisms when present at very high concentrations in soil (USEPA, 1994), resulting in avoidance by the earthworm or aestivation followed by mortality. It has been reported that concentration of petroleum hydrocarbons greater than 50,000 mg Kg^{-1} completely inhibits microbial growth which in turn would lead to mortality of earthworms (Beskoski et al., 2012; Khan et al., 2012). In situations with such concentrations of PAHs, vermiremediation would not be an ideal approach in remediating the contamination.

2.8.3 FORMATION OF BY-PRODUCTS

Most if not all biological remediation techniques (vermiremediation included) leads to formation of metabolites which might be more toxic than the parent compound and could be as a result of incomplete remediation processes (Sayara, 2010; Malia and Cloete, 2004; Singh, 2012).

2.8.4 MOISTURE REQUIREMENTS

Earthworms are semi-aquatic organisms; hence they are moisture loving organisms that require soils with a good water holding capacity for optimal activity. In a vermiremediation setup, the moisture content of the soil is required to be kept between 65-70% (Eijsacker et al., 2001; Contreras-Ramos et al., 2005).

2.8.5 BIOAVAILABILITY OF RECALCITRANT POLLUTANT

The ability of earthworms to remediate HMW PAHs is important, due to the hydrophobic nature of these PAHs which decreases their solubility hence making them less available for earthworms and their associated microorganisms for remediation (Contreras-Ramos et al., 2008; Dendooven et al., 2011; Rorat et al., 2017). Considering that remediation can only occur if contaminants are readily available for uptake by earthworms and the associated microorganisms, reduced availability would impact the time of remediation and could lead to a persistence of the contaminants in the environment leading to potential biomagnification effect (Zhang et al., 2011; Cui et al., 2013; Potter et al., 1999).

2.9 ENHANCING VERMIREMEDIATION WITH APPLICATION OF (BIO)SURFACTANTS

2.9.1 PROPERTIES OF SURFACTANTS

Surfactants are amphipathic molecules that have both hydrophilic and hydrophobic moieties which partition preferentially at the interface between fluid phases that has different polarities and hydrogen bonding, such as in oil and water (Georgiou et al., 1992). The hydrophobic phase tends to be the hydrocarbon while the hydrophilic phase is non-ionic, either positively or negatively charged or it could be amphoteric (Georgiou et al., 1992; Desai and Bannat, 1997). The dual nature causes the surfactants to adsorb at interface thereby reducing the interfacial energies (Ron and Rosenberg, 2002; Rosenberg and Ron, 2001).

Depending on the head group of the surfactant, they are classified as anionic, non-ionic, cationic and zwitterionic (cation and anionic groups).

When in low concentrations, surfactants exist as monomers where they accumulate at the interfaces present in the medium. As the interface areas are partitioned and the concentration of aqueous surfactants increases, monomers aggregate and form micelles. The concentration at which micelles are formed is the *"critical micelle concentration"* (CMC). At any concentration above the CMC, surfactants solubilize the hydrophobic compound which in water are not soluble (Rosenberg and Ron, 1999).

Volkering et al. (1997) suggested that a good surfactant should have the capacity to lower the surface tension of water from 72 to 30 ± 5 mN/m. There is also a correlation between the surface tension and concentration of the surface-active compound until the CMC is reached. Makkar and Rockne (2003) define CMC as *"the characteristic concentration of surfactant in solution above which the appearance and the development of micelles brings about a sudden variation in the relation between the concentration and certain physicochemical properties of the solution."* Thus, above certain concentration of surfactants (CMC), stable aggregates of 10 – 200 molecules/micelles are formed. This could possibly increase the rate of solubilisation of heavy organic compounds (HOC) (Edwards et al., 1991; Volkering et al., 1997). Paria (2008) observed that physical properties of surfactants such as surface and interfacial tension and detergency changes below CMC (Fig 2.8). The CMC can be used to measure the surfactant efficiency in solubilising HMW (Pacwa-Plociniczak et al., 2011).

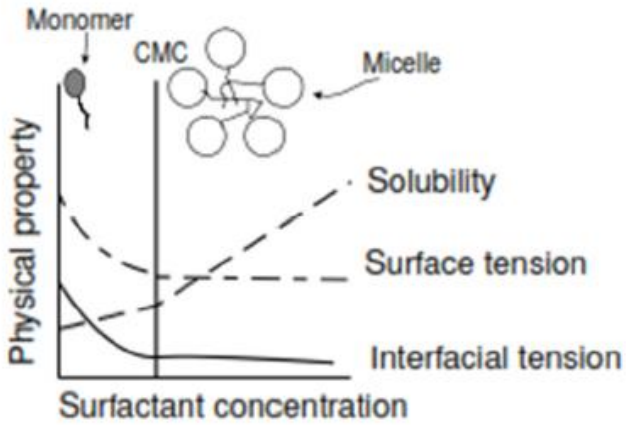


Figure 2. 8 Graphical representation of critical micelle concentration (source: Mulligan et al., 2001).

All of the above properties make surfactants a vital commodity in industries. Their use includes: emulsification, wetting and phase dispersion, detergency, foaming or solubilisation. Table 2.13 illustrates the areas of their application. The petroleum industry is one of the major industries that makes use of surfactants in monitoring the capacity of oil removal (Makker and Rockne, 2003: Banat et al., 2000: Cameotra and Makkar, 1998). Their uses are further extended into the pharmaceutical, food as well as cosmetic industries (Cameotra and Bollag, 2003-; Van Hamme et al., 2003; Van hamme et al., 2006; Singh et al., 2007).

However, with the view of creating and supporting a green environment, the toxicity of chemical surfactants as well as the costs of their production pose major problems in applying them to solve environmental challenges (Singh et al., 2007).

Table 2. 13 Types of modern surfactants used in industries

Surfactant type	Examples	% of total production	Major uses
Anionic	Carboxylates, sulfonates, sulfuric acid esters	66	Washing powder
Cationic	Amine oxides, moniamines, quaternary ammonium salts	9	Fabric softeners, shampoos
Nonionic	Carboxylic acids and carbohydrate esters, glyceride, and their ethoxylated deriviatives	24	Laundry cosurfactants, washing up liquids, personal care products, and food
Amphoteric	Alkyl betaines, alkyl dimethylamines, imidazonilinum deriviatives	1	Speciality uses

(source: Cameotra and Makkar, 2010)

2.9.2 THE USE OF SURFACTANT IN REMEDIATION

The effectiveness of remediation of crude oil could be affected by a whole range of factors, but the major factor is the bioavailability of the contaminant compound to the degrading microbes (Makker et al., 2003; Zhong et al., 2014). This is because of their low solubility, strong, and/or irreversible sorption to the soil particles (Rockne et al., 2002). A suitable solution in making compounds bioavailable could be addition of surfactants which helps to increase desorption as well as solubility in the aqueous phase thus making it readily available to degrading microbes. Several authors have reported addition of surfactants both chemical (synthetic) surfactants and biosurfactants increase the removal rate of contaminants (Aronstein et al., 1991; Dobler et al., 2016). Though HMW hydrocarbons persist longer in the soil than lower molecular counterparts, it has been reported that over a long period of time, there is often some form of degradation of HMW compounds in soils, which shows that HMW PAHs can actually be degraded. Furthermore, several HMW compounds are susceptible to partial degradation by bacteria after they derive their energy and carbon by degrading other LMW compounds (Leahy and Colwell 1990). With crude oil and most of its components, the two major obstacles for biodegradation are the absence of microbial metabolic capabilities and the hydrophobicity of molecules which reduces their bioavailability to microbes responsible for biodegradation (Prince, 1997; Franzetti et al., 2008). Of these two factors, the latter is of more importance as the low solubility rate would result in slow degradation. Furthermore, the molecules of this HMW PAHs tends to bind strongly to soil as well as soil organic matter (Rockne et al., 2002). The binding of these molecules to soil organic matter (SOM) could reduce the bioavailability of crude oil or its constituents as this causes alterations in the SOM followed by formation of tighter bonds to the already altered SOM (Rockne et al., 2002).

Thus, to increase the solubility of crude oil, addition of surface-active agents (surfactants) are used, acting as mobilizing agents (Miller, 1995). Overall, the addition of biosurfactant improves the likelihood of biodegradation by increasing the solubility of hydrophobic organic contaminants (HOC)

2.9.3 MECHANISM OF ACTION

Surfactants impact the availability via three mechanisms:

- dispersion of non-aqueous phase lipids (NAPL) organics, which causes an increase in contact area as a result of the interfacial tension between the aqueous and non-aqueous phase
- increase in the solubility of pollutant caused by the presence of micelles and the micelles contain large concentrations of HOCs, and
- active transport of pollutant from solid phase that can come about by the interaction of the pollutant with a single surfactant molecule, by introducing surfactant to a solid phase, as well as reducing surface tension of soil water particle

Due to the impact of the first two mechanisms on the transfer of pollutant to the aqueous phase, it is unclear as to the precise contributions of these two mechanisms in increasing the bioavailability of contaminants (Makkar, 2003; Rebelo et al., 2013). Schipper et al. (2000) suggested three approaches as to how biodegradation of HMW PAHs are aided by surfactant namely:

- Bacteria collecting the pollutant from the micellar core
- Surfactants increase the mass transfer of pollutant to aqueous phase where they become available to pollutants for further use, and
- Addition of surfactants induces a change in the hydrophobicity of the pollutant cell which enhances the contact between the cells and NAPL

For bioremediation to occur, three factors need to be in place, and they are:

- Availability of microbes
- Accessibility of contaminants and
- A conducive environment

Furthermore, for bioremediation to be successful the microbes have to degrade the contaminant into less toxic form, and ultimately to carbon dioxide and water (Margesin et al; 2003; Margesin and Schinner, 2001; Liu et al., 2017). However, the rate at which

biodegradation occurs is dependent on several factors including: nutrient concentration, pH, oxygen concentration, concentration and bioavailability of the contaminant, the pollution history of the environment, physical and chemical characteristics of contaminant (Margesin and Schinner, 2001).

Also, to be classified as successful bioremediation, it must be shown that the biodegradation is the primary function of the degrading community and also the level of degradation must surpass that of natural degradation (Calvo et al., 2009).

2.9.4 DISADVANTAGES OF CHEMICAL SURFACTANTS IN BIOREMEDIATION

Darvishi et al. (2011) suggested that conventional treatment methods of contaminated land are not sufficient in thoroughly remediating them. Volkering et al. (1998) reported that the introduction of chemical surfactant into the environment on its own becomes a contamination of the said environment, thus the toxicity of the surfactant as well as its possible degraded by-product are of major concern. Chemical surfactant could be toxic to the indigenous microbes (Darvishi et al., 2011) which was suggested by Volkering et al. (1997) may be due to disruption of the cellular membranes and the interaction with key functional proteins.

2.10 BIOSURFACTANTS

2.10.1 DEFINITION OF BIOSURFACTANT

As defined by Martins et al. (2009), *'biosurfactant is an isolated or non-isolated compound obtained from a microorganism that has the capacity to influence interfaces and to significantly reduce the amount of work required to overcome surface tension'*. Though an abstract definition, biosurfactants are basically surfactants that are of biological origin. Several microorganisms such as bacteria and fungi have been reported to produce biosurfactants (Abdel-Mawgoud et al., 2010).

Biological molecules are amphiphilic compounds that possess hydrophobic and hydrophilic moieties, where they partition at interphases (Banat et al., 2000; Darvishi et al., 2011), and

microbial compounds that exhibit very high surface activities as well as emulsifying activity are categorized as biosurfactant (Banat et al., 2000).

2.10.2 BIOLOGICAL ALTERNATIVES TO CHEMICAL SURFACTANTS

There is a strong contrast between the disadvantages of using synthetic surfactants and the advantages of applying biosurfactant. Due to the natural occurrence of biosurfactants in the soil, their environmental application is more acceptable (Volkering et al., 1997). They have been one of the top choices as ecological alternatives to the conventional chemical surfactants in bioremediation (Paria, 2008). Due to their biological origin, biosurfactants exhibit better compatibility to bioremediation and to microbial biodegradation (Calvo et al., 2009). In addition to this, it has been reported that they exhibit lower toxicity, high activity and stabily at extreme temperatures, pH, as well as salinity (Abdel-Mawgoud et al., 2010). Biosurfactants possess several advantages over synthetic surfactant among which are their unusual structural diversity and unique properties.

2.10.3 CLASSIFICATION OF BIOSURFACTANTS

Biosurfactants are categorized according to several factors such as their molecular weight, chemical composition, mode of action, physico-chemical properties and origin of microbes (Pacwa-Plociniczak et al., 2011). Biosurfactants can be classed into two categories based on their molecular weight. The term biosurfactant is meant to be used for low molecular weight microbial surfactants, and bioemulsifiers used for high molecular weight microbial surfactants.

Low molecular weight microbial surfactants include lipopeptides, rhamnolipids, glycolipids and proteins while higher molecular weight microbial surfactants include polysaccharides, lipoproteins and lipopolysaccharides (Banat et al., 2010; Darvishi et al., 2011). Of all the low molecular weight surfactants, rhamnolipids and glycolipids are the most studied. The low molecular weight biosurfactants possess molecules that reduces the surface and interfacial tension, while high molecular weight bioemulsifiers do not lower the surface and interfacial tension but are more effective in stabilizing oil in water emulsions (Darvishi et al., 2011).

High molecular weight bioemulsifiers have the potential to stabilize the emulsion between water and liquid hydrocarbons, which increases surface area for microbial degradation. However there has been little to no reported information on their performance in enhancing bioremediation of hydrocarbons by microorganisms (Banat et al., 2010). Low molecular weight biosurfactants have the potential of reducing the surface tension hence increasing surface area of insoluble organic compound. Above the CMC, low molecular weight biosurfactants have the abilities to trap the insoluble organic contaminant in their micelle, which would then result in enhanced bioavailability of the hydrophobic contaminants to microbes for degradation.

The diversity exhibited by biosurfactants far surpasses that of chemical surfactants of the wide range of microorganisms that can produce them (Ron and Rosenberg, 2002; Rosenberg and Ron, 1999; Bodour et al., 2003). These biosurfactants play different natural roles in supporting the growth of the organisms which produces them (see table 2.14).

Biosurfactant aids increase in surface area of hydrophobic interface thereby increasing bioavailability of hydrophobic compounds, heavy metal binding, quorum sensing, biofilm formation, bacterial pathogenesis (Mulligan and Eftekhari, 2003; Mulligan, 2005; Singh and Cameotra, 2004). There are several microbes producing different biosurfactants, but no single agent that is suitable to solve all problems (Mulligan and Eftekhari, 2003; Mulligan, 2005; Singh and Cameotra, 2004).

Table 2. 14 Major types of glycolipids produced by microorganisms (source: Cameotra and Makkar, 2010)

Biosurfactant type	Producing microbial species	Application
Sophorolipids	*Candida bambicola* ATCC 22214	Emulsifier
Trehalose lipids	*Rhodococcus sp*	Bioremediation
Rhamnolipids	*Pseudomonas aeruginosa 57SJ*	Bioremediation
	Renobacterium salmoninarum 27BN	
	P. putida Z1 BN	Bioremediation
	P. aeruginosa PA1	
	P. chlorophis	Bioremediation
	P. aeruginosa GL1	Bioremediation
	P. aeruginosa GL1	Biocontrol agent
	Pseudozyma fusiformata VKM Y-2821	Hydrocarbon assimilation
	Bacillus	Surface-active agent
	Subtilis 22BN	Antifungal activity
Liposan	*Candida lipolytica*	Emulsifier
Mannosylerythritol lipids	*Candida antartica*	Neuro-receptor antagonist,
		Antimicrobial agent
	Kurtzmanomyces sp 1-11	Biomedical applications
Ustilipids	*Ustilago maydis* and *Geotrichum candidum*	Dopamine D3 receptors antagonist
Flocculosin	*P. floculosa*	Antifungal, biocontrol agent
Anionic glucose lipid	*Alcanovorax borkumensis*	Biomarkers

2.10.4 IMPACTS OF BIOSURFACTANTS ON REMEDIATION

Contamination of soil by crude oil has shown to be a complex process Degradation of crude oil contaminants are limited by the low solubility of these compounds in water (Banat 2000; Desai and Banat 1997), compounds adsorb onto the soil matrix, thus are not readily bioavailable to microbes for degradation. Hydrocarbon in soil remain in separate non-aqueous phase liquid (NAPL) which may be present as film or droplets on soil particles which usually makes them unavailable (Hommel, 1997).

Pacwa-Plociniczak et al. (2011) highlighted microbial cell interact with liquid alkanes via adhesion to large oil droplets and cellular assimilation of emulsified small oil droplets by pseudosolubulization (Fig 2.9) (Rosenberg and Ron, 1999).

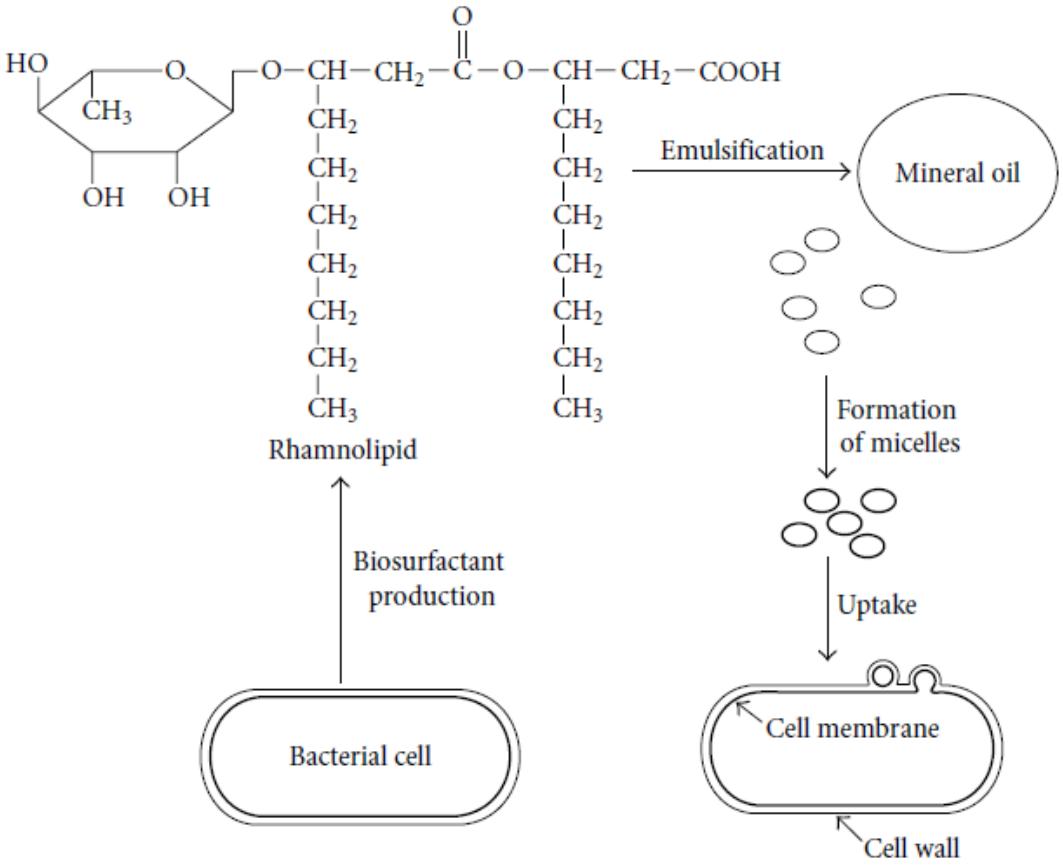


Figure 2. 9 Biosurfactant (rhamnolipid) produced by Pseudomonas sp. uptake of hydrocarbons (Source: Fritsche and Hofritcher, 2000).

There is a strong tendency for organic contaminants to adhere onto to soil matrices which results in a longer period of time for remediation to occur. Franzetti et al. (2010) reported that the extended remediation time can be overcome by the application of biosurfactants, and this is closely linked to the process of mobilization of contaminant molecules. Mobilization of molecules would occur at concentrations below the CMC.

Franzetti et al. (2010) suggested that the application of biosurfactants tends to increase the hydrophobicity of degrading microbial community, thus enabling cells to access more hydrophobic substrates. The more hydrophobic the cells are, the easier the access of microorganisms to come in contact with oil drops and solid hydrocarbons (Pacwa-Plociniczak et al., 2011).

Further to application of biosurfactants in the remediation of organic waste, they have also been applied in remediating inorganic waste such as heavy metals contaminated sites. Metals such as cadmium and lead could be very toxic in the environment with even minute concentrations having potent effects. One of the biggest challenges faced with heavy metals is that they are not biodegradable, they can only be bio-transformed from one state to another, which would subsequently aim to reduce their mobility and potency (Pacwa-Plociniczak et al., 2011). Biosurfactants became very advantageous in heavy metal treatment because of their ability to form complexes with heavy metals, e.g. an anionic biosurfactant would form a complex by creating an ionic bond. There is a very strong bond between an anionic biosurfactant and heavy metal which is stronger than that between soil and heavy metals. There is a direct competition between heavy metals and cationic biosurfactant due to them both wanting to bind with negatively loaded surfaces. Formation of micelles could also result in heavy metal removal. They can be mobilised in water and this happen by the heavy metals' bonds with biosurfactant polar heads. Heavy metal complexes could be removed after chelating by a washing process as reported by Banat et al. (2010).

Several authors have reported a successful use of biosurfactants in remediating heavy metal contamination (Mulligan, 2009; Banat et al., 2010; Pacwa-Plociniczak et al., 2011).

Several authors as indicated in Liu et al. (2018)’s review have reported the use of biosurfactant and biosurfactant producing microorganisms in remediation (Table 2.15).

Table 2. 15 Studies on rhamnolipid assisted bioremediation by increasing bioavailability

Contaminant	Microorganism	% removal	Reference
PAHs	*P. chrysoporium*	71.5	Wang et al. (2014)
PAHs	Indigenous microbes	95	Nikolopoulou et al. (2013)
PAHs	Indigenous microbes	90	Sponza and Gouk (2010)
Petroleum hydrocarbons	*Burkolderia multivorans* NG1	87	Mohanty and Mukherji (2013)
PAHS	*Sphingobacterium* sp. QPH-3	75.6	Zhu et al. (2013)
Crude oil	*P. aeruginosa* DN1	90.52	Ma et al. (2016)
Diesel oil	Indigenous microbes	100	Whang et al. (2008)
Phenanthrene	*P. aeruginosa* BP9	92	Bezza and Chirwa (2014)
Fluoranthene	*P. aeruginosa* DVP20	47	Sharma et al. (2015)
Hexachlorocyclohexane	*Sphingomaonas* sp. NM05	95	Manickham et al. (2012)

Mechanisms in which rhamnolipid biosurfactant can increase bioavailability of contaminants for biodegradation are highlighted in Table 2.16.

Table 2. 16 A summary of studies highlighting the different mechanisms rhamnolipids increase bioavailability.

Mechanism	Contaminant	Microorganism	Report	Reference
Solubilisation	Phenanthrene	*Spingomonas* sp. GF2B	A comparative study between rhamnolipid and Tween 80 on bioremediation, where rhamnolipid removed 99.5% of PH compared to 33.5% removed by Tween 80	Pie et al. (2010)
	Hexachlorohexane (HCH)	Indigenous microbes	Solubilisation of HCH was increased between 3-9 folds, resulting in 30-50% higher removal compared to control.	Manickam et al. (2012)
Emulsification	Hexadecane	Indigenous microbes	Bioavailability was increased as a result of rhamnolipid forming emulsions with hexadecane, reducing droplet size to <0.22 µm.	Cameotra and Singh (2008)
Interfacial area	Hexadecane	*Candida tropicalis*	Three different concentrations of mono-rhamnolipid (11.4, 19 and 38 mg L^{-1}) to treat hexadecane, all 3 concentrations induced increase in cell surface hydrophobicity increasing bioavailability of hexadecane for uptake.	Zeng et al. (2011)
Increased permeability of cell plasma membrane	Hexadecane	*P. aeruginosa*	They proposed that the increased degradation was as a result of rhamnolipid enhanced cell surface permeability, allowing interfacial uptake of contaminant.	Zhong et al. (2014
Enhanced enzyme activity	PAHs	Indigenous microbes	Authors reported that rhamnolipid at 0.2 g Kg^{-1} removed 71.5% compared to controls' 52% in 60 days, they proposed that rhamnolipids promotes soil enzyme activity increasing bioavailability.	Wong et al. (2004)

2.10.5 ENHANCED VERMIREMEDIATION INTEGRATED WITH APPLICATION OF RHAMNOLIPID BIOSURFACTANT

Several factors will impact on the efficiency of degradation of PAHs in soil by earthworms amended with rhamnolipid biosurfactants, including physical, chemical, biological and environmental factors (Hernandez-Castellanos et al., 2013). Biosurfactant have also been reported to make humic substances in soil more bioavailable to serve as nutrients available for earthworm and microbial activity. Microorganisms also use these nutrients as a carbon source (Wick et al., 2011; Okere and Semple, 2012; Hernandez-Castellanos et al., 2013). Hence vermiremediation integrated with application of biosurfactant could be a potential improvement in biodegradation of HMW PAHs. Not only is it an eco-friendly approach, it improves soil health through burrowing activities and soil composting.

A summary of the possible fate and behaviour of organic contaminates coupled with their intermediate products is illustrated in Fig. 2.10. The processes which interacts with the fate of contaminants in soil can be listed as follows; bound to soil matrix (persistence), volatilization (loss), biodegradation (loss) and leaching (loss) (Stokes et al., 2005). The ultimate goal of every bioremediation technique is mineralization of contaminants into CO_2 and H_2O which is dependent on the bioavailability of the contaminant.

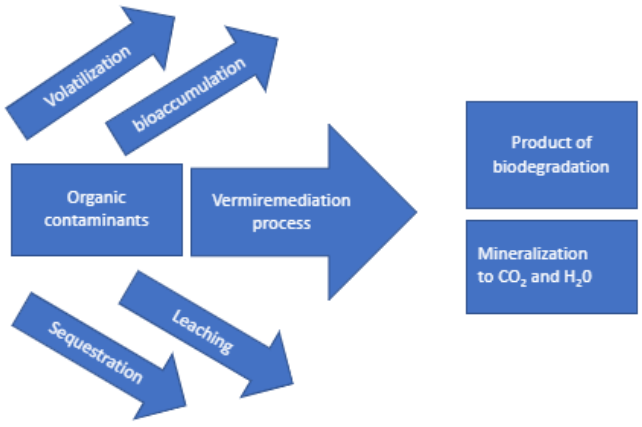


Figure 2. 10 Fate of organic contaminants in soil during vermiremediation

2.11 RESEARCH OBJECTIVES

I. Conduct an in-depth review of established remediation technologies and economic analysis to justify bioremediation technique selected for this study.

II. Investigate the removal capacity of BAP by *Eisenia hortensis* and *Lumbricus terrestris* with or without biosurfactant

III. Determine the removal capacity of combined PAHs (PH, FL and BAP) by *Eisenia hortensis* and *Lumbricus terrestris* with or without biosurfactant.

IV. Determine the synergistic or antagonistic effect of both PAHs and biosurfactant and examine the response of on the oxidative enzymes including glutathione *S*-transferase activity and monooxygenase enzymes (ethoxyresorufin O-deethylase and methoxyresorufin *O*-deethylase activities of CYP1A) in both species.

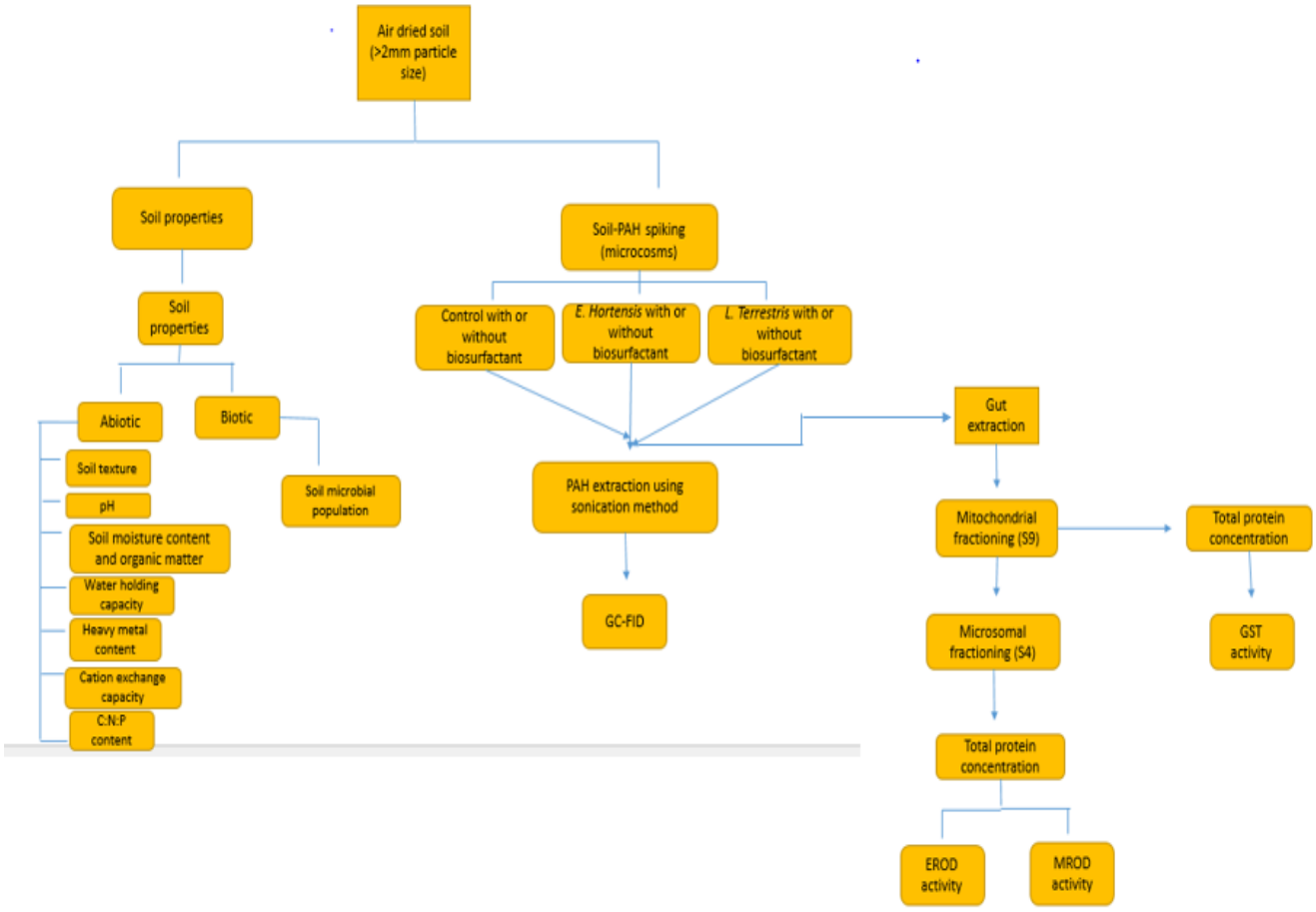


Figure 2. 11 Flowchart highlighting complete methodology

2.12 NOVELTY OF RESEARCH

Vermiremediation technology as well as biostimulation (application of biosurfactant) as individual approaches have been explored separately with timeframes of 56 days and above (Rorat et al., 2017; Contreras-Ramos et al., 2005; Contreras-Ramos et al., 2008; Franco et al., 2006). However, there has not been a systematic approach of an integration of vermiremediation with biosurfactant which this research aims to achieve by using both epigeic and anecic species of earthworms in remediating PAHs using an application of biosurfactant to stimulate bioavailability of PAHs which will accelerate the process of biodegradation.

This novel approach could be governed by sustainable development principles owing to the fact that the whole setup is biological in nature and an eco-friendly approach that will remove PAHs from contaminated land.

This study also illustrates the individual and joint effect (either synergistic, antagonistic or no effect) of combined rhamnolipid biosurfactant and PAHs (PH, FL and BAP), as well as BAP and biosurfactant on biochemical pathways in both the epigeic and anecic species of earthworms (*E. hortensis* and *L. terrestris*), examining the effect they have on monooxygenase (EROD and MROD activity) and antioxidase (GST) enzyme pathways to understand the effect they have on the metabolic pathways in both earthworm species, thus further understanding the toxicological effect of biosurfactant. Thus, results obtained from this study should optimize and improve our knowledge on PAH removal from contaminated land.

CHAPTER 3- ENHANCED VERMIREMEDIATION OF 3-, 4- AND 5-RING POLYAROMATIC HYDROCARBON CONTAMINATION USING EPIGEIC AND ANECIC EARTHWORM SPECIES AND BIOSURFACTANT.

3.1 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are components of crude oil that are recalcitrant pollutants and have negative impact on the environment and human health (Lyu et al., 2014). PAHs are organic compounds comprise -three or more fused aromatic rings and could be of various structural configurations. As mentioned in chapter 2 (Section 2.3), over 120 PAHs have been classified by the U.S. Environmental Protection Agency (EPA) of which 16 are listed as priority pollutants (Bisht et al., 2015).

Soil is a medium and habitat for a diversity of life; for instance, nematodes, oligochaetes, bacteria, actinomycetes, fungi, algae, protozoa, and lower invertebrates called mesofauna. These organisms either exist naturally in soil or are introduced by other means such as by man for a variety of purposes one of which include bioremediation where organisms biodegrade contaminants in contaminated land (Abioye, 2011).

As reviewed above (Section 2.5), earthworms generally inhabit a wide range of soil types and could represent up to 60-80% of the soil total biomass (Hickman et al., 2008a). These organisms play a key role in the fertility of the soil by their burrowing activity that keeps the soil in a continuous turning state that improves the drainage capacity and aeration of the soil (Bogan and Sullivan, 2003). Earthworms are the largest and easily assessable inhabitants of the soil, they were referred to by Charles Darwin (1881) as engineers of the soil ecosystem due to their burrowing activity which constantly changes the medium of the soil (Thompson et al., 1993) hence their potentials as viable remediation species.

In a study examining the removal of three polycyclic aromatic hydrocarbons at different concentrations using *Eisenia fetida,* Contreras-Ramos et al. (2008) observed a higher removal percentage of the PAHs in soils amended with earthworms. Ma et al. (1995) also reported an increased removal of fluoranthene (FL) and phenanthrene (PH) from soils contained earthworms. Eijsacker et al. (2001) demonstrated a steady decrease of 71% in the

concentration of phenanthrene (180 mg Kg^{-1}) when soils were amended with earthworms over a period of 150 days.

It has been reported that earthworm cast (vermicast) are very rich in nutrients, such as nitrogen and phosphorus, as well as microorganisms such as *Pseudomonas* species that accelerates the biodegradation of PAH (Calvo et al., 2009). In addition, bacteria found in the gut of the earthworm are of variable species and capable of remediation, these species include *Pseudomonas, Azoarcus, Acidobacterium* (Singleton et al., 2003).

Bioremediation would be a preferred path to remediation of PAHs, due to the eco-friendly mechanism of degrading contaminants. However bioavailability of hydrocarbons remains a huge challenge in remediation which is controlled by conditions such as physical and chemical processes. Hydrocarbons adsorb strongly to soil particles, they are hydrophobic and have low solubility in water (Margesin et al., 2001). To cope with this, microbes have developed mechanisms to increase the bioavailability of these compounds so as to make use of them as their carbon source as well as energy source (Kumari et al., 2012). Strategies developed by the microbes to aid bioavailability include the formation of bio-films as well as the production of biosurfactant increase solubility of contaminants (Kumari et al., 2012).

Biosurfactant decrease the tension of hydrocarbon-water interface which can result in the pseudosolubulization of the hydrocarbon through the micelle and in turn lead to an increase in mobility and bioavailability thus increasing biodegradation (Kumari et al., 2012; Janbandhu and Fulekar, 2011; Banat et al., 2000).

Urum et al. (2006) investigated the removal of crude oil from the soil using biosurfactant (rhamnolipid and saponin) and a chemical surfactant sodium dodecyl sulfate (SDS). They reported that SDS performed best at removing most of the crude oil, followed by rhamnolipids and saponin. However, the chemical surfactant appeared to remove more of the aliphatic compounds present, while the rhamnolipid removed more of the aromatics present in the soil sample (Urum et al., 2006; Kumari et al., 2012).

The application of earthworms or biosurfactant in the removal of organic pollutant (PAH) as well as inorganic pollutants (heavy metals) have been studied widely (refer to Section 2.3.1) (Rorat et al., 2016; Rorat et al., 2017; Dominguez et al., 2000). However, there is a dearth of

information on the application of both technologies to optimize the removal of PAH. Although *Eisenia* species are the most commonly used sentinel organisms in vermiremediation and ecotoxicology due to their easy handling, their short term reproduction rate and short lifecycle as well as ecological indicators of early malfunctions in the environment (Dominguez et al., 2000; Pauwels et al., 2013), they are epigeic and it can be argued that they are not true inhabitants of the soil like the endogeic and anecic species such as *Aporrectodea longa* and *Lumbricus terrestris* that actually lives in the soil (Butt and Lowe, 2007).

This study was undertaken to investigate 1 – the soil physico-chemical and biological parameters as well as characterising biosurfactant microtoxicity; 2 - the removal of phenanthrene (PH), fluoranthene (FL) and benzo(a)pyrene (BAP) combined by epigeic and anecic earthworm species enhanced with rhamnolipid (biosurfactant) in microcosm experiment; 3- bioaccumulation of PAH in the body of both species of earthworms (if any).

3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

Analytical grade hydrocarbons used were purchased from Sigma-Aldrich (UK) with purity 96% PH 97% purity FL and 96% purity BAP, solvents were procured from Fisher-Scientific (UK) with 99% purity for both acetone and hexane. Syringeless filter was procured from GE Healthcare (UK), rhamnolipid biosurfactant was procured from AGAE technologies, USA. All other chemicals, glassware and instruments used were of analytical grade.

3.2.1.1 EARTHWORMS

Adult *L. terrestris* were purchased from Blade Biological Ltd UK, and adult *E. hortensis,* wormery and bedding were purchased from WormsDirect UK. A total of 500 *E. hortensis*, and 800 *L. terrestris* earthworms was procured to which there were 300 cases of mortality during method development and optimization. Both species were eventually stored in plastic containers containing Kettering soil (see Section 3.2.2) amended with 5% compost (w/w), and moisture content of 70% was maintained by watering each plastic container twice every week. Same procedure was carried out an all microcosm experiments.

3.2.2 SOIL PROPERTIES AND PREPARATION

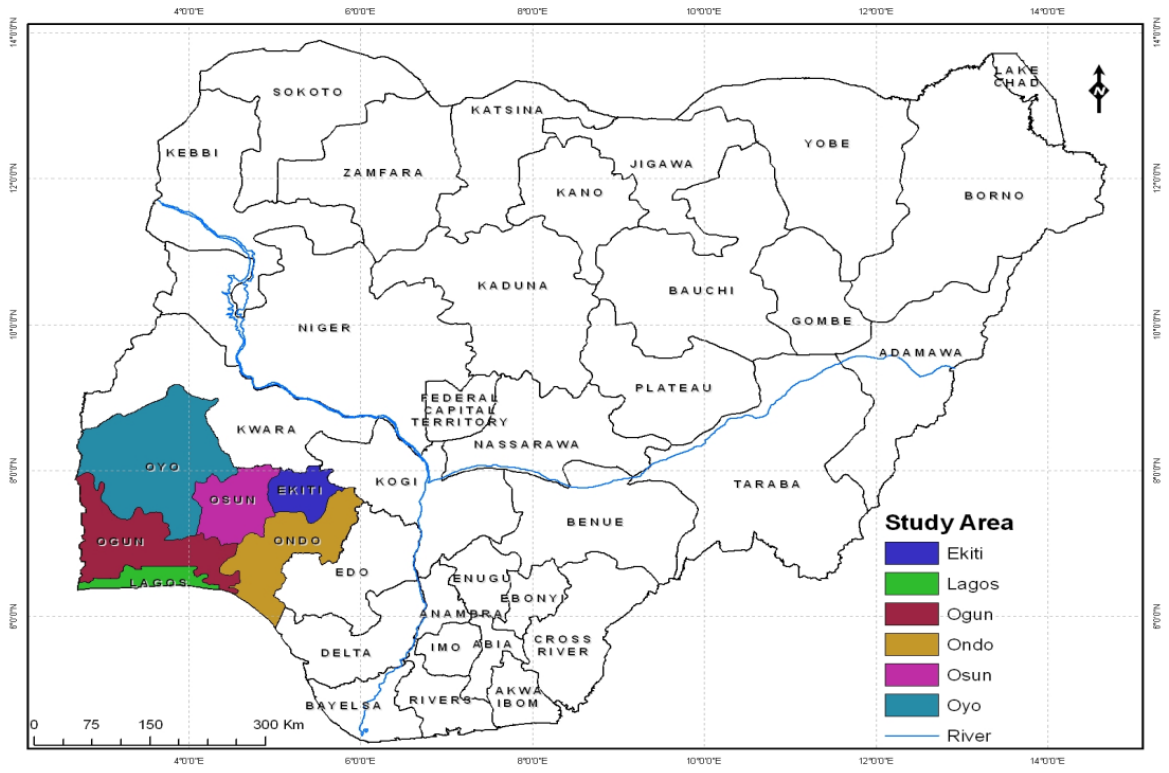


Figure 3. 1 Map of western Nigeria where reference samples were collected (Source: Ayotamuno et al., 2010)

The sampling site is located in Lagos state western Nigeria (Northern Latitude $6^0$24' Western Latitude $3^0$23) (Fig 3.1) characterised by its humid temperature climate with an average annual temperature of 30 ˚C. Soil was collected within a mile radius using a soil auger. Soil samples (1Kg each) were collected between 0 and 20 cm from soil surface from 3 random locations within the radius. Each soil sample was crumbled by hands to break the clumps present in the soil. A 2mm sieve was used to sieve soil samples to remove unwanted debris such as stones, pieces of wood etc. Soil was then left to air dry for 48 hours, after which it was weighed and stored in a fridge at 4 ˚C. To set-up a laboratory scale microcosm experiment, physico-chemical parametric tests were conducted on reference soil obtained from Nigeria and locally obtained soil from Kettering Loam Soil Limited UK. Kettering Loam soil was eventually used for all laboratory experiments.

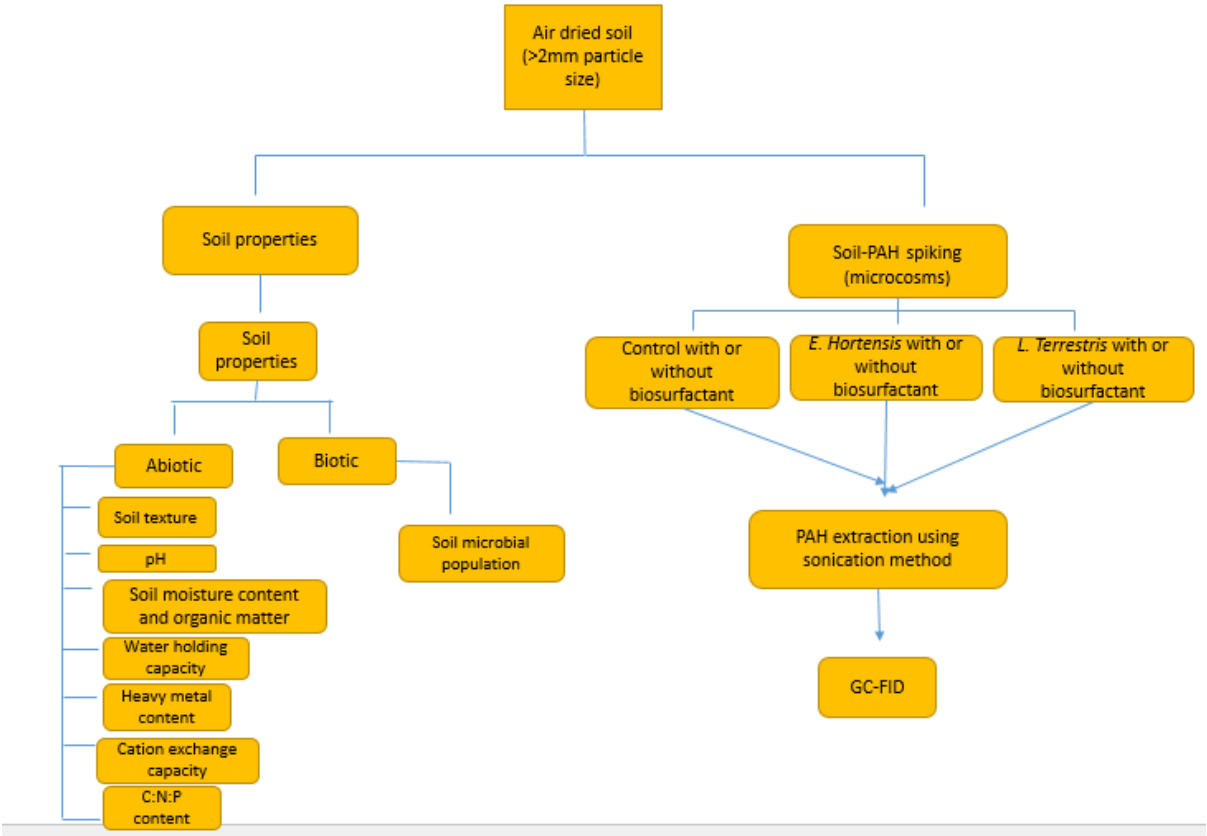


Figure 3. 2 A flowchart highlighting overview of methodology

3.2.3 SOIL AND BIOSURFACTANT ANALYSIS

Kettering Loam Soil was air dried and homogenized. The following experiments were performed pre-contamination (Fig 3.2): water holding capacity (WHC), soil moisture content (SMC) and soil organic matter (SOM) analysis, soil texture determination (Burke et al., 1989), pH measurement using a meter probe following the protocol as described by Burke et al. (989), toxicity analysis of the soil using microtoxicity analysis and a contact test (OECD, 2000), as well as soil microbial population estimation using pour plate technique. Soil microbial population was repeated at the end of the 28-day experiment to investigate the relationship between vermiremediation and microbial population growth.

3.2.3.1. SOIL TEXTURE AND COMPOSITION

Burnham (1980) reported guidelines which could be used for finger assessment of soil as compared to the texture triangle. To analyse the soil texture, finger assessment technique was used as described by Burnham (1980).

3.2.3.2. SOIL pH

The pH of soil was measured using a Fisher brand Hydrus 300 pH meter with the glass electrode probe. The pH meter was calibrated using pH 4, 7 and 10 buffers. Soil (10 g) was measured and transferred into a clean beaker, deionized water was added to the soil in the ratio of 1:2 (soil to water), and the mixture was stirred with a sterilized glass rod after which the calibrated pH meter electrode was placed in the soil-water mixture to read the pH. This process was repeated twice.

3.2.3.3. SOIL MOISTURE CONTENT (SMC)

Soil moisture content was measured by weighing three empty crucibles, 20 g of soil was weighed into each crucible and transferred to an oven at 105 ˚C overnight followed by cooling in a desiccator as described by Helalia (1993). Moisture content% was calculated thus: (initial weight – final weight)/final weight x 100

3.2.3.4. SOIL ORGANIC MATTER (SOM)

Soil organic matter influences soil properties in several ways which includes: infiltration and retention of water, CEC ability, soil colour and temperature, bioavailability of soil nutrients to plants etc. to determine organic matter content, it has to be separated from the inorganic constituent which can be achieved by destroying the organic content in soil and accounting for the loss as the difference in reduced measurement (Nelson and Sommers, 1982).

Soil organic matter (SOM) was measured taking the same soil sample from soil moisture content and burning overnight in the furnace at 450 ˚C and then cooled in the desiccator.

SOM% = (initial weight of sample – final weight of sample) / final weight of sample x 100

3.2.3.5. WATER HOLDING CAPACITY (WHC)

WHC represents the maximum percentage of water available for plants, this includes both the hygroscopic moisture and capillary moisture (Helalia, 1993). To measure WHC of the soil, 0.5g of soil on a Whatman filter paper was subjected to a pressure of 3000 psi for 3 minutes. This led to the formation of two distinct areas commonly referred to as the meat and flesh area (Helalia, 1993). WHC is thus represented as the ratio of water to meat area, where a lower ratio is desirable as this indicates the ability of soil to bind water.

3.2.3.6. CATION EXCHANGE CAPACITY (CEC)

CEC represents a measure of total negative charge density; thus, it is a measure of cation retention capacity of the soil. This method follows three key steps: 1) cation exchange sites are saturated with specific cations, 2) excess saturation solution is removed and 3) saturated cations are replaced (Rhoades, 1982).

Approximately 5 ± 0.05g of air dried Kettering Loam soil was weighed and transferred into 50 mL centrifuge tubes, 1 M sodium acetate (30 mL) was added to each tube and sonicated for 10 mins in an ultrasonic water bath, then centrifuged at 3000 x g for 5 mins. The supernatant was decanted, and 30 mL ethanol was then added to each tube, placed in ultrasonic water bath for 5 mins, centrifuged and then decanted. This procedure was repeated twice to ensure total removal of remnant sodium acetate. Cation from soil was then extracted using 20 mL of 1 M ammonium acetate, repeating the procedure as described earlier. The procedure was repeated 3 times, supernatant was filtered and collected in volumetric flasks and made up with deionized water. Cation concentrations was then determined by an already calibrated inductive coupled plasma optical emission (ICP-OES) (Thermo Scientific iCAP 6000 series).

Data Analysis

Sample concentration = {(C*D*V)/S}/R

Where:

C= Concentration from extract (ppm or mg L^{-1})

D= dilution factor

V= Volume of extractant

S= soil dry weight (g)

R= relative atomic mass of element (Na- 22.99, Mg= 24.3, K= 39.1)

Cation exchange capacity ($cmol_c$ Kg L^{-1}) = $Na^{+}+Mg^{2+}+K^{+}+Ca^{2+}$

3.2.3.7. TOTAL NITROGEN

Kjeldahl method is a wet oxidation procedure, where organic N in soil sample is converted to NH_4^+-Cl by digestion using sulphuric acid (H_2SO_4). The concentration of NH_3 released from the distillation of the digest determines the amount of NH_4^+-Cl (Bremner and Mulvaney, 1982).

Approximately 5 ± 0.05 g of soil was oxidised in 10 mL of sulphuric acid in a Teflon tube, mixture was cooled using distilled water and finally made alkaline using NaOH to produce ammonia (NH_3). NaOH solution was carefully added to create a separate layer from sulphuric acid. The ammonia produced was separated by distillation, after which it was followed by another reaction using hydrochloric acid in the distillation flask. The flask was agitated to obtain homogeneity of layers. This was followed by heating the flask for distillation of both ammonia and water. Distilled ammonia and water was collected in a separate flask that contained excess hydrochloric acid aimed at preventing loss by volatilization. As the ammonia is collected, it undergoes a reaction with hydrochloric acid to form NH_4Cl salts. NH_4^+ was titrated with HCL using methyl red as an indicator.

Calculation: %N = (a*M*14*100)1000* 5g

Where a + used volume of HCl, M = Molarity of HCl solution.

3.2.3.8. TOTAL ORGANIC CARBON (TOC)

Approximately 5 ± 0.5 g soil was weighed and dried for 1 hour at 103 $^{\circ}$C. TOC was measured using Shimadzu 2000 TOC-5000A total organic carbon analyser. The instrument was calibrated using glucose (organic carbon) and sodium bicarbonate (inorganic carbon)

3.2.3.9. SOIL MICROBIAL POPULATION (POUR PLATE TECHNIQUE)

Three incubation bottles containing 99 mL sterile water labelled 10^{-2}, 10^{-4}, and 10^{-6} respectively were prepared, to which Kettering loam soil (1 g) was weighed and deposited into the 10^{-2}, bottle then covered and whirled to obtain a homogenous solution without air bubbles. Using a pipette and pipette filler, 1 mL of the 10^{-2} solution was pipetted into 10^{-4} bottle, covered and whirled, with the aid of another pipette, 1mL from 10^{-4} was pipette into 10^{-6} and mixed well to get homogenous solution. four Petri dishes were labelled TSA 10^{-4} to 10^{-7}, another four was labelled GYEA 10^{-3} to 10^{-6}, and the last four were labelled SDA 10^{-2} to 10^{-5}. Using the 1000 µL Gilson pipette and sterile tips, 1000 µL was pipette each into the Petri

dish 10^{-4} and 10^{-6} with different tips from the respective dilution bottles, while 100 µL was pipette into 10^{-5} and 10^{-7} from the respective dilutions. TSA agar bottle was withdrawn from boiling water bath, approximately 20 mL was measured into each plate, each dish was whirled and partially covered to allow steam out and medium to solidify. Same method was applied to GYEA and SDA with respective dilutions. On solidification, dishes were inverted and incubated at 25 °C for 7 days.

The same procedure was repeated after 28 days for all the controls and experiments.

3.2.3.10 BACKGROUND HEAVY METAL AND PHOSPHORUS

Background heavy metal and phosphorus concentrations were measured with an inductive coupled plasma optical emission spectrometer (ICP-OES; Thermo Scientific iCAP 600 series) upon the digestion of 0.5 ± 0.05 g of air dried soil in 20 mL of nitric acid and transferred to the microwave for digestion (as described by USEPA method 3051A). Certified reference material BGS-102 was used in validating laboratory method and the ICP was calibrated with individual metals standards in deionized water.

3.2.3.11 MICROTOXICITY TEST

The toxic effect of rhamnolipid on the indigenous microbial population was tested using the microtox model 500 toxicity analyser using the 95% microtox test method. This was done by using frozen dry bacteria *Vibrio fischeri* as a test specimen for the impact. The bacteria were exposed for 5 and 15 minutes respectively.

On exposure of the bacteria *Vibrio fischeri* to the rhamnolipid, the amount of light emitted by the bacteria was measured. The more toxic the rhamnolipid, the less the light is emitted by the bacteria.

The concentration was then measured after 5 and 15 minutes of exposure which is the EC_{50}, the point where the light emitted by the bacterium is reduced by 50%.

3.2.4 METHOD DEVELOPMENT AND VALIDATION

The gas chromatography instrument used was a Thermo-Scientific brand connected to a flame ionization detector. Data acquisition was done using the Thermo-Scientific Chromeleon software. Column used was a non-polar stationary phase Zebron ZB-5MS GC capillary column (30 m x 0.25 mm

x 0.25 µm). FDA. (2007) international accepted criteria was used in validating methods. The selected parameters evaluated were; linearity, recovery, accuracy, limits of detection and quantification.

3.2.4.1 STANDARD CURVE

Stock solutions of PH, FL and BAP was prepared by dissolving individual PAHs in acetone to make separate stock solutions of 60 mg Kg^{-1} each. A 5- point standard curve (2, 10, 40, 200 and 1000 mg Kg^{-1}) by adding known concentrations of all three PAHs to acetone. Linear regression of peak area of each PAH against nominal concentration of the analyte was used to plot the calibration curve.

3.2.4.2 RECOVERY, PRECISION AND ACCURACY

Analyte recovery was determined using spiked soil samples from quality control. Peak areas of the PAHs at three combined concentrations (1:1:1) 60, 90 and 120 mg Kg^{-1} obtained from three replicates of quality control samples. These were compared to extracted blank soils to derive percentage extraction. Same quality control samples as above were used to evaluate the precision and accuracy. Using three replicates of the quality control samples prepared on three non-consecutive days, the precision was calculated using coefficient of variation (C.V. %), with accuracy being expressed as percentage difference between concentration added and concentration recovered from spiked soil sample.

3.2.4.3 LIMITS OF DETECTION AND QUANTIFICATION

FDA (2007) accepted criteria for the limit of quantification was defined as the precision and accuracy for three extracted samples with a C.V. ≤20%. Limit of detection was determined using the lowest concentration of the analyte that resulted in a signal to noise ratio of 9.6.

3.2.5 PRELIMINARY STUDIES

An in-depth pilot study was conducted to validate viability of both earthworm species at different concentrations. Both species were subjected to two of the three PAHs (FL and PH at combined concentrations of 60 mg Kg^{-1} and 120 mg Kg^{-1}) for the duration of 28 days. Same spiking procedures in following Sections 3.2.6 and 3.2.7 were used to conduct this study with exception of BAP.

3.2.6 INDIGENOUS MICROBIAL DEGRADATION (control)

Approximately 5 ± 0.05 g air-dried Kettering Loam soil was weighed and transferred into twelve 200 mL beakers. PAH concentration (PH, FL and BAP at 180 mg Kg^{-1} combined (60 mg Kg^{-1} each) and 60 mg Kg^{-1} individual BAP) were selected based on what has already been reported in literature (Contreras-Ramos et al, 2005; Rorat et al, 2017; Rodriguez-Campos et al, 2019). PAHs were dissolved in acetone and mixed thoroughly in with the soil 180 mg Kg^{-1} combined PAHs (PH, FL and BAPc) to one part, and 60 mg Kg^{-1} individual BAP (BAPi) to the other, then left in a desiccator for 16 hours for solvent to completely evaporate, 20 g soil was then added to each container and mixed thoroughly (Brinch et al., 2002). Half the containers were amended with 100 mg L^{-1} rhamnolipid biosurfactant (PAHB) while the other half was left unamended (PAH). Soil water holding capacity (WHC) was adjusted to 70% with distilled water and properly homogenized to ensure even distribution of hydrocarbons throughout the medium. The containers were placed in a Gratnell tray containing 10mL water and kept in a dark room at 22 ± 2 °C for 28 days (Fig 3.2).

3.2.7 EARTHWORM DEGRADATION

EPIGEIC/ANECIC EARTHWOMRMS (*Eisenia hortensis/Lumbricus terrestris)*

Approximately 125 ± 0.05g of soil was weighed into 24 transparent 1.5 L plastic containers (height 9 x diameter 18.5 cm). The same spiking conditions and parameters as described in Section 3.2.5.1 were repeated in this Section: five adult earthworms (*Eisenia hortensis/Lumbricus terrestris*) identified by their clitellum, were weighed and added on top of soils in each container, covered with an air-tight lid perforated with holes for ventilation and stored in a dark room at temperatures of 22 ± 2 °C. Twice a week soils were watered to maintain optimal soil water content. Treatments were soil contaminated with different concentrations of PAH with *Eisenia/Lumbricus* (PAHE/PAHL) and soils contaminated with PAH amended with 0.1 g L^{-1} biosurfactant and *Eisenia/Lumbricus* (PAHBE/PAHBL). Samples were collected and analysed every week using a destructive microcosm technique.

3.2.8 PAH EXTRACTION FROM SOILS BY GC-FID

Soil samples (5.0 g) were collected randomLy from treatment in triplicates at day 0, 7, 14 and 28 and air dried. Phenanthrene, Fluoranthene and Benzo(a)pyrene in soil was extracted using the sonication method as developed by Song et al. (2002). Approximately 1.5 ± 0.5 g of soil was measured and mixed with 3 g anhydrous sodium sulphate to form a fine powder, the mixture was then transferred into amber vials. Hexane (10 mL) was added to each vial and transferred into an ultrasonic bath set at 40 ˚C for 20 mins, the samples were vortexed for 15 seconds then sonicated again for another 20 mins. Supernatant was collected in a 50 mL centrifuge, the process was repeated twice. Extracts were then centrifuged at 6,000 x g for 15 min, then pooled and passed through a 0.2 µm syringed filter. Extracts were transferred to a Turbo-Vap nitrogen evaporator where the solvent was evaporated to dryness. Samples were reconstituted with 1 mL hexane, aspirated and transferred for analysis with a Thermo-Scientific GC-FID with a Zebron ZB-5MS GC capillary column (30 m x 0.25 mm x 0.25 µm). Targeted separation was achieved with the following conditions: initial oven temperature 170 ˚C (1 minute holding time), then increased to 240 ˚C at 15 ˚C, held for a min; then to 275 ˚C at 4 ˚C held for a minute then to 320 ˚C at 10 ˚C and held for 5 mins. the flow rate of the carrier gas (hydrogen) was 1 mL/min. 1 µL of the sample was injected through a split-less mode into the oven at 310 ˚C and into the FID at 330 ˚C. The GC was calibrated with individual standards before use, QA/QC was ran by spiking 5g of soil with a known amount of PH, FL and BAP and extracting after 24 hours to validate extraction methods were satisfactory.

The GC functions in measuring the analyte by taking up a small volume of the sample into the GC syringe. The syringe is inserted in a hot injector port of the GC where the sample injected. The temperature of the injector is set higher than the boiling point of the analyte in order to vaporize the analyte component into gaseous form in the injector. Carrier gas (helium) flows through the injector where it forces the gaseous analyte into the GC column. Separation of the analyte component occurs in the column, molecules separate between the stationary phase (high boiling point liquids) and mobile phase (carrier gas) in the column. The analyte travels through the GC column and eventually reaches the detector, they analyte reaches the detector at different times due to the separation in the column which is dependent on their molecular weight. A signal is sent to the chart recorder from the flame ionization detector

which results in a peak. The peak area is proportional to the concentration of the analyte generating the peak.

3.2.9 EARTHWORM ANALYSIS

3.2.9.1 SAMPLE PREPARATION

E. hortensis and *L. terrestris* in amended and unamended microcosms from destroyed microcosms (day 2, 7, 14 and 28) were randomLy selected, washed weighed and placed in separate petri dish with filter papers to purge their guts for 12 hours. The earthworms were sacrificed by immobilizing in ice cold glycerol solution 20% (v/v) for 3 minutes after which their gut was excised and washed in ice-cold (0.15 M) KCl solution to remove haemocytes and homogenized in liquid nitrogen.

3.2.9.2 PAH CONCENTRATION ANALYSIS

PH, FL and BAP concentrations from homogenized earthworms (Section 3.2.9.1) were determined as described in Section 3.2.8 after acid digestion as described by Demuynck et al. (2007). 50 mg ± 0.05 dry mass was digested at room temperature in 0.5 mL 70% nitric acid for 12 hours, samples were heated in a water bath at 120 °C to remove nitrous vapour as well as reduce the volume by half. The temperature was increased to 180 °C upon addition of 1 mL solution of nitric, perchloric acid and sulfuric (10:3:2 v/v). Samples were collected, filtered and made up to 20 mL with deionized water. Filtrate were then passed through a solid phase column packed with a C18 silica gel. The analyte was eluted from the column using acetonitrile. Body accumulation factor was calculated using the equation below:

$$BAF_{PAH} = \frac{C_{PAH}\ \text{earthworm}}{C_{PAH}\ \text{soil}}$$

Where C_{PAH} earthworm is the concentration of selected PAH in the body of the earthworm (mg Kg^{-1}) and C_{PAH} soil is the concentration of same PAH in soil (mg Kg^{-1}).

3.2.10 STATISTICAL ANALYSIS

PH, FL and BAP concentrations were subjected to a one-way or two-way analysis of variance (ANOVA) with *post-hoc* Tukey's test to test for significance of difference between the treatments using GraphPad Prism 6.

3.3 RESULTS

Kettering soil used in vermiremediation was characterized with a substantial organic matter that serves as source of nutrition to the worms, pH of soil was 7.1 ± 0.02. Derived Microtox EC_{50} concentration after 15 minutes was 0.1 g L^{-1}, microbial population for bacteria fungi and actinomyces were 3.6 x 10^4 5.6 x 10^3 and 5.5 x 10^4 (cfu g^{-1}) (Table 3.1) respectively indicating the soil was well characterized for remediation.

Table 3. 1 Soil physico-chemical and biological parameters and microbial count

Soil sample	**Method**	**Nig**	**Ket**
pH	3.2.3.2	6.7 ± 0.5	7.1 ± 0.02
Bacteria (cfu g^{-1})	3.2.3.10	5.4 x 10^4	3.6 x 10^4
Fungi (cfu g^{-1})		3.5 x 10^4	5.6 x 10^3
Actinomyces (cfu g^{-1})		1.4 x 10^4	5.5 x 10^4
Water holding capacity (%)	3.2.3.5	46.3 ± 0.1	40.1 ± 0.3
Soil composition (clay-silt-loam) (%)	3.2.3.1	27, 33, 40	23, 35, 42
Soil organic matter (%)	3.2.3.4	5.4 ± 0.5	3.9 ± 0.3
Soil moisture content (%)	3.2.3.3	7.9 ± 0.4	4.9 ± 0.2
CEC (cmol (+) Kg^{-1})	3.2.3.6	27.9 ± 0.2	30.1 ± 0.3
Total nitrogen (%)	3.2.3.7	0.2 ± 0.03	0.2 ± 0.01

Total phosphorus (mg g^{-1})	3.2.3.8	0.4 ± 0.01	0.42 ± 0.01
Total organic carbon (mg Kg^{-1})	3.2.3.9	40 ± 0.5	40.3 ± 0.5

Where Nig = Nigerian soil and Ket = Kettering soil

3.3.1 DEVELOPMENT AND VALIDATION OF SOIL EXTRACTION METHOD FOR QUANTIFICATION OF PAHs

Extraction method was validated using quality control soils that were spiked at three known concentrations.

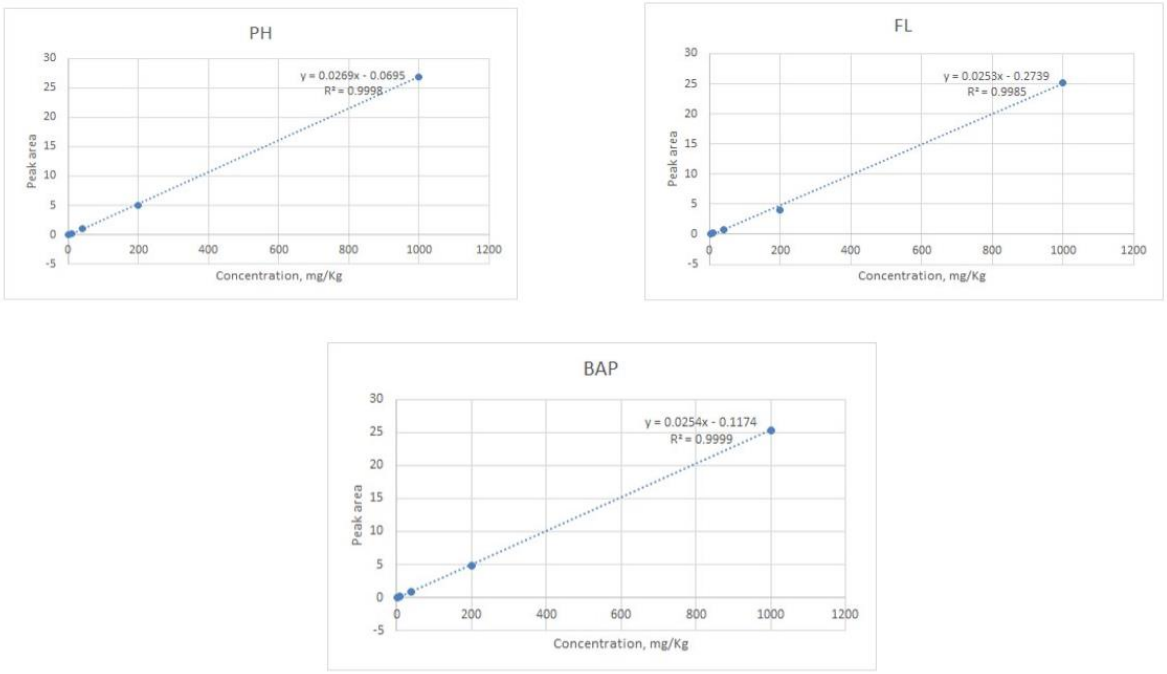


Figure 3. 3 Standard curve for all three PAHs were linear from 2 to 1000 mg Kg^{-1}, with the C.V in the range of 1.9 - 13.4% and an accuracy of 95 – 105.2% (n=3).

Selected quality control concentrations were used for inter and intra-day precision and accuracy. The precision at all three concentrations for all three PAHs was below 15% C.V, accuracy was calculated as derived percentage of the initial spiked concentration (Table 3.2). same concentrations used for quality control were used for calculating recovery. This was done by comparing extracted PAHs from spiked soil samples to blank soil samples. A good recovery rate of the PAHs within the range of 80 – 90% was derived, with limits of detection 2 mg Kg^{-1} and limit of quantification 5 mg Kg^{-1}.

Table 3. 2 Accuracy, intraday (n=3) and intraday (n=9) and recovery (n=3) for PAH (PH, FL and BAP) from spiked soil

PAH (mg Kg^{-1})		1st day		2^{nd} day		3^{rd} day		Recovery (%)
		Accuracy (%)	C.V. (%)	Accuracy (%)	C.V. (%)	Accuracy (%)	C.V. (%)	
60	PH	99	2.4	101.3	6.6	100	1.3	86.2 ± 1.3
	FL	97.2	4.6	99.7	4.3	97.6	4.2	86 ± 1.9
	BAP	99.5	6.2	99.8	1.3	99.4	0.4	81.8 ± 1.3
90	PH	99.6	2.8	101.1	5.1	100.3	1.7	87 ± 3.3
	FL	95.2	3.6	93.7	2.3	97.1	3.2	85 ± 1.3
	BAP	97.7	4.2	98.8	4.7	98.4	1.4	82 ± 1.7
120	PH	101.1	1.7	99.3	3.6	100	1.3	85.3 ± 3.3
	FL	99.3	3.1	98.9	4.1	99.6	2.2	85.1 ± 1.3
	BAP	101.2	4.8	99.3	2.3	98.4	2.4	80.0 ± 1.7

PRELIMINARY STUDY

Results from preliminary experiments (pilot) showed no traces of PH or FL by day 28 in experiments amended with either species of earthworm and biosurfactant, or earthworm alone compared to controls with neither earthworm or biosurfactant amendment and biosurfactant amendment only (refer to Appendix 1 for data from preliminary studies).

3.3.2 TRACE HEAVY METAL CONCENTRATION IN SOIL

Seven trace metals Cd, Cr, Cu, Mn, Ni, Pb and Zn were examined before and after the duration of 28 days, Ni was not detected in any of the samples. Furthermore, there was no statistical difference for any of the trace metals before and after except Mn which increased in C_L and C_{LB} (Table 3.3).

Table 3. 3 Trace heavy metal concentration in con, conB, E, EB, L, LB at day 0 and day 28.

	Concentration (µg Kg^{-1})						
	Day zero (control)	No biosurfactant	Biosurfactant only	E	EB	L	LB
Cd	0.27 ± 0.06 [a]	0.13 ± 0.06 [a]	0.3 ± 0.01 [a]	0.32 ± 0.05 [a]	0.21 ± 0.08 [a]	0.52 ± 0.03 [a]	0.71 ± 0.02 [a]
Cr	160.11 ± 1.74 [a]	156.93 ± 2.70 [a]	158.02 ± 51.5 [a]	161.89 ± 1.13 [a]	161.07 ± 0.6 [a]	158.29 ± 2.2 [a]	159.53 ± 1.33 [a]
Cu	35.51 ± 1.69 [a]	32.39 ± 2.02 [a]	40.39 ± 0.77 [b]	37.95 ± 0.77 [a]	30.67 ± 2.17 [a]	25.59 ± 1.39 [a]	33.97 ± 1.5 [a]
Mn	348.73 ± 5.32 [a]	388.64 ± 7.07 [b]	391.57 ± 0.75 [b]	316.11 ± 1.77 [c]	388.99 ± 3.12 [b]	354.35 ± 1.73 [a]	394.24 ± 3.34 [b]
Ni	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Pb	83.64 ± 0.56 [a]	86.26 ± 1.19 [a]	84.27 ± 0.3 [a]	82.63 ± 0.49 [a]	83.89 ± 0.44 [a]	80.5 ± 0.41 [a]	83.35 ± 0.86 [a]

Results are represented as means ± SD, n = 3. Means not sharing the same letter are statistically different according to ANOVA test ($p<0.05$) and N.D = not detected.

3.3.3 BODY WEIGHT AND FECUNDITY IN EARTHWORMS

Both *E. hortensis* and *L. terrestris* in unamended experiment (60 mg Kg^{-1} BAP and 180 mg Kg^{-1} PH, FL and BAP) showed no significant increase in body weight (Fig 3.4). Both species showed significant increase in body weight within the first 7 days in experiments amended with biosurfactant however all showed loss of weight by day 28.

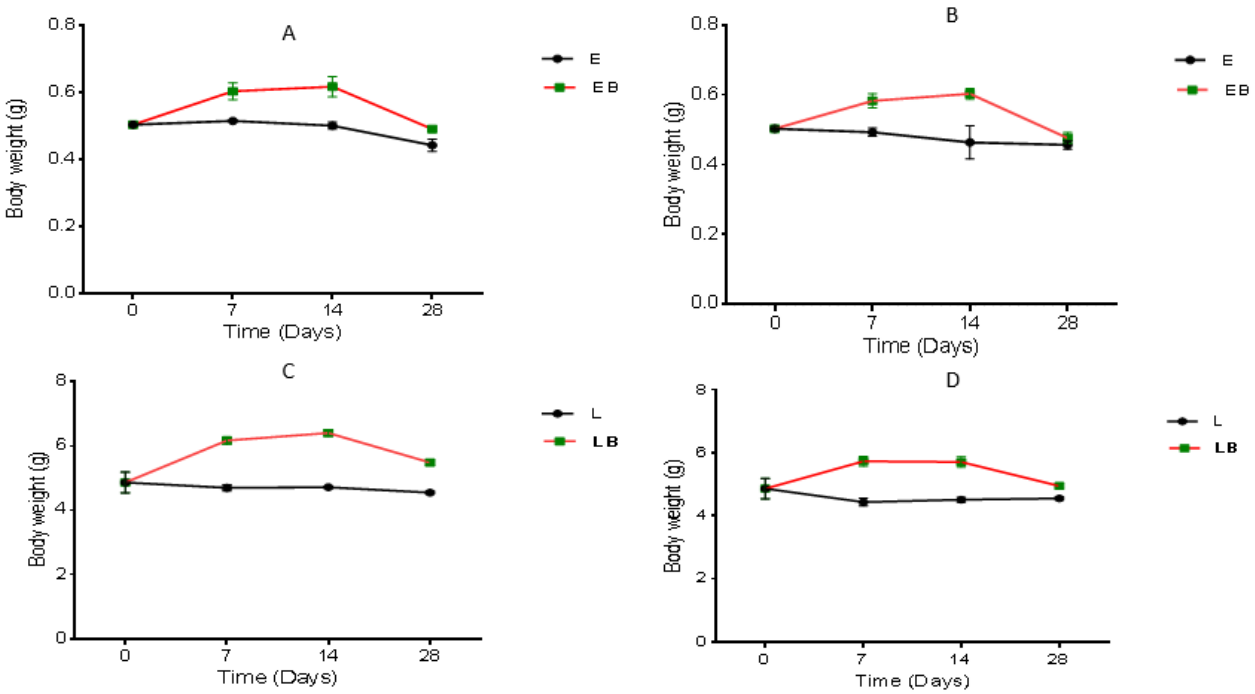


Figure 3. 4 Change in body weight of earthworms within the course of a 28-day experiment, results are represented as means ± SD, n = 160. A and B represent body weight of *E. hortensis* in soil containing 180 mg Kg^{-1} PAH (PH, FL and BAP) and 60 mg Kg^{-1} BAP amended with 0.1 g L^{-1} biosurfactant (EB) and unamended (E) respectively. C and D represent *L. terrestris* in the same conditions as A and B.

No mortality was recorded for the duration of exposure of PAHs in all experiments with the exception of *L. terrestris* at day 28 where there was complete mortality. The cocoon production in both *E. hortensis* and *L. terrestris* were higher in number in experiments amended with biosurfactant and at combined concentration of PAH (180 mg Kg^{-1} PH, FL and BAP) than unamended experiments and individual concentration of PAH (60 mg Kg^{-1} BAP), suggesting the application of biosurfactant influenced the production of cocoons in both species (Table 3.4).

Table 3. 4 Fecundity and mortality after 28 days.

	C_0 (mg Kg^{-1})	E	EB	L	LB
FECUNDITY (cocoons):	180	2 ± 0.8	3.25 ± 0.4	2.25 ± 1.5	4.5 ± 1.7
	60	1 ± 2	2.75 ± 1.1	2 ± 1.4	3.25 ± 1.2
MORTALITY (%):	180	0	0	100	100
	60	0	0	100	100

C_0 represents total spiked concentration of PAH both *E. hortensis* and *L. terrestris* were exposed to. Results are represented as means ± SD, n = 3, *p-value* <0.01 in cocoon production from biosurfactant amended soil in both species at both concentrations

3.3.4 PAH ANALYSIS IN SOIL

Overall approximately 85% of the introduced PH and FL and 80% of BAP introduced were recovered from spiked soil at day zero (Table 3.5). This concentration varied little over the 28-day treatment period, with an average removal of 30% of both contaminants after 4 weeks of exposure. The concentration of the contaminants added did not appear to affect the removal of all three PAHs. The average removal of both concentrations upon amendment with biosurfactant was 60% PH and 55% FL after 4 weeks (Table 3.5).

Both earthworm species removed more PH, FL and BAP (p<0.001) in the presence of biosurfactant. Epigeic worms (*E. hortensis*), tended to remove more of the phenanthrene compared to anecic worms *(L. terrestris*) after 4 weeks (Fig 3.5). Removal of PH, FL, BAPc and BAPi after four weeks in unamended soil with *E. hortensis* was an average of 67%, 59%, 55% and 51% respectively. On addition of biosurfactant amendment, PH was undetectable after 4 weeks of exposure, FL was removed by 99%, BAPc by 80% and BAPi by 61%. *L. terrestris* removed an average of 54% PH, 55% FL, and 56% both BAPc and BAPi without biosurfactant amendment and both PH and FL were not detected after 4 weeks, with 72% removal in BAPc and 65% in BAPi (Fig 3.5), indicating that biosurfactant did accelerate the removal of PH FL and BAP by *E. hortensis* and *L. terrestris*. Survival in both species was not affected by the concentrations of PAHs and biosurfactant.

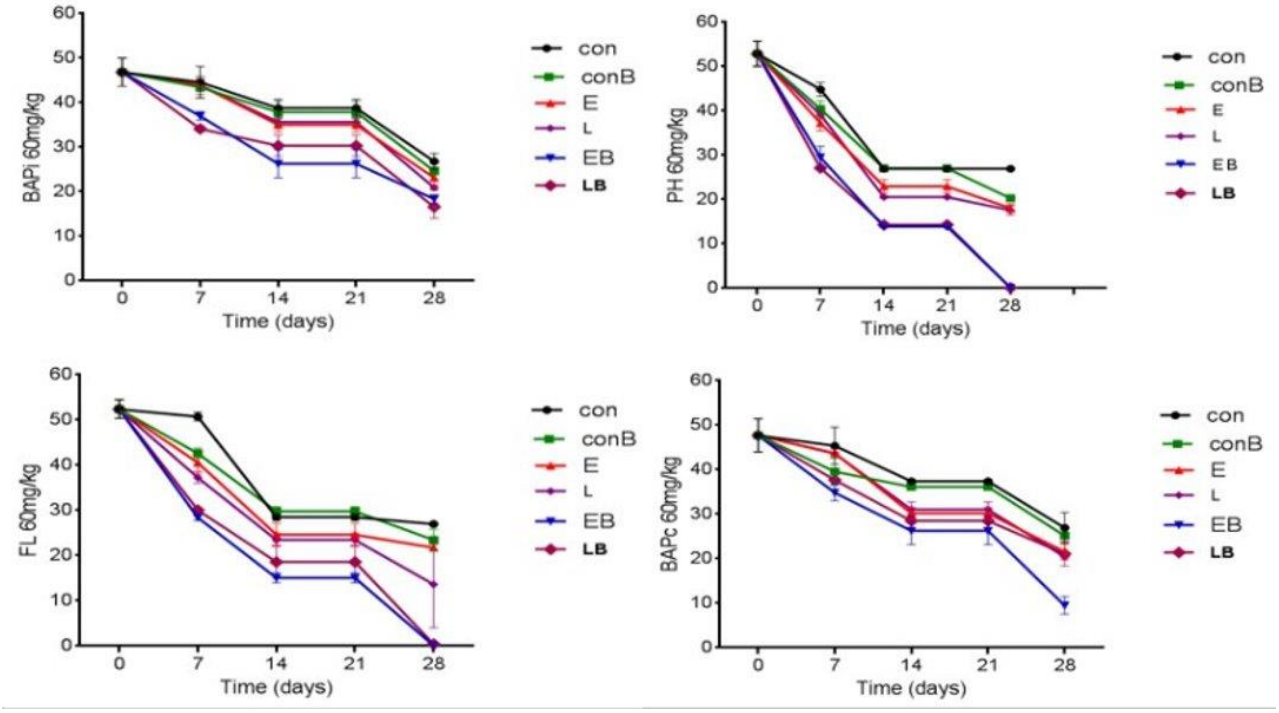


Figure 3. 5 Comparative removal of PH, FL and BAP from treatment after 4 weeks in both *Eisenia hortensis* and *Lumbricus terrestris.* All data are represented as means ± SD, n = 3.

Table 3. 5 PAH concentration and percentage removed at day 0 and day 28. Results are represented as means ± SD, n = 3, ($p < 0.05$) and N.D = not detected.

Compounds	0	Con		conB		E		EB		L		LB	
	0d	28d	%	28d	%	28d	%	28d	%	28d	%	28d	%
PH 60mg Kg^{-1}	49.8±1.3	31.6±3.5	36.6	23.3±2.9	53	16.2±2.98	67.5	N.D.	100	23.1±0.8	53.6	N.D.	100
FL 60mg Kg^{-1}	49.8±1.3	29.4±3.13	41	27.1±0.5	45.6	20.4±0.5	59	N.D.	100	22.51±0.54	54.8	N.D.	100
BAPc 60mg Kg^{-1}	47.6±3.7	26.9±3.4	43.5	25.1±0.9	47.3	21.5±1.8	54.8	9.4± 1.9	80.3	21±2.7	55.9	13.4±9.3	71.9
BAPi 60mg Kg^{-1}	46.8±3.1	26.7±1.8	43	24.6±0.9	47.4	23.1±2.4	50.6	18.3 ±1	60.9	20.81±0.63	55.5	16.5±2.6	64.7

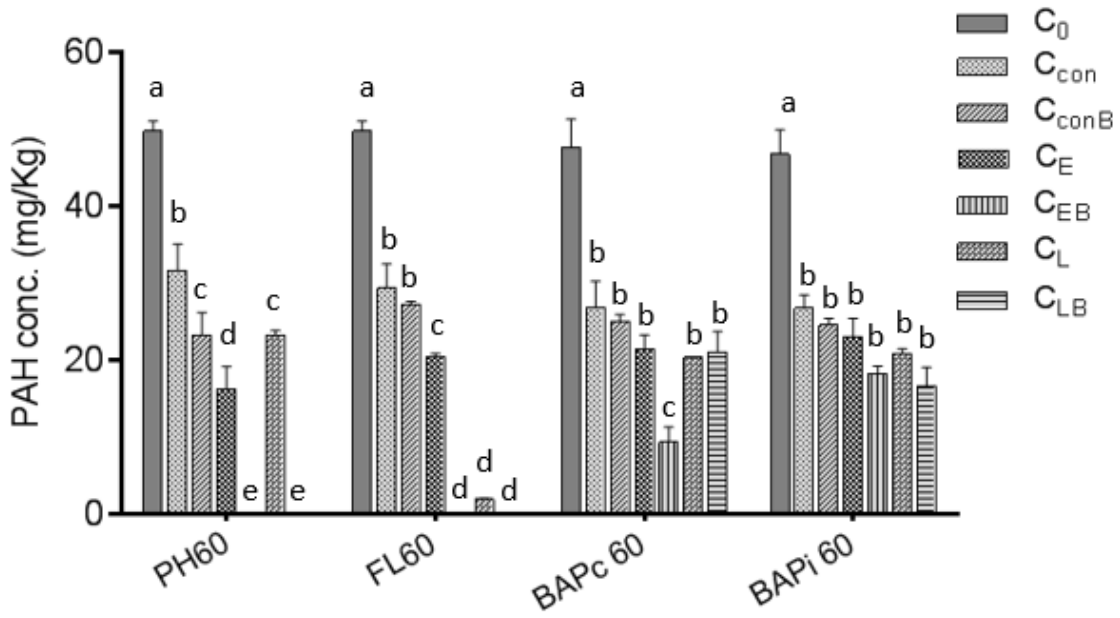


Figure 3. 6 A comparative overview of combined PAHs (PH, FL and BAP) and individual BAP removal between treatments in both *Eisenia hortensis* and *Lumbricus terrestris* after 28 days. All data are represented as means ± SD, n = 3. Means not sharing the same letter are statistically different according to ANOVA test (p<0.05).

$C_{0 =}$ amount of PAH extractable at day zero as a percentage of PAH added

C_{con} = amount of PAH extractable from unamended soil without earthworms after 28 days as a percentage of amount extractable at day zero

C_{conB} = amount of PAH extractable from biosurfactant amended soil without earthworms after 28 days as a percentage of amount extractable at day zero

C_E = amount of PAH extractable from unamended soil with *Eisenia* after 28 days as a percentage of amount extractable at day zero

C_{EB} = amount of PAH extractable from biosurfactant amended soil with *Eisenia* after 28 days as a percentage of amount extractable at day zero

C_L = amount of PAH extractable from unamended soil with *Lumbricus* after 28 days as a percentage of amount extractable at day zero

C_{LB} = amount of PAH extractable from biosurfactant amended soil with *Lumbricus* after 28 days as a percentage of amount extractable at day zero

3.3.5 PAH ANALYSIS IN EARTHWORMS

Body accumulation factor calculated across all experiments in both *E. hortensis* and *L. terrestris* were non-detectable as all three PAHs were not detected in homogenized earthworm tissue.

3.6 DISCUSSION

3.6.1 SOIL PROPERTIES

Several soil physico-chemical parameters determine the environmental conditions soil micro and macro-biota are subjected to (Parr et al., 1983). Typically, mineral soils consist of an average 45% mineral material with different proportions of sand silt and clay, 25% water and 25% air and approximately 5% of organic matter. Results of soil physico-chemical and biological characteristics with similar properties are highlighted in Table 3.1.

Soil texture and structure play a crucial role in the degradation of PAHs. Karimi-Lotfabad et al. (1996) reported a 25% non-extractable percentage of phenanthrene exposed to soils with high clay content in them, Manilal and Alexander (1991) also reported a quicker degradation of PAHs in liquid medium compared to soil which could be associated with the sorption of contaminants to organic matter in the soil. Hence for optimised remediation of contaminants it is very important to understand the structure and texture of the soil. In this particular soil sample used in this study clay fraction was the least.

For degradation to occur in soil, soil nutrients are to be abundant for microbial consumption (Arora et al., 1982). Most frequent nutrients that are limited in abundance are nitrogen and phosphorus in soil which they come in contact with through their burrowing activities (USEPA, 1994). Addition of these nutrients to contaminated soils could further enhance microbial degradation of organic contaminants into carbon dioxide and water.

Generally, the carbon, nitrogen and phosphorus ratio in bacteria cells are 100:15:3 (Zitrides, 1983), but is variable according to soil types. Nitrogen and phosphorus should however be made available for optimised degradation of contaminants (Alexander, 1994). Measuring soil

C:N:P allows evaluation of the nutrient availability in the soil which is important in degradation of organic contaminants.

Saturated moisture conditions could lead to reduced hydrocarbon degradation because of the low supply of oxygen. However, with low moisture conditions, soil micro and macro-biota activities suffer because they require moisture to function (CONCAWE, 1980). Soils such as sandy soils have low WHC because of its large pore sizes hence it rapidly loses water, whereas soils with a mixture of pore sizes such as loam holds and retains water and loses it very slowly hence suitable for bioremediation (Turco and Sadowsky, 1995).

WHC in Kettering soil was 40% with a 4.9% moisture content, WHC was optimized between 60-70% (desired earthworm moisture conditions) to remediate PAHs. Riser-Roberts (1998) reported a WHC between 30-90% would aid bioremediation of different organic contaminants, although the moisture content required for the mineralisation of contaminants vary from contaminant to contaminants. Holman and Tsang suggested a WHC between 50-70% was best suited for the degradation of PAHs which falls in line with the optimised WHC used in this study.

Cernigilia (1992) indicated that soils generally have organic matter content between 5-12%, organic matter content in this study was on average 4% which is just under the average limit. The adsorption of contaminants to soil particles influences and changes the molecular character of the compound.

In this study, all soil treatments were amended with garden compost to boost the organic matter in soil hence aiding bioremediation. Generally, soil organic material has high amounts of humic substances that consist of several functional groups that function by binding both inorganic and organic substances to the soil. These same properties function in maintaining indigenous soil microorganisms that also aid remediation of contaminants (Godbout et al., 1995).

pH of soil is generally in the range of 5 to 9 (Sommers et al., 1981). The pH in soils used in this study was 7 which is neutral which favourable conditions for bacterial growth and activities are. pH plays crucial roles in biological activities of the soil in terms of adaptation of soil micro and macro-biota, and bioavailability of nutrients as well as contaminants.

3.6.2 PAH RECOVERY

Ramos-Contreras et al. (2006) reported 79% recovery of PH from Acolman soil whilst Eijsackers et al. (2001) reported 80% recovery of FL from sandy soil and Song et al. (2002) reported 74% recovery of PH from sandy soil with earthworms, all using ultrasonic method. Similar result was achieved in this research with even higher recovery of both PH and FL with both hydrocarbons showing same pattern of removal as seen in Fig 3.5. This could be attributed to the change of extracting solvent to hexane which has been reported to be the most effective solvent in extracting hydrocarbons, compared to the most used solvent acetone (Xing and Pignatello, 1997). Fractions that were extractable were on average 90% PH, 85% FL and 80% BAP, hence it could be argued that the extracting technique even though time and solvent consuming, is an effective one.

The application of different species of earthworms in vermiremediation and vermicomposting of PAHs and heavy metals have been widely studied. Rodriguez-Campos et al. (2014) discussed the possibilities of earthworms in metabolizing organic contaminants by feeding on them. Furthermore, they discussed the symbiotic relationship between earthworms and the microbes which upon contact could stimulate their growth and activity, it can be said that the microbial community that goes into the earthworms and passes through their gut is far less than what is egested in the vermicast after passage through the gut. This was well represented in this work with the microbial communities of bacteria as high as 8.7×10^7 cfu g^{-1} compared to the 7.2×10^4 cfu g^{-1} in the surrounding soil by the end of the 28-day exposure ($p<0.001$). Similar reports were discussed by Teotia and Dahm (1950) where they found 3.2×10^7 cfu g^{-1} microflaura cells compared to the 6×10^5 cfu g^{-1} they found in the surrounding medium which is due to the intestinal interaction of the microorganisms or possibly the high organic matter contents which results in an increased rate of microbial activities and proliferation (Brown et al., 2004). The microorganism succession and dynamics in the earthworm cast have been reported to be quite complex, and they are dependent on several factors such as food ingested, the time it passes through the gut, as well as ingested and indigenous gut microorganisms.

E. fetida survival rates of 97% in a PAH mixture was reported by Gomez-Eyles et al. (2011), though there was a significant decrease in body weight after 56 days of exposure. Contreras-Ramos et al. (2008) also recorded a complete inhibition in production of cocoons after 70 days of exposure to a mixture of PAHs. This study showed similar results with diminished cocoon production and body weight in both *E. hortensis* and *L. terrestris* (Table 3.4). A possible explanation to the inhibition of reproduction as well as diminished body weight could be explained by oxidative damages caused by PAHs (Owen et al., 2008). Sforzini et al. (2015) identified biomarkers that demonstrated PAHs caused several changes at cellular and tissue levels which could possibly have affected reproduction and body weight. Furthermore, the concentrations of PAHs or possibly the bioaccumulation of the trace heavy metals (that might have been more bio-accessible with the application of biosurfactant) could have also played a role in the inhibition. However, concentrations of trace metals from this study were minute and below interaction limits.

3.6.3 INDIGENOUS MICROBIAL REMOVAL OF PAHs

In this study, removal of phenanthrene (59%) was slightly higher than that of fluoranthene (54%) and benzo(a)pyrene (47%) after 4 weeks in the presence of biosurfactant compared to the 30% removal in treatment without biosurfactant. This result agrees with findings reported by Urum et al. (2006) and Rodriguez-Campoz et al. (2014) on the impact of aqueous biosurfactant on washing crude oil (PAHs) contaminated soil compared to the impact of chemical surfactant, where biosurfactant removed an average of 80% of the PAHs in crude oil at different concentrations. They demonstrated that most of the hydrocarbons were removed due to solubilising and mobilization of molecules.

Banat et al. (2000) argued that the ability of a surfactant to mobilize or solubilize contaminants is dependent on its concentration. Rhamnolipid was able to solubilize contaminants above its critical micelle concentration (CMC) unlike some surfactants such as lecithin, aescin and tannin (Pirollo et al., 2008; Urum et al., 2004). Biosurfactant could however pose toxic threats to the microbial community present in the soil, hence the necessity for a Microtox test in this study to determine the EC_{50} which give a reading 0f 0.1 g L^{-1}. This correlates with the observation of Lai et al. (2008) where surfactants at mass

concentrations of 0.2% and below did aid solubilisation of TPH contaminants from soil, but at concentrations above 0.2%, no significant difference in degradation of total petroleum hydrocarbon (TPH) was observed compared to TPH degradation at lower concentrations. It could also be argued that an increase in the concentration could enhance the removal of PAHs further. Rahman et al. (2003) reported that rhamnolipid added at concentrations above their CMC tend to enhance solubility and biodegradation of hexadecane, n-paraffin, octadecane and PAHs in soils. Santa Anna et al. (2007) also suggested optimization of biosurfactant in PAH removal by mixing sandy soils containing mostly PAHs with 6.3-7.9 g L^{-1} biosurfactant, achieved PAH removal of up to 91%. Findings in this study upon application of 0.1 g L^{-1} biosurfactant (CMC) to FL and PH spiked loam soil (60 and 120 mg Kg^{-1} combined) with both earthworm species, a removal of 95% of both hydrocarbons (Fig 3.5) was observed.

The result from removal of PAHs from unamended soils agree with Atagana et al. (2003) and Eijsackers et al. (2001) where tetrachloride was used as solvent for the ultrasonication extraction. They reported 42% removal of fluoranthene, this could be related to its low solubility in aqueous phase (260 µg L^{-1}), and this reduces their bioavailability and bio-accessibility for degradation to occur.

Overall the degradation of soil contaminants is dependent on the entire soil biota. Even though a great diversity of soil biota has been reported to be capable of degrading lower molecular weight PAH, there are only few genera capable of degrading high molecular weight PAHs of four, five and six rings (Juhasz and Naidu, 2000). It has however been reported that bacteria are capable of degrading these high molecular weight PAHs when they are cultured on alternative carbon sources such as phenanthrene (Juhasz and Naidu, 2000; Chen and Aitken, 1999). Hence the co-exposure of microorganisms to contaminants with phenanthrene may aid the induction of enzyme synthesis for the degradation of high molecular weight PAHs (Chen and Aitken, 1999).

3.6.4 EFFECT OF EPIGEIC AND ANECIC EARTHWORMS ON THE REMOVAL OF PAH

Higher invertebrates or multicellular organisms such as arthropods, nematodes or annelids (earthworms) are ubiquitous in soils. Due to their constant movement in soils, these organisms are constantly exposed to contaminants in the soil such as PAH. As discussed

earlier, they could take up these contaminants via different routes such as orally or dermally since PAHs are well known for attaching soil surfaces which the earthworms eventually feed on (Johnsen et al., 2005). Several authors have studied the effect of contaminants on *Eisenia* species because of their vast tolerance limits, but as argued by Lowe and Butt (2016) it was of interest to study the comparison between epigeic and anecic species.

OECD (2000) have established two major terminologies for standard toxicity methods and this are "*no observed effect concentration* (NOEC)" which is the concentration at which there is no significant visible adverse effect when compared to the untreated samples, and the second no "*pathological symptoms"* toxic effect such as swellings around the clitellum or lesions on the body surface of worms.

The removal of PAHs in treatments with both species that were amended with biosurfactant was greater in unamended soil treatment though they also removed a notable percentage of contaminants. Removal of PH and FL was reported by Ma et al. (1995), where they found 86% removal PH and 70% FL after 56 days of treatment. Eijsackers et al. (2001) reported similar findings of steady PH removal upon addition of earthworms with less than 1% PH found after 50 days of treatment, their findings and results obtained from this research were not only similar but also a shorter lag phase period with no detection in treatments at 180 mg Kg^{-1} and 99% degradation in treatments after 4 weeks (Fig 3.5 and 3.6).

The removal of BAP was lower compared to PH and FL; however, removal of BAP was higher in combined PAH treatment compared with individual BAP treatment (Table 3.5). Although the difference was not statistically significant, the result agrees with Eijsacker et al. (2001) that remediation comprising a mixture of PAHs could result in a higher degradation of HMW PAHs. This may be due to degradation of lower ring PAHs such as PH that yields a growth in the population of microorganisms capable of degrading PAHs with more rings. Furthermore, the amendment with biosurfactant significantly increased the removal of BAP ranging from 60-80% in both earthworm species. five-ring hydrocarbons compared to 3- and 4-ringed hydrocarbons are generally less bioavailable hence more resistant to degradation (Piskonen and Itavaara, 2004). Similar results were seen in unamended treatments where only 43% of BAP was removed in both combined and individual BAP treatment. Interestingly, biosurfactant amendment improved the removal of BAP up to 80% with epigeic species (*E.*

hortensis). The hydrophobic properties of BAP (Log K_{ow}) and its large octanol-water partition coefficient are related to its adsorption and stability in soil which in turn is responsible for its reduced biodegradability (Juhasz and Naidu, 2000). It could be possible that a higher concentration or perhaps a longer incubation period could result in complete mineralization of BAP, however as earlier discussed that the chosen concentration of 0.1 mg L^{-1} was the EC_{50} derived and any concentration above this could possibly be toxic to microbes and subsequently to the earthworms who are dependent on microorganisms.

The mechanism earthworms use in degradation of hydrocarbons is yet to be fully understood. A potential external mechanism is the vermicast which is very rich in nutrients, such as N and P, as well as microorganisms capable of degrading PAHs (Marinari et al., 2000; Beffa et al., 1995). In addition, earthworms increase aeration in soil through their constant burrowing which possibly could increase the conditions for microbial activity thus accelerating the biodegradation of the PAHs. Several authors have argued that earthworms not only disperse indigenous microorganisms in the soil and/or the stimulation of their metabolic activity, their constant burrowing also plays a role in degradation. However, Schaefer et al. (2005) investigated the role of earthworm burrowing activities on degradation by simulating burrowing with mechanical mixing. This however did not show changes in the levels of hydrocarbon nor did it increase the soil microbial biomass concluding that manual mixing is not properly representative of actual burrowing of worms, nor that the effects of burrowing of worms mirrors the effect of microbial degradation. Singleton et al. (2003) investigated the microbial community (bacteria) present in the gut of earthworms such as *Pseudomonas, Azoarcus, Burkholderia, Paenibacillus, Acidobacterium* and *Spiroplasm.* Some of the bacteria studied such as *Pseudomonas, Acidobacterium* and *Alcaligenes* have been reported to have the capabilities in degrading PAHs (Cerniglia, 1992; Juahsz and Naidu, 2000; Johnsen et al., 2005). Furthermore, Morrison et al. (2000) found that the internal fluids present in the cocoons of *Eisenia* hosts bacteria of the genera *Nocardia, Pseudomonas* as well as *Alcaligenes*. In addition, Tiunov and Dobrovolskaya (2002) reported the presence of bacteria (*Rhodococcus* and *Azotobacter*) in burrows of *Lumbricus terrestris.* It has been argued by several authors that *Rhodococcus* can use PAHs as a sole source of carbon and can hence degrade them (Dean-Ross et al., 2001; Dendooven, 2011; Contreras-Ramos, 2008; Brown et al., 2004). The

drillosphere (walls of burrows) could possibly be laced with detritus and coprolites, burrows could also accumulate casts which contains microproteinaceous slime as well as products of nitrogen metabolism which in itself could aid mobility of PAHs and enhance degradation (Brown et al., 2004). Scheu (1991) argued that endogeic and anecic earthworms (*Octolasion lacteum, L. terrestris)* exude mucus from body surface and cast which does correspond to 0.2% and 0.5% of their daily body weight. This mucus has been argued to be metabolized by the microorganisms easily thus stimulating their catabolic activity and growth, Gevao et al. (2001) went on to suggest that these activities could possible lead to an increased bioavailability which is brought about by the actions of the earthworms on the soil and the gut activities could accelerate microbial mineralization of contaminants and be totally dependent on the gut passage time (Brown et al., 2004). In addition, the gut of both *E. hortensis* and *L. terrestris* could act as a form of stimulant for microorganisms ingested alongside the contaminant as well as organic matter during passage through the system, thus awakening the dormant gut flora (Fischer et al., 1997; Edwards and Bohlen, 1996; Brown et al., 2004). It is necessary to note that these micro-fauna associated with earthworm guts are then excreted via casts as well as the microbial community that adheres on to earthworm skin surfaces (Edward and Bohlen, 1996), possibly then being transmitted or further dispersed by water flow which further spreads the microbial community (Kretzshmar, 2004). This represents one of the key routes by which microbial distribution in soils occurs for degradation.

3.7 CONCLUSION

Polyaromatic hydrocarbons (PAH) contamination in soil continues to be one of the biggest environmental challenges because of the persistence and carcinogenicities of the contaminants. Vermiremediation provides a sustainable and environmental friendly solution to remediate these pollutants. As biosurfactant has also been demonstrated to enhance hydrocarbon remediation, this work examined possible synergistic effect of biosurfactant on vermiremediation to improve the efficacy in hydrocarbon removal in contaminated soil. Overall, the time response relationship study showed a very strong significance of application of earthworms and biosurfactant in removing PH, FL and BAP with significant regression coefficient twice the concentration from controls (p-value = 0.001) indicating that this

approach proves to be promising and should be further investigated in scaling to industrial applications

Both epigeic and anecic species of earthworms have very good potentials for the removal of PAHs both 3-, 4- and 5-ringed hydrocarbons in the presence of rhamnolipid biosurfactant. Epigeic species appeared to be more tolerant to increased concentrations and environmental conditions compared to anecic species that were extremely sensitive to concentrations and environmental conditions. Our data suggests vermiremediation using epigeic species with biosurfactant could be a promising and environmental friendly technique in removing hydrocarbons from soils. However, a major limitation for using anecic earthworm species would be environmental conditions to which they are exposed, and also a large amount of both species would probably be needed on the field as well as sufficient substrate for their growth and general activity where a possible solution to this is further discussed in the future studies (Chapter 5, Section 5.3).

Chapter 4: MONOOXYGENASE AND ANTIOXIDASE ENZYMATIC RESPONSE IN *Eisenia hortensis* AND *Lumbricus terrestris* EXPOSED TO 3-, 4-, AND 5-RING HYDROCARBONS AMENDED WITH BIOSURFACTANT

4.1 INTRODUCTION

Biochemical processes involve physical and chemical changes on a continuous basis. Most biochemical reactions require biological catalysts (enzymes) (Bennett and Frieden, 1962; Holum, 1975). Contrary to previous reports (Brown et al., 2004), lower invertebrates possess enzymes capable of digesting polysaccharides. Enzyme activity can provide information on status of ecosystem or soil fertility as they are involved in the transformation of biological and foreign compounds in soils (Tate, 2000).

To date, there is little information and understanding of the roles which earthworms play in the degradation of xenobiotic compounds such as PAHs (as discussed in chapter 3, Section 3.4). However, understanding the biochemical processes involved in different parts of the earthworm including the presence of phase I (monooxygenase) and II (antioxidase) enzymes as well as possible relationships between both phases could enhance the present understanding of the biochemical pathways earthworms employ in assisting the metabolism of xenobiotic compounds in their tissues.

CYP1A inducers in the environment are largely studied by using CYP1A enzymes, measurement of CYP1A proteins, mRNA or catabolic activity of the Ah-receptor response to CYP1A. Two major catalysed CYP1A enzyme reactions are ethoxyresorufin-*O*-deethylase (EROD) and methoxyresorufin-*O*-deethylase (MROD) whose substrates are 7-ethoxy/methoxyresorufin. The substrates are metabolised to a final product of resorufin usually using a fluorescence detector and in some cases ultraviolet detector. These reactions occur in the smooth endoplasmic reticulum where catalytic reactions are in the microsomes that are formed from homogenizing the tissue of the organism. Though EROD and MROD activities are typically classified as biomarkers of CYP1A, it is useful to also note that several other CYP1 isoenzymes such as CYP1A1, 1A2 and 1B1, could also be the catalyst in this reaction in human (Shimada et al., 1997). However, this might not be the case in lower organisms.

Cao et al. (2012) in their comparative study of EROD and CYP3A4 as biomarkers in *E. fetida* following an exposure to heavy metal contaminants reported that EROD activity was significantly more sensitive with an average increase of 80% in EROD activity compared to the controls.

Several researchers investigated biochemical responses to different compounds in *E. fetida* and they are; Pb (Saint-Denis et al., 2001), carbaryl (Ribera et al., 2001), and BAP (Saint-Denis et al., 1999). Overall there was no clear dose-response relationship established for most biomarkers assayed. The results derived showed that Pb, carbaryl and BAP induced a biochemical response in *E. fetida* far more than has been reported in other organisms such as fish (Van Der Oost et al., 2003). Where carbaryl inhibited acholinetserase activities significantly, exposure to Pb greatly induced lipid peroxidation and went on further by inhibiting other phase I and II enzymes that are involved in detoxification of xenobiotics such as MROD and GST activities, whereas exposure to BAP caused an induction of MROD and catalase activities.

There are several cofounding factors that may influence biochemical response (EROD and MROD activities) and they include but are not limited to; organotins and other metals, effects in mixtures of Ah-receptor ligands with synergistic and antagonistic effects, as well as prolonged exposure to pollutants resulting in alternation in CYP1A response. In designing an *in vitro* study of these biomarkers, it is very important to take into account all of these factors and more and ensure that both exposed and non-exposed groups are subjected to the same conditions for accurate results.

The biotransformation of PAH compounds is the major cause of the toxicity to Ah-receptor ligands. There are possibilities of production of highly reactive metabolites by CYP1A enzymes which may form DNA adducts thus inducing carcinogenicity in the cells (Conney, 1982). E.g. the oxidation of BAP to epoxides in cells of species that are highly reactive (BAP-7,8-dihydrodiol-9,10-epoxide) is catalysed by CYP1A enzymes (Conney, 1982; Xue and Warshawsky, 2005). Other possible mechanisms in the carcinogenicity of PAHs is metabolite formation e.g. o-quinones that are susceptible to undergoing redox reactions that yields the production of reactive oxygen species (ROS) followed by the breaking of DNA strands (Flower et al., 1997). The effects which BAP has in carcinogenicity has been widely studied where mice

that have no Ah-receptors have been reported to be resistant to the carcinogenic effect of BAP (Shimizu et al., 2000).

The main routes of PAH uptake in earthworms are orally and through the skin due to its permeability leading to the induction of CYP1A enzymes. Sundberg et al. (2005) reported that in early life of fishes the toxicity that accompanies exposure to a mixture of PAHs was most like caused by the PAHs that are not ligands to the Ah-receptor pathway, inducing CYP1A enzymes which could have protective responsibilities against toxicity. However, this does not necessarily mean early stage toxicity of PAHs (Billiard et al., 2006; Incardona et al., 2006). Ah-receptors play a very important role in mediating the toxicity of agonists and induction of enzymes.

4.2 AIMS

The aim of this experiment is to investigate the combined effect of phenanthrene (PH), fluoranthene (FL) and benzo(a)pyrene (BAP) with rhamnolipid biosurfactant, as well as individual effect BAP and biosurfactant from vermiremediation (Chapter 3) on biochemical processes in both epigeic (*E. hortensis*) and anecic earthworm species (*L. terrestris*).

4.3 OBJECTIVES

- To assess the effect, synergistic, antagonistic or no effect of PAHs and biosurfactant on the levels of total proteins in the mitochondria and the microsomes in both species
- Determine the synergistic or antagonistic effect of both PAHs and biosurfactant on the oxidative stress enzymes measuring glutathione S-transferase and catalase activities in both species; and monooxygenase enzymes measuring the ethoxyresorufin O-deethylase (EROD) and methoxyresorufin O-deethylase (MROD) activities of CYP1A in both species.

4.4 MATERIALS AND METHODS

4.4.1 MATERIALS

Methanol (HPLC grade), was obtained from Fisher-Scientific UK, monopotassium phosphate (KH_2PO_4), dipotassium phosphate (K_2HPO_4), Resorufin, 7-ethoxyresorufin, phenylmethanesulfonyl fluoride (PMSF), reduced β-nicotinamide adenine diculeotide

phosphate (NADPH), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), reduced L-glutathione (GSH), 1-Chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma-Aldrich UK. Bovine serum albumin (BSA) and Bradford reagent were obtained from Bio-Rad UK. All other reagents used were of analytical grade.

4.4.2 EARTHWORM PREPARATION

Procedure described in chapter 3, Section 3.2.9. All the process was undertaken at temperature of 4 ˚C to prevent denaturation of enzymes.

4.4.3 MITOCHONDRIAL AND MICROSOMAL FRACTIONAL EXTRACTION

A modified method based on Yang et al. (2012), Zamaratskaia and Zlabek. (2009) and Belaz and Oliveira (2013) was used for mitochondrial and microsomal isolation.

Excised guts from each treatment were pooled and homogenized using liquid nitrogen. Guts were afterwards further homogenized using 5 mL of homogenization buffer [ice-cold phosphate buffer saline (KH_2PO_4 10 mmol L^{-1}, K_2HPO_4 10 mmol L^{-1}, sucrose 250 mmol L^{-1}, and PMSF 0.134 mmol L^{-1})]. (w/v). Samples were homogenized for 15s and transferred into Eppendorf tubes for centrifuging. Homogenates were transferred into a pre-chilled Eppendorf centrifuge 5430R and centrifuged at 4 ˚C for 15 minutes at 15,000 x g for the production of post-mitochondrial fraction. Supernatant were collected and divided into two parts, one part for analysis of GST activities, and the other was further centrifuged at 150,000 x g for 90 mins for the pelletisation of microsomal fractions for measuring EROD activities of CYP1A1. The supernatant were aliquoted and stored at -80 ˚C (Cryotube F570H Eppendorf freezer).

Quality control was conducted by validating the extraction methods using a fresh pig liver, using the same procedure.

4.4.4 TOTAL PROTEIN CONTENT (using Bradford assay)

Total protein contents in the mitochondrial and microsomal fractions were measured using the Bradford assay (Bradford, 1976), adjusted for 96-well plates using bovine serum albumin.

Stock solution of BSA was prepared at 1.0 mg mL^{-1} in distilled water and calibration standard solution ranging from 0.1– 1 mg mL^{-1}.

In a 96-well plate, 5 µL of the standard, samples (mitochondrial and microsomal fractions) and water were pipetted into separate wells and into each well, 250 µL of Bradford reagent was added swirled and stored in a dark room for 5 min and absorbance was read using Fluostar Omega microplate reader (BMG LABTECH) at 595 nm using UV.

4.4.5 METHOD DEVELOPMENT AND VALIDATION (using UPLC-FLR)

Ultra-performance liquid chromatography (UPLC) system employed to detect resorufin and its derivatives was an Acquity Waters H-class system (Singapore) that is connected to a fluorescence (FLR) detector (λ_{exc} 535 and λ_{em} 586 nm). Acquisition of data was done using Acquity Waters MassLynx software. A C18 chromatography column was used (2.1 x 50 mm) (Acquity UPLC® 1.7 µm). The column was supported by an Acquity UPLC BEH C18 guard column, 130 Å, 1.7 µm, 2.1 mm x 5 mm).

Method validation was done following the internationally accepted criteria (FDA, 2007). Linearity, selectivity, recovery, accuracy and limits of quantification and detection were parameters that were evaluated.

4.4.5.1 PREPARATION OF SPIKED SAMPLES

Preparation of quality control, calibration standard and unknown microsome solution was done by aliquoting 50 µL of resorufin stock solution into Eppendorf tubes, and reconstituting with 350 µL of buffer solution which consisted of HEPES 100 mmol L^{-1}; pH 7.8 and magnesium chloride ($MgCl_2$) (5 mmol L^{-1}), 25 µL of substrate (ethoxyresorufin) (0.1 mmol L^{-1}) 25 µL NADPH (5.0 mmol L^{-1}), and 50 µL of inactivated microsome samples. Eppendorf tubes were then vortexed for 30s, followed by the addition of 400 µL ice-cold methanol (HPLC grade) for protein precipitation. Tubes were placed on crushed ice for 15 min and then centrifuged at 10,000 x g for another 15 min at 4 ˚C in an Eppendorf centrifuge 5430R. Derived supernatant was decanted, and 250 µL of supernatant were transferred into vials for UPLC analysis. Injection volume was 10 µL. The preparation of blank was the same without the addition of

resorufin, for the development and validation of this method, microsomal fractions used were inactivated by heating in a dry bath at 55 ˚C for 5 min.

4.4.5.2 STANDARD CURVE

A 7-point standard curve (0.05, 0.1, 0.2, 0.4, 0.8, 1 and 2 pmol L^{-1}) was prepared by adding known concentrations of resorufin to buffer-methanol solution (1:1 v/v). A linear regression analysis of the peak area of resorufin against nominal concentration of the analyte was used for the calibration curve.

4.4.5.3 SELECTIVITY, PRECISION, RECOVERY AND ACCURACY

For the purpose of selectivity, there was a daily assessment of resorufin in blank and spiked microsome samples. Preparation of blank microsomes is as described in Section 4.9.5.1, where the volume of resorufin (50 µL) was replaced with the buffer solution HEPES 100 mmol L^{-1}; pH 7.8 and magnesium chloride ($MgCl_2$) (5 mmol L^{-1}).

Determination of analyte recovery from the spiked samples was carried out by analysing quality control samples at three chosen concentrations: 0.5, 15.5 and 31 pmol L^{-1}. Peak area obtained from three replicates of the extracted quality control samples was compared with four replicates of quality control which was prepared using blank supernatant using the same concentration so as to obtain a percentage extraction. The precision and accuracy were determined by evaluating the same quality control samples as above. Each quality control sample concentration had three replicates and were prepared on three non-consecutive days. The precision was then evaluated using coefficient of variation (C.V. %) while accuracy was expressed as a percentage difference between added concentrations recovered concentration from the microsomal samples.

4.4.5.4 LIMITS OF DETECTION AND QUANTIFICATION

FDA (2007) - accepted criteria for the limit of quantification was defined as the precision and accuracy for three extracted samples with a C.V. ≤20%. Limit of detection was determined using the lowest concentration of the analyte that resulted in a signal to noise ratio of 9.6.

4.4.6 PRELIMINARY STUDIES

Earthworms removed from pilot studies in chapter 3 (Section 3.3.5) were washed and sacrificed for mitochondrial and microsomal fractions following A method-based on Waxman and Chang (1993), Yang et al. (2012) and Belaz and Oliveira (2013) with slight modifications as described in Section 4.9.6 below. Total protein in both mitochondrial and microsomal fraction was measured using Bradford assay as described Section 4.9.4, and GST activity in mitochondrial fraction was measured following Habig et al (1974) method described in Section 4.9.7.1.

4.4.6.1 FILTER PAPER CONTACT TEST

Acute toxicity test was conducted following OECD guidelines. Different concentrations of PAHs (combined 180 mg L^{-1} PH, FL and BAP (1:1:1), 60 and 180 mg L^{-1} BAP) was dissolved in acetone to make three separate stock solutions. Filter paper was placed in a 10 mL glass vial, to which 1 mL of either concentration of PAH was added. Solvent was allowed to evaporate overnight followed by the addition of either 1 mL of rhamnolipid biosurfactant solution (0.1 g L^{-1}) or 1 mL distilled water. One earthworm (*E.hortensis* /*L. terrestris*) was placed in each vial, covered with perforated parafilm and placed in a dark cupboard at 22 $^{\circ}$C ± 2 for 72 hours. EROD, MROD and GST activities were measured following the same procedures as above.

4.4.7 MONOOXYGENASE ENZYMES: CYP1A; EROD AND MROD ACTIVITY

A method-based on Waxman and Chang (1993), Yang et al. (2012) and Belaz and Oliveira (2013) with slight modifications was used for EROD and MROD activity measurement in the gut of *Eisenia hortensis* and *Lumbricus terrestris.* The concentration of substrate used was based on the result reported by Belaz and Oliveira (2013) that measured the EROD activity of CYP1A in Wister rat livers.

For the microsomal incubation; 400 µL buffer solution which consisted of HEPES 100 mmol L^{-1}; pH 7.8 and magnesium chloride ($MgCl_2$) (5 mmol L^{-1}), 25 µL of substrate (ethoxyresorufin/methoxyresorufin) (0.1 mmol L^{-1}) and 50 µL of microsome samples. Tubes were vortexed and pre-incubated 37 $^{\circ}$C for 1 min, afterwards the reaction was initiated by the addition of 25 µL NADPH (5.0 mmol L^{-1}), tubes were left in a shaking water bath for 5 min at

37 ˚C. The reaction was terminated by the addition of 400 µL ice-cold methanol (HPLC grade). Tubes were tubes were then vortexed and left to cool on crushed ice for 15 mins and then centrifuged at 10,000 x g for another 15 min at 4 ˚C in an Eppendorf centrifuge 5430R. Derived supernatant was decanted, and 250 µL of supernatant were transferred into vials for UPLC analysis. Injection volume was 1 µL. UPLC was calibrated using resorufin standards.

QA/QC was conducted during method validation. This was carried out by spiking microsomes with three known concentrations of resorufin using an intra and inter day recovery analysis.

4.4.8 ANTIOXIDASE ENZYMES

4.4.8.1 GLUTATHIONE S-TRANSFERASE (GST) ACTIVITY

This assay was carried out using the Habig et al. (1974) method adjusted for the 96-well plate, using 1-chloro-2,4-dinitrobenzene as the substrate that is conjugated to reduced L-Glutathione (GSH) by GST enzymes. The reaction solution was prepared by adding 4950 µL of 0.2M phosphate buffer at pH 6.5, followed by 900 µL of GSH 10 mM and 150 µL CDNB 60 mM, homogenized and placed on ice in a dark box. The first row of the 96-well plate was left empty to avoid interference in reading and into the second row, 50 µL of phosphate buffer (blank) was added, followed by 50 µL samples to the subsequent rows. 250 µL of the reaction solution was then added to the wells with the blank and the samples, and absorbance was read at 340 nm after 3 min standing in the dark.

Calculation

GST activity = $[(BC/t) / Є] \times (tv/va) \times df = U\ mL^{-1}$

Where (BC = blank corrected, t = time (minute), Є = extinction coefficient 0.0096 $\mu M^{-1}cm^{-1}$, tv = total volume per well (mL), va= volume of analyte (mL), U = unit ($\mu mol\ mL^{-1}$).

4.4.9 STATISTICAL ANALYSIS

A one-way or two-way ANOVA was used to analyse the significant difference between the treatments and earthworms followed by a *post-hoc* Tukey test. A Pearson correlation was used to evaluate the degree of relationship between the enzyme activities as well as between

the bioaccumulated concentrations in earthworms and the enzymatic responses. GraphPad Prism6 and R was used to conduct the above analysis.

4.5 RESULTS

To evaluate the enzyme activities of CYP1A1 and CYP1A2, the microsomal fractions was extracted from both earthworm species used.

4.5.1 TOTAL PROTEIN CONCENTRATION

Standard curve for quantification of total protein concentration was linear from 0.1 – 1 mg mL^{-1} using BSA as standard. The regression equation and correlation coefficient derived was

$$y = 0.2797x + 0.0366$$

R^2 = 0.9964, while the C.V. at individual concentration of analysis was between 0.4 - 17.5%.

The total concentration of proteins found in the microsomal fraction from pooled *E. hortensis* and *L. terrestris* gut was an average of 0.24 mg mL^{-1} (±0.03) and 0.28 mg mL^{-1} (±0.02) respectively, which is in line with most *in-vitro* assays requiring protein concentrations that requires protein concentrations within the range of 20 – 200 µg mL^{-1} (Burke et al., 1989; Walawalkar et al., 2006; Cao et al., 2012; Belaz and Oliveira, 2013).

4.5.2 DEVELOPMENT AND VALIDATION OF METHOD ON UPLC FOR DETERMINATION OF RESORUFIN, 7-ETHOXYRESORUFIN AND 7-METHOXYRESORUFIN

Biochemical processes in the isolated gut of *E. hortensis* and *L. terrestris* were evaluated by monitoring their individual ability to biotransform 7-ethoxyresorufin and 7-methoxyresorufin to resorufin, which is mediated by CYP1A1 and CYP1A2 respectively.

Two mobile phases were compared using a C18 chromatography column (2.1 x 50 mm) (Acquity UPLC® 1.7 µm). MeOH/water (50/50 v/v) and a combination of potassium phosphate buffers at pH 2.5 and 7.5 respectively, and MeOH/water (50/50 v/v) and ammonium acetate buffer using the same pH as the former. C18 columns have a hydrophobic phase containing silica that are of different purity and acidity, due to the crystallization of potassium phosphate which could affect the chromatographic performance of the column (Snyder et al., 1997). However, potassium phosphate buffers under similar conditions with ammonium acetate

provided better resolution with a more defined resorufin retention even though ammonium acetate had higher gains, but peaks were not well resolved (Fig 4.1), hence it was the preferred buffer in this particular study.

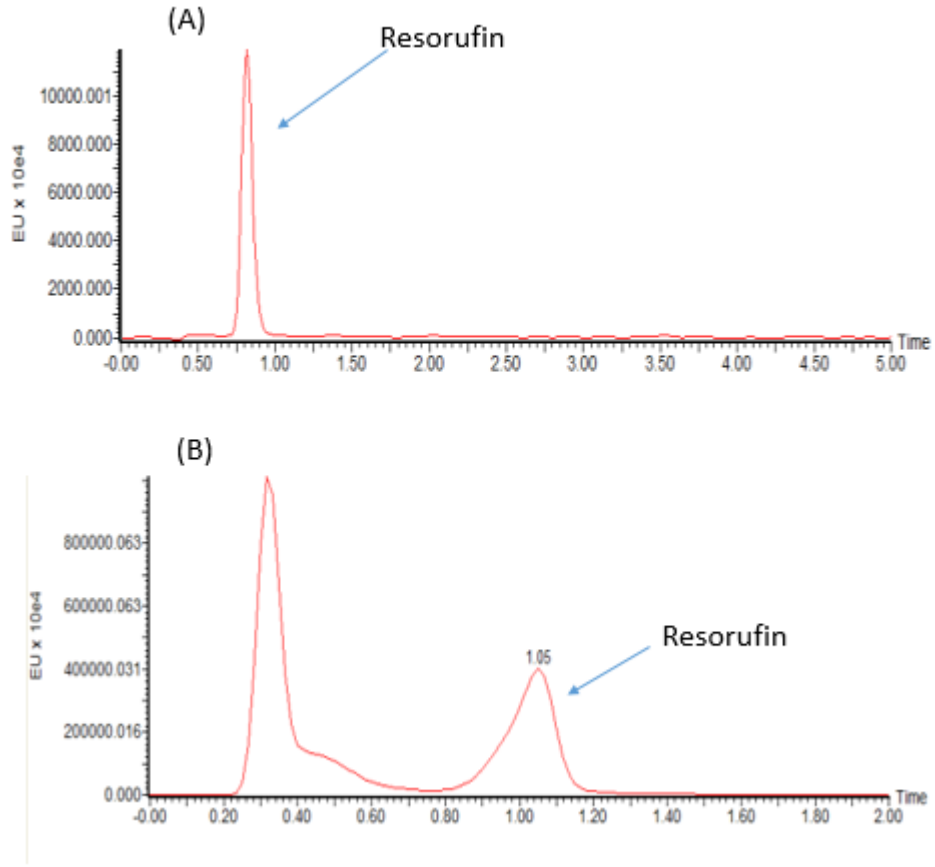


Figure 4. 1 Chromatograph of resorufin separation using mobile phase (A) MeOH:KH2PO4 as buffer (10 mmol/L; pH 2.5) (50:50 v/v) and (B) MeOH:C2H7NO2 (10 mmol/L; pH 2.5) (50:50 v/v), chromatographic conditions set were; column a C18 chromatography column (2.1 x 50 mm) (Acquity UPLC® 1.7 µm). Flow rate was 0.4 mL/min and injection volume was 10 µL. Detection parameters; fluorescent detection at λ_{exc} 535 and λ_{em} 586 nm

Resorufin compounds have ionisable functional groups, and temperature plays a key role in influencing the protonation or deprotonation of the ionic compounds hence the retention factor (Snyder et al., 1997). For this study three separate temperatures were investigated 35 °C, 40 °C and 45 °C and the initial temperature of 35 °C was used for this study (Fig 4.2)

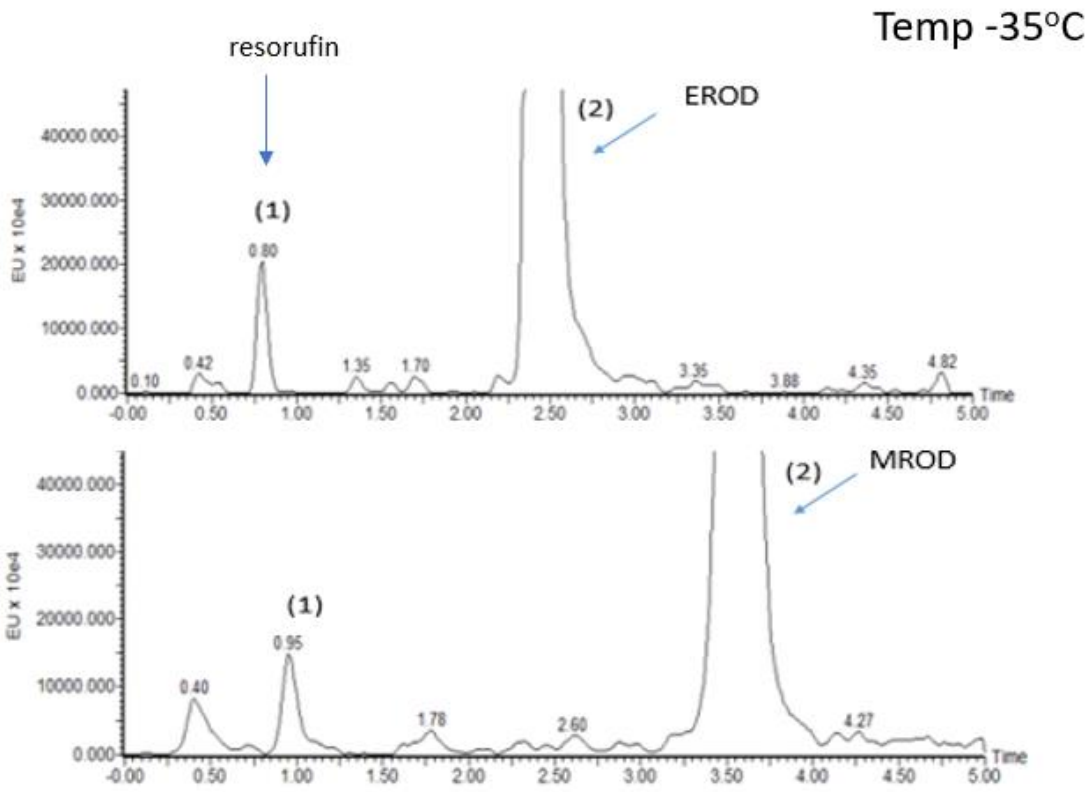


Figure 4. 2 Chromatograph of resorufin (1) standard in the presence of 100 µm L^{-1} 7-ethoxyresorufin and 100 µm L^{-1} 7-methoxyresorufin (2), using a fluorescent detection at λ_{exc} 535 and λ_{em} 586 nm, chromatographic conditions set were; column C18 chromatography column (2.1 x 50 mm) (Acquity UPLC® 1.7 µm) with MeOH:KH_2PO_4 as buffer (10 mmol L^{-1}; pH 2.5) (50:50 v/v). Flow rate was 0.4 mL/min and injection volume was 10 µL.

Different chromatographic conditions were investigated with the column C18 (2.1 x 50 mm) (Acquity UPLC® 1.7 µm) to optimize the retention time. Overall the preferred chromatographic conditions were using potassium buffers:MeOH (10 mmol L^{-1}; pH 2.5) (50:50 v/v), using a fluorescent detection at λ_{exc} 535 and λ_{em} 586 nm and temperature at 35 ˚C, with proper validation of the UPLC method following international accepted criteria (FDA, 2007).

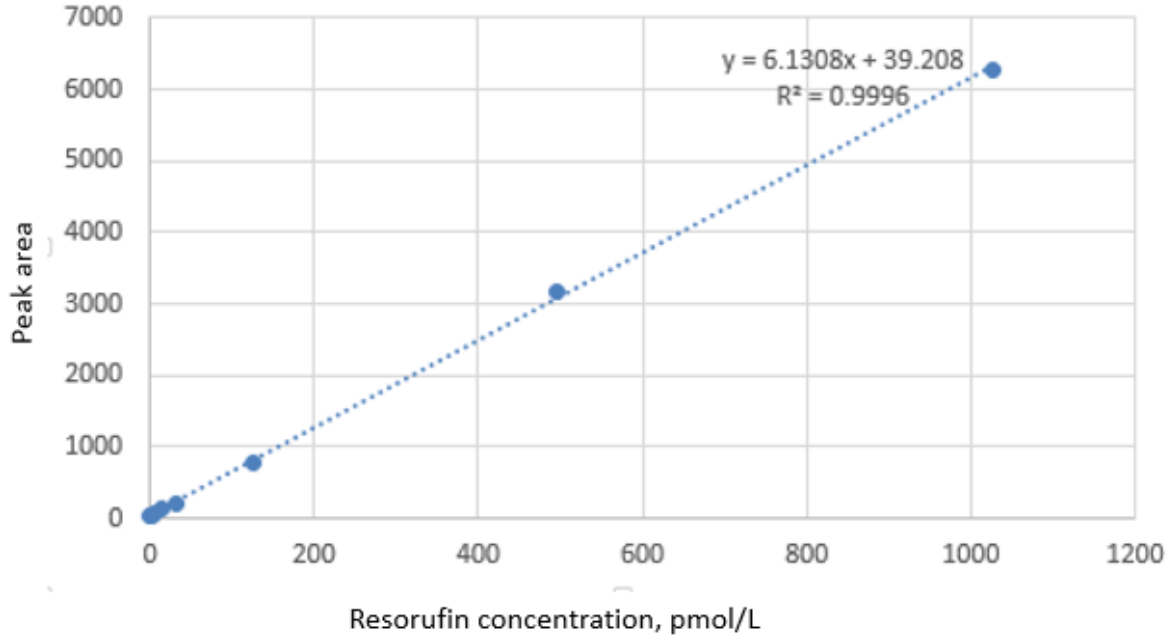


Figure 4. 3 Standard curve for resorufin was linear from 0.488 to 1000 pmol mL^{-1}, the range of the C.V. was in the range of 1.7 to 11.1% with an accuracy of 92.1 to 110.4% (n=3).

Three concentrations were used as quality control for inter and intra-day precision and accuracy, all samples were prepared in triplicates and analysed on 3 randomized days. The precision at all the concentration levels was below 15% of C.V. while the accuracy was calculated as percentage derived from what was initially added (Table 4.1).

Table 4. 1 Accuracy, intraday (n=3) and intraday (n=9) and recovery (n=3) for resorufin assay in the gut of *E. hortensis* and *L. terrestris* microsomes.

Resorufin (pmol/L)	1st day		2nd day		3rd day		Recovery (%)
	Accuracy (%)	C.V. (%)	Accuracy (%)	C.V. (%)	Accuracy (%)	C.V. (%)	
0.5	104.7	3.9	100.6	8.6	100	4.3	107.3 ± 7.3
15.5	99.1	1.6	98.7	2.8	99.6	1.2	98.7 ±-1.3
31	99.1	1.2	99.8	1.3	101.4	0.4	99.8 ±-0.2

Concentration used for recovery was same as that used for quality control. Results were calculated using comparison to blank supernatant that contained the same concentrations of resorufin. The rate of recovery was very high ranging from 99 to 107%, the limit of detection 0.2 pmol mL^{-1} and limit of quantification was 0.3 pmol mL^{-1}.

4.5.2.1 PRELIMINARY STUDIES

EROD and MROD activities showed a 2-fold increase across all concentrations, with GST activity showing no significant changes. However, there was no significance difference from data obtained from soil contact test and filter paper test (p >0.05). Refer to appendix 1 for study data

4.5.3 ENZYMATIC RESPONSE TO PAH IN SOIL

Enzymatic activities in homogenised guts of earthworms exposed to 180 mg Kg^{-1} combined PAHs and 60 mg Kg^{-1} individual BAP for 28 days with or without biosurfactant amendment from Chapter 3 showed induction and inhibition of enzyme activities at different time points between day 2 – 28

4.5.3.1. CYP RESPONSE

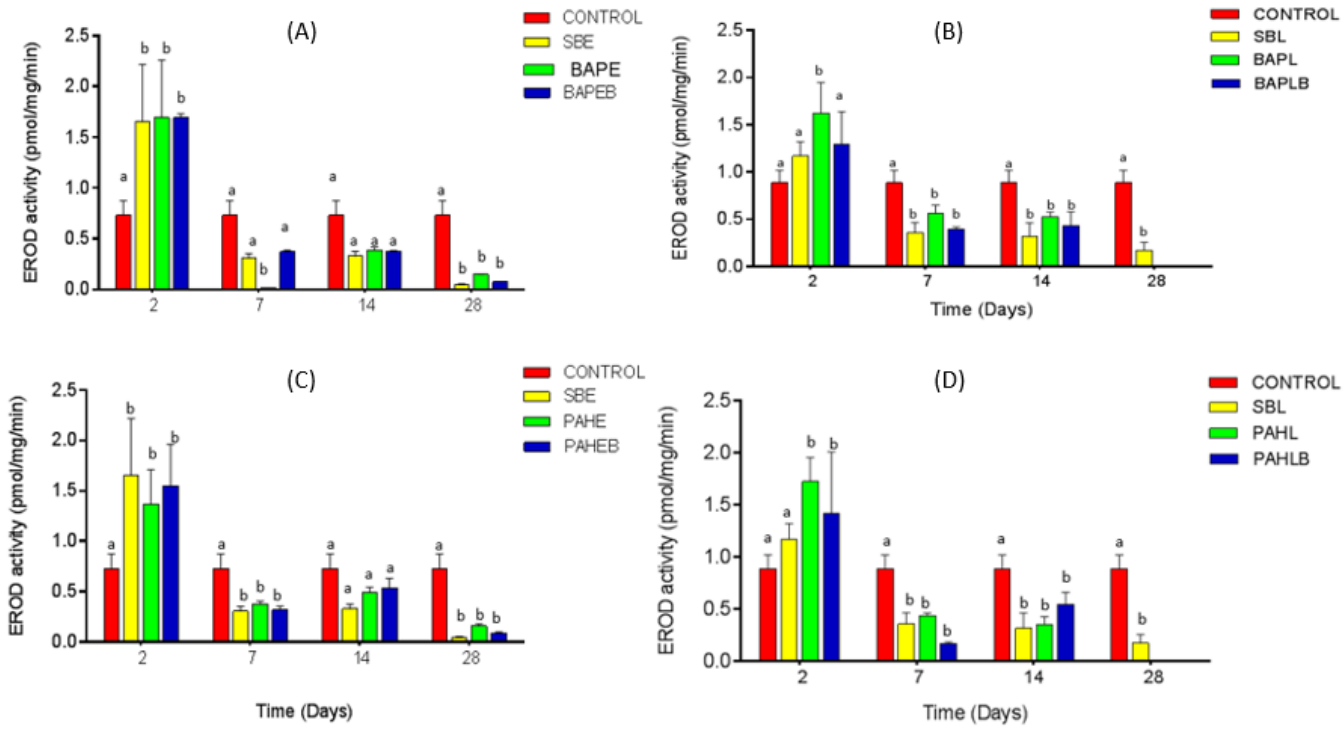


Figure 4. 4 Combined effect of 3-, 4- and 5-ring PAH amended with biosurfactant on EROD activity in *Eisenia hortensis* and *Lumbricus terrestris* after a 28-day exposure. Values are represented as means ± SD (n = 3). Data not sharing same letter are significantly different with $p<0.05$.

Where:

Control – *E. hortensis* or *L. terrestris* from non-spiked and unamended soil

SBE/SBL - *E. hortensis* or *L. terrestris* from non-spiked soil amended with biosurfactant.

BAPE/BAPL - *E. hortensis* or *L. terrestris* from BAP spiked soil unamended with biosurfactant

BAPEB/BAPLB - *E. hortensis* or *L. terrestris* from BAP spiked soil amended with biosurfactant

PAHE/PAHL - *E. hortensis* or *L. terrestris* from PH, FL and BAP spiked soil unamended with biosurfactant

PAHEB/PAHLB - *E. hortensis* or *L. terrestris* from PH, FL and BAP spiked soil amended with biosurfactant.

Fig 4.4A illustrates EROD activity response in *E. hortensis* to BAP (60 mg Kg^{-1}) with or without rhamnolipid biosurfactant amendment from day 2-28. A significant amount of enzyme induced was approximately 2-fold higher than control was recorded at day 2 in both amended and unamended treatment (*p-value* = 0.01) which then saw a significant reduction (*p-value* = 0.01) in the enzymatic activity between day 7 and 28.

Fig 4.4B illustrates same the activity in *L. terrestris* to BAP in similar conditions as *E. hortensis*. Similarly, there was an induction in EROD activity at day however, BAP yielded a significant increase of approximately 2-fold increase at day 2. Like *E. hortensis*, EROD activities decreased significantly from day 2-28 and complete mortality in treatments with BAP at day 28.

PAH at 180 mg Kg^{-1} (combined PH, FL and BAP) in *E. hortensis* resulted in higher EROD activity ranging between 2-2.5 higher than the control, from day 7-28 EROD activity was decreased across all treatment compared to the control (Fig 4.4C).

EROD activity in *L. terrestris* exposed to PAH at 180 mg Kg^{-1} (combined PH, FL and BAP) were induced between 0.2-1.5 folds higher than the controls at day 2 with the amendment of biosurfactant alone not causing an induction that is significantly different from control. From day 7-28 EROD activity decreased across all treatment and complete mortality recorded at day 28 in treatments including PAHs (Fig 4.4D).

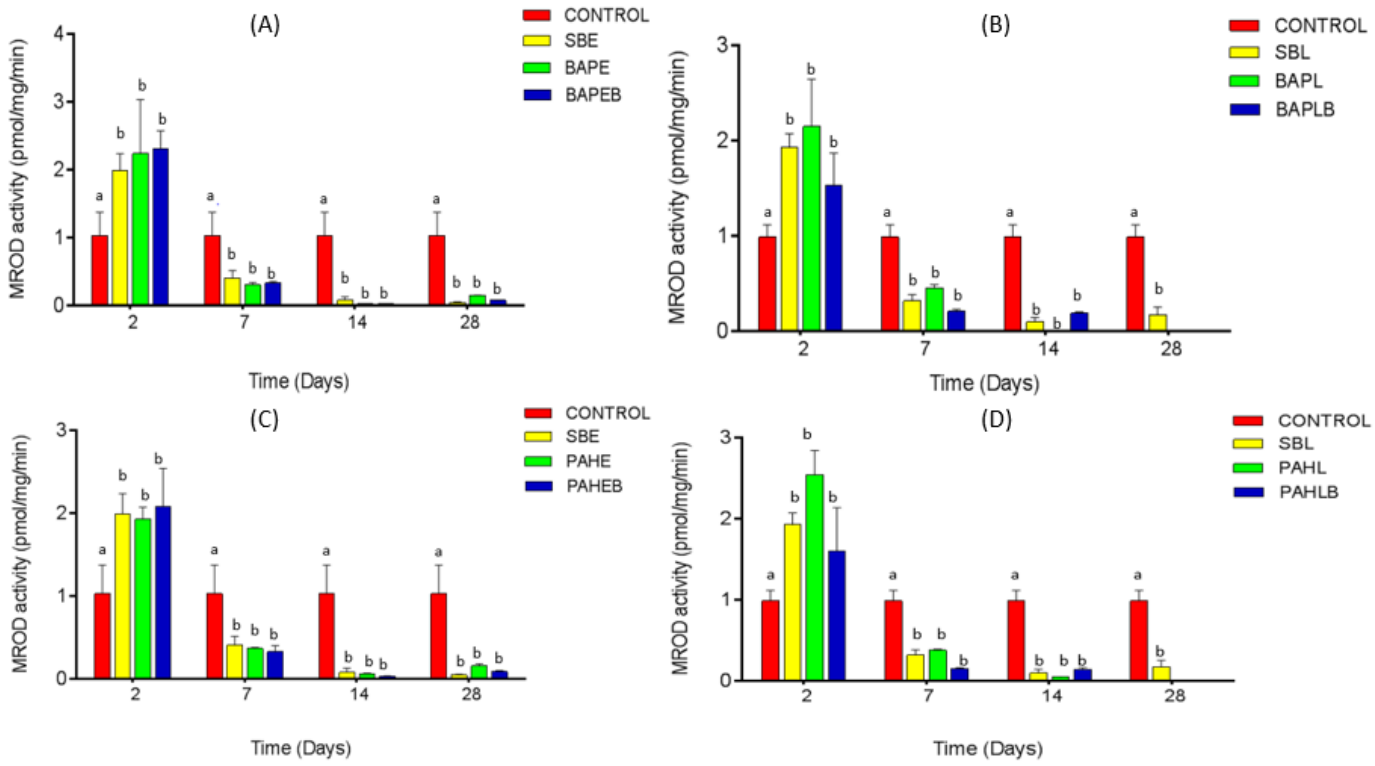


Figure 4. 5 Combined effect of 3-, 4- and 5-ring PAH amended with biosurfactant on MROD activity in *Eisenia hortensis* and *Lumbricus terrestris* after a 28-day exposure. Values are represented as means ± SD (n + 3). Data not sharing same letter are significantly different with $p<0.05$.

MROD activities in *E. hortensis* were significantly (*p-value* = 0.01) induced on day 2 with a 2-fold increase in the biosurfactant treatment, BAP and BAP amended with biosurfactant treatment up to 2-folds higher than the control, and a significant decrease was recorded from day 7-28 across all the treatments (Fig 4.5A).

Fig.4.5B. illustrates MROD activities in *L. terrestris* exposed to BAP with or without biosurfactant amendment, MROD activity was induced between 1.5-2.2 folds on day 2 and significantly decreased from day 7-14, complete mortality was recorded for earthworms exposed to both BAP and BAP amended with biosurfactant on day 28.

MROD activities similarly were significantly (*p-value* = 0.01) induced in PAH 180 mg Kg^{-1} (combined PH, FL and BAP) up to 2-fold higher across all treatment at day 2 in *E. hortensis* compared to the control and significantly reduced between day 7-28 (Fig. 4.5C), and in *L. terrestris* induction of MROD was between 1.7-2.5 folds higher by day 2 and also significantly reduced between day 7 and 28 across all treatments (Fig 4.5D).

4.5.3.2 ANTIOXIDASE ACTIVITY

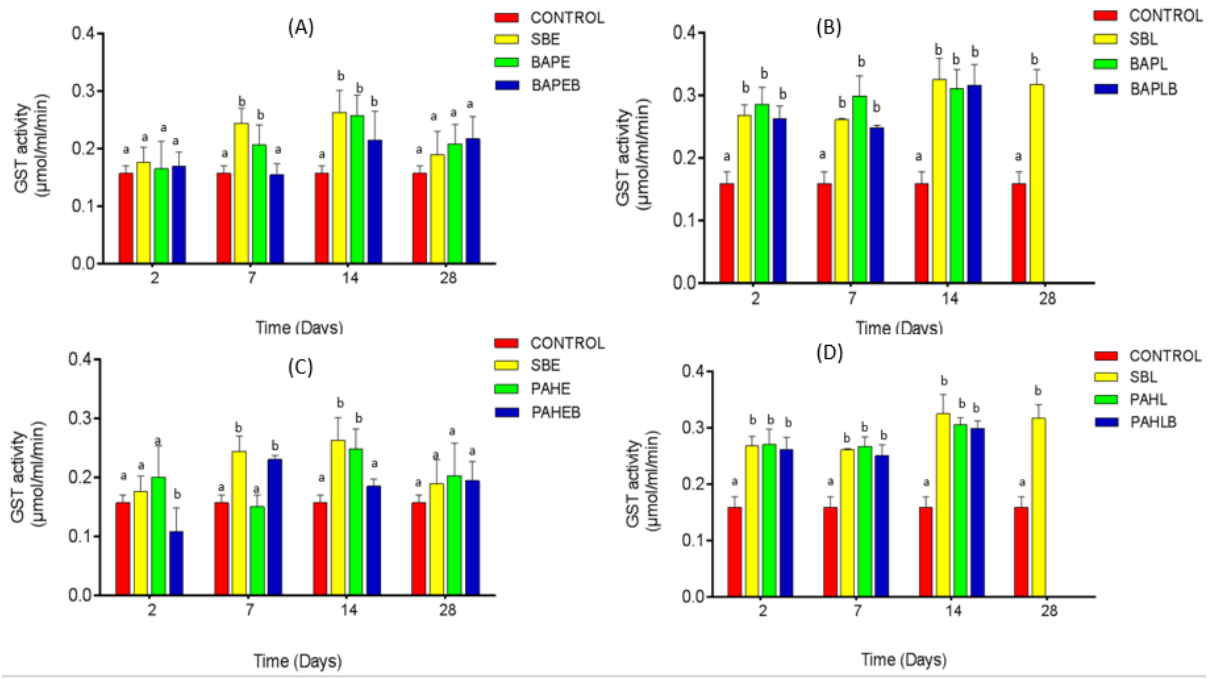


Figure 4. 6 Combined effect of 3-, 4- and 5-ring PAH amended with biosurfactant on GST activity in *Eisenia hortensis* and *Lumbricus terrestris* after a 28-day exposure. Values are represented as means ± SD (n + 3). Data not sharing same letter are significantly different with $p<0.05$.

Fig 4.6A and C illustrates GST activity in *E. hortensis* exposed to BAP (60 mg Kg^{-1}) and PAH 180mg Kg^{-1} (combined PH, FL and BAP). Activity was not induced at day 2, however between day 7 and 28 activity was induced up to 2-fold higher than the control. Contrary to induction pattern in *E. hortensis*, GST activities in *L. terrestris* across all treatments were induced 2-2.5 folds higher than the controls from day 2 right across till day 28.

A correlation analysis was conducted to investigate if there was any specific correlation in response characteristics between EROD and MROD in earthworms from the soil. There was a significant positive correlation between both EROD and MROD enzyme activity across all treatments in the soil ($p < 0.05$) while there was no correlation between monooxygenase and antioxidase enzymes (Fig 4.7, 4.9 and 4.9).

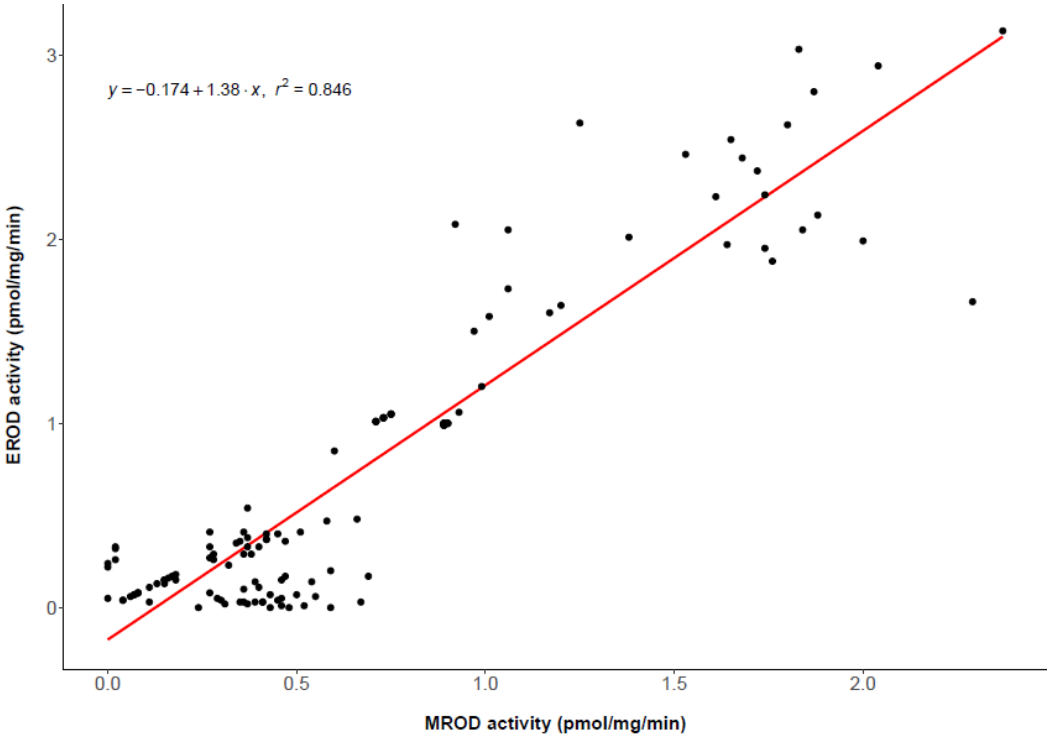


Figure 4. 7 Overall correlation analysis between EROD and MROD in earthworms exposed to spiked PAHs ($p < 0.05$).

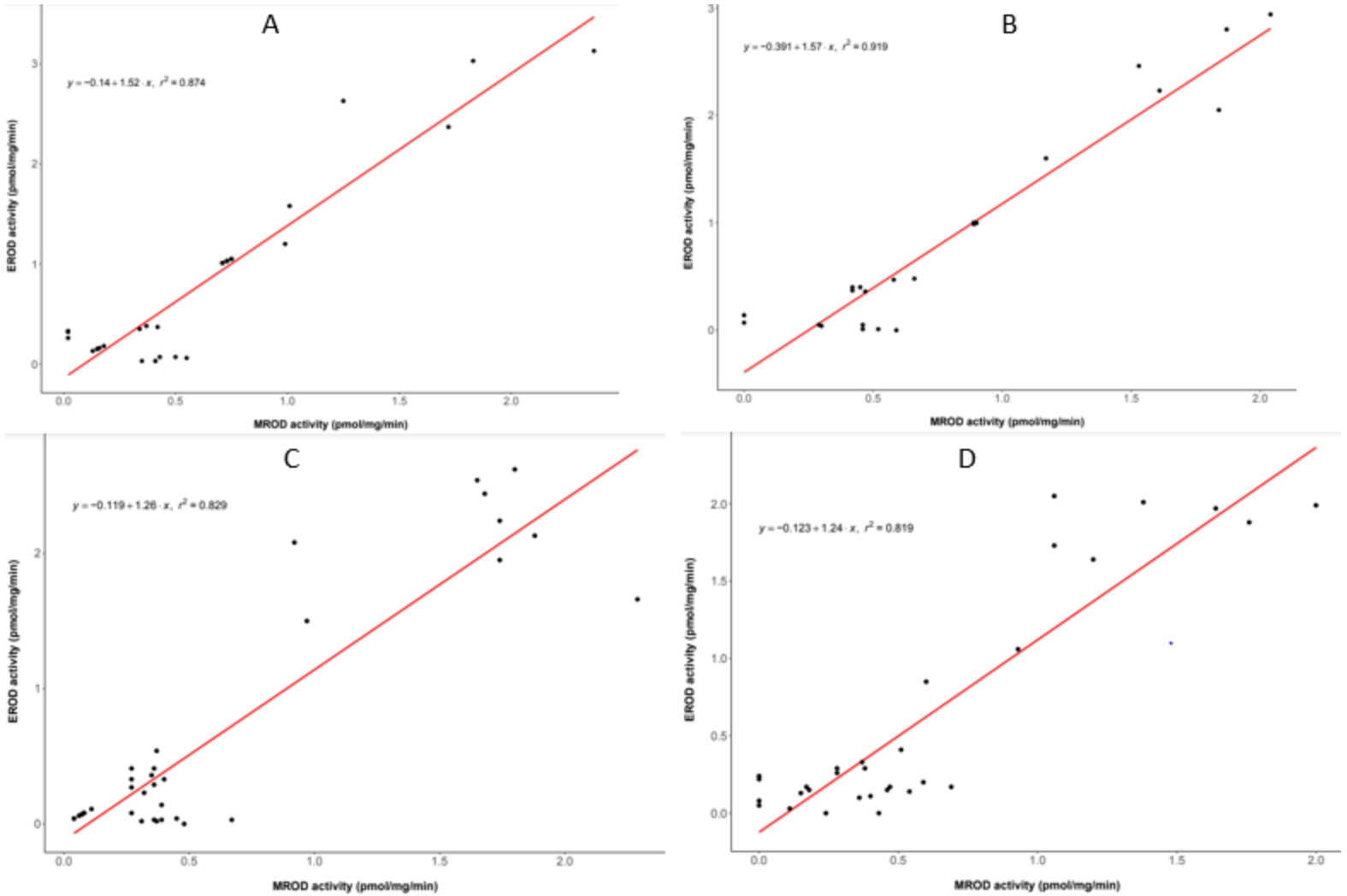


Figure 4. 8 Correlation analysis between EROD and MROD in earthworms exposed to spiked PAHs ($p < 0.05$).

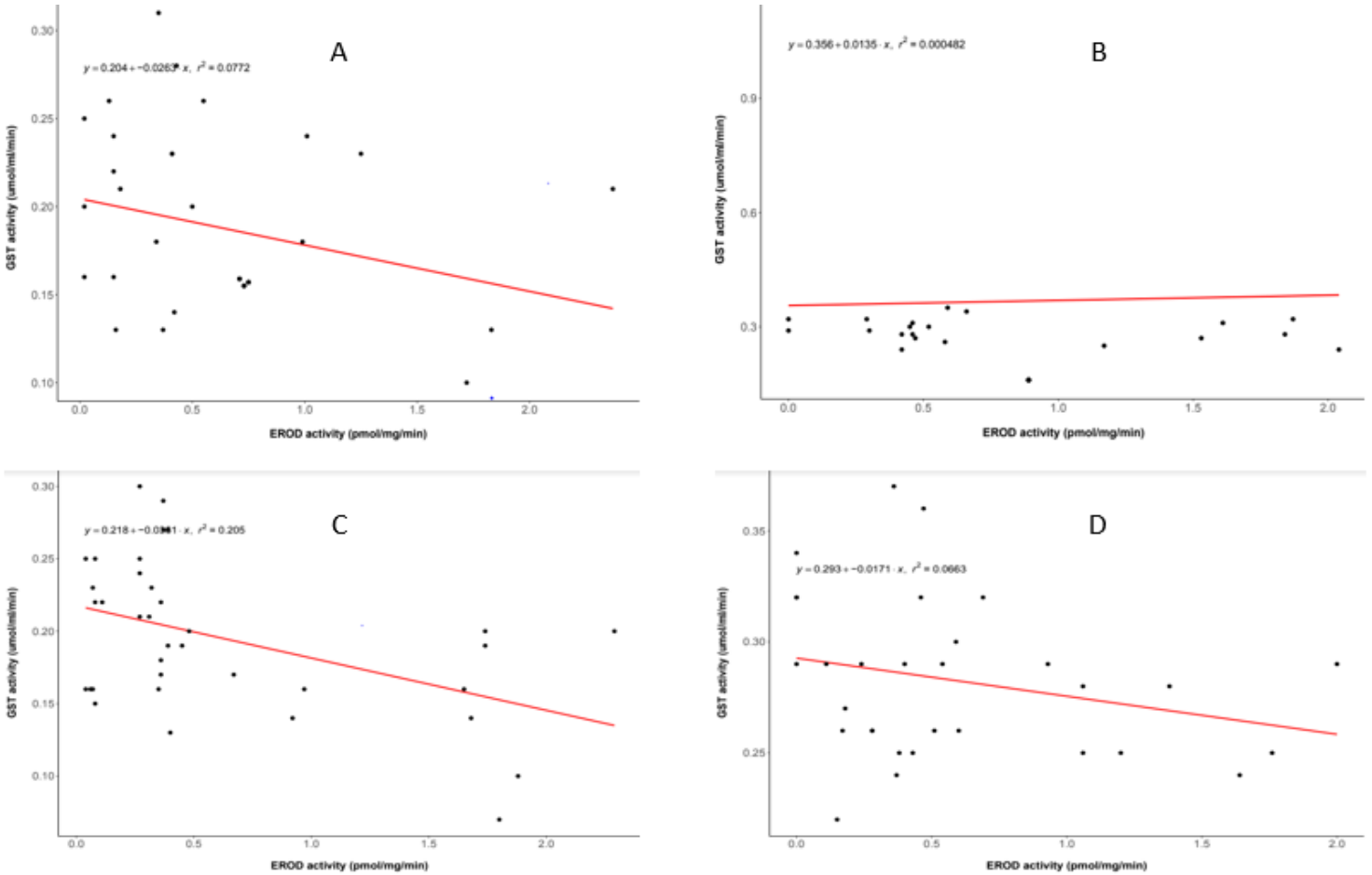


Figure 4. 9 Correlation analysis between EROD and GST in earthworms exposed to spiked PAHs ($p < 0.05$).

Where A = treatments with *E. hortensis* unamended with biosurfactant, B = treatments with *L. terrestris* unamended with biosurfactant, C = treatments with *E. hortensis* amended with biosurfactant and D = treatments with *L. terrestris* amended with biosurfactant.

4.6 DISCUSSION

Organic and inorganic contaminants have the potential to have strong inductive and inhibitory effects on biochemical activities in organisms (Xu et al., 2015). PAHs have been reported to be highly toxic and carcinogenic in nature even at minute concentrations. However, to date the biological adverse effects of PAHs and mechanism of their toxicity is unclear (Wu et al., 2012; Cao et al., 2016). In this regard, the biomarkers from phase I and II were investigated to study the activities of these enzymes to elucidate the enzyme mechanism upon exposure to PAHs.

4.6.1 CYP INDUCTION

Cytochrome enzymes have been reported to play major roles in metabolism of endogenous compounds as well as biotransformation of xenobiotics (Isin and Guengerich, 2006; Cao et al., 2017). Several studies have proven that expression and enzymatic activities of CYPs in various organism can give a reflection of the presence of environmental contaminant (Allen and Moore, 2004; Tabrez and Ahmad, 2013). There have been different reports of various alterations in CYP expressions and enzyme activities in various organisms exposed to different xenobiotic; e.g. PAHs, heavy metals, TCDD and dexamethasone (Abrahamson et al., 2007; Cao et al., 2012; Brown et al., 2004). The present study aimed to evaluate the possible synergistic or antagonistic effect combination of PAHs and surface-active agents (rhamnolipid biosurfactant) have on CYP family CYP1A1 and 1A2 in the gut of *E. hortensis* and *L. terrestris* upon exposure for 28 days.

CYP1A1 (EROD) and 1A2 (MROD) activities are widely used as biomarkers of environmental contamination such as PAHs and PCBs due to the fact that both these activities can be induced significantly in organisms on exposure to various xenobiotics (Tabrez and Ahmad, 2013; Han et al., 2009; Devier et al., 2013; Jones et al., 2014; Kennedy et al., 1996). However, there are several cases where EROD activity has either been inhibited in cells or bidirectional in cell function (Elbekai and El-Kadi, 2004, Bruschweiler et al., 1996; Amara et al., 2013). This study recorded elevation in EROD activities in both earthworm species (*E. hortensis* and *L. terrestris*) exposed to a combination of PH, FL and BAP, as well as individual BAP both amended and unamended with biosurfactant at day 2. Following a time response, EROD activity declined

from day 7 right up till day 28, recording activities similar to or lower than that of the controls in most treatments. It can be deduced that the induction of CYP1A enzymes upon exposure to PAHs resulted in the induction of EROD activity irrespective of the biosurfactant amendment, and to date the cellular regulation of this particular enzyme in earthworms remains unknown. The decrease in the activity of EROD could be attributed to an inhibition in CYP1A metabolism that has been widely reported in vertebrates (Sanchez et al., 2005; Sen and Semiz, 2007; Vieira et al., 2009) but very little in invertebrates (Cao et al., 2012; Gottardi et al., 2016; Kim et al., 2010). Generally, organic pollutants are believed to bind to AH-receptors which triggers the induction of CYP1A enzymes resulting in altering changes in conformation of the cell as well as the turnover of proteins (Pereira et al., 2009) this resuls in induction or inhibition in CYP1A synthesis in organisms. The significant induction followed by inhibition of EROD activities in both *E. hortensis* and *L. terrestris* could indicate onset of metabolic reactions of phase I enzymes as well as an adaptive mechanism. Similarly, MROD activities in both earthworm species were significantly induced at day 2 across all treatments and inhibited from day 7 through day 28. The sensitivity of response in both EROD and MROD could also give an insight into induction of phase 1 metabolism of xenobiotics (Cao et al., 2017).

The necessity to evaluate the consistency between soil contact and filter paper contact tests has been suggested by several authors since both methods are used for toxicity testing. In several studies it has been reported that results obtained both from soil and filter paper contact tests were very similar (Miyazaki et al., 2002; Saint-Denis et al., 2001; Brown et al., 2004). However, some researchers argued the use of biochemical endpoints could indicate different results in both tests. Cao et al. (2017) reported no significance in CYP3A4 activities exposed to copper or zinc. Labrot et al. (1996) also reported a different biochemical response in *E. fetida* exposed to lead compared to that from soil contact test. In this study, no significant difference in the biochemical activities (EROD, MROD and GST) in both earthworm species were recorded, the results obtained being similar to that of filter paper contact test (data shown in Appendix 2).

The bioavailability of PAHs on entry into soil media has been a major challenge in land remediation and soil testing, and the higher the number of benzene rings, the lower the

bioavailability and bioaccesibility as seen in Chapter 2 (Section 2.3). The reduced bioavailability of PAHs to earthworms and microorganisms as previously discussed can be attributed to soil complexity (Cook and Hendershot, 1996) as well as soil physicochemical parameters such as pH which does play an important role in PAH bioavailability, hence the application of surface active agent (rhamnolipid biosurfactant 0.1 g L^{-1}) as amendment to increase the bioavailability of the PAH (please refer to chapter 3, Fig 3.5). Also reported in chapter four is the bioaccumulation factor of PAHs in earthworms where we did not detect any hydrocarbon in the earthworm homogenised tissue even with the amendment with biosurfactant. This is possibly attributed to the AH-receptors induction of enzyme activity that could hydrolyse contaminants and excrete them back into soil medium or possibly in circulation in the earthworm stream as bio transformed conjugates (Baron et al., 1994). This accords with reports from Malev et al. (2016) who reported limits below detection of PAHs in earthworms in their study also indicating possibilities of high detection limits for quantification.

4.6.2 ANTIOXIDASE DEFENCE

Oxidative stress is known to be the imbalance in the antioxidant system and formation of free oxyradicals, which could be due to an increase in the formation of oxygen reactive species or a weakened defence mechanism (Leon et al., 2004), which as a result, could affect the structure-function dynamics in systems, cells or organs (Laszczyca et al., 2004; Leon et al., 2004). Bivalves, fishes, oligochaetes are some of the classes of organisms whose antioxidant systems have been well studied, whether collected from contaminated sites or exposed to contamination (Martin et al., 2008). Selected antioxidant parameters in this particular study could be a potential biomarker where it not only reflects the exposure to contaminants but also the toxicity of the contaminants.

Both combined and individual PAHs significantly induced GST activity in both earthworm species which is consistent with other reports of GST induction when exposed to organic/inorganic pollutants (Maity et al., 2018) that showed induction of just up to 5-fold higher than control levels when *E. fetida* was exposed to cadmium.

These results showed that GST in *L. terrestris* can be used as a potential biomarker of short-term PAH exposure. Due to a delayed response of GST activities in *E. hortensis* (day 14 compared to day 2 *L. terrestris*), it most likely would be more suitable as a long-term exposure biomarker. This result suggests the involvement of an enzymatic pathway (phase I and II) in the metabolism of PAHs. The induction of GST showed that the regulation of free radicals formed was possible by glutathione metabolism and by enzymatic conjugation because GSTs were induced across all treatments in both species of earthworms. It can also be argued that the initial unaltered state of GST activities in *E. hortensis* on day 2 signifies the ability of GSH to bind to PAHs and ROS non-enzymatically in order to remove them, thus can be qualified as a protective mechanism of the cell (Klaassen et al., 1985; Wu et al., 2012). Typically, the amount of GSH content present in the cell is inversely proportional to the amount of contaminant present in the earthworm as well as the enzyme activities such as GST and GPx (glutathione peroxidase) which indicates the activation of an antioxidant defence system (Radu et al., 2010). Active utilization of enzymes such as GST and GPx is crucial in the maintenance of homeostasis of both internal and external cell environment (Zhang et al., 2009; Maity et al., 2018).

A medium to strong correlation was observed between the concentration of PAHs in soil and the biochemical responses investigated (r= EROD – 0.52, MROD - 0.54 and GST – 0.22) using a time response relationship. This result agrees with reports from Cao et al. (2017) where a time and dose dependent relationship between CYP3A4 enzyme activities and heavy metal concentration was in *E. fetida*. The result obtained indicates the potential that these enzymes could serve as oxidative stress indicators in the environment. A very strong correlation was noted between EROD and MROD activities across all treatments in both earthworm species (r = 0.9) indicating a similar metabolic pathway of both CYP1A enzymes in both earthworms' species. This provides a new insight and understanding on the interferences and overlaps between CYP1A1 (EROD) and CYP1A2 (MROD). These interferences in pathways are yet to be studied in lower organisms such as earthworms, hence the importance of these particular results. Understanding the relationship between both enzyme pathways will result in a better detailed knowledge in induction of enzymes and cellular regulation of enzymes which as discussed earlier is not well understood even in higher vertebrates, hence this could be a

promising beginning into further research in this area. Even though both isoenzymes have distinct substrate specificity, they do tend to overlap due to the strong similarities between both enzymes active sites which was portrayed in our findings (Tassaneeyakul et al., 1993; Zamaratskaia and Zablek, 2009).

This research showed that the application of biosurfactant did not play a significant role in effecting the enzyme activities (Fig 4.7 A-D, Fig 4.8 A-D and Fig 4.9 A-D). Their interactions with the enzyme activities was similar to earthworms exposed to PAHs. This can be explained by a cells' natural metabolising/detoxifying any foreign substance which it has been exposed to from its system. However, no mortality or weight loss was recorded in controls with biosurfactant only, indicating that biosurfactant might impose cellular stress in organisms solely because they are foreign substances, but they do not pose any physiological stress or damage to both earthworm species for the duration of exposure. Hence this result agrees with the research hypothesis that biosurfactants even though they have been characterised as non-toxic using physiological parameters as forms of measurement, also are non-toxic at cellular levels as indicated from the findings of this study.

Overall, the bioavailability of HMW PAHs vary from PAH to PAH (Contreras-Ramos et al., 2006). Results obtained from the bioavailability of PAHs in this study agrees with other authors where the order of bioavailability of PAHs to earthworms was PH>FL>BAP. The low bioavailability could be as a result of the adsorption properties of HMW PAHs in soil as well as the physico-chemical properties of the soil such as organic matter content of the soil or soil pH which could influence the availability of PAH for degradation (Gaw et al., 2012).

The application of rhamnolipid biosurfactant in this study as reported in chapter 3 increased bioavailability of PAHs which in turn resulted in an increased and accelerated removal of the PAHs by both earthworm species. Furthermore, no PAH was detected in either earthworm species from soil contact test. This result agrees with other researchers who reported no accumulation of PAHs in earthworms (Malev et al., 2016). The absence of PAHs in the earthworm could indicate the ability of the earthworm to biodegrade organic pollutant that are bioavailable to them. In addition, the ability of biosurfactant to solubilise and mobilize HMW PAHs where the concentration of the PAHs were subsequently reduced as reported in

chapter three further provides evidence that earthworms and their associated microorganisms are capable of degrading contaminants that are readily available to them.

4.7 CONCLUSION

The laboratory-scale study of enzymatic activities in a time response relationship showed that both EROD and MROD activities were induced between days 0 and 2 of exposure, the level of increase in enzymatic activities were similar in both genera (between 2 and 2.5-fold higher in EROD and MROD activities) indicating the presence of AH receptors which further indicates that the earthworms have the ability to degrade HMW PAHs. Enzymatic activities decreased by day 7, with complete mortality of *L. terrestris* by day 28 hence enzymatic activities could not be measured in *L. terrestris* at day 28. While an increase in GST activity was observed from day 7 in both genera. The application of rhamnolipid did not appear to affect the levels of indicator enzymes. We can conclude from this study that biosurfactant at 0.1g L^{-1} is non-toxic to either earthworm species but can enhance remediation of HMW PAHs significantly.

Chapter 5- OVERALL DISCUSSION AND CONCLUSION

5.1 RESEARCH OVERVIEW

This research was designed to study the efficacy of an integrated approach using rhamnolipid biosurfactant and vermiremediation for the treatment of PAHs contaminated land. Specifically, the use of both epigeic species (*E. hortensis*) of earthworms and anecic species (*L. terrestris*) in biodegrading 3-, 4- and 5-ring PAHs was explored. Based on this approach, it was expected that the treated soil would become richer in organic contaminants, due to solubilisation of not only the PAHs in the soil but as also the soil nutrients and organic matter in addition to the earthworm burrowing activities, thus improving soil structure, physico-chemical properties and biogeochemical cycle. Thus, this integrated approach stands as a promising solution to optimize and enhance remediation of HMW PAHs and also improve soil health and quality.

5.2 EFFECTIVENESS OF BIOSURFACTANT IN VERMIREMEDIATION

Experiments were designed to evaluate the performance of *E. hortensis* and *L. terrestris* in degrading PAHs in the presence of surface-active agents (biosurfactant). Rhamnolipid biosurfactant was selected following principles of environmental sustainability. Soil used for this experiment was Kettering loam soil which is an artificial soil, artificially spiked with a combined concentration of 180 mg Kg^{-1} PAH. The spiked concentration accordingly was 60 mg Kg^{-1} PH, 60 mg Kg^{-1} FL and 60 mg Kg^{-1} BAP, an additional experiment with only BAP 60 mg Kg^{-1} was also undertaken. Results showed that biosurfactant-amended treatment performed better in removing PAHs, while the combination of both biosurfactant and earthworm performed best in removing PAHs. PH and FL were completely removed, and 80% BAP was also removed. Highlights of the experiments are listed as follows:

1. Potentials of successfully degrading PAHs is associated with their hydrophobicity, which surface active agents enhances by increasing the solubility of hydrophobic contaminants (Section 3.2.5)
2. The presence of biosurfactant improves the mobility of nutrients in the soil to microbes and earthworms thus improving soil structure (Section 3.2.3.10, 3.2.4 and 3.2.5).

3. Nutrients mobility as well as solubility of PAHs yields production and growth of microbial community and earthworms which in turn resulted in optimized complete mineralisation of PH and FL and 80% degradation of BAP (Section 3.2.3.10).
4. Biochemical activities in earthworms used to monitor the possible toxicity of biosurfactants at molecular levels indicated little to no toxicity to earthworms further indicating the suitability of biosurfactant application in combination with other bioremediation approaches.

A time response relationship between the concentration of PH, FL and BAP, and length of exposure was established; In the presence of biosurfactant (0.1 g L^{-1}), *E. hortensis* removed on average 91% ± 5.2 PH, 70% ± 8.4 FL and 27% ± 4 BAP after 7 days whereas *L. terrestris* removed approximately 89% ± 0.5 PH, 67% ± 6.4 FL and 30% ± 1.4 BAP, while in the absence of biosurfactant, *E. hortensis* removed only 26% ± 1.7 PH, 17% ± 6.2 FL and 8.4% ± 0.7 and *L. terrestris* 25% ± 4.5 PH 24% ± 8.8 FL and 6.4% ± 3 BAP after 7 days of treatment. A one-way ANOVA test between treatments and control showed that there was a significant difference ($p<0.05$) in the degradation of FL and PH in control soils in the presence of biosurfactant compared with the control soils without biosurfactant. In addition, a significant difference was observed between degradation of PH and FL by *E. hortensis* in the presence of biosurfactant, compared to its control (absence of biosurfactant, similar results between degradation by *L. terrestris* with biosurfactant and its control (without biosurfactant). However, there was no statistically significant ($p>0.05$) difference between FL and PH degradation by *E. hortensis* with biosurfactant and *L. terrestris* with biosurfactant.

Table 5. 1 Research overview (Vermiremediation)

Experimental design	Target contaminants	Experimental design	Results	Conclusion
Vermiremediation of HMW PAHs via biostimulation	Phenanthrene (PL) Fluoranthene (FL) Benzo(a)pyrene (BAP)	Treatment time: 28 days Soil: artificially spiked soil (Kettering loam soil). Treatment: Soil + BAP Soil + BAP + B(iosurfactant) Soil + BAP + E(*isenia hortensis*) Soil + BAP + L(*umbricus terrestris*) Soil + BAP + E + B Soil + BAP + L + B Soil + PAH (PL+FL) Soil + PAH + B Soil + PAH + E Soil + PAH + L Soil + PAH + E + B Soil + PAH + L + B	Compared to controls, earthworms removed 3 and 4- ringed hydrocarbons the most and a certain percentage of 5-ring hydrocarbon, however, in the presence of biosurfactant amendment, 3- and 4-ring hydrocarbons were completely removed, and a high percentage of 5-ring hydrocarbon was removed. Overall, combined PAHs was had a greater degradation compared to singular 5-ring PAHs.	Biosurfactant amendment enhanced remediation of PAHs across all treatments. There was no significant difference in the performance of both epigeic and anecic species; however, epigeic species are easier to handle and monitor.

Table 5. 2 Regression equation of PAH removal in a time response analysis.

Biosurfactant	Earthworm	PAH (mg Kg^{-1} day^{-1})			*p*-value
Y	N	PH	=	43.7 *(3.61)* - 0.9 *(0.5)* T	>0.05
Y	Y		=	47.5 *(3.61)* - 1.9 *(0.27)* T	<0.001
N	N		=	44.7 *(3.61)* - 0.7 *(0.5)* T	>0.05
N	Y		=	49.6 *(3.61)* - 1.4 *(0.27)* T	<0.001
Y	N	FL	=	45.7 *(0.35)* - 0.9 *(0.5)* T	>0.05
Y	Y		=	48.1 *(0.35)* - 1.9 *(0.26)* T	<0.001
N	N		=	51.2 *(0.35)* - 1 *(0.5)* T	>0.05
N	Y		=	48.5 *(0.35)* - 1.1 *(0.26)* T	<0.001
Y	N	BAP	=	47.5 *(0.93)* - 0.8 *(0.13)* T	<0.001
Y	Y		=	46.1 *(0.93)* - 1.1 *(0.07)* T	<0.001
N	N		=	50.5 *(0.93)* - 0.9 *(0.13)* T	<0.001
N	Y		=	49.3 *(0.93)* - 1 *(0.07)* T	<0.001
Y	N	Total PAH	=	92.2 *(12.4)* - 1.7 *(1.66)* T	>0.05
Y	Y		=	111.2 *(12.4)* - 3.8 *(0.1)* T	<0.001
N	N		=	98.5 *(12.4)* - 1.7 *(1.66)* T	>0.05
N	Y		=	118.1 *(12.4)* - 3.2 *(0.1)* T	<0.05

Where B = biosurfactant, NB = no biosurfactant, N = no earthworm, Y = earthworm present, T = time (days), standard error of coefficients are represented in brackets accompanying coefficients and *p*-value indicating statistical significance of PAH removal with time.

The regression equation illustrated in Table 5.2 indicated the coefficient of present PAHs and the predicted concentration that is removed in all treatments using biosurfactant and earthworms as variables. Overall a time response analysis showed the application of earthworms and biosurfactant in degrading PAHs to be very significant. The removal of PH, FL and BAP by *E. hortensis* and *L. terrestris* was much enhanced in the presence of biosurfactant, where the average rate of decrease of the total PAHs was 3.78 mg Kg^{-1} day^{-1}. This significantly outperformed the decrease of 1.70 mg Kg^{-1} day^{-1} observed in the absence of earworms and biosurfactant. Presence of earthworms and biosurfactant on individual PAHs also had an average decrease of 1.86, 1.85 and 1.12 mg Kg^{-1} day^{-1} PH, FL and BAP compared to the 0.73, 0.97 and 0.85 mg Kg^{-1} day^{-1} observed in the absence of both biosurfactant and earthworms (Table 5.2). The degradation recorded in this study was at a faster rate compared to studies in literature using earthworms alone where they took a longer period to metabolise contaminants. The previous studies using biosurfactant alone for remediating contaminated soils did remove at an even faster rate, however application was at a very high concentration

of 156 g L^{-1} (10,000 times above the CMC and 1000-fold above the current study) to achieve a quicker removal (Pinto and Moore, 2000).

The rationale of using the microtox EC_{50} limit was based on the symbiotic relationship between earthworms and microorganisms, hence choosing the toxicity limits that the microorganisms could adapt to. However, it may be possible to raise the concentration of biosurfactant with the knowledge that earthworms are resistant organisms where authors like Contreras-Ramos et al. (2006) reported *E. fetida* survival in PAH concentrations of >1000 mg Kg^{-1} of soil and survival rate of 80%. *Eisenia* species did remove a larger percentage in the first two weeks which could be because of their resistant nature and ability to quickly acclimatize themselves to the environmental conditions. One limitation from method development (pilot study) was collection of time-point samples from same microcosm. Soils could not be further homogenized to avoid upsetting the pseudo-natural earthworm habitat, hence the solution devised for further experiments to set up individual time series microcosm independently (implemented in Section 3.2.5) to achieve termination and optimal homogenization at desired time points.

Compared to conventional bioremediation technologies that achieved >90% removal efficiencies (Rodriguez-Campos et al., 2014; Contreras-Ramos et al., 2006; Ma et al., 2005), the integration of vermiremediation with biosurfactant in this study are comparable. Although, the integrated approach has been demonstrated in the laboratory successfully, it needs to be scaled up into field studies to demonstrate real world applicability. A possible limitation to the extrapolation of these results in field studies could be the variation of results and earthworm behaviour in the field such as avoidance of contaminated areas, soil type and temperature, thus this needs to be further studies in the field.

The complete removal of PAHs like PH in these studies after 28 days with application of biosurfactant compared to controls without earthworms or biosurfactant, indicates two important aspects; biosurfactants increases the bioavailability of the contaminants, and earthworms have the potentials of aiding remediation of PAHs. The significant reduction in the concentration of HMW PAHs like BAP compared to treatments without earthworms and/or biosurfactant shows the ability of earthworms in aiding remediation of these PAHs in soil. The removal or reduction of these PAHs in soil could be attributed to mineralization of

these PAHs into CO_2 and H_2O. These results are in agreement with findings from other researchers such as Tejada and Masciandaro (2011), Contreras-Ramos et al. (2008), Azizi et al. (2013) and many others. Contreras-Ramos et al. (2006) reported similar findings with removal of >90% PH over 90 days whereas in the currently reported study, not only was there a higher percentage of removal of PH (100%), the mineralization of PH was achieved within 28 days of exposure which could be associated with the solubilisation of the PAHs by rhamnolipid biosurfactant, enhancing the bioavailability to earthworm species and their associated microorganisms.

As discussed earlier (in chapter 3 Section 3.4.4), there remains a considerable knowledge gap of the specific mechanism that earthworms use in remediating PAHs. Rodriguez-Campos et al. (2014) suggested that the improved soil quality produced by earthworm activity indicated that activities associated with the production of the improved soil quality must be associated with the digestive system of the earthworms. There have been three possible mechanisms associated with earthworms in vermiremediating of PAHs and they are; enhancing oxidation processes by soil aerating, enhancing microbial activity, and increasing mobility or bioavailability of the contaminants (Schaefer and Juliane., 2007).

For industrial scale vermiremediation, there are several factors to be considered such as cost of implementation as well as potential savings from the removal and disposal of waste, soil amendment and fertilizer expenditure. Several institutions have discovered that it is more cost-effective to use vermiculture as an eco-friendly and sustainable approach to organic waste degradation on-site.

Most species used in vermiremediation/vermicomposting and ecotoxicology are *Eisenia* sp. (mostly *E. fetida*) because of the ease in handling and culturing. The selected species used in this study *E. hortensis* (European night crawler) performed similarly to its "sister specie" *E. fetida*. Furthermore, vermiculture is beginning to gain some attention in countries like Nigeria, however it is still very small scaled and research-based applications in this part of the world. It is noteworthy because African sister specie of *E. hortensis* (European night crawler), *Eudrilus eugeniae* (African night crawler) is a potential species that can be considered for remediation and composting. Also, there have been reports of the presence of epigeic species (*E. fetida*) in Nigerian soils capable of remediating PAHs in contaminated soils (Njoku et al., 2016), thus

indicating that both species of earthworms could be potentially applied in remediation of HMW PAHs in Nigerian soils.

The second research focus of this study was to investigate the potential toxicity biosurfactant has at molecular levels (Table 5.3). Banat et al. (2010) highlighted that biosurfactant is more sustainable than chemical surfactants but that its toxicity was not fully understood. The toxicity of PAHs was well represented in this study, this is significant because PAHs such as BAP have a soil half-life between to 14-18 months in aerobic conditions with maintained temperatures between 10-30^{o}C (Coover and Sims, 1987). They therefore persist in the environment for a long time, posing toxic stress on soils and its associated micro and macro flora and fauna. With bioavailability being the major focus of remediation of PAHs, which is greatly influenced by the soil organic matter present could be explained that different soil types with different levels of soil organic matter content could affect toxicity of PAHs in soil. Due to earthworm species such as *L. terrestris'* need for nutrient rich soils for optimum activity, soil used in this study was amended with approximately 16% organic matter (compost). However, it has been discussed by several authors that soils with high organic content reduces the bioavailability of PAHs in soil hence reducing their uptake for degradation (Jager et al, 2003; Veethak et al, 2005). This challenge was overcome in this study with the use of biosurfactant where there was ≥80 recovery of PAHs in soils.

Monitoring weight change in earthworms is a good indicator to observe changes in stress levels imposed on them. Decrease in body weight of earthworms signified the presence of contaminants which is posing toxic stress on the worms hence inhibiting the growth. Significant increase in body weight of both earthworm specie in biosurfactant amended treatment on the other hand was rather interesting, possibly explained by the solubilisation of soil nutrients which earthworms also need for their metabolic processes, which could also explain the higher production of cocoons in treated microcosms. This would be in line with Eijsackers et al. (2001) findings where earthworms exposed to 10 mg Kg^{-1} PH amended with 40% organic matter yielded 39 cocoons compared to 14 produced in same conditions amended with 10% organic matter after 77 days of exposure. This indicates that organic matter influences both growth and reproduction of worms, where the bioavailability of the organic matter could be influenced by the addition of biosurfactant.

5.3 BIOCHEMICAL RESPONSE IN EARTHWORMS

Some authors have reported the presence of monooxygenase enzymes in worms (CYP enzymes) which suggest the presence of AhR receptors in worms, where this would infer the ability to degrade PAHs by earthworms (Azedah and Zarabi, 2015). However, the presence of biosurfactant did not induce or inhibit both EROD and MROD activities any different from how PAHs induced or inhibited the activities of both CYP1A1 and CYP1A2 EROD and MROD activities. Authors such as Brown et al. (2004) could not detect the presence of EROD activities in *E. fetida*, but they suggested that the enzymes were present because *E. fetida* proteins cross reacted with AhR antibody where this receptor (as discussed in chapter 4) is the key regulator of CYP1A activity. Eason et al. (1998) were also not able to detect the presence of these enzymes, a possible reason for this is the method of analysis where several researchers use spectrofluorometric detection, even though this method is very sensitive, it has been reported to be not very selective, and CYP450 enzymes are prone to several interferences which could interfere with the peak at 450 nm leading to false readings (Oliveira et al., 2013). Hence the development and validation of a sensitive and selective method on the liquid chromatography using Oliveira et al. (2013) method that resulted in detection of EROD and MROD activities. Furthermore, the unaltered GST activity across all treatments between day 0 and 14 conforms to findings of Saint-Dennis et al. (1999) who found unaltered GST activity in *E. fetida* exposed to BAP between day 0 and 14, which could suggest that these enzymes were not induced because monooxygenase enzymes were being induced at the exact period executing phase one detoxification. However, the induction of these enzymes from day 14 onwards indicates the induction of phase II transformation.

Overall, there is substantial evidence that:

1) The integration of vermiremediation with application of biosurfactant could potentially accelerate the removal and mineralisation of PAHs.

2) the application of both epigeic and anecic species were individually successful in remediating HMW PAHs however, epigeic species are easier to handle more cost effective and have a shorter lifecycle compared to anecic species.

3) The application of biosurfactant poses little or no toxic effects to both earthworm species at molecular levels and

4) A positive relationship between monooxygenase activities and antioxidase enzyme activities suggesting a similar relationship could exist between phase I and II degradation.

Generally, the results obtained from this research demonstrate a potential avenue into integrating vermiremediation with biosurfactant in treating PAH contaminated soils. However, the work also has some limitations that should be considered as follows:

1. The chosen biosurfactant concentration was based on derived EC_{50}, but it is reasonable to suggest that tolerant species such as *E. hortensis* could withstand higher concentrations. However, due to the known symbiotic relationship between earthworms and microorganisms, it was decided not to exceed biosurfactant concentrations above the EC_{50}.
2. Sensitivity of *L. terrestris* compared to more resistant *E. hortensis* reduced the limits of how far *E. hortensis* could be investigated in this research as it was important for both species to be subjected to the laboratory same conditions before further individual investigations, hence suggesting that *E. hortensis* could potentially perform better even in harsher conditions to which they are subjected.

In conclusion, remediation of contaminated land requires intense evaluation and careful planning for execution. All remediation options need to be specifically tailored towards site-specific parametric condition by conducting small scale pilot studies prior to actual remediation process. However, as highlighted by Kuppusamy et al. (2017), it is very important that both characterization of contaminated site as well as the pilot study for treatability answer few questions before upscaling into field;

a) Can it be treated naturally (natural attenuation)? If so, are metabolites more toxic?
b) Can the above pose post-remediation risks?
c) Are environmental conditions appropriate to support optimum remediation?
d) Does remediation result in secondary waste that needs further treatment?
e) If there is incomplete remediation, where do the leftover pollutant go?

Table 5. 3 Research overview (biochemical response)

Experimental research	Target enzymatic response	Experimental design	Results	Conclusion
Vermiremediation of HMW PAHs via biostimulation	EROD MROD and GST	This is an extension of work done in chapter 3. Earthworms were collected from experiments in chapter 3 and isolated after which their guts were removed for biochemical analysis.	EROD and MROD activity were induced across all treatment between day 0 and day 2 and subsequently inhibited from day 7 while GST activity were not altered until day 7 where the activity was induced across all treatments.	Toxicity of biosurfactant at the molecular levels in *E. hortensis* and *L. terrestris* shows little or no toxicity where all three activities monitored were similar to that of the PAHs.

5.4 FUTURE STUDIES

It is important to note that there are several criteria and parameters such as magnitude of contamination, toxicity and mobility of contaminants, geochemical, geophysical and biological characteristics of the soil, planned use of the site etc. to take into account when selecting vermiremediation as a bioremediation technique, as the biodegradation of PAHs in contaminated land is a complex procedure. The extrapolation of laboratory scale vermiremediation of PAHs to the field could be challenging, which could result in different efficiencies. This is attributed to the fact that microcosms might not represent to an adequate extent the intricate interactions between different soil types, the nature of pollutant and earthworm behaviour. There are several unforeseen factors in field studies that could affect successful extrapolation of laboratory results to field study, this goes beyond the known conditions which could affect the extrapolation of laboratory studies to the field such as soil homogeneity, avoidance, or contact between nutrient and soil matrix (Atagana, 2004). There remains a gap for further research into mitigating the unforeseen conditions associated with extrapolating laboratory to field studies. Additionally, soil toxicity should be carried out in field to measure the performance of vermiremediation. Several studies reported that the behaviour of spiked soil with contaminants could be very different from actual contaminated soils such as that of the Ogoni land in the Niger-Delta regions of Nigeria, thus results obtained from laboratory remediation of spiked soil could at times be misleading when faced with field study results. Hence the study of vermiremediation technique integrated with biosurfactant needs to be verified with actual contaminated soil in the field.

Further to the integrated approach of vermiremediation with biosurfactant in this study, another interesting approach is amendment with organic wastes. Major cities in Nigeria like Lagos generate tons of organic waste daily with inadequate means of disposal, earthworms are known natural degraders of organic waste such as kitchen waste, and this waste materials consist of their microbial community which also in turn can make use of the HMW PAHs as source of carbon hence having a 2-way action which is:

1) Earthworms composting the organic waste generated and transported to contaminated sites and
2) Earthworms assisting in remediation of contaminated soil which would be rich in organic matter from compost created previously.

In addition, several studies on vermicomposting/vermiremediation are carried out on a small scale where the reactors are centred on the entire medium (mixture oi soil bedding and earthworms) (Suthar, 2010), with little emphasis in the earthworm itself as a reactor. Due to their participation in redesigning soil profiles, physical and chemical properties of soil through feeding on soil particles and organic waste, earthworms are regarded as natural reactors (Ansari, 2011). The benefits associated with application of earthworms are several, some of which are stimulating and increasing biological activities in the soil by fragmentation and uptake of organic matter which in turn increases surface area to microorganisms (Nasiru et al., 2013). However, there is still an inadequate understanding of the digestive kinetics of an earthworm in vermitechnology which results in situations such as poor cast management that could in itself be of adverse effects. Earthworm casts have been shown to produce some greenhouse gases, nitrous oxide in particular when not properly managed (Majeed et al., 2013). The passage of the substrate through the gut of earthworms and activities associated with the substrate transformation in the gut is important to understand (Li et al., 2010; Sinha, 2009; Lavelle, 1988). This needs to be further explored and investigated from vermicast produced from this study. Hence understanding earthworm digestive kinetics could provide better understanding of the effectiveness of earthworms as reactors as well as mitigating any negative impacts.

Isolating the gut in earthworms to determine exposure of soil borne Ah receptor agonist has its potentials because it is the digestive hub in earthworms. Both monooxygenases (EROD and MROD) and antioxidases (GST) enzyme activity were induced on exposure to PAHS indicating ability to degrade PAHs. Again, these results need to be extrapolated to field studies, with all enzymes induced in artificially-spiked soil, this would give rise to possible questions such as would the enzymes be induced in the same pattern in laboratory studies? If so, could the induction in monooxygenases and antioxidases be attributed to external factors such as air pollution, or more-so, could there be other inducers of monooxygenases and antioxidases present in the soil? Further research is required to answer these questions where other parts of the earthworm such as the skin or juvenile stages as well as the gut need to be investigated.

Combination of both epigeic and anecic species in similar media holds a promising prospect in vermiremediation, however the selection of a more resistant anecic specie

other than *L. terrestris* needs to be further explored this would allow for expanding the subjecting conditions of earthworms to harsher conditions which they could thrive in such as increased concentrations of PAHs or biosurfactant without having any direct deleterious effects on them. This could potentially allow for an industrial scale vermiremediation/vermicomposting plant in Nigeria and globally.

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APPENDIX

Appendix 1

PRELIMINARY STUDY DATA

1.1 Change in body weight of earthworms after the course of a 28-day experiment

Type of treatment	PAH (mg Kg^{-1})	E	EB	L	LB
Soil contact	0 (control)	0.6 ± 0.03 [a]	0.6 ± 0.01 [a]	4.7 ± 0.1 [a]	4.7 ± 0.3 [a]
	60 (PH & FL)	0.55 ± 0.04 [a]	0.57 ± 0.02 [a]	4.6 ± 0.2 [a]	4.5 ± 1 [a]
	120 (PH & FL)	0.58 ± 0.01 [a]	0.55 ± 0.04 [a]	4.6 ± 0.1 [a]	4.7 ± 0.7 [a]
Filter paper contact	0 (control)	0.6 ± 0.03 [a]	0.7 ± 0.01 [a]	4.7 ± 0.1 [a]	4.9 ± 0.06 [b]
	180 (PH, FL & BAP)	0.51 ± 0.02 [a]	0.5 ± 0.02 [a]	4.2 ± 0.1 [a]	4.1 ± 0.1 [a]
	60 (BAP)	0.46 ± 0.07 [a]	0.45 ± 0.03 [a]	4 ± 0.7 [a]	4 ± 0.6 [a]
	180 (BAP)	0.4 ± 0.05[a]	0.4 ± 0.03 [a]	4 ± 0.1 [a]	3.94 ± 0.5 [a]

results are represented as means ± SD, n = 3, means not sharing the same letter are statistically different according to ANOVA test ($p<0.05$)

1.2 Fecundity and mortality after 28 days.

	C_0 (mg Kg^{-1})	E	EB	L	LB
FECUNDITY (cocoons):	60	3.75 ± 0.6[a]	3.25 ± 0.3[b]	2.25 ± 0.5[a]	3 ± 0.4[b]
	120	2.5 ± 0.5[a]	4.25 ± 0.5[b]	2 ± 0.6[a]	2.75 ± 0.7[a]
MORTALITY (%):	60	0	0	0	0
	120	0	0	0	0
PAH in earthworm	60	-	-	-	-
	120	-	-	-	-

C_0 represents total spiked concentration of PAH both *E. hortensis* and *L. terrestris* were exposed to. Results are represented as means ± SD, n = 3, means not sharing the same letter are statistically different according to ANOVA test ($p<0.01$) and - = not detected.

1.3 Microbial population present in Kettering soil before (C_0) and after treatment

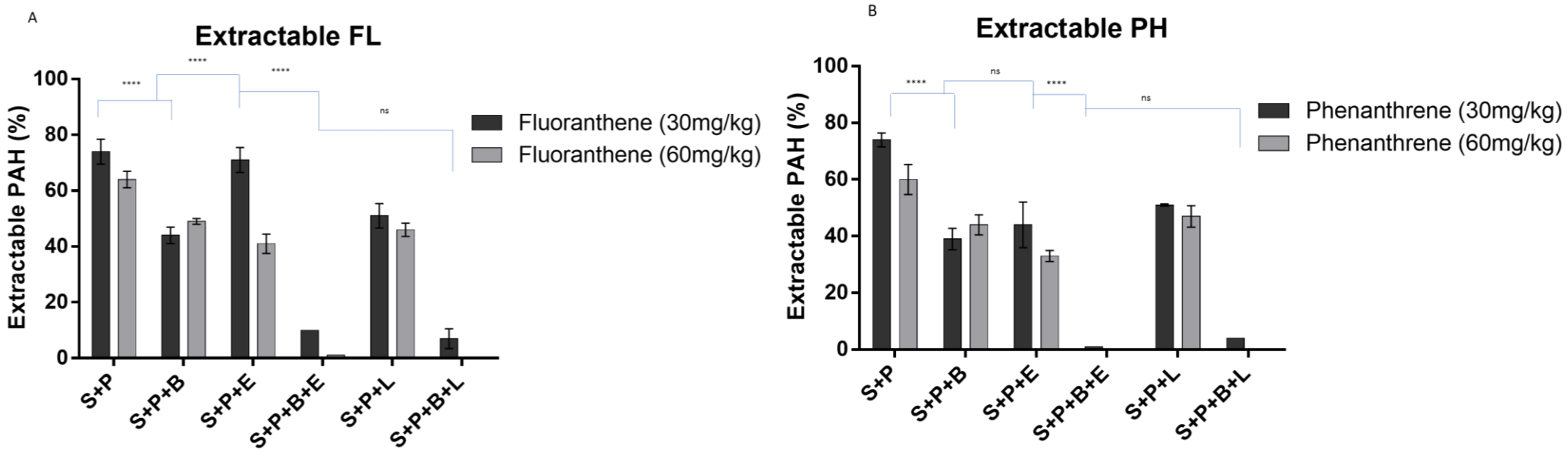
TREATMENT	Bacteria (cfu/g)	Fungi (cfu/g)	Actinomycetes (cfu/g)
C_0	$1.2 \times 10^{4(a)}$	$3.6 \times 10^{3(a)}$	$7.2 \times 10^{4(a)}$
B	$7.7 \times 10^{4(b)}$	$2.3 \times 10^{4(b)}$	$9.5 \times 10^{5(b)}$
PAHE	$3.3 \times 10^{5(c)}$	$2.7 \times 10^{4(b)}$	$4.2 \times 10^{5(b)}$
PAHL	$5.1 \times 10^{5(c)}$	$1.7 \times 10^{4(b)}$	$1.3 \times 10^{5(b)}$
PAHEB	$8.7 \times 10^{7(d)}$	$1.4 \times 10^{5(c)}$	$5.5 \times 10^{6(c)}$
PAHLB	$5.2 \times 10^{7(d)}$	$3.24 \times 10^{5(c)}$	$7.3 \times 10^{6(c)}$

Where C_0 = microbial population at day zero, results are represented as means ± SD, n = 3, means not sharing the same letter are statistically different according to ANOVA test ($p<0.05$

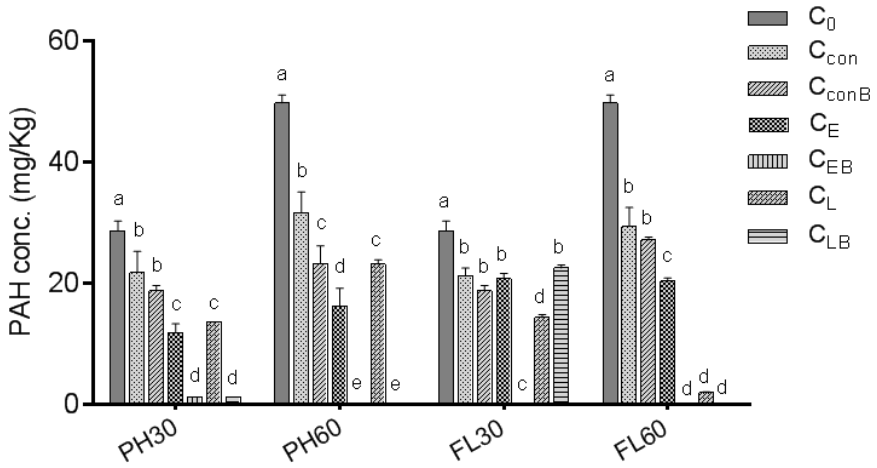
1.4 Extractable PAH (%) in experimental samples after 28 days

PAH	Amount added (mg/Kg)	Extractable at Day (mg/Kg)	C_0 (%)	Extractable PAH					
				Con	ConB	E	EB	L	LB
				Soil after 4 weeks (%)	Soil after 4 weeks (%)	Soil with *Eisenia* after 4 weeks (%)	Soil with *Eisenia* after 4 weeks (%)	Soil with *Lumbricus* after 4 weeks (%)	Soil with *Lumbricus* after 4 weeks (%)
FL	30	29	95	74	44	71	10	51	7
	60	50	83	64	49	41	1	46	N.D
PH	30	29	95	74	39	44	1	51	4
	60	50	83	60	44	33	N.D	47	N.D

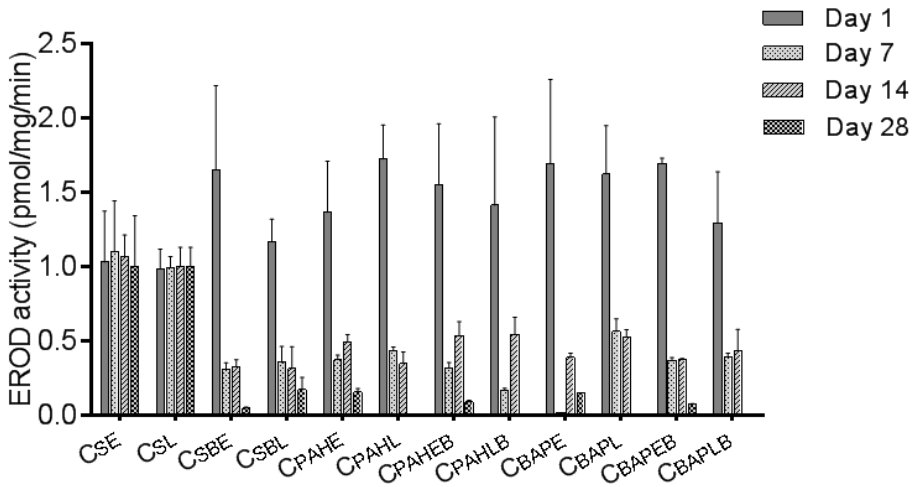
(Where N.D means not detectable)



1.1 A comparative overview of FL and PH removal between treatments in both *Eisenia hortensis* and *Lumbricus terrestris.* All data are represented as means ± SD of triplicates. **** represents a significant difference at $P < 0.0001$, * represents $p < 0.05$ and no represents no significance.



1.3 A comparative overview of combined PAHs PH and FL (60 and 120 mg Kg^{-1}) removal between treatments in both *Eisenia hortensis* and *Lumbricus terrestris* after 28 days. All data are represented as means ± SD, n = 3. Means not sharing the same letter are statistically different according to ANOVA test ($p<0.05$).



1.4 Combined effect of 3-, 4- and 5-ring PAH amended with biosurfactant on EROD activity in *Eisenia hortensis* and *Lumbricus terrestris* after a 28-day exposure. Values are represented as means ± SD (n = 3). Data not sharing same letter are significantly different with $p<0.05$.

1.5 Table Relative activity of GST, EROD and MROD in earthworms from filter paper contact test

Treatment	GST (µmol/mg/min)	EROD (pmol/mg/min)	MROD (pmol/mg/min)
Control E	1 ± 0.15[a]	1 ± 0.3 [a]	1 ± 0.1 [a]
Control L	1 ± 0.06 [a]	1 ± 0.13 [a]	1 ± 0.07 [a]
Control E + Bio	1.14 ± 0.261 [a]	0.83 ± 0.25 [a]	1.2 ± 0.25 [a]
Control L + Bio	1.01 ± 0.1 [a]	1.48 ± 0.13 [b]	1.1 ± 0.1 [a]
PAHE	0.62 ± 0.3 [b]	0.94 ± 0.26 [a]	1.04 ± 0.2 [a]
PAHL	0.98 ± 0.08 [a]	1.2 ± 0.4 [a]	0.8 ± 0.2 [a]
PAHEB	0.94 ± 0.29 [a]	1.03 ± 0.34 [a]	1.1 ± 0.36 [a]
PAHLB	1.06 ± 0.1 [a]	1.39 ± 0.2 [b]	1.1 ± 0.2 [a]
BAPE (60 mg/Kg)	0.96 ± 0.15 [a]	1.03 ± 0.02 [a]	1.2 ± 0.1 [a]
BAPL (60 mg/Kg)	0.98 ± 0.08 [a]	1.11 ± 0.27 [a]	0.8 ± 0.2 [a]
BAPEB (60 mg/Kg)	1.38 ± 0.11 [c]	0.19 ± 0.06 [c]	0.2 ± 0.02 [b]
BAPLB (60 mg/Kg)	1.57 ± 0.11 [c]	0.2 ± 0.01 [c]	0.2 ± 0.01 [b]
BAPE (180 mg/Kg)	0.97 ± 0.01 [a]	0.3 ± 0.03 [c]	0.2 ± 0.02 [b]
BAPL (180 mg/Kg)	0.86 ± 0.13 [a]	0.2 ± 0.09 [c]	0.18 ± 0.03 [b]
BAPEB (180 mg/Kg)	0.99 ± 0.06 [a]	0.4 ± 0.05 [c]	0.2 ± 0.04 [b]
BAPLB (180 mg/Kg)	1.5 ± 0.14 [c]	0.2 ± 0.1 [c]	0.4 ± 0.06 [b]

APPENDIX 2

General Linear Model: Ph (mg/Kg) Mean versus Time biosurfactant, Earthworm Method

Factor coding (-1, 0, +1)

Rows unused 38

Factor Information

Factor	Type	Levels	Values
Biosurfactant	Random	2	B, NB
Earthworm	Random	2	N, Y

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biosurfactant	1	2.79	2.79	8.77	0.207
Earthworm	1	20.53	20.53	64.59	0.079
Biosurfactant*Earthworm	1	0.32	0.32	0.01	0.941
Time (days) (Biosurfactant, Earthworm)	4	4442.90	1110.72	19.52	0.000
Error	14	796.50	56.89		
Lack-of-Fit	6	769.23	128.21	37.62	0.000
Pure Error	8	27.26	3.41		
Total	21	5565.51			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
7.54272	85.69%	78.53%	37.21%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	46.39	3.61	12.83	0.000	
Biosurfactant					
B	-0.80	3.61	-0.22	0.828	*
Earthworm					
N	-2.17	3.61	-0.60	0.558	*
Biosurfactant*Earthworm					
B N	0.27	3.61	0.07	0.941	*
Time (days) (Biosurfactant, Earthworm)					
(B, N)	-0.889	0.499	-1.78	0.096	4.02
(B, Y)	-1.861	0.273	-6.83	0.000	2.08
(NB, N)	-0.731	0.499	-1.46	0.165	4.02
(NB, Y)	-1.394	0.273	-5.11	0.000	2.08

General Linear Model: FL (mg/kg) Mean versus Time ... ant, Earthworm
Method

Factor coding	(-1, 0, +1)
Rows unused	38

Factor Information

Factor	Type	Levels	Values
Biosurfactant	Random	2	B, NB
Earthworm	Random	2	N, Y

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biosurfactant	1	9.59	9.593	1.34	0.453
Earthworm	1	0.02	0.020	0.00	0.966
Biosurfactant*Earthworm	1	7.14	7.142	0.14	0.717
Time (days) (Biosurfactant, Earthworm)	4	3974.88	993.720	19.07	0.000
Error	14	729.48	52.106		
Lack-of-Fit	6	714.77	119.128	64.79	0.000
Pure Error	8	14.71	1.839		
Total	21	5162.60			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
7.21842	85.87%	78.80%	21.22%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	48.39	3.46	13.99	0.000	
Biosurfactant					
B	-1.48	3.46	-0.43	0.674	*
Earthworm					
N	0.07	3.46	0.02	0.985	*
Biosurfactant*Earthworm					
B N	-1.28	3.46	-0.37	0.717	*
Time (days) (Biosurfactant, Earthworm)					
(B, N)	-0.847	0.477	-1.77	0.098	4.02
(B, Y)	-1.846	0.261	-7.07	0.000	2.08
(NB, N)	-0.966	0.477	-2.02	0.063	4.02
(NB, Y)	-1.137	0.261	-4.36	0.001	2.08

General Linear Model: BAP (mg/kg) Mean versus Time ... t, Earthworm

Method

Factor coding	(-1, 0, +1)
Rows unused	16

Factor Information

Factor	Type	Levels	Values
Biosurfactant	Random	2	B, NB
Earthworm	Random	2	N, Y

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biosurfactant	1	20.87	20.87	1361.90	0.017
Earthworm	1	3.64	3.64	237.52	0.041
Biosurfactant*Earthworm	1	0.02	0.02	0.00	0.964
Time (days) (Biosurfactant, Earthworm)	4	4134.41	1033.60	137.68	0.000
Error	36	270.26	7.51		
Lack-of-Fit	6	138.59	23.10	5.26	0.001
Pure Error	30	131.67	4.39		
Total	43	4603.77			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.73993	94.13%	92.99%	91.84%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	48.332	0.928	52.05	0.000	
Biosurfactant					
B	-1.548	0.928	-1.67	0.104	*
Earthworm					
N	0.647	0.928	0.70	0.491	*
Biosurfactant*Earthworm					
B N	0.042	0.928	0.05	0.964	*
Time (days) (Biosurfactant, Earthworm)					
(B, N)	-0.801	0.128	-6.25	0.000	4.02
(B, Y)	-1.1237	0.0700	-16.05	0.000	2.08
(NB, N)	-0.853	0.128	-6.66	0.000	4.02
(NB, Y)	-1.0142	0.0700	-14.48	0.000	2.08

General Linear Model: total PAH 1 versus Time (days), ... t, Earthworm

Method

Factor coding	(-1, 0, +1)
Rows unused	20

Factor Information

Factor	Type	Levels	Values
Biosurfactant	Random	2	B, NB
Earthworm	Random	2	N, Y

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biosurfactant	1	88.9	88.91	481.05	0.029
Earthworm	1	760.8	760.84	4116.68	0.010
Biosurfactant*Earthworm	1	0.2	0.18	0.00	0.990
Time (days) (Biosurfactant, Earthworm)	4	33570.2	8392.54	6.66	0.001
Error	32	40324.6	1260.14		
Lack-of-Fit	6	13974.5	2329.09	2.30	0.065
Pure Error	26	26350.0	1013.46		
Total	39	75694.2			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
35.4985	46.73%	35.07%	9.12%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	105.0	12.4	8.44	0.000	
Biosurfactant					
B	-3.3	12.4	-0.27	0.792	*
Earthworm					
N	-9.7	12.4	-0.78	0.443	*
Biosurfactant*Earthworm					
B N	0.2	12.4	0.01	0.990	*
Time (days) (Biosurfactant, Earthworm)					
(B, N)	-1.67	1.66	-1.01	0.322	3.97
(B, Y)	-3.776	0.997	-3.79	0.001	2.47
(NB, N)	-1.70	1.66	-1.02	0.313	3.98
(NB, Y)	-3.192	0.997	-3.20	0.003	2.47

Appendix 3

General Linear Model: EROD activity (pmol/mg/min) ... hworm species

Method

Factor coding	(-1, 0, +1)
Rows unused	36

Factor Information

Factor	Type	Levels	Values
Biosurfactant	Random	2	B, NB
Earthworm species	Random	2	E, L

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value	
Earthworm species	1	0.05027	0.050267	0.39	0.638	x
Biosurfactant	1	0.00544	0.005440	0.04	0.872	x
Biosurfactant*Earthworm species	1	0.13059	0.130593	1.12	0.307	
Time (days)(Biosurfactant, Earthworm species)	4	0.13179	0.032948	0.28	0.885	
total PAH 1	1	0.99741	0.997408	8.56	0.010	
Error	15	1.74849	0.116566			
Total	23	5.17401				

x Not an exact F-test.

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.341418	66.21%	48.18%	2.72%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.200	0.326	0.61	0.549	
Earthworm species					
E	-0.096	0.146	-0.66	0.521	*
Biosurfactant					
B	-0.031	0.143	-0.22	0.832	*
Biosurfactant*Earthworm species					
B E	0.151	0.142	1.06	0.307	*
Time (days) (Biosurfactant, Earthworm species)					
(B, E)	-0.0082	0.0162	-0.51	0.619	3.70
(B, L)	-0.0022	0.0371	-0.06	0.954	4.89
(NB, E)	-0.0032	0.0153	-0.21	0.835	3.29
(NB, L)	-0.0370	0.0368	-1.01	0.331	4.80
total PAH 1	0.00665	0.00227	2.93	0.010	2.32

General Linear Model: MROD activity (pmol/mg/min) ... worm species

Method

Factor coding	(-1, 0, +1)
Rows unused	36

Factor Information

Factor	Type	Levels	Values
Biosurfactant	Random	2	B, NB
Earthworm species	Random	2	E, L

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value	
Earthworm species	1	0.1122	0.1122	0.48	0.601	x
Biosurfactant	1	0.2973	0.2973	1.29	0.458	x
Biosurfactant*Earthworm species	1	0.2295	0.2295	0.84	0.373	
Time (days) (Biosurfactant, Earthworm species)	4	0.8171	0.2043	0.75	0.573	
total PAH 1	1	2.8899	2.8899	10.61	0.005	
Error	15	4.0855	0.2724			
Total	23	14.0123				

x Not an exact F-test.

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.521890	70.84%	55.29%	19.49%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	-0.034	0.499	-0.07	0.947	
Earthworm species					
E	-0.143	0.223	-0.64	0.531	*
Biosurfactant					
B	-0.228	0.218	-1.04	0.313	*
Biosurfactant*Earthworm species					
B E	0.200	0.218	0.92	0.373	*
Time (days) (Biosurfactant, Earthworm species)					
(B, E)	-0.0004	0.0247	-0.02	0.988	3.70
(B, L)	-0.0077	0.0568	-0.14	0.893	4.89
(NB, E)	-0.0130	0.0233	-0.56	0.587	3.29
(NB, L)	-0.0927	0.0562	-1.65	0.120	4.80

total PAH 1	0.01132	0.00347	3.26	0.005	2.32

General Linear Model: GST activity (µmol/mL/min) versus ... rm species

Method

Factor coding	(-1, 0, +1)
Rows unused	36

Factor Information

Factor	Type	Levels	Values
Biosurfactant	Random	2	B, NB
Earthworm species	Random	2	E, L

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value	
Earthworm species	1	0.008075	0.008075	60.20	0.007	x
Biosurfactant	1	0.002233	0.002233	25.25	0.113	x
Biosurfactant*Earthworm species	1	0.000086	0.000086	0.08	0.775	
Time (days) (Biosurfactant, Earthworm species)	4	0.001264	0.000316	0.31	0.865	
total PAH 1	1	0.001523	0.001523	1.51	0.239	
Error	15	0.015162	0.001011			
Total	23	0.067698				

x Not an exact F-test.

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0317930	77.60%	65.66%	45.29%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	0.2461	0.0304	8.10	0.000	
Earthworm species					
E	-0.0385	0.0136	-2.83	0.013	*
Biosurfactant					
B	-0.0197	0.0133	-1.49	0.158	*
Biosurfactant*Earthworm species					

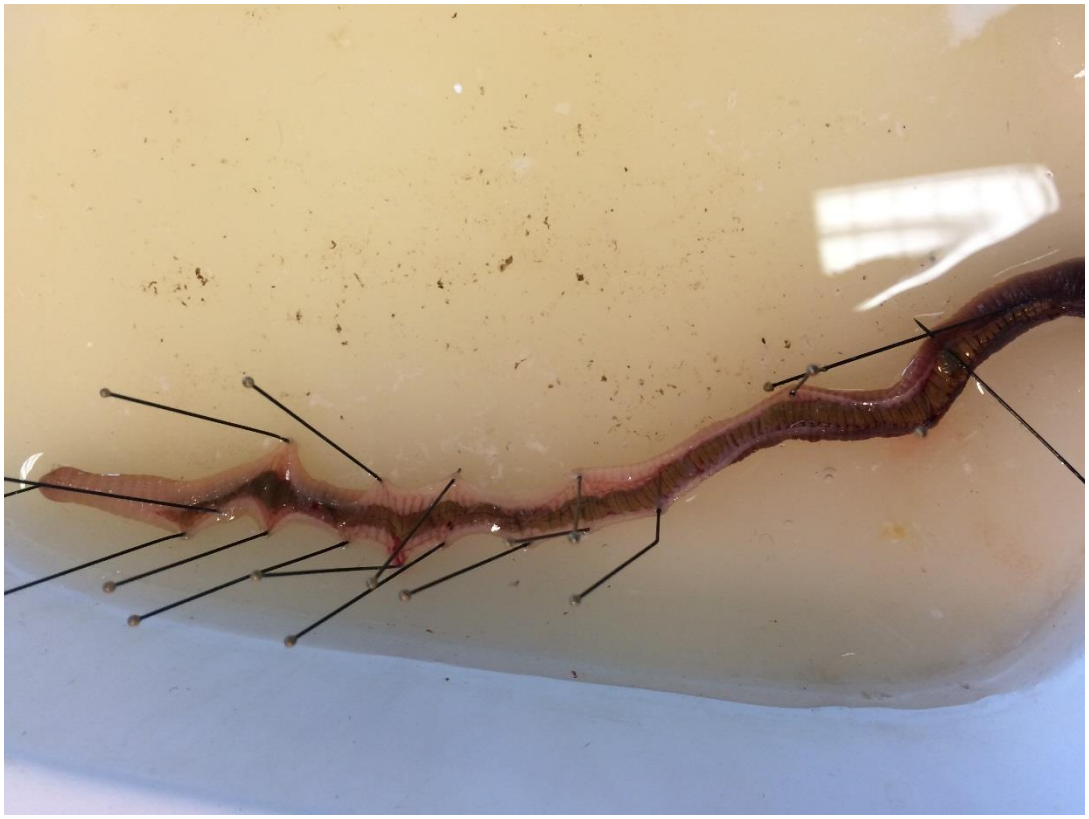
B E	-0.0039	0.0133	-0.29	0.775	*
Time (days) (Biosurfactant, Earthworm species)					
(B, E)	0.00117	0.00151	0.78	0.449	3.70
(B, L)	0.00292	0.00346	0.84	0.411	4.89
(NB, E)	-0.00013	0.00142	-0.09	0.928	3.29
(NB, L)	0.00135	0.00342	0.39	0.699	4.80
total PAH 1	-0.000260	0.000212	-1.23	0.239	2.32

Appendix 3: Supplementary images

Site sampling

Setting up wormery

Setting up microcosms



Gut extraction

Microsomes pellet from ultracentrifugation

Appendix 4:

List of Conferences

Research student summer conference 2015, Middlesex University London, UK.

Poster presentation: Enhanced vermiremediation of hydrocarbon contaminated land with biosurfactants

Research student summer conference 2016, Middlesex University London, UK.

Poster presentation: Enhanced vermiremediation of hydrocarbon contaminated land with biosurfactants: An ecotoxicological approach

Society of environmental toxicology and chemistry UK branch student meeting 2016, Oxford, UK

Poster presentation: A comparative study of impacts of *Eisenia hortensis* and *Lumbricus. terrestris* in PAH remediation

Research student summer conference 2017, Middlesex University London, UK.

Oral presentation: Enhanced vermiremediation of hydrocarbon contaminated land with biosurfactants: An integrated approach

Society of environmental toxicology and chemistry 57th annual meeting 2017 Belgium

Poster presentation: Enhanced vermiremediation of hydrocarbon contaminated land with biosurfactants

6th International symposium of environmental biotechnology and engineering 2018, Obregon Mexico

Oral presentation: Enzymatic response in *E. hortensis* and L*. terrestris* exposed to 3-, 4-, and 5-ring hydrocarbons amended with biosurfactant