Biosurfactant Enhanced Biodegradation of High Molecular Weight Polycyclic Aromatic Hydrocarbons in a Two-Stage Continuous Stirred Tank Bioreactors and Biofilm Tank

TSHILIDZI BRIDGET LUTSINGE

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) do not dissolve easily in water, due to their hydrophobic properties. PAHs are unavailable to most aromatic compound degrading organisms, due to these properties. In this study, a biosurfactant producing culture enhancing dissolution of PAHs was isolated, to make them bioavailable. The culture was introduced to the system to improve the dissolution of PAHs and degrade the PAHs thereafter. The aim of the study was to use a strategy with a biofilm process, subsequent to a continuous stirred tank bio-reactors (CSTRs) to successfully remove PAHs from water, with microorganisms that can degrade these pollutants.

The open system could easily be controlled and set to optimum conditions, stimulating the growth of PAH degraders. The feed rate and influent concentration can be controlled and the system can easily be cleaned. Biodegradation was achieved, using optimum conditions obtained from the conducted batch studies in a CSTR process ensuring a feasible biodegradation process. Two cultures, *Pseudomonas aeruginosa* and microbial consortia, were used during the biosurfactant production and PAHs degradation preliminary batch studies. The biosurfactants produced, were identified as Lipopeptides and degradation results indicated great degradation of fluoranthene and triphenylene with a mixed culture consortium present in the system. 90.1% of fluoranthene and 79.6% of triphenylene was degraded after 22 d of incubation in the batch system.

Degradation of fluoranthene was studied using biosurfactants and microbial consortium in a three-stage continuous flow system. Reactor 2A fluoranthene influent (60.89%) was degraded, 70.02% of Reactor 2B fluoranthene influent was degraded and 77.17% of biofilm tank fluoranthene influent was degraded.

Kinetic studies were conducted, using a Monod model to describe the substrates degradation for batch systems. The highest degradation rate for fluoranthene was determined to be 0.29 h^{-1} and for triphenylene was 0.13 h^{-1} with half saturation values of 991.84 mg/L and 451 mg/L respectively, indicating that fluoranthene was degraded faster than triphenylene, when incubated for 22 d.

The study demonstrated that biosurfactant production and biodegradation of fluoranthene can be achieved in an open CSTR system, as much as it can be done in a batch system. The biological remediation of PAHs in wastewater plants can be introduced and applied for wastewaters rich, with PAHs.

Declaration

I, **TSHILIDZI BRIDGET LUTSINGE,** hereby declare that the work provided in this dissertation is to the best of my knowledge original (except where cited) has never been submitted for another degree at this or any other tertiary education institution.

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Signature of candidate

This …….. day of ……. 2017

Dedication

This dissertation is dedicated to

My grandparents Mr Ntshavheni Abel Lutsinge and Mrs Takalani Lucy Lutsinge who raised me, believed in me and encouraged me to always aim high and do my ultimate best to achieve my dreams.

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Firstly, I would like to thank God for giving me the knowledge and wisdom to complete this study. Without His guidance and protection this dissertation would not have been possible.

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List of abbreviations

PAH	Polycyclic Aromatic Hydrocarbon
LMW	Low Molecular Weight
HMW	High Molecular Weight
CSTR	Continuous Stirred Tank Reactor
US EPA	United States Environmental Protection Egency
GC/MS	Gas Chromatography/ Mass Spectrometry
EPS	Extracellular Polymeric Substances
CMC	Critical Micelle Concentration
MSM	Mineral Salts Medium
E_{24}	Emulsification Index
CMD	Critical Micelle Dilution
HPLC	High Performance Liquid Chromatograph
TLC	Thin Layer Chromatography
FTIR	Fourier Transform Infrared Spectroscopy

List of symbols

C	Substrate concentration (mg/L)
t	Time
d	Days
h	Hours
min	Minutes
μ_{max}	Monod maximum specific utilisation rate of substrate (h^{-1} or d^{-1})
K_s	Monod half-velocity coefficient (mg/L)
X	Biomass concentratoion (mg/L)
Y	Yield coefficient
k_d	Monod maximum specific utilisation rate of substrate (h^{-1} or d^{-1})
D	Diffusion coefficient (m^2)
Z	Radical distance in biofilm (m)
V	Volume of the reactor (L)
ε	Reactor porosity
Q	Flow rate (L/h)
A	Total surface area of the media (m^3)
L	Length (m)
j_f	Flux at the biofilm surface ($mg/m^2.h$)
r	Substrate volumetric conversion rate (mg/h)
q_{max}	Maximum specific conversion rate (h^{-1} or d^{-1})
K_i	Affinity constant of substrate (mg/L)

CHAPTER 1: INTRODUCTION

1.1 Background

Polycyclic aromatic hydrocarbons (PAHs) are compounds comprising two or more fused benzene rings (Haritash & Kaushik 2009). PAHs are discharged from both industrial human activities and natural sources. Polycyclic aromatic hydrocarbons combined with other chlorinated and non-chlorinated aromatic organics, are classified as carcinogenic micro-pollutants. They are generally resistant to environmental degradation due to their greater hydrophobicity (Gan *et al*. 2009). PAHs are composed of carbon and hydrogen atoms, arranged in the form of fused aromatic rings (linear, cluster or angular arrangement) belonging to the group of persistent organic pollutants (Maliszewska-Kordybach 1999). Compounds comprising two or three benzene rings are classified as low molecular weight (LMW) PAHs. Those indicating four or more benzene rings are classified as high molecular weight (HMW) PAHs. HMW PAHs are more likely to adsorb to soil particles or solid surfaces due to their hydrophobicity resulting higher surface affinity and are less biodegradable than the LMW compounds (Ukiwe *et al*. 2013).

Several countries globally, studied and developed remediation strategies for PAH contaminated sites including physical, chemical and biological technology. Methods used indicate, incineration (Wheatley & Sadhra 2004; Sato *et al*. 2011; Chen *et al*. 2013), excavation and landfilling (Vidali 2001; Das & Chandran 2011), and land-farming (Wilson & Jones 1993; Vidali 2001; Bamforth & Singleton 2005). These methods were used in a variety of contexts. It was found to be expensive, difficult to execute, and inefficient, applying in treatment of large flows of contaminated wastewater or decontamination of large land sites (Banat 1995; Vidali 2001; Das & Chandran 2011).

Bioremediation offers several advantages over conventional traditional technologies as it is environmental friendly, economically versatile and can completely mineralise the organic pollutant to carbon dioxide and water, in certain instances (Bustamante *et al*. 2012). The process of bioremediation uses microorganisms to detoxify or remove pollutants, relying on microbial enzymatic activities to transform or degrade the offending contaminants in the environment. Biosurfactants are used to enhance the bioavailability of the hydrophobic substrates by increasing solubility of the compounds in water, achieving higher degradation rates for hydrophobic

compounds, (Benincasa 2007). Sarubbo *et al*. (2015) reported favourable properties of biosurfactant, produced by *Pseudomonas sp.* in the bioremediation of hydrophobic compounds in soil and water.

Degradation of HMW PAHs is mostly achieved in batch experiments (Bezza & Chirwa 2016; Patowary et al. 2015). Saiu et al. (2016) efficiently removed greater than 90 % of pyrene in a batch system using *Pleurotus sajor-caju*. The present study is aimed at degrading fluoranthene by means of colonies of *Pseudomonas aeruginosa* bred in a stirred tank reactor.

Earlier studies indicated, PAHs can be degraded either via microorganisms' metabolism or co-metabolism (Churchill *et al*. 1999; Vital-Lopez *et al*. 2015; Adams *et al*. 2015). Metabolism is a process which degrades or transforms a substrate or compound using a living cell and co-metabolism is a process in which a substrate is degraded or transformed by an enzyme or cofactor produced during microbial metabolism of another substrate (Horvath 1972). A wide array of bacterial, fungal and algal species were found to degrade PAH compounds, with bacteria holding the most important group of degraders. Some PAH-degrading microorganisms, primarily bacteria, are capable of using the PAHs as the carbon and energy source, and thus transform the contaminants into molecules that can enter the organisms' central metabolic pathways (Riser-Robert , 1998). Bacteria, such as *Pseudomonas, Mycobacterium, Rhodococcus, Bacillus, Arthrobacter, Nocardia, Acinetobacter* and *Flavobacterium* are the primary degraders of polycyclic aromatic hydrocarbons in soil. Several studies were conducted on the degradation of LMW PAHs and more information is available on this topic, though few studies were conducted on HMW PAHs and limited information exists, regarding the bacterial degradation of HMW PAHs.

1.2 Problem Statement

Polycyclic Aromatic Hydrocarbons are hydrophobic compounds that are recalcitrant to degradation and are persistent and accumulative in the environment as a results. Due to their carcinogenic, mutagenic and toxic nature, these compounds become of human health and environmental concern and must be removed from any environmental site. PAHs enter the surface water mainly through industrial effluents, municipal effluents, oil spillage or leakage and atmospheric fallouts. Industrial activities contribute a significant amount of PAHs to surface water

through industrial effluents. Remediation strategies for PAH contaminated sites include physical, chemical and biological technology have been studied and developed in many countries over the world. Methods such as incineration, excavation and landfilling, and land-farming have been used in a variety of contexts. However, these methods have been found to be expensive, difficult to execute, and inefficient when applied for treatment of large flows of contaminated wastewater or decontamination of large land sites. Alternatively, bioremediation offers several advantages over conventional traditional technologies because it is environmental friendly, economically versatile, and in some cases, can completely mineralise the organic pollutant to carbon dioxide and water. As one of the emerging class of pollutants, PAHs needs to be removed from the industrial wastewater before it is discharged to ensure that contamination of effluent pathways and the receiving water bodies is eliminated. The proposed technology for this study can also be suitable to treat PAH contamination closer to the source of pollution in case of industrial activities to decrease the levels of PAHs in the highly industrially polluted rivers.

1.3 Aims

Aim 1 – Enrich, Isolate, Purify and Identify Microorganisms

Isolate, purify and characterize microbial strains sourced from contaminated soil. The strain should be grown in the presence of HMW PAHs and therefore degrade PAHs.

Aim 2 – Biosurfactant Production

Screen for microbial strain capable of producing biosurfactants and characterize them. This stage also includes optimising the production of biosurfactants by evaluation of different mineral salts medium contents.

Aim 3 – Dissolution of PAHs using Biosurfactants

Aim 3 involves dissolving HMW PAHs using the produced biosurfactants

Aim 4 – Degradation of PAHs

Final stage involves degradation of HMW PAHs in a batch and CSTR system. This stage also involves determining optimum conditions which PAH-degrading microbial consortium exercises their full capacity to degrade the water.

1.4 Methodology

Microbial Enrichment, Isolation, Purification and Identification:

PAH degrading microorganisms isolated from a known PAH contaminated soil, were enriched in a batch system using a technique adopted from Bezza and Chirwa (2016). Microbial strains were isolated and purified using a streak and spread technique on an agar plates. Identification of produced microbial strains was conducted in the Department of Microbiology and Plant Pathology at the University of Pretoria using a 16S rRNA gene sequence analysis.

Biosurfactant Production:

Screening for potential biosurfactant producing cultures, was accomplished, using a drop and collapse and emulsification index techniques. Isolates with positive results, were used to produce biosurfactants, with various kinds of mineral salt mediums to evaluate the optimum conditions. Producing biosurfactants were physically and chemically characterised. Foaming and surface tension analysis were used for physical characterisation. Thin chromatography layer (TCL) and Fourier transform infrared spectroscopy (FTIR) were used for chemical characterisation.

PAH Degradation

Fluoranthene and triphenylene were dissolved and degraded in a batch system, with produced biosurfactants and degrading cultures, respectively. Only fluoranthene was dissolved and degraded using a biofilm reactor, subsequent to a continuous stirred tank bio-reactor (CSTR).

1.5 Main Findings of the Study

A biosurfactant producing culture was isolated from the engine oil contaminated site, purified and identified as *Pseudomonas aeruginosa* HE978271.1. During the biosurfactant production, *Pseudomonas aeruginosa* pure isolates produced more biosurfactants, compared with a mixed microbial consortium. Fluoranthene and triphenylene were both successfully dissolved, using biosurfactants with fluoranthene with a higher dissolving capacity, compared with triphenylene. In batch studies, 90.1% of fluoranthene was degraded after 22 d of incubation; 79.6% of triphenylene was degraded after 22 d. In a continuous flow, 60.89% of Reactor 2A fluoranthene influent was degraded, 70.02% of Reactor 2B fluoranthene influent was degraded and 77.17% of biofilm tank fluoranthene influent was degraded.

CHAPTER 2: LITERATURE REVIEW

2.1. Background

In the early 1900s, Polycyclic Aromatic Hydrocarbons' (PAHs) existence in the environment was considered non-hazardous due to its lower concentrations. Human and environmental health was not endangered, as PAHs concentrations were considered insignificant and were mainly contributed through natural sources. With increasing populations and new discoveries, human activities, such as industrial processes became a major contributor of PAHs in the environment. The appearance of elevated PAH levels became a concern when Kennaway (1930) made the first discovery linking hydrocarbons as the producer of cancers in mice (Fieser 1938). This discovery initiated a new scientific research, intensively studied and continues to be studied in the human and environmental health fields. With no remediation solutions in place, accumulation and persistence of these compounds became a challenge to the environment and human population. Scientists had to suggest solutions to the emerging concerns. A study on a remediation technique for polycyclic aromatic hydrocarbon, using osmium tetroxide was conducted and reported in the late 1940s by Cook and Schoental (1949).

The study proved that cancer-producing compounds were oxidised by osmium tetroxide in benzene-pyride to diols, enduring facile dehydration to hydroxyl-derivetives of the original hydrocarbons. Such process could only degrade chemically available compounds and those sorbed in soul could not be degraded. Poglazova *et al*, (1976) studied the possibility of biological remediation of PAHs by assumption, considering the high biochemical activity of microorganisms in soil nature, with microorganisms participating in the metabolism of PAHs. The penetration and accumulation of hydrocarbons into the microorganism cells were discovered and studied with fluorescence microscopy. The results indicated that the presence of capable bacteria in the soil, lead to the bacteria accumulating and destroying some of the hydrocarbons (Poglazova *et al*. 1967).

Conventional treatment technologies, such as physical and chemical remediation strategies, were studied and documented. A more diverse non-conventional treatment technology, biological treatment technology, proved the best solution to solve the challenge of PAHs contamination due to its numerous advantages compared with the conventional method. Biological degradation, also referred to as bioremediation, of PAHs, was intensively studied and reported mostly in batch

studies (Boldrin *et al*. 1993; Obayori *et al*. 2008; Bezza & Chirwa 2015; Rodriguez-mateus *et al*. 2016) with few continuous flow (Villemur *et al*. 2000; Mahanty *et al*. 2008) and biofilm (Stach & Burns 2002; Singh *et al*. 2006) degradation involved. To effectively overcome the challenge of the unavailability of PAHs to degradation, surfactants technique was introduced to dissolve these compounds, prior to degradation.

Literature delineated the advantages of using biologically produced surfactants (biosurfactants) over chemically synthesised surfactants in promoting availability of PAHs contaminants to degradation (Randhir, Makkar & Rockne 2003; Sachdev & Cameotra 2013; Singh & Cameotra 2014).

The current study aims at increasing the effectiveness of microorganisms in degrading High Molecular Weight PAHs in a controlled CSTR system with biofilm reactor as a final polishing stage.

2.2. Sources of PAHs

Complete combustion of hydrocarbons in oxygen, results in the complete oxidation of the carbon and hydrogen present to carbon dioxide and water, though it is not easy to completely combust organic substances. In the natural environment, it becomes more difficult to achieve complete combustion, due to factors such as, temperature and the oxygen presence (Connell, 1997). Incomplete combustion of organic substances at high temperatures and under pyrolytic conditions results in formation of PAHs (Samanta *et al*. 2002; Kabziński 2002). At temperature range of 400-500^{o}C, organic components are partly creaked smaller and becomes unstable molecules, recombining to form larger and more PAHs (Kabziński 2002). Natural or anthropogenic sources produce PAHs, indicating widespread environmental pollutants.

Natural production of PAHs in the environment, occurs through several pathways including: vegetation burning in forest and bush fires; thermal geological reactions associated with fossil-fuel and mineral production; volcanic eruptions and biosynthesis (Wilson & Jones 1993). Natural sources of PAHs, such as volcanic activities and forest fires do not significantly contribute to the PAH emission (Maliszewska-Kordybach 1999).

Anthropogenic production of PAHs includes incomplete combustion of fossil fuels, coke production, and several industrial processes (Wang *et al*. 2010). Anthropogenic processes resulting in the formation of PAHs are often classified as either petrogenic or pyrogenic sources. A process called petrogenic refers to hydrocarbon compounds formation associated with petroleum products such as creosote, fuel oil, crude oil, etc. Pyrogenic refers to hydrocarbon compounds formation associated with combustion of petroleum, wood or coal (Abdel-shafy 2015, Okodeyi 2013). Some of these processes are presented in Figure 2-1. According to Ukiwe *et al*. (2013) it was reported that large concentration of PAHs are expected to be present in the urban areas where bush burning for agricultural farming is commonly practiced as well as in petroleum exploitation and refining operations (Ukiwe *et al*. 2013).

PAHs can be detected in surface water, groundwater, road runoff, soil, sediment and air. These PAHs are spread from atmosphere to vegetation which can also lead to contamination of food (Kanaly & Harayama 2000). Generation and distribution of HMW and LMW PAHs was discovered in the process of medical waste incineration and distributed in the surrounding water and soil environment (Chen *et al*. 2013; Wheatley & Sadhra 2004).

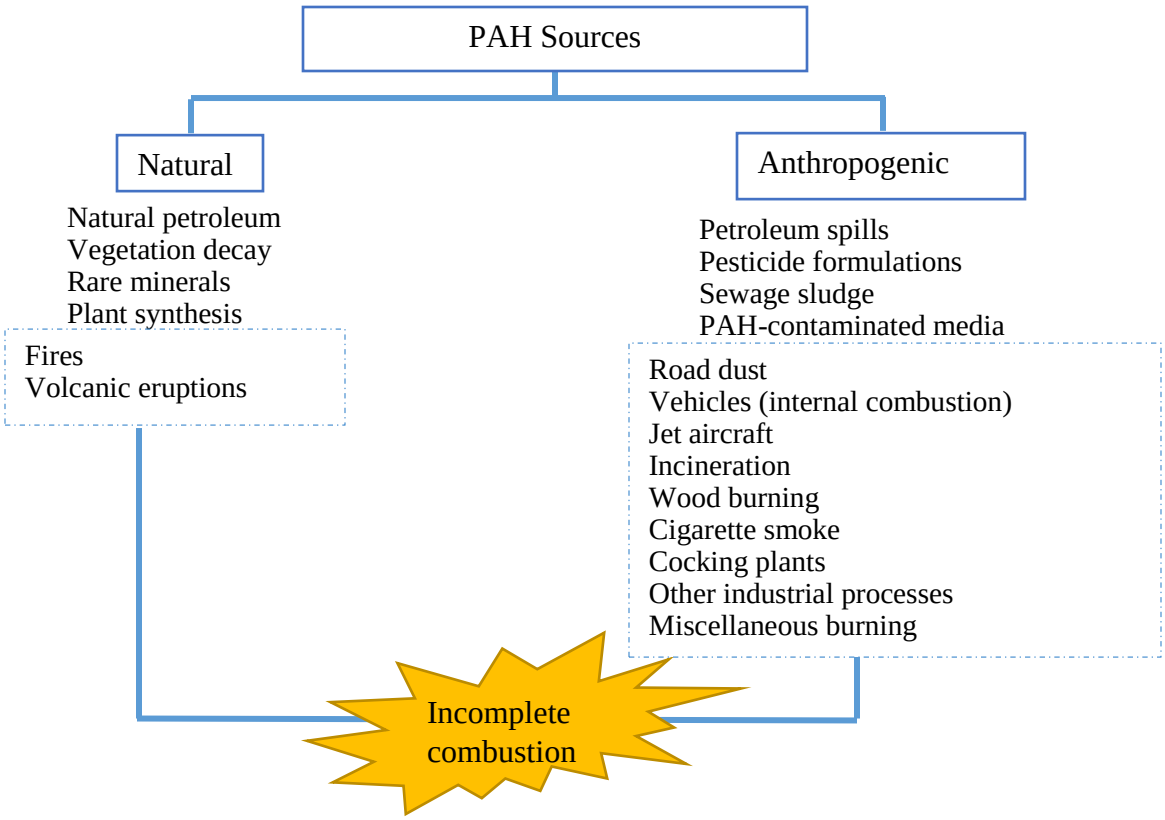


Figure 2-1: PAHs sources (figure source:(Abdel-Shafy & Mansour 2016)*)*

2.3. Classification of PAHs

Polycyclic aromatic hydrocarbons constitute a large and diverse class of organic compounds comprising three or more fused aromatic rings in various structural configurations (Randhir S Makkar & Rockne 2003). Figure 2-2 presents the various kinds of rings PAHs may possess. Most PAHs occur as hybrids, which leads to various components occurrence with the same ring number (i.e. anthracene and phenanthrene both have 3-ring number but their arrangement is different, making them two different compounds). Environmental face and chemical properties of a PAH molecule are dependent in part upon the molecular weight (the number of rings) and the ring linkage pattern (Kanaly & Harayama 2000).

PAHs compounds may also be classified according to their molecular weight. Compounds comprising two or three benzene rings are classified as low molecular weights (LMW), while

compounds with four of more benzene rings are classified as high molecular weights (HMW) (Ukiwe *et al*. 2013; Ghosh *et al*. 2014).

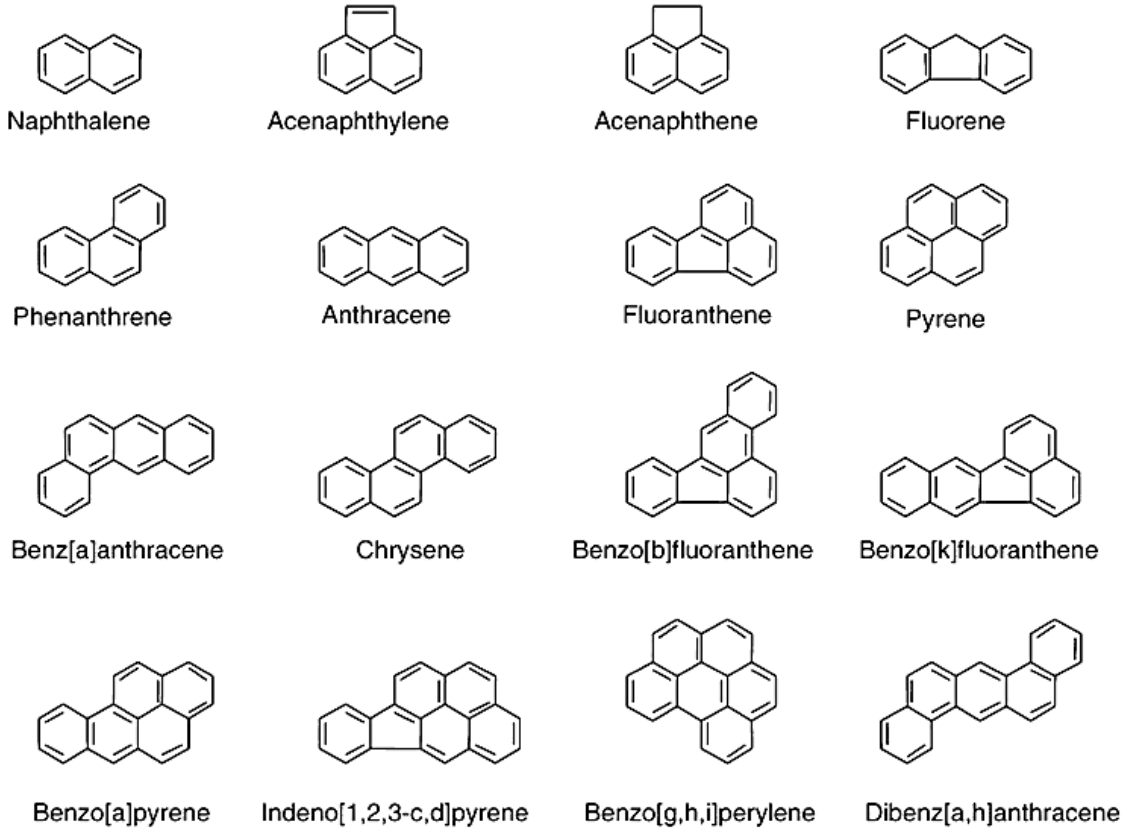


Figure 2-2: Structure configurations of the 16 PAHs which are considered priority pollutants by the US EPA (Shukla et al. 2014).

2.4. PAHs Occurrence and Persistence

LMW PAHs are relatively soluble in water and more concentrated in water, however, HMW PAHs are hydrophobic and are more likely to adsorb to soil organic matter (Wang *et al*. 2010; Chen *et al*. 2013). PAHs with low water solubility tend to be resistant to biological, chemical and photolytic breakdown, which makes it difficult to remove these pollutants from the environment (Bamforth & Singleton 2005). PAHs with HMW in contaminated soil tend to move deeper recesses of soil particles, soil aggregates and the organic matter as the soil ages (Bezza & Chirwa, 2015), resulting in these HMW PAHs with higher hydrophobility and toxity to have longer environmental persistence (Bamforth & Singleton 2005).

The persistence of PAHs in the environment was reported to be dependent on variety of factors such as chemical structure of the PAH, the concentration and dispersion of the PAH and the bioavailability of the contaminant (Bamforth & Singleton 2005). Environmental factors, such as soil type and structure, pH, temperature, adequate levels of oxygen, nutrients and water for the activity of the pollutant-degrading microbial community will control the time that PAHs persist in the environment (Bamforth & Singleton 2005). The relationship between PAH persistence in the environment and an increase of benzene rings number is consistent with the results of several studies conducted correlating PAH molecular size and environmental biodegradation rates (Kanaly & Harayama 2000).

2.5. PAHs Distribution

PAHs are widely distributed in soils and sediments, surface water and groundwater, and the atmosphere. They have been detected in marine sediments, gas works site soils, automobile garages, sewage sludge- contaminated soils, sea waters, rivers, wastewaters, groundwater and in atmospheric air.

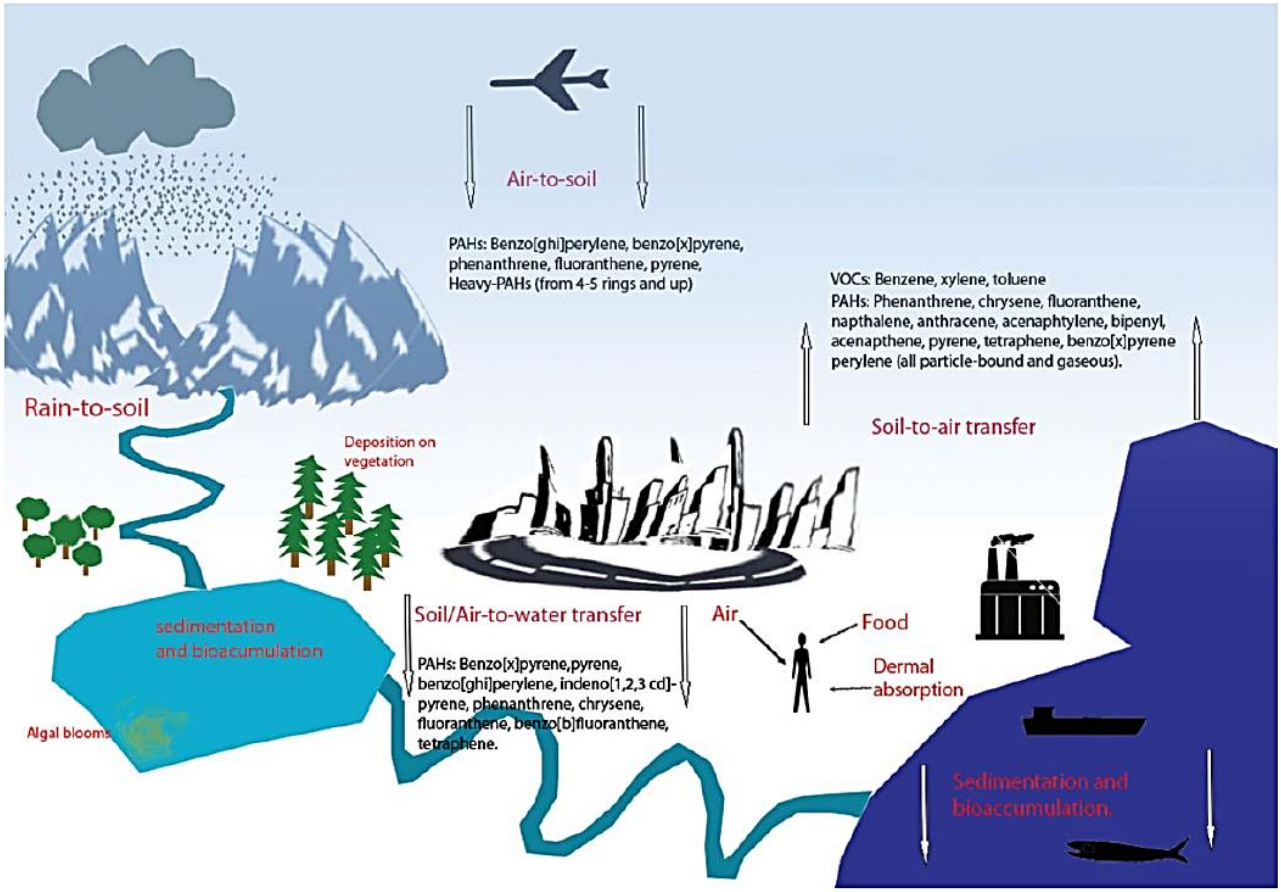


Figure 2-3: PAH distribution in the environment (Image source: (Manzetti 2013)*)*

2.5.1. PAHs in Air

The occurrence of PAHs in the atmosphere is mainly attributed to emissions from, natural sources such as volcanic activities as well as forest fires, and anthropogenic sources such as agricultural fires, power plants, vehicle exhaust and other industrial activities (Abdel-Shafy & Mansour 2016). PAHs exist in two phases in the atmosphere, namely vapor phase and/or solid phase in which PAHs are sorted onto particulate matter. High molecular weight PAHs are dominantly found in

particulate phase and low molecular weight PAHs are commonly found in vapor phase although they can also exist in particulate phase through condensation after emission (Kim *et al*. 2013).

2.5.2. PAHs in Water

PAHs enter the surface water mainly through industrial effluents, municipal effluents, oil spillage or leakage and atmospheric fallouts (Manoli & Samara 1999). Industrial activities contribute a significant number of PAHs to surface water through industrial effluents. The removal of PAHs from the industrial wastewater before its discharge is vital to ensure that contamination of effluent pathways and the receiving water bodies is eliminated. Presence of PAHs in municipal or domestic wastewater effluents is due to usage of solid fuels such as wood, coal, dung, agricultural residues as a means of cooking or heat generation. In case of oil spills in an aquatic environment, lighter hydrocarbons components will volatize, the polar hydrocarbons components will dissolve in water, and most of the oil components will remain on the water surface due to their low solubility (<1 ppm) (Karath, et al., 1999). Atmospheric fallout contribution of PAHs to the surface water occurs through vehicle exhaust and industrial emission and have been reported to also cause a significant accumulation of PAHs pollutants in the environment (Doong & Lin 2004).

2.5.3. PAHs in Soil

The accumulation of PAHs in soil is mainly due to the direct and indirect deposition of anthropogenic sources such as town gas sites, solid fuel domestic heating, aircraft exhaust, car exhaust, etc. natural sources such as volcanic eruptions and forest fires may also contribute towards PAHs contamination in soil (Smith *et al*. 2006). Industrial and domestic wastewaters contribute towards soil contamination through their channels if the water channels are not protected to avoid contaminations. Oil spills and runoffs from contaminated sites (motor service stations, roads paved with a mixture of creosote material, poles preserved with creosote to prevent decomposition, wood processing sites, etc.) will contaminate soil if it is being exposed to it.

2.6. PAHs Pollution

2.6.1. PAHs Pollution in South Africa

Few studies have been conducted on potential sources, distribution and persistence of PAHs in South Africa. Soil and sediment from industrial, residential, and agricultural areas in central South

Africa (between Vereeniging and Vanderbijlpark) were analysed using gas chromatography/mass spectrometry (GC/MS) to determine levels of PAHs (Nieuwoudt *et al*. 2011). The total concentration of the PAHs from the studies ranged between 0.044 µg/g and 39 µg/g, with the concentration of carcinogenic PAHs ranging from 0.019 µg/g to 19 µg/g. United States Agency for Toxic Substances and Disease Registry depicts that the PAH concentration range for significantly contaminated sites should be below 1.0 µg/g (Okedeyi *et al*. 2013). PAHs concentration in water and sediment samples from Mvudi and Nzhelele rivers in Limpopo province, ranged from 13.174 mg/L to 26.382 mg/L and 27.10 mg/kg to 55.93 mg/kg, respectively (Edokpayi *et al*. 2016). The detection limit for PAH in water is 0.01 µg/L, stipulated by South Africa drinking water standards (Mamba *et al*. 2008). Soil samples from Matla power plant, located in the Mpumalanga province, Lethabo power plant in Free State Province and Rooiwal power plant in Gauteng, held a total PAH concentration range from 9.73 µg/g to 61.24 µg/g (Okedeyi *et al*. 2013).

2.6.2. PAHs Pollution Globally

In 2004, global atmospheric emissions of the 16 PAHs listed as the US EPA priority pollutants were estimated using reported emission activity and emission factor data. It was estimated that the total global atmospheric emission of these 16 PAHs was 520 giga grams per year (Gg/y) (Figure) with biofuel (56.7%), wildfire (17.0%) and consumer product usage (6.9%) as the major sources. China (114 Gg/y), India (90 Gg/y) and the United States (32 Gg/y) were the top three countries with the highest PAH emissions (Zhang & Tau 2008 _emailed). Benzo[a]pyrene global emission results were also reported.

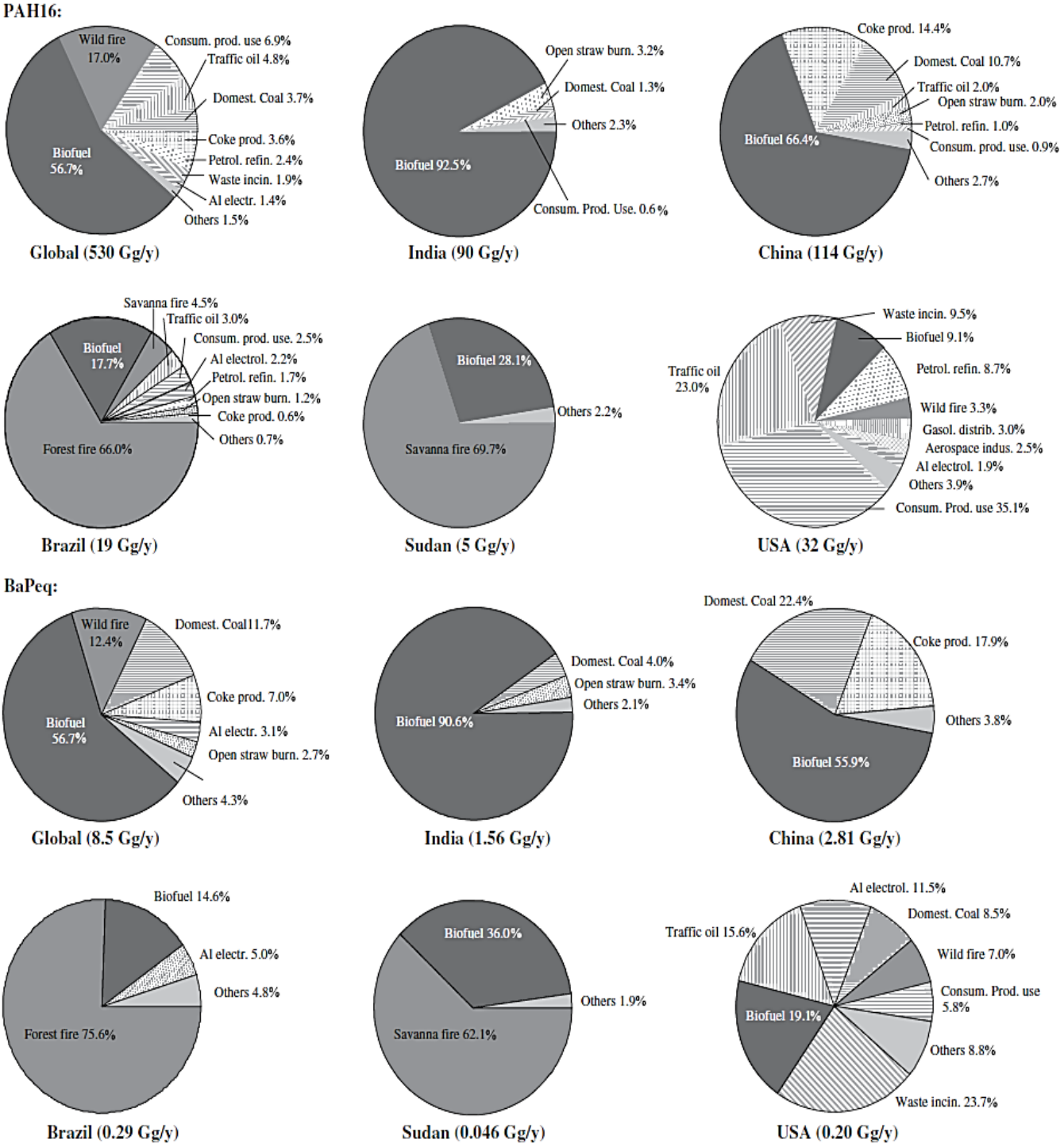


Figure 2-4: Global PAH pollution [figure source:(Zhang & Tao 2009)*]*

2.7. Health Concerns

2.7.1. Environmental Health Concerns

From Figure, it can be observed that PAHs are distributed from one environmental compartment to another, leading to contamination and pollution of the environment, resulting in environmental damage and the living organisms thereof. PAHs pollution (air – water – soil and sediments) and the effects on environmental health was studied and reported in literature. Several studies have reported correlations between the induction of cytochrome P450 (CYP), elevated 7-ethoxyresorufin-O-deethylase (EROD) activities, lysosomal membrane destabilization, DNA damage, and endocrine and reproductive effects in fish and invertebrates with PAH contamination in the water and sediments they inhabit (Zhang *et al*. 2016) . An experiment on the effects of PAHs on plant was conducted by Desalme et al. (2013), it was observed with obtained data from vascular plant leaves that contamination occurs by both direct (air-leaf) and indirect (air-soil-root) pathways. Furthermore, experimental studies demonstrated that PAHs affect plant growth, interfering with plant carbon allocation and root symbioses (Desalme *et al*. 2013). In general, plants uptake atmospheric and soil PAHs. This contamination induced leaf injuries, decreased biomass production, indicating additional adverse biological and physiological effects. The microbial community is strongly influenced by PAH contamination levels in soil; this has a major effect on the microbial activities including bioremediation of other pollutants.

2.7.2. Human Health Concerns

Polycyclic aromatic hydrocarbons signify a threat and of human concern, because of their mutagenic and carcinogenic effects (Kumar *et al*. 2006a). Toxicity of PAHs and their widespread distribution led to an increased interest, due to the presence of these compounds in the aquatic and terrestrial environment (Nekhavhambe *et al*. 2014). Table 2-1 indicates, numerous studies conducted, connecting short-term and long-term effects on human health because of PAHs exposure through consumption, dermal absorption and inhalation. According to Menzie *et al*., quoted by Maliszewska-Kordybach (1999), the estimated potential doses of carcinogenic PAHs by inhalation range between 0.02 µg/day and 3 µg/day; nearly 20 times less than the calculated food dose and about 25 times more than the potential dose with drinkable water (Maliszewska-Kordybach 1999).

Table 2-1: PAH pollutants with their human health effects and anthropogenic sources

Pollutant	Health Effects	Use/Source	refence
Napthalene	Carcinogenic, hemolytic anemia	Industrial application	(Shukla et al. 2014)
Phenantherene	Carcinogenic, dye	Coal tar, pedticides	(Shukla et al. 2014)
Anthracene			
Pyrene	Toxic to kidneys and livers. Carcinogenic	Dye and dye precusor, combustion, pigments manufacturing	(Shukla et al. 2014)
Fluoranthene	Tumors, cancer,	Rice straw, bituminous coal, metalurgical coking, firewood, alluminium production	(Li et al. 2016) (Long, Margaret, et al. 2016) (Misaki et al. 2016)
Benzo(a)pyrene	DNA damage, lung and skin cancer, transgene mutation, chest pains, irritation	Industries, coal tar, automobile exhaust fumes	(Long, Lemieux, et al. 2016) (Long, Margaret, et al. 2016) (Collins et al. 1991)

Experimental tests on the effects of PAH on human health are usually conducted on mice, using a lethal dose technique. The lethal dose can, in 50% of cases (LD_{50}) measure the relative toxicity of PAHs. Table 2-2 present the toxicity of some PAHs expressed as milligrams of toxic material per kilogram, of the subject's body weight, that will cause death in 50% of cases (Bamforth & Singleton 2005). Bamforth and Singleton (2005) emphasised the importance of specifying the way in which the test animal inject the toxic material (such as oral) and the animal used to test the material.

Benzo[a]pyrene was reported to be one of the most intoxicating carcinogenic PAHs and became one of the most studied PAH in the PAHs compound class (Juhasz & Naidu 2000). Human cancer risk was estimated, using data extrapolated from studies, with inhalation, feeding and intratracheal administration as an injection route of benzo[a]pyrene into animals (Collins *et al*. 1991).

The U.S. Environmental Protection Agency (U.S. EPA) classified 16 of the PAHs presented in Table 2-3, as priority pollutants, based on toxicity, potential for human exposure, frequency of occurrence at hazardous waste sites and the extent of information available (Bojes & Pope 2007).

Table 2-2: Toxicity of some PAHs. Source (Bamforth & Singleton, 2005)

Material	Number of rings	LD_{50} value (mg/kg)	Test subject	Exposure route
Naphthalene	2	533-710	Mice	Oral
Phenanthrene	3	750	Mice	Oral
Anthracene	3	>430	Mice	Intraperitoneal
Fluoranthene	4	100	Mice	Intravenous
Pyrene	4	514	Mice	Intraperitoneal
Benzo[a]pyrene	5	232	Mice	intraperitoneal

Table 2-3: 16 Properties of biodegradable priority PAHs important to bioavailability.

PAH	No. of rings	Molecular weight	Aqueous solubility (µg/L)	Log K_{ow}	Cancer class U.S. EPA[a]	Cancer class IARC[b]
Naphthalene	2	128	31,000	3.4	D	3
Phenanthrene	3	178	1,300	4.6	D	3
Anthracene	3	178	70	4.5	D	3
Fluoranthene	4	202	260	5.2	D	3
Pyrene	4	202	135	5.2	D	3
Benzo[a]anthracene	4	228	14	5.9	B2	2A
Chrysene	4	228	2	5.9	B2	3
Benzo[b]fluoranthene	5	252	2	6.5	B2	2B
Benzo[k]fluoranthene	5	252	1	6.8	B2	2B
Benzo[a]pyrene	5	252	4	6.5	B2	2B
Dibenz[a,h]anthracene	5	278	0.5	7.2	B2	N/A

[a] Cancer class from U.S. Environmental Protection Agency (U.S. EPA) weight-of-evidence classifications D = not classifiable; B2 = probable human carcinogen.

[b] Cancer class from International Agency for Research on Cancer (IARC)

3 = not classifiable; 2A = probable human carcinogen; 2B = possible human carcinogen; N/A = not available

2.8. Remediation Strategies

PAHs pollutants can be remediated, with the following technologies: chemical oxidation; phytoremediation; solvent extraction and bioremediation. Remediation strategy of focus in this study indicates, biosurfactant-assisted bioremediation of PAHs.

2.8.1 Chemical Oxidation

In chemical processes, synthesised chemicals are used to remove these pollutants. Fenton's reagent and ozone are the two most commonly oxidants used in the oxidation reaction, to remediate soils contaminated with PAHs (Gan *et al*. 2009). *The fenton's reaction* is based on the hydrogen peroxide decomposition in the presence of ferrous iron, producing a hydroxyle radical, signifying the main oxidising species (Flotron *et al*. 2005). *Ozone (O3)* is a highly reactive and powerful oxidant used in the chemical industry as an oxidising agent, used widely, treating drinking water (O'Mahony *et al*. 2006).

In a study conducted by (Jonsson, et al., 2006) it was reported that the efficiency of degrading PAHs by both Fenton's reagent and ozone, is highly dependent on the initial concentration of PAHs in the soils and the particular components present. The study indicated that both methods yielded a higher degradation of LMW PAHs. The remaining PAHs were strongly sorbed to soil matrix and is chemically unavailable for degradation.

The shortcomings of these methods include: introducing chemicals into the environment, also being pollutants, needing further treatment; the costs may be too high; the pollutants may be chemically unavailable to treatment, due to the hydrophobic nature of PAHs.

Table 2-4: PAH degradation studies using chemical oxidation method

Study	PAH degraded	Notes	Reference
Degradation of PAHs in contaminated soil using Fenton's reagent	Anthracene, benzo[a]pyrene and pyrene	Coarse soil with high percentage of LMW PAHs, low organic matter and low percentage of iron aluminium, manganese oxides and chlorides are suitable for Fenton's treatment especially if the contamination occurred recently	(Jonsson et al. 2007)
Removal of sorbed PAHs from soil, sludge and sediment samples using the Fenton's reagent process	Benzo[a]pyrene	Oxidation of sorbed PAHs from solid environment samples can be achieved using large excess of hydrogen peroxide without any pH adjustment. However, not all PAHs were reactive, PAHs containing more than five-atom carbon cycle were observed to be non-reactive to the hydrogen peroxide	(Flotron et al. 2005)
The use of ozone in remediation of PAH contaminated soil	Phenanthrene	Due to the negative effect on ozonation exerted by water, ozone is more effective in sandy soils than in clay soil. The nature of soil and potential soil microorganisms play a vital role in the effectiveness of the combined ozone-biodegradation process	(O'Mahony et al. 2006)
Efficacy of in-situ for remediation of PAH contaminated soils	Phenanthrene, pyrene, and chrysene	More than 95% of phenanthrene was removed with an ozonation time of 2.3 h at an ozone flux of 250 mg/h. 91% of pyrene was removed after 4 h of treatment with an ozone flux of 600 mg/h. Chrysene concentration decreased from 100 to 50 mg/kg after 4 h of being exposed to ozone.	(Masten & Davies 1997)

2.8.2 Phytoremediation

Phytoremediation involves, using plants with their associated microorganisms to extract or detoxify pollutants from contaminated systems (Ukiwe, et al., 2013). Plants are known to enhance remediation of soil through biophysical and biochemical processes, such as manipulation of plant uptake of pollutants by promoting a more acidic soil environment, absorption of nutrients bound with pollutants, and secretion of enzymes from plants, acting as surfactants to increase the bioavailability of pollutants (Gan, *et al*. 2009). This process to remove pollutants from the environment, may be time-consuming.

Table 2-5:PAH degradation studies using phytoremediation method

Study	PAH oxidised	Notes	Reference
Biodegradation aspects of PAHs	Benzo[a]pyrene, benzo[a]anthracene, dibenzo[a,h]anthracene and chrysene	PAHs degradation was studied using a mix of 8 prairie grasses in loam sand. PAH concentration was observed to be reducing in planted areas as compared to unplanted areas, indicating that the removal of these compounds from contaminated sites was enhanced by phytoremediation. The biodegradation was greater from benzo[a]pyrene followed by chrysene, then benzo[a]anthracene and finally dibenzo[a,h]anthracene.	(Haritash & Kaushik 2009)
Degradation of phenanthrene and pyrene in rhizosphere of grasses and lagumes.	Phenanthrene pyrene	After 80 d of experiment, soil that was contaminated was analyzed for PAH concentration. The results showed that more PAH were dissipated in planted soil than in unplanted soil.	(Lee et al. 2008)

2.8.3 Solvent Extraction

Solvent extraction technology involves removal of PAHs from the soil using an individual solvent or mixture of solvents (Gan *et al*. 2009). Extraction agents, such as acetone, n-hexane, n-heptane, methanol, toluene, trichloroethylene, sunflower oil and peanut oil, are some of the agents used in the solvent extraction technology (Li *et al*. 2012; Gan *et al*. 2009).

The shortcomings of these methods include: introducing chemicals into the environment, being pollutants, needing further treatment; and the costs for treatment may be too high.

Table 2-6: PAH degradation studies using solvent extraction method

Study	Extraction agent used	PAH removed	Notes	Reference
Solvent extraction for heavy crude oil removal from contaminated soils	Acetone, n-haxane, n-heptane, methanol, toluene, trichloroethylene aluminium oxide	Nephthene aromatics, polar aromatics	The mixture of acetone and hexane showed to be the most effective in removal of petroleum hydrocarbons from contaminated soil. The ratio between solvent and soil was studied and results demonstrated an increased efficiency with higher solvent soil ratio.	(Li et al. 2012)
Removal of PAHs from contaminated soils using sunflower oil.	Sunflower oil	FLU, PHE, ANT, FLA, PYR, BaA, CHR, BbF, BkF, BaP, DahA, BghiP, IP	More than 90% of total PAHs (total of 13 PAHs concentration of 4721 mg/kg) was removed from a heavily contaminated soil using 4L of sunflower oil	(Gong et al. 2006)
Vegetable oil as a contaminated soil remediation amendment.	Peanut oil	Anthracene and other PAHs	Extraction efficiency was 90% when 2.5 – 20% peanut oil was used to remove anthracene. Total PAH extraction efficiency increased from 51.5% at 20^0C to 81.4% at 60^0C	(Pannu et al. 2004)

2.8.4 Bioremediation

The process of bioremediation can be defined, using microorganisms to detoxify or remove pollutants, relying on microbial enzymatic activities to transform or degrade the offending contaminants in the environment (Atlas & Philp, 2005). According to Gan *et al*. (2009) this is nature's way to recycle waste, by breaking down organic or inorganic matter into nutrients, using living organisms (Gan *et al*. 2009). The ultimate goal of the remediation process conducted in the environment, should be to resort the ability of the water, soil and sediments to function according to their potential, other than just removing the contaminants from the polluted substrates (Ukiwe *et al*. 2013). Information on chemical or the mixture of petroleum-derived chemicals (such as oil spills and process wastes) can be learned and provided in the environment, through bioremediation studies, stimulating scientific development on clean-up methods suited for the environment, following the procedure below (Speight & Arjoon, 2012):

- Analyzing the contaminated sites,
- Determining the best method suited for the environment, and

- Optimizing the cleanup techniques leading to the emergence of new process.

2.8.4.1 Bioremediation technologies

Most of the available information on the treatment of the HMW PAHs, focusses on the bioremediation of soil due the hydrophobic nature of these pollutants. Technologies, such as land treatment or farming, composting and bioreactors, amongst others, are documented as treatment technologies for PAHs contaminants. Land treatment can be viewed as an *in situ* treatment, substituting contaminated soil, without needing to move or relocate the soil (Wilson & Jones 1993).

This is a cheap method that is mostly limited by the depth of soil that can be treated and soil factors such as pH, oxygen, moisture, temperature and nutrients can be difficult to control (Riser-Robert , 1998). Composting method indicates an *on-site* treatment, utilising a prepared bed to treat highly contaminated material under controlled conditions (Wilson & Jones 1993). Composting methods combine several land farming strategies, minimising its disadvantages. Bioreactor treatment requires excavation of contaminated material and constructing reactors used during treatment (Riser-Robert, 1998). Bioreactor treatment is more expensive, compared with the mentioned treatment technologies. An advantage of bioreactor above the conventional methods indicates: if the potential hazards from discharges and emissions are very toxic and possess serious threats to the environment, they can easily be contained and managed using an in-vessel system (Riser-Robert, 1998).

Biological, physical and chemical processes can be combined and utilised in a controlled, cost-effective and efficient manner, using bioreactors. Technology used in remediation, depends on the nature and concentration of the contaminants in the soil. Prior implementing a treatment process, a feasibility study should be conducted determining the most appropriate treatment process. Bioreactor technology can also be adopted and used to treat contaminants in aquatic phase by firstly, conducting a feasibility study on the contaminant nature; thereafter optimise the bioreactor conditions to effectively remove contaminants from water.

2.8.4.2 Bioremediation mechanism

Natural attenuation: A natural process utilising the environment natural ability to degrade the contaminants (Fatima M. Bento *et al*. 2005). This is a very slow process and is often accelerated by biostimulation and bioaugmentation.

Biostimulation: A process involving identifying and adjusting certain physical and chemical properties (such as temperature, pH, nutrient content or substrate and oxygen) of the contaminated environment, important to the growth of indigenous microorganism which will accelerate the degradation of pollutants (Abdulsalam *et al*. 2011).

Bioaugmentation: A process involving inoculation of highly concentrated and specialised microorganisms with desired degradation capability into the contaminated environment, enhancing the biodegradation rate of the contaminants (Abdulsalam *et al*. 2011).

2.8.4.3 Advantages and disadvantages of bioremediation

Advantages of bioremediation include: (i) the microbes, capable of degrading contaminant increase in number when the contaminant is present and decrease when the contaminant is degraded; the by-products after the treatment are usually harmless products, such as water, carbon dioxide and sell biomass; (ii) public acceptance of the treatment due to the natural process involved; (iii) a complete destruction of the contaminant is possible; (iv) and it also prove to be less expensive than other technologies used for clean-up of hazardous waste (Vidali, 2001).

Disadvantages of bioremediation include but is not limited to: (i) the process may be longer compared with the other technologies; (ii) difficulty in achieving optimal environmental conditions suitable for the growth of microbial population; (iii) bioremediation is limited to biodegradable compounds (Vidali, 2001).

2.8.4.4 Microorganisms involved in PAHs degradation

Metabolic versatility of microorganisms is vital in bioremediation and is utilised to degrade hazardous pollutants (Seo *et al*. 2009). Microorganisms should be able to use pollutants as substrate, to successfully remove pollutants from contaminated media. Microorganisms capable of degrading certain contaminants are usually isolated from a site previously contaminated with

that specific contaminant, readily found in abundance (Whyte *et al*. 1996). Bacteria that can degrade petroleum products, include species of *Pseudomonas, Aeromonas, Moraxella, Beijerinckia, Flavobacteria, Chrobacteria, Nocardia, Corynebacteria, Modococci, Streptomyces, Bacilli, Arthrobacter, Aeromonas,* and *Cyanobacteria* (Das & chandran 2011).

2.8.4.5 Factors affecting bioremediation

Control on environment conditions for the bioremediation of polluted water may be direct, compared with the bioremediation of contaminated soil due to the soil environment being heterogeneous with mixed gaseous, solid and liquid phases associated with interfaces. (Atlas & Philp, 2005). Professionals responsible for decontaminating any contaminated sites, should ensure success by optimising the physical and chemical factors important to the growth of the microorganisms (Atlas & Philp, 2005). Environmental factors directly affecting the efficiency of bioremediation include: bioavailability of the contaminant to be degraded; temperature at which the degradation process is occurring; pH; oxygen availability for aerobic processes or its depletion in anaerobic processes; moisture content and the availability of nutrients for microbial growth amongst others (Ghosal *et al*. 2016).

2.8.4.5.1 Bioavailability

The effect of physic-chemical and microbiological factors on the rate and extent of biodegradation can determine bioavailability of compounds (Bamforth & Singleton 2005). As previously mentioned, biodegradation of HMW PAHs adsorbed in soil, is not possible without the assistance from surface active agents, such as biosurfactants, bioavailability indicating important factors determining the success of bioremediation, when dealing with PAHs with more than three benzene ring structures. The rate at which particular organic compound, in this case PAHs, dissolves in water, is critical to its biodegradability as it governs the rate of transfer to the organism (Atlas & Philp, 2005). The rate of HMW PAH biodegradation is severely reduced by bioavailability limitations, though cultures that can degrade these PAHs effectively, overcome the bioavailability limitations, using the following mechanisms: (i) increasing the solubility of substrates by producing biosurfactants (ii) promoting direct attachment to hydrophobic substrate by modifying their cell surface hydrophobicity (Ghosh *et al*. 2014).

2.8.4.5.2 Temperature

Temperature is an important factor determining the production and survival of microorganisms suitable and capable of bioremediations. Temperature influences the rate of biodegradation, controlling the rate of enzymatic reaction within the microorganism. The speed of enzymatic reaction in the microorganism cell approximately doubles for each 10^{o}C rise in temperature, though this reaction has its upper temperature limit, microorganisms can withstand (Speight & Arjoon, 2012). A study conducted by (Antizar-Ladislao *et al*. 2007) on the influence of various temperature conditions on the bioremediation of PAHs in contaminated soil, demonstrate that a greater removal of PAHs was achieved when temperature was 38^{o}C.

2.8.4.5.3 Nutrients availability

Microorganisms require nitrogen and phosphorus and other nutrients for incorporation into biomass; availability of these nutrients becomes an important factor concerning hydrocarbon degradation (Atlas 1995). Degradation can be hindered or can be relatively slow due to a lack of sufficient nutrients, such as nitrogen and phosphorus with electron acceptors, such as oxygen and other substrates. This would indicate the environment has to be manipulated in a way that biodegradation is stimulated and the reaction rates increased (Iwamoto & Nasu 2001). Atlas (1995) reports an analysis, indicating the difference in biodegradation rates resulting difference in nutrients levels in sediment pore waters. Greater nutrient concentrations attributed higher rates observed (Atlas, 1995).

2.8.4.5.4 Oxygen

The presence of oxygen is vital for aerobic degradation. The initial intracellular attack on organic hydrocarbons during their catabolism by bacteria, involves the oxidation of the substrate by oxygenises, requiring the presence of oxygen (Varjani & Upasani 2017) as illustrated in Figure *5* in Section 2.11. Although aerobic degradation of hydrocarbons was studied intensively, the anaerobic degradation of hydrocarbons studies emerged and proved to degrade hydrocarbons. A diverse physiological group of microorganism grown under chlorate-reducing, nitrate-reducing, sulfidogenic or methanogenic conditions can utilise hydrocarbons as their carbon and energy source in anaerobic degradation (Mbadinga *et al*. 2011). For a technology designed for an aerobic

degradation, it is important to ensure that oxygen is not depleted in the system for significant rates of hydrocarbon degradation.

Degradation process may also depend on microbial competence. Microbial as a limiting factors may be due to the insufficient of biomass concentration, enzyme activity and population diversity.

2.8.4.6 Optimizing bioremediation

Contaminants of concern can be degraded in appropriate conditions. The success of the degradation process depends highly on the ability to determine these conditions and establish them in the contaminated environment. Table 2-7 outlines the essential factors for microbial degradation or bioremediation success.

Table 2-7: Essential factors for bioremediation. Source (Speight & Arjoon, 2012)

Factors	Optimal conditions
Microbial population	Suitable kinds of organisms that can degrade all of the contaminants. Microorganisms can be added to the existing native degrading population via seeding to get enough microbial population in the system.
Oxygen	Enough oxygen to support aerobic biodegradation (about 2% oxygen in gas phase or 0.4 mg/L in the soil water.
Water	Soil moisture should be from 50-70% of the water holding capacity of the soil.
Nutrients	Nitrogen, phosphorus, sulfur, and other nutrients to support good microbial growth are essential. Contaminated site can be enriched through fertilization to stimulate the growth of the microorganisms capable of biodegradation.
Temperature	Appropriate temperature for microbial growth range is $0 – 40^0$C.
pH	Best range for pH is from 6.5 to 7.5

2.9. Biofilms Reactors

Microorganisms in natural environments are generally found in close proximity with surfaces and interfaces, in the form of multicellular aggregates attached combined with the slime they produce (Singh *et al*. 2006). Accumulation of these microorganisms on interfaces to form assemblages of single or multiple populations, through extracellular polymeric substances (EPS), results in

formation of biofilms (Wimpenny *et al*. 2000). Biofilm formation and EPS were associated with higher bioavailability of PAHs and their increased degradation rate because of their microbial biomass, bacterial chemotaxis and the ability to immobilise compounds (Meliani & Bensoltane 2014; Singh *et al*. 2006). Various microorganisms (or various nutritional conditions), will affect the type of biofilms produced; not all kinds of biofilms can degrade and tolerate PAHs (Rasamiravaka *et al*. 2015). Biofilms produced by pseudomonas aeruginosa can synthesise biosurfactant compounds and tolerate aromatic hydrocarbon compounds (Meliani & Bensoltane 2014).

2.10. Biosurfactants

Prokaryotes and eukaryotes produce surface-active molecules known as, biosurfactants. Biosurfactants are defined as a heterogeneous group of surface-active molecules, produced by microorganisms presenting two different moieties, hydrophilic and hydrophobic. As a result, this combination allows microorganisms to interact with hydrophobic substrates or surfaces. These molecules reduce surface tension, critical micelle concentration (CMC) and interfacial tension in both aqueous solutions and hydrocarbon mixtures (Banat 1995). When the source of carbon and energy for microorganisms' growth signifies an insoluble substrate such as hydrocarbon, microorganisms facilitate their diffusion into the cell, producing biosurfactants (Karath, *et al*., 1999). Biosurfactants can produce stable emulsions with insoluble substrates such as hydrocarbons. Microorganisms implying pseudomonas aeruginosa, are best known to produce biosurfactants on compounds, both alkanes and water-soluble and their produced biosurfactants are classified as Rhamnolipids (Sineriz, et al., 2009). Table 2-8 provides a list of microorganisms, capable of producing their respective biosurfactants.

Some of the advantages of biosurfactants, compared with their chemically synthesised counterparts are (Kosaric 1992):

- Biodegradability.
- Generally low toxicity.
- Biocompatibility and digestibility, allowing their application in cosmetics, pharmaceuticals and as functional food additives.

- Availability of raw materials. Biosurfactants can be produced from cheap raw materials, available in large quantities. The carbon source may be derived from hydrocarbons, carbohydrates and lipids, used separately or combined.
- Acceptable production economics. Depending on the application, biosurfactants can also be produced from industrial waste and by-products; this is of interest for bulk production (in petroleum related technologies).
- Use in environmental control. Biosurfactants can be efficiently used in handling industrial emulsions, control of oil spills, biodegradation and detoxifying industrial effluents and in bioremediation of contaminated soil.
- Specificity. Biosurfactants, being complex organic molecules with specific functional groups, are often specific in their action. This would be of particular interest in detoxifying specific pollutants, de-emulsification of industrial emulsions, specific cosmetic, pharmaceutical and food applications.

Table 2-8: List of biosurfactant producing bacteria. Source (Shekhar *et al*. 2015)

No	Biosurfactants	Microorganism source
1	Glycolipids	
	Trehalolipids	*Rhodococcus erythropolis*, *erythropolis*, *Arthrobacter* sp., *Nocardia Mycobacterium* sp.
	Trehalose dimycolates	*Mycobacterium* sp., *Nocardia* sp.
	Trehalose dicorynomycolates	*Arthrobacter* sp., *Corynebacterium* sp.
	Rhamnolipids	*Pseudomonas aeruginosa*, *Pseudomonas* sp.
	Sophorolipids	*Torulopsis bombicola*, *Torulopsis apicola*, *Torulopsis petrophilum Torulopsis* sp.
	Cellobiolipids	*Ustilago zeae*, *Ustilago maydis*
2	Lipopeptides and lipoproteins	
	Peptide-lipid	*Bacillus licheniformis*
	Serrawettin	*Serratia marcescens*
	Viscosin	*Pseudomonas fluorescens*
	Surfactin	*Bacillus subtilis*
	Fengycin	*Bacillus* sp.
	Arthrofactin	*Arthrobacter* sp.
	Subtilisin	*Bacillus subtilis*
	Gramicidins	*Bacillus brevis*, *Brevibacterium brevis*
	Polymyxins	*Bacillus polymyxa*, *Brevibacterium polymyxa*
	Lichenysin	*Bacillus licheniformis*
	Ornithine lipids	*Myroides* sp. *SM1*, *Pseudomonas* sp., *Thiobacillus* sp., *Agrobacterium* sp., *Gluconobacter* sp.

3	Fatty acids, phospholipids, and neutral lipids	
	Neutral lipids	*Nocardia erythropolis*
	Phospholipids	*Thiobacillus thiooxidans*
	Bile salts	*Myroides* sp.
	Fatty acids	*Candida lepus*, *Acinetobacter* sp., *Pseudomonas* sp., *Micrococcus* sp., *Mycococcus* sp., *Candida* sp., *Penicillium* sp., *Aspergillus* sp.
4	Polymeric surfactants	
	Emulsan	*Arethrobacter calcoaceticus*
	Biodispersan	*Arethrobacter calcoaceticus*
	Mannan lipid protein	*Candida tropicalis*
	Liposan	*Candida lipolytica*
	Carbohydrate protein lipid	*Pseudomonas fluorescens*, *Debaryomyces polymorphus*
	Protein PA	*Pseudomonas aeruginosa*
5	Particulate biosurfactants	
	Vesicles and fimbriae whole cells	*Arthrobacter calcoaceticus*

2.11. PAH Biodegradation

A general PAH biodegradation adopted from (Juhasz & Naidu 2000) involves the following process and is illustrated in Figure 2-5:

- An initial step involves bacteria oxidising aromatic hydrocarbons to *cis*-dihydrodiols. The conversion step is performed with various enzymes where atmospheric oxygen is incorporated into the substrate. Bacteria produces dioxygenases, incorporating two oxygen atoms into the aromatic nucleus.

- *cis*-dihydrodiols are re-aromatised through a *cis*-dihydrodiols dehydrogenase, yielding a dihydroxylated derivative. If oxidation of *cis*-dihydrodiols continues, formation of catechols, substrate for other deoxygenases impedes, instigating enzymatic cleavage of the aromatic ring.

- Catechols can either be processed through a *meta* cleavage pathway or an *ortho* cleavage pathway.*Meta* pathway involves, cleavage of the bond between a carbon atom with a hydroxyl group and the adjacent carbon with a hydroxyl group, while an *ortho* pathway involves cleavage of the bond between carbon atoms of the two hydroxyl groups, producing *cis*,*cis*-mucosic acid.
- Ring cleavage results in producing pyruvic, acetic, succinic, fumaric acids and aldehydes. All these compounds are used by microorganisms, for the synthesis of cellular constituents and energy. Carbon dioxide and water are by-products, resulting from these reactions.

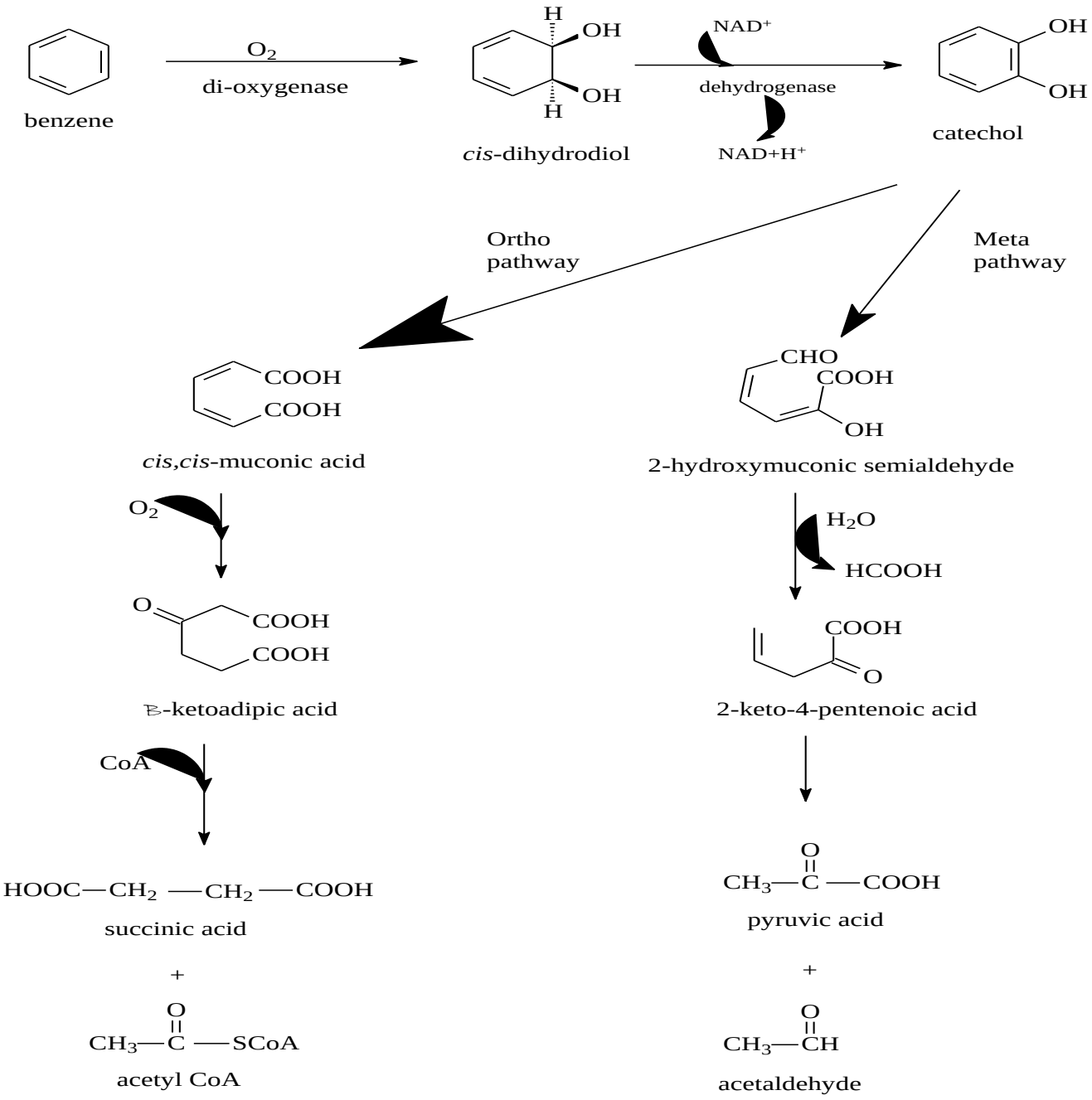


Figure 2-5: ortho or meta cleavage pathway for microbial metabolism of the aromatic ring. Source (Juhasz & Naidu 2000)

Table 2-9 provides results of some of the experiments conducted with bioremediation technology. Most of the experiments were conducted in a batch setup and very few in a reactor setup. The results also show the involvement of biosurfactants' presence, improving the degradation effectiveness of the compounds.

Table 2-9: PAH biodegradation studies conducted previously

PAH pollutant	Microorganism involved	Biosurfactant used (if any)	Metabolic pathway	notes	Reference
Pyrene (Batch study)	Pseudomonas putida	Unidentified biosurfactant	In catechol it produced a yellow coloring suggesting a meta-cleavage product of catechol.	68% pyrene degradation after 5 d of incubation. Biosurfactants lowered the surface tension of medium from 54.9 dN cm^{-1} to 35.4 dN cm^{-1} with E24 of 74%	(Kumar *et al*. 2006b)
Pyrene, Benzo(a)anthracene, Benzo(a)pyrene (Batch study)	Mycobacterium sp. strain RJGII-135		Three metabolites : 4,5-phenanthrene-dicarboxylic acid, 4-phenanthrene-carboxylic Acid, and 4,5-pyrene-dihydrodiol. (proposed pathway was dioxygenase enzymatic processes)	Mycobacterium sp. strain RJGII-135 appears to degrade both large-ring com-pounds such as PYR, BAA, and BaP and small-ring com-pounds such as phenanthrene and anthracene	(Schneider *et al*. 1996)
Motor oil PAHs (Batch study)	Bacillus subtilis	Lipopeptide	Not identified	Up to 82% of PAHs in the used motor oil was degraded after 18 d of incubation. More than Two fold compared to the degradation with no biosurfactant supplementation.	(Bezza & Chirwa 2015)

Table 2-9: PAH biodegradation studies conducted previously (continued…)

PAH pollutant	Microorganism involved	Biosurfactant used (if any)	Metabolic pathway	notes	Reference
Benzo[a]pyrene, Dibenz[u,h]anthracene and Coronene (Batch study)	Pseudomonas cepacia	No biosurfactants (PAHs were dissolved in dimethylformamide)	Not identified	After 63 d incubation, there was a 20 to 30% decrease in the concentration of benzo[u]pyrene and dibenz[u,h]anthracene and a 65 to 70% decrease in coronene concentration.	(Juhasz *et al*. 1996)
Pyrene, Chrysene, Benzo(a)pyrene, Perylene (Two-liquid-phase bioreactor)	Micriobial consortia (mixture of different cultures)	Rhamnolipids (biosurfactant) Triton X-100, Witconol SN70, Brij 35 (surfacta	Not identified	Degradation rates were 27 $mg.L^{-1}d^{-1}$ for pyrene, 8.9 $mg.L^{-1}d^{-1}$ for chrysene, 1.8 $mg.L^{-1}d^{-1}$ for benzo(a)pyrene and 0.37 $mg.L^{-1}d^{-1}$ for peryle	(Marcoux *et al*. 2000)
Phenanthrene, Pyrene, Chrysene, and Benzo[a]pyrene (Two-liquid-phase bioreactor)	Micriobial consortia (mixture of different cultures)	No biosurfactants (PAHs were transferred into silicon oil)	Not identified	The rate of pyrene degradation was 19 $mg.L^{-1}$ $.d^{-1}$ and no pyrene was detected after 4. The degradation rates of chrysene and benzo[a]pyrene were 3.5 and 0.94 $mg.L^{-1}.day^{-1}$ respectively.	(Villemur *et al*. 2000)
phenanthrene, anthracene, acenaphthene, fluorene, fluoranthene, and pyrene. (Aerated Bioslurry Reactors)	Paenibacillus validus PR-P1, Sphingomonas sp. PR-P12, and Arthrobacter sp. PR-P3	No biosurfactants (Salt marsh grass was used to enhance the biodegradation of PAHs)	Not identified	In all bioreactors a rapid reduction.of greater than 95% of the initial phenanthrene, acenaphthene, and fluorene occurred within 14 d. Pyrene and fluoranthene reductions of 70 to 90% were achieved by day 77 of treatment. Anthracene was more recalcitrant and reductions ranged from 30 to 85%.	(Launen *et al*. 2002)

CHAPTER 3: METHODS AND MATERIALS

3.1 Microorganism and Culture Enrichment

Two cultures were used in this study. A mixed culture was obtained from a study conducted at the University of Pretoria (Bezza & Chirwa, 2016), and the other bacterial strain used was isolated from a working motor service yard in Pretoria West, South Africa. Using the engine oil contaminated soil, microbial culture was prepared in a 250mL Erlenmeyer flask, adding 5g of soil to 100mL nutrient broth and left in a rotary shaker with a temperature 34^{o}C and rotary speed 150 rpm for 24 h. Nutrient broth with composition (g/L): 1.0 meat extract, 2.0 yeast extract, 5.0 peptone, and 8.0 sodium chloride, was prepared by dissolving 16g of nutrient broth solid in 1L distilled water and autoclaving the solution at 121^{o}C for 15 min.

After 24 h, 10mL of microbial culture was transferred to a fresh sterile mineral salt medium (MSM) supplemented with 25 mg of PAHs as sole carbon and energy source. Four sets of samples, A, B, C, and D were prepared with fluoranthene, triphenylene, pyrene and a mixture of the three PAHs. Samples were incubated in a rotary shaker with a temperature of 34^{o}C and rotary speed of 150 rpm for 14 d. After 14 d, 10mL from each sample was transferred into a new sterile MSM solution supplemented, with PAHs as sole carbon and energy source respectively. The culture enrichment was performed over 29 d.

MSM solution was prepared by adding the following chemicals in a 1L of distilled water: 6.0 g $(NH_4)_2SO_4$, 0.4 g $MgSO_4.7H_2O$, 0.4 g $CaCl_2.2H_2O$, 7.8 g $Na_2HPO_4.2H_2O$, 4.5 g KH_2PO_4 and 2 mL of trace element solution. Trace element solution (L^{-1}) contained: 50 µM $CaCl_2$, 25 µM $FeSO_4$, 0.1 µM $ZnCl_2$, 0.2 µM $CuCl_2$, 0.1 µM NaBr, 0.05 µM Na_2MoO_2, 0.1 µM $MnCl_2$, 0.1 µM KI, 0.2 µM H_3BO_3, 0.1 µM $CoCl_2$, and 0.1 µM $NiCl_2$. The MSM preparation method was adopted from (Trummler *et al*. 2003).

3.2 Culture Isolation and Purification

After culture enrichment, 1ml of each sample was extracted and serially diluted in sterile mineral salt medium, using glass test tubes; 100 µl from the 4th test tube was transferred into a prepared nutrient agar plate and incubated at 30^{o}C for 48 h, for bacterial colonies to grow. Colonies were

streaked on new nutrient agar plates twice, further purifying bacterial isolates, using a spread and streak plate technique.

Nutrient agar solution, comprising the composition (g/L): 1.0 meat extract, 2.0 yeast extract, 5.0 peptone, 8.0 sodium chloride, and 15.0 agar, was prepared by dissolving 31g of nutrient agar solid in 1L distilled water. The solution was placed on a stirrer and stirred until the mixture was completely dissolved. The solution was then autoclaved at 121^{o}C for 15 min and left to cool until it was possible to touch the flask without burning. After mixing well, 25-30 ml was poured unto petri dishes to settle.

3.3 Screening for Potential Biosurfactants-Producing Bacteria

Few methods were used for screening bacteria isolated with the potential to produce biosurfactants.

3.3.1 Drop-collapse Method

This method indicates using the following procedure adopted from research (Bodour & Miller-Maier 1998): qualitative method was performed on a 96-micro well plate. Before the test was conducted, the plate was coated with mineral oil and incubated for 24 h at room temperature; 5 µL aliquot of a sample was delivered into the centre of the well, using a pipette. The drop would bead up, spread slightly or collapse depending on the quantity surfactant in the sample. If the drop collapse or spreads, it indicates the presence of surfactant and if the drop remains bead up, it indicates the absence of the surfactants.

3.3.2 Emulsification Index Test (E24)

E_{24} can be defined as the percentage of the height of an emulsified layer, divided by the total height of the liquid column (Fátima Menezes Bento *et al*. 2005). Cell free supernatant (2 mL) of the cultured sample was added to 2mL of hexane. The mixture was then mixed for 2 min with a vortex mixer and allowed to stand for 24 hrs.

$$E_{24} = \frac{height\ of\ emulsified\ layer}{total\ height\ of\ liquid\ column}\ X\ 100 \qquad [3\text{-}1]$$

3.3.3 Oil Spreading Method

30mL of distilled water was poured onto a petridish and 1mL of oil was added to the plate containing distilled water using a pipette. 2 µL of the culture supernatant is carefully placed at the centre of the oil surface. The clear zone on the oil surface indicates the presence of biosurfactant producing isolates.

3.4 Species Identification by 16S rRNA Fingerprint

The purified colonies were streaked on nutrient agar, followed by incubating at 30°C for 18 h preparing for 16S rRNA gene sequence analysis. Microbial pure cultures were grown from loop-fuls from individual colonies, transferred to fresh media, containing low quantities (2-5 mg/L) of fluoranthene. The process was repeated at least three times for each colony type to achieve an almost pure culture of each identified species.

Genomic DNA was extracted from purified colonies, according to the protocol described for the Wizard Genomic DNA purification kit (Promega Corporation, Madison, WI, USA); 16S rRNA genes were amplified by a reverse transcriptase-polymerase chain reaction (RT-PCR) using primers pA and pH1 (Primer pA corresponds to position 8-27; Primer pH to position 1541-1522 of the 16S gene under the following reaction conditions: 1 min at 94ºC, 30 cycles of 30 s at 94ºC, 1 min at 50ºC and 2 min at 72ºC, and a final extension step of 10 min at 72ºC). PCR fragments were then cloned into pGEM-T-easy (Promega) [Promega Wizard® Genomic DNA Purification Kit (Version 12/2010)]. The 16S rRNA gene sequences of the strains were aligned with reference sequences from phenol and benzoate, degrading Pseudomonads using Ribosomal Database Project II programs. Sequence alignment was verified manually, using the program BIOEDIT. Pairwise evolutionary distances based on an unambiguous stretch of 1274 bp, were computed by using the method by Jukes and Cantor (1969).

To present the identified strains, a phylogenetic tree was constructed based on the average sequence similarities of the whole gemone sequence (Satoh *et al*. 2013).

The sample indicates that the presence of surfactants were preserved in a 30% glycerol solution prepared:

Solution with 30% glycerol and 70% distilled water was autoclaved at 121^{o}C for 15 min. 500 µL of bacterial culture was added to 500 µL of 30% glycerol solution in a 1.5 mL tubes. Samples were stored at -70^{o}C for future use

3.5 Biosurfactant Production

3.5.1. Pre-culture

To prepare an inoculum from the stored cultures, the following procedure was performed for microorganisms' cultivation: 50mL of nutrient broth was added to a 250mL Erlenmeyer flask and inoculated with 1mL of pseudomonas aeruginosa. The media was incubated for 24 h in a rotary shaker with 160 rpm at 34^{o}C.

3.5.2. Method 1

Biosurfactant production, using pseudomonas aeruginosa was reported to be optimally produced upon exhaustion of nitrogen in the medium with pH range of 6.5-6.7 indicating a temperature of 37^{o}C. A two-step process was used according to (Trummler *et al*. 2003), for optimised production of biosurfactants.

STEP 1: Cell overproduction

A growth medium with composition of (g/L): 3.63 g $(NH_4)NO_3$, 0.4 g $MgSO_4.7H_2O$, 0.4 g $CaCl_2.2H_2O$, 7.8 g $Na_2HPO_4.2H_2O$, 4.5 g KH_2PO_4 and 2 mL of trace element solution was used for cell overproduction.

A growth medium (1000 mL) was added to a 3L Erlenmeyer flask, supplemented with 10% (v/v) glycerol as a substrate and inoculated with 4% preculture. The flasks were shaken for 72 h at 34^{o}C, 160 rpm. Cell biomass was harvested after 72 h by centrifugation (6000 rpm, 20 min, 4^{o}C). Cell free supernatant surface activity was checked and used to examine the Emulsification Index (E_{24}) measurements.

Step 2: Biosurfactant production

A nitrogen limited growth medium with composition of (g/L): 0.4 g $MgSO_4.7H_2O$, 0.4 g $CaCl_2.2H_2O$, 7.8 g $Na_2HPO_4.2H_2O$, 4.5 g KH_2PO_4 and 2 mL of trace element solution was used for biosurfactant production.

With a final wet biomass, less than 5% (w/v), cell mass was suspended in the growth medium, using 3L Erlenmeyer flasks, supplemented with olive oil as a substrate. The experiments were performed in triplicates. To avoid thick coating oil layers at the top of the solution, the oil was added into the flasks daily until 4% was reached. The experiments were performed in triplicates and left in a shaker for 6 d at 33^oC, 160 rpm and pH of 7. Periodically (at 0, 24, 48, 72, 96, 120 and 144 h) emulsification index, biomass concentration, surface tension and biosurfactant concentration were measured.

3.5.3. Method 2

Biosurfactants were produced in shaking flasks over a period of 12 d. Growth medium comprised nutrient broth (8 g/L) and NaCl (5 g/L), used for growth optimisation on biosurfactant production. In a 1L Erlenmeyer flask, 400mL of growth media was added and supplemented with 10% olive oil or glycerol, as substrate. FourmL preculture was inoculated in the media and incubated at room temperature of 30^oC on a rotary shaker (150 rpm) for 12 d. The experiments were performed in triplicates. To avoid thick coating oil layer at the top of the solution, the oil was added into the flasks daily until 10% was reached. Periodically (at 0, 24, 48, 72, 96, 120 and 144 h) emulsification index, biomass concentration, surface tension and biosurfactant concentration, were measured.

3.5.4. Method 3

This method is similar to Method 1, indicating a two-step process for optimised production of biosurfactants (Trummler *et al*. 2003).

STEP 1: Cell overproduction

Nutrient broth was used for cell overproduction; 400mL of broth was added into a 1L Erlenmeyer flask and inoculated with 4% of preculture. The flasks were shaken for 24 h at 30^oC, 160 rpm. Cell biomass was harvested after 24 h by centrifugation (6000 rpm, 20 min, 4^oC). Cell free supernatant was checked for surface activity and used to check for Emulsification Index (E_{24}) measurements.

Step 2: Biosurfactant production

A growth medium adopted from (George & Jayachandran 2013) with a composition of (g/L): 0.1 g $MgSO_4.7H_2O$, 4.5 g $Na_2HPO_4.2H_2O$, 0.68 g KH_2PO_4, 4.5 g $NaNO_3$ and 0.5 g yeast extract, was used for biosurfactant production.

With a final wet biomass less than 5% (w/v), cell mass was suspended in 400mL growth medium, using 1L Erlenmeyer flasks supplemented with 4% olive oil as a substrate. To avoid thick coating oil layer at the top of the solution, the oil was added into the flasks daily until 4% was reached. The experiments were performed in triplicates and left in a shaker for 6 d at 33^oC, 160 rpm and pH of 7. Periodically (at 0, 24, 48, 72, 96, 120 and 144 h) emulsification index, biomass concentration, surface tension and biosurfactant concentration were measured.

3.6 Surface Tension

The surface tension of the culture cell free supernatant was measured with a Du Nouy tensiometer (Kruss Tensiometer, K11 model – Germany) equipped with a 1.9 cm platinum ring. For each sample, three independent measurements were taken at room temperature of 25^oC; their average was recorded as the surface tension value.

3.7 Critical Micelle Concentration

To estimate the concentration of biosurfactants, expressed in critical micelle dilution (CMD), surface tension for various dilutions of the sample were measured, using the above surface tension method. To measure CMD, the cell free culture supernatants were diluted 10 times CMD^{-1} to 100 times CMD^{-2} with ultrapure water. Surface tension versus biosurfactant concentration graph was plotted to determine the CMC. The strong cohesive force amongst the water molecules, due to the extensive intermolecular hydrogen bonding, resulted in high surface tension. With addition of surfactants to water, surfactants monomers hinder the degree of intermolecular hydrogen bonding amongst water molecules at the solution interface, because of their preferential adsorption. As a result, addition of surfactant decreased the surface tension drastically until it reached the CMC point, signifying a point at which the solution interface becomes saturated with amphiphiles. The addition of surfactants beyond the CMC point resulted in self-assembled surfactants, to form

micelles and populate the bulk phase without much effect on the surface tension (Chakraborty *et al*. 2011).

3.8 Biosurfactant Extraction

Two extraction methods were used to extract crude biosurfactants after production. The first method was adopted (Santhini & Parthasarathi 2014). From the biosurfactant solution produced, cells were removed by centrifugation at 6000 rpm, 4^{o}C for 20 min. Cell free supernatant was acidified by using 6N HCl by adjusting the pH to 2, the solution was then kept overnight at 4^{o}C. The precipitates obtained was pelleted by centrifugation for 20 min at 4^{o}C; dried and determining the quantity of crude biosurfactant produced. The second method was adopted from (Satpute *et al*. 2010). Cell free supernatant was added to equal amount of an ice-cold acetone and kept overnight at 4^{o}C. The precipitates obtained was pelleted by centrifugation for 20 min at 4^{o}C, dried and weighed to determine the quantity crude biosurfactant produced.

3.9 Continuous Stirred-Tank Bioreactor

A three stage system with Continuous Stirred Tank Bio-reactors and biofilm tank was designed and developed to test and analyze the dissolution and degradation of PAHs in a more controlled environment.

Three stages in the process are;

- ***Stage 1***: Producing biosurfactants in Reactor 1, using the methodology described in Section 3.5.4, aiming using these biosurfactants to dissolve HMW PAHs.
- ***Stage 2***: Growing microbial consortia capable of degrading dissolved PAHs from the first stage.
- ***Stage 3***: Growing biofilm on stone media aimed at further degradation of PAHs. This stage also involved aeration, provided through air injection in a recycled flow.

3.9.1. Reactor setup

A – Reactor 1: dissolution tank

B – Feed tank: diluted mineral salts medium

C – Reactor 2 with buffer separating compartment

2A (stirred side) and 2B

D – Safety factor tank

E – Aeration tank

F – Biofilm tube

G – Air injector

H – Effluent tank

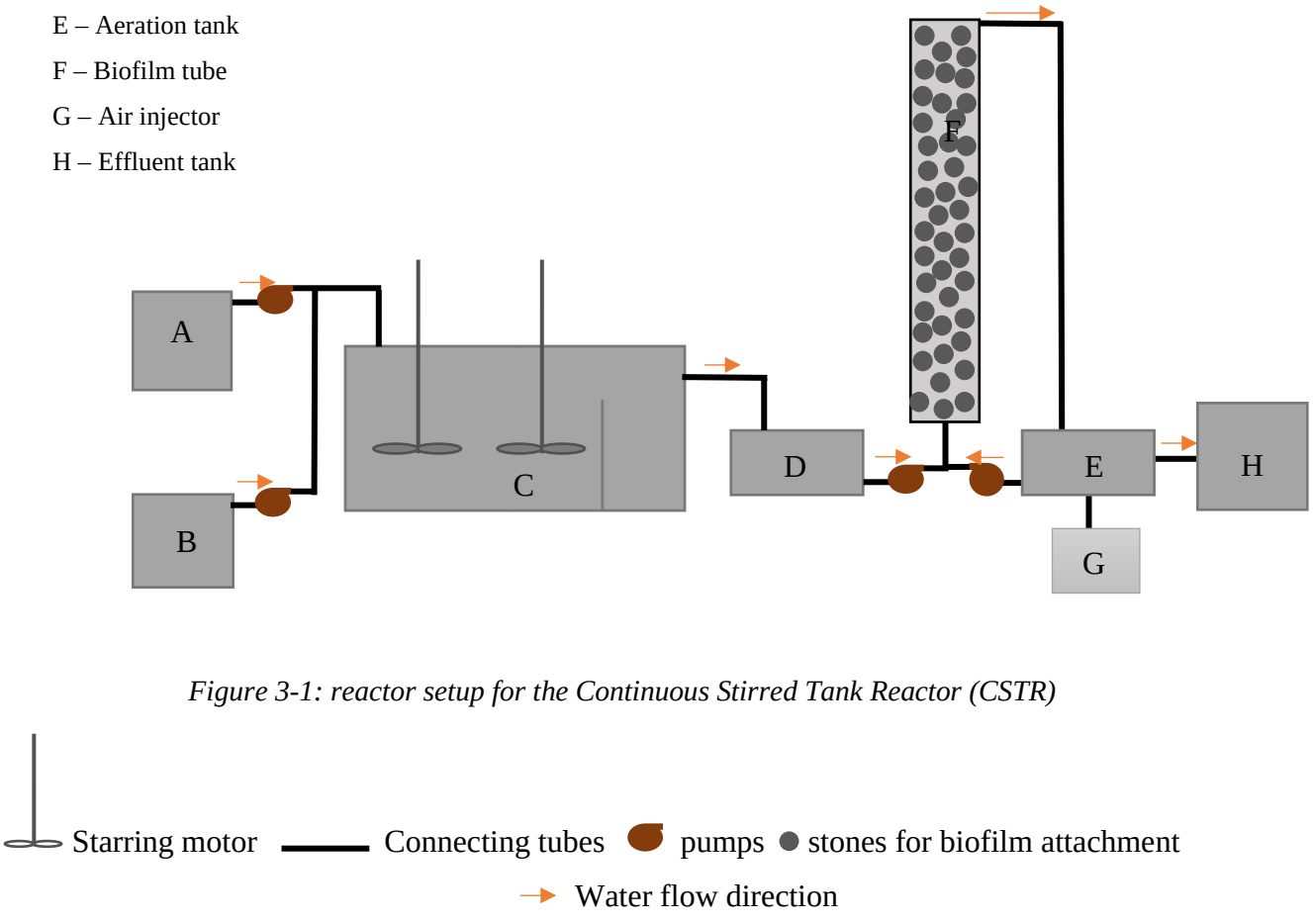


Figure 3-1: reactor setup for the Continuous Stirred Tank Reactor (CSTR)

3.9.2. Reactor configurations

Table 3-1: Tank sizes

	Reactor 1	Reactor 2		Biofilm tube
		Compartment A	Compartment B	
Tank size (L)	36.75 L	63 L (overall reactor 2 volume)		3.82 L
Water volume (L)	24.84 L	38.7 L	9 L	1.001 L
Surface area (m^3)	-	-		0.676 m^3
Flow rates (L/h)	0.276 (from PAHs) 0.567 (from media)	0.863 (average)	0.860	0.863 influent 6.529 recycle
Retention time (h)		43.83	10.19	1.19

3.9.3. Reactor Tracer Studies

Tracer studies were conducted to evaluate the performance of the reactor. NaCl with Na concentration of 150 mg/L was dissolved in1 L of distilled water and used to feed in Reactor 2. Samples were extracted periodically to determine the effluent concentration from Reactor 2A, 2B and biofilm reactor. Sodium concentration was then measured using Atomic Absorption Spectroscopy to determine the concentrations. The inflow rate of the feed was 0.8 L/h and was kept constant throughout the run.

3.10 HMW PAHs Dissolution

3.10.1. Batch Dissolution

To determine the solubility of fluoranthene, various concentrations of biosurfactants were added to three 250mL Erlenmeyer flasks with 100mL of MSM and supplemented with 70 mg of fluoranthene. 25mL, 50 mL, and 100mL of biosurfactants were added to the three flasks respectively to determine the solubility of fluoranthene. A control flask was also prepared with 70

mg/L of fluoranthene but not amended with any biosurfactants. Same procedure was followed to determine the solubility of triphenylene using biosurfactants. From the solutions, 2mL samples were drawn from the reactor, using a pipette and vortexed vigorously with hexane to extract the PAHs in the sample. Hexane was extracted and evaporated with a separation funnel, leaving PAHs coating at the bottom of test tubes. PAHs were re-dissolved using acetonitrile and filtered into vials for analysing using a high-performance liquid chromatograph (HPLC).

3.10.2. CSTR Dissolution

Two runs were performed for experimental purposes. A preliminary run was performed using a feed-batch into the reactor instead of feeding the dissolved PAHs from the Reactor tank 1. A 400mL solution with PAHs was prepared in a 1L flask, adding 200mL of dissolved PAHs from stock solution (463.89 mg/L) prepared during PAH solubility studies during batch experiments to 200mL of MSM. The initial concentration in the fed-batch experiment was 156.94 mg/L. For the actual experimental CSTR, biosurfactants were produced in Reactor tank 1 over a period of 12 d. After production of biosurfactants, 15 g of fluoranthene was added to the reactor Tank 1 with biosurfactant solution of 29.12 L. Four samples were taken to determine the concentrations after the addition of fluoranthene to the tank within the first 8 h period.

3.11 Degradation of HMW PAHs

3.11.1. Degradation of PAHs in Batch System

Experiments for PAHs degradation were conducted in triplicates. From the dissolved PAHs during the dissolution stage, PAHs were added to 250mL Erlenmeyer flasks with 100mL of growth media. The flasks were inoculated with PAH degrader microbial consortia and loosely capped with aluminium foil to avoid oxygen depletion, and thereafter incubated for 12 d at 37^{o}C in a rotary shaker with 160 rpm. 2mL aliquots were sampled from the flask and analysed for PAH concentration and cell biomass after each 24-hour period for 12 d. 2mL samples were drawn from the reactor using a pipette and vortexed vigorously with hexane to extract the PAHs in the sample. Hexane was extracted and evaporated with a separation funnel, to leave PAHs coating at the bottom of test tubes. PAHs were re-dissolved using acetonitrile and filtered into vials for analysing, using a high-performance liquid chromatograph (HPLC).

3.11.2. Degradation of Fluoranthene in CSTR System

Prior to operating the continuous experimental run, PAH degraders were grown in Reactor 2 and the biofilm tube for a period of 7 d. 2 L of Nutrient broth solution and 2 L of mineral salt mediums were diluted into Reactor 2 with tap water to fill up to a volume of 47.7 L. 1 L of pre-cultured inoculum was added to the reactor to introduce the PAH degrades and grow them in the system. 250mL of glycerol was added to the tank to serve as carbon and energy source for the microorganisms. The solution from Reactor 2 was pumped into the biofilm to allow biofilm formation on the media surface. For 7 days, aeration and agitation was provided by overhead stirrers in Reactor 2 and through recycled flow infused with air through aeration tank in the biofilm tank. After 2 d, 1 L of MSM supplemented with glycerol was added directly to Reactor 2 and 100mL aeration tank, providing nutrients for the microorganisms.

To evaluate the degradation of fluoranthene, dissolved fluoranthene from the biosurfactant production reactor, was pumped into Reactor 2 containing PAH-degraders with a retention time of 44 h to allow partial degradation of fluoranthene and thereafter pumped into the biofilm reactor stage for further degradation. The reactor was run for 12 d while sampling after 4 h for the first 3 d and after 6 h for the remaining running period. 2mL samples were drawn from the reactor with a pipette and vortexed vigorously with hexane to extract the PAHs in the sample. Hexane was extracted and evaporated with a separation funnel, to leave PAHs coatings at the bottom of test tubes. PAHs were re-dissolved using acetonitrile and filtered into vials for analysing, using a high-performance liquid chromatograph (HPLC).

3.12 Analytical methods

3.12.1. HPLC Analysis

A high performance liquid chromatograph (HPLC) Waters 2695 separation module equipped with waters Photodiode Array Detector Model 2998 was used. The PAHs were separated using a reverse phase mode, a Waters PAH C_{18} column (250 mm × 4.6 mm, 5 µm particle size), temperature of 25^{o}C and pressure of 4000 psi. The detection wavelength operated at 254 nm. The mobile phase (Acetonitrile:Water) was programmed with a reciprocating pump.

Table 3-2: HPLC gradient elution program

Time (min)	Acetonitrile %	Water %	Flow rate (mL/min)
0-1	70	30	1
1-25	70	30	1
25-35	100	0	1
35-40	100	0	1
40-45	70	30	1

3.12.2. Kinetic Modeling

In experiments where PAH concentrations is large, the rate of reaction is zero order (Knightes & Peters 2003). Zero order reactions proceed at a rate independent of the concentration of the reactants and is described using the equation:

$$\frac{dC}{dt} = -k_d\, X \qquad [3\text{-}2]$$

In experiments where PAH concentrations is low, the rate of reaction is first order. First order reactions proceed at a rate directly proportional to the concentration of the reactants and is described using the equation:

$$\frac{dC}{dt} = -k_d\, CX \qquad [3\text{-}3]$$

The monod model is a transitional phase between the zero order and first order (high and low substrate concentration) and is described using the equation:

$$\frac{dC}{dt} = -\frac{\mu_{max} C}{K_s + C} X \qquad [3\text{-}4]$$

And the associated biomass growth is described by the following equation:

$$\frac{dX}{dt} = Y \frac{\mu_{max} C}{K_s + C} X \qquad [3\text{-}5]$$

Where variables are:
C (mg/L) = substrate concentration
X (mg carbon/L) = biomass concentration
t (h) = time
μ_{max} (mg substrate/ mg carbon/h) = the maximum substrate utilisation rate per unit biomass
K_s (mg/L) = half-saturation coefficient
Y (mg carbon/mg substrate) = yield coefficient

CHAPTER 4: CULTURE AND BIOSURFACTANT CHARACTERIZATION

4.1. Screening and Identification of Biosurfactant Producing Bacteria

Three colonies were isolated from soil sample P1 and five from soil sample P2, after enrichment of the contaminated soil. The colonies were tested for their biosurfactant production potential, using drop collapse and oil spread screening methods. All the isolates from soil sample P1 tested negative for both methods. From soil sample P2, three strains (Strain A, B, and D) tested positive on both methods and were selected to hold high biosurfactant producing potential. The strains were sent for identification using 16 rRNA analysis; the results indicated that the Strain A, B, and D belong to members of the genus Pseudomonas. It further indicated a high sequence similarity (100%) to pseudomonas aeruginosa as presented in Figure 4-1.

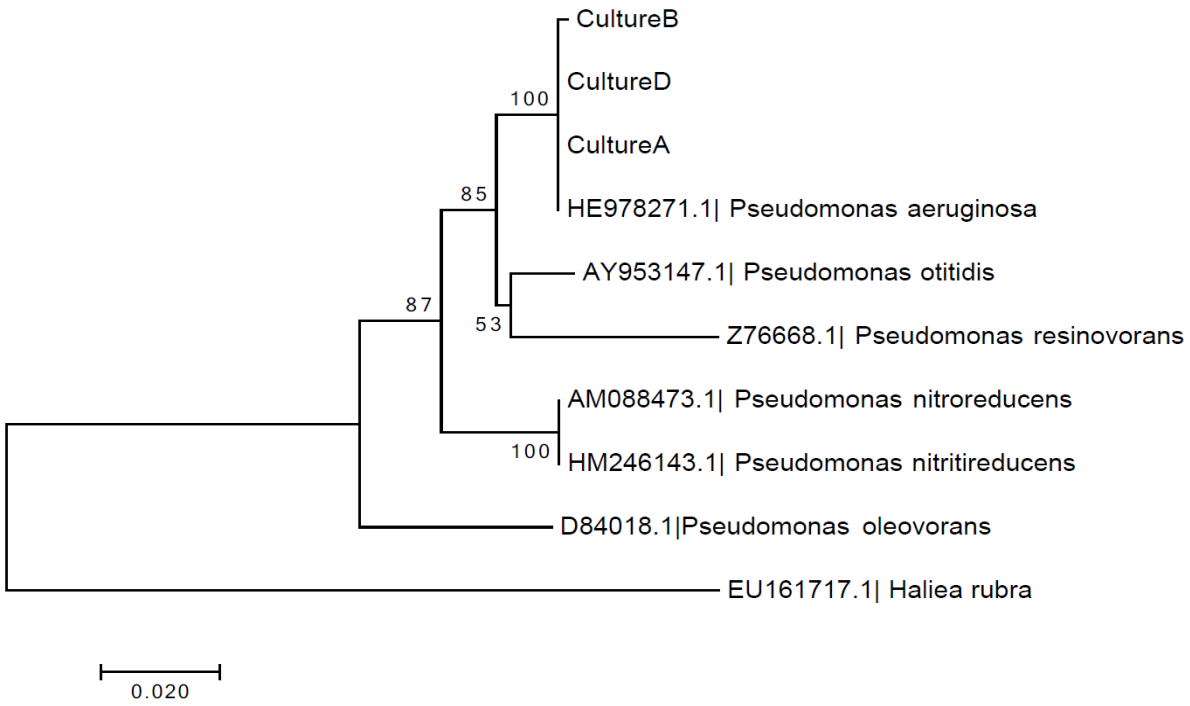


Figure 4-1: Phylogenetic tree generated from the 16S rRNA genotype fingerprinting analysis of the Gram-positive colonies using Haliea rubra as an outgroup.

4.2. Physical Properties of Biosurfactant

4.2.1. Surface Tension

During biosurfactant production, surface tension of cell free supernatant was measured periodically for 12 d. It was observed as represented in Table 4-1, that in the first 5 d of incubation surface tension dropped significantly from 55 mN/m to 28 mN/. Between day six and day nine, a slight surface tension decrease was observed (day 12 surface tension was 27 mN/m), meaning that the quantity of biosurfactants produced was sufficient to reach the possible lowest surface tension. This proves that the biosurfactants produced, was enough for the medium to reach the lowest possible surface tension, the critical micelle concentration (CMC).

Table 4-1: Surface tension of the media during biosurfactants production

Time (d)	0	1	2	3	4	5	6	7	8	9
Surface tension (mN/n)	55	53.69	50.09	42.13	30.89	28.46	28.34	28.46	28.09	27.82

The quantity biosurfactants produced in a medium, can be expressed in Critical Micelle Dilutions (CMD). CMD indicates the dilution necessary for the medium to reach the critical Micelle concentration, a point where the solution interface becomes saturated with amphiphiles and the addition of surfactants beyond that point results in surfactants self-assemble, forming Micelles and populate the bulk phase with slight effort, effecting the surface tension (Chakraborty *et al*. 2011). Figure 4-2 indicates a graph representing the biosurfactant concentration required to reach the lowest surface tension of the medium. Dilution factor of 49 or 49 x CMD of biosurfactant concentration, is required to reach surface tension average of 29.326 mN/m, corresponding with 28 mN/m lowest surface tension average measured during producing biosurfactants.

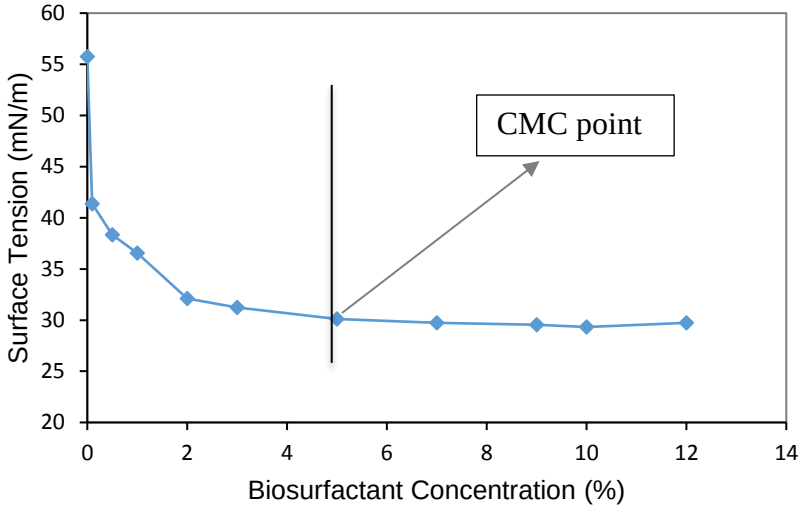


Figure 4-2: Critical micelle dilution graph

4.2.2. Emulsification Index (E24)

To examine the quality of culture producing biosurfactant, their ability to form and stabilise emulsions is evaluated. Figure 4-3 indicates the emulsification formed after 48 h with E24% of 64.29%, 52% and 55.3% from left to right respectively. Culture CB held the most emulsification and was chosen for producing biosurfactants.

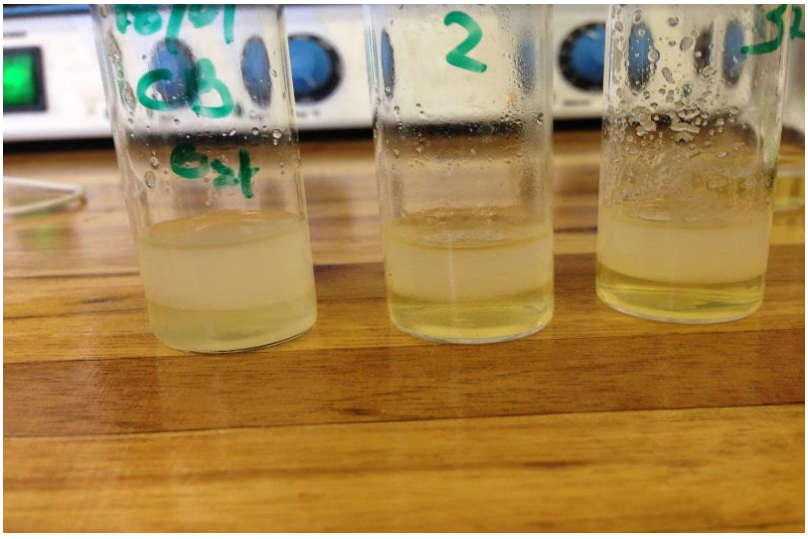


Figure 4-3: E24 % results showing microbial potential to produce biosurfactant

4.2.3. Foaming

The presence of the foam indicates the availability of surfactants in the solution. Foam formation results when surfactants become concentrated at a gas-liquid interface, leading to formation of bubbles through the liquid and on the interface (Satpute *et al*. 2010). Figure 4-4 presents foam formation results for both batch and CSTR experiments.

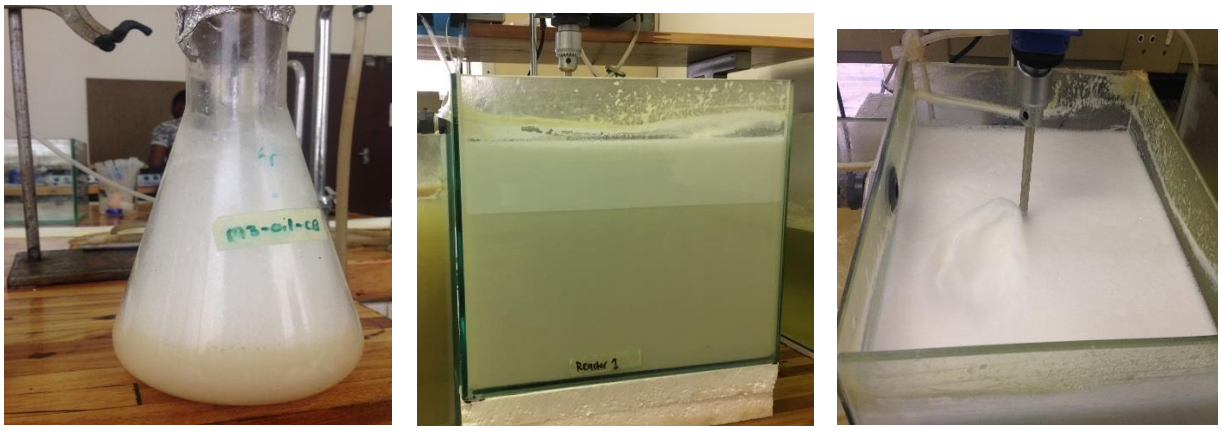


Figure 4-4: Foaming results (left=batch, center = CSTR side view, right = CSTR top view)

4.3. Chemical Characterization of Biosurfactants

4.3.1. Thin Layer Chromatography

The composition of biosurfactant was determined, using TLC plates. The method to characterise biosurfactants, was adopted from Sharma *et al*., (2014). OnemL aliquot of partially purified crude biosurfactant was separated on a pre-coated silica gel plate, using chloroform methanol:glacial acetic acid (65:15:2 v/v) as a solvent system. The plate was sprayed with a prepared ninhydrin solution (2 g of ninhydrin dissolved in 100mL of acetone) and dried in an oven at 110 ^{o}C for two minutes. The appearance of a pink spot on the TLC indicated a positive reaction, when biosurfactant reacted with ninhydrin, signifying the presence of peptide moieties that could be the lipopeptide. Figure 4-5 indicates a picture from the TLC test of the biosurfactants, used for dissolving fluoranthene and triphenylene.

Figure 4-5: TCL plate with pink spot from reaction of biosurfactant with ninhydrin

4.3.2. Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) is used to explain the chemical structure of unknown samples by identifying types of functional groups. These infrared absorption bands, identify specific molecular components and structures (Pornsunthorntawee *et al*. 2008). Partially purified biosurfactant was analysed and the FTIR spectrum indicated –NH- (peptide group) broad absorbance peak centred on 3264 cm^{-1}, C-H (alkane group) sharp peak around 2925 cm^{-1}, C-ON (amide group) strong stretching mode peak around 1713 cm^{-1}, $-CH_3$,CH_2- (aliphatic chains) medium weak multiple bands from around 1457 to 1418 cm^{-1} and C-N (amine group) strong stretching mode peak around 1084 cm^{-1}. A wide range of literature indicates these as characteristic of lipopeptide.

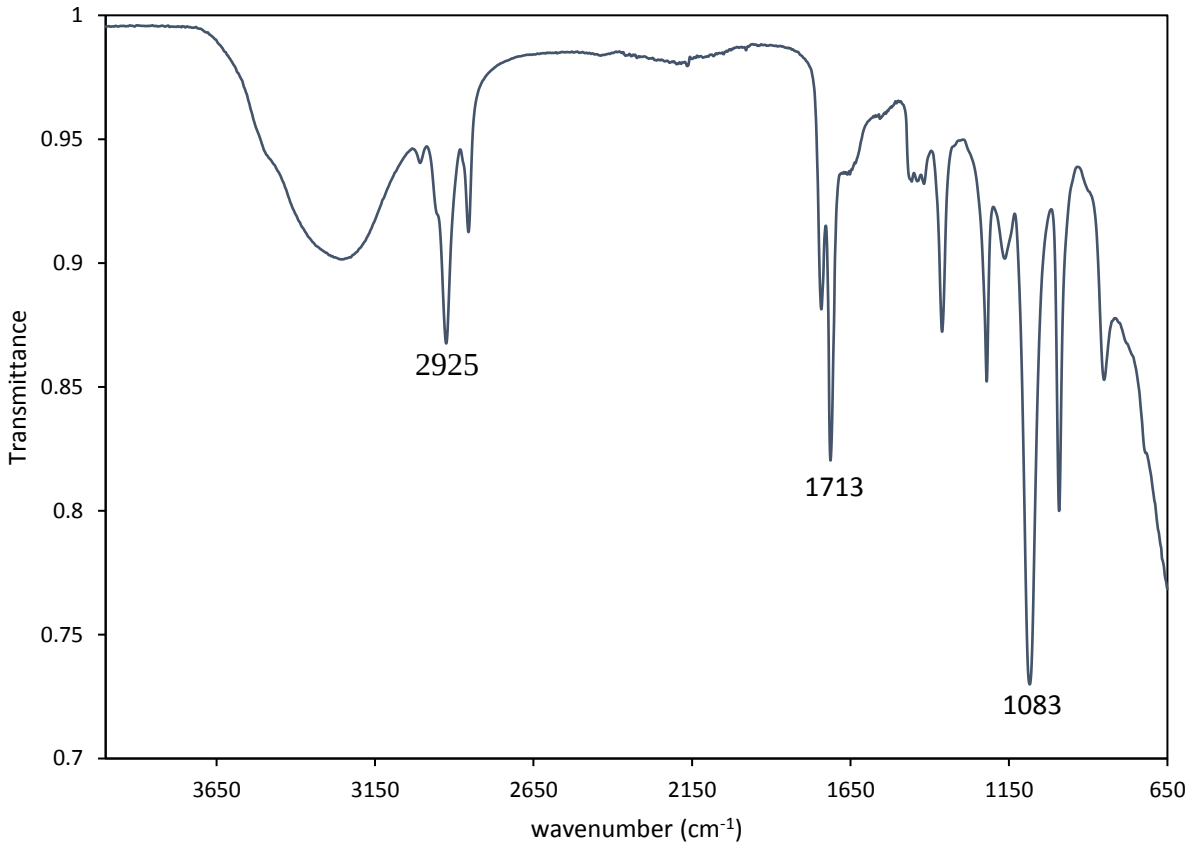


Figure 4-61: Fourier transform infrared (FTIR) absorption spectrum of biosurfactant produced by Pseudomonas aeruginosa strain

4.4. PAHs Dissolution Dynamics With and Without Biosurfactants

4.4.1. Fluoranthene Dissolution Studies in Batch

Various concentrations of biosurfactants were added to a 100mL Erlenmeyer flask supplemented with 70mg of fluoranthene and triphenylene. 25mL, 50 mL, and 100mL of biosurfactants were added to three flasks to determine the solubility of these compounds; 105.92, 126.758, and 240.911 mg/L fluoranthene was dissolved with above-mentioned biosurfactants concentrations respectively. 46.863, 51.079, and 65.045 mg/L of triphenylene was dissolved with above-mentioned biosurfactants concentrations respectively. The concentration of dissolved PAHs increased linearly with increasing concentration of biosurfactants (R^2 = 0.9906 for fluoranthene and R^2 = 0.9447 for triphenylene). At 25mL of biosurfactants, dissolved fluoranthene was 407.38

times higher solubility than its normal aqueous solubility at room temperature (0.26 mg/L. From Figure 4-7, it can be concluded that the effect of biosurfactants is more prominent on less soluble PAHs.

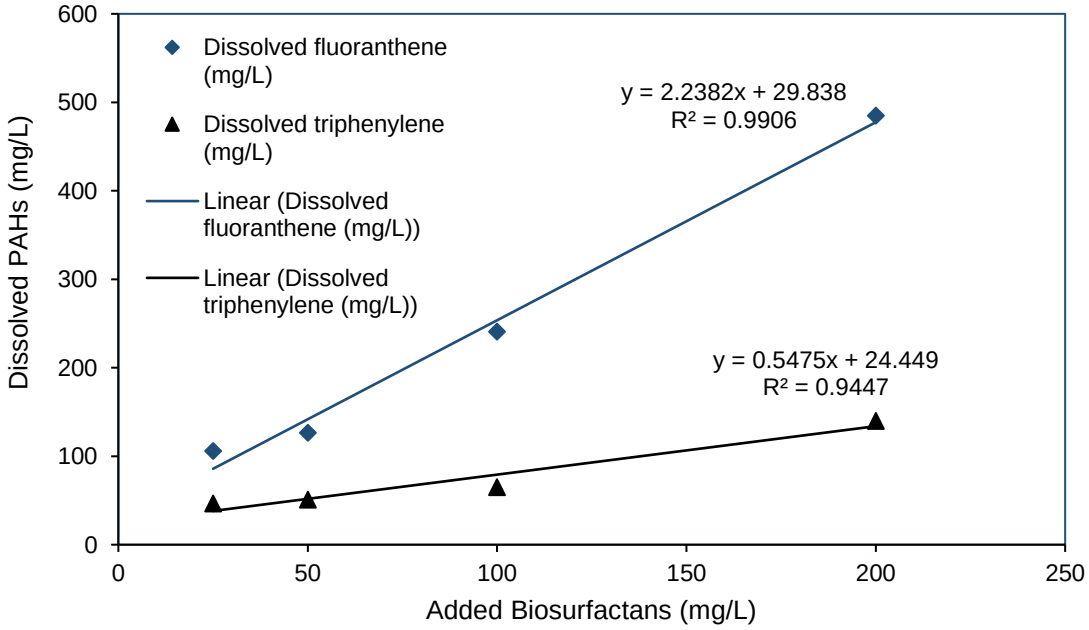


Figure 4-7: Dissolution curves for fluoranthene and triphenylene

4.4.2. Fluoranthene Dissolution in CSTR System

Two runs were performed for the experimental purposes. A preliminary run was performed, using a feed-batch into the reactor instead of feeding the dissolved PAHs from the Reactor tank 1. A 400mL solution with 157mg/L of biosurfactants-dissolved PAHs was prepared in a 1L flask, adding 200mL of dissolved PAHs from stock solution prepared during PAH solubility studies during batch experiments to 200mL of MSM. For the actual experimental CSTR run, biosurfactants were produced in reactor Tank 1 over a period of 12 d. After production of biosurfactants, 15 g of fluoranthene was added to the reactor Tank 1 with biosurfactant solution of 29.12 L. An average of 350 mg/L was measured after taking multiple initial concentrations after the addition of fluoranthene to the tank within the first eight-hour period.

4.5. Summary

Isolated cultures were identified as Pseudomonas aeruginosa using 16S rRNA genotype fingerprinting analysis. The isolated strains also indicated great potential of producing biosurfactants by positive results from drop-collapse and oil displacement. Biosurfactants were successfully produced using the Pseudomonas aeruginosa strains and were physically examined by measuring the surface tension and the emulsification index (E_{24}). Surface tension of ultrapure water was dropped from 55.76 to ± 28 mN/m with the presence of biosurfactants. Biosurfactants were chemically characterised, using the FTIR and the TLC analysis and were determined to be lipopeptide. Dissolution tests were conducted, using fluoranthene and triphenylene. It was proven that the biosurfactants produced could dissolve both compounds, respectively.

CHAPTER 5: BIOSURFACTANT-ASSISTED DEGRADATION OF HMW PAHS

5.1 Preliminary Fluoranthene Degradation Studies

5.1.1 Fluoranthene Degradation in Batch Studies using Different Cultures

Preliminary experiments were conducted using pure isolated and mixed culture to evaluate the effectiveness of each culture in reducing fluoranthene. Pseudomonas aeruginosa and a mixed culture degraded the fluoranthene. Fourteen mg/L of biosurfactant-dissolved fluoranthene was added to 100mL of MSM inoculated with 5% culture biomass in a 250mL Erlenmeyer flask. Samples were drawn from the flasks periodically (0, 6, 12, 24, 48, 96, 114 h) to measure the concentration of the remaining fluoranthene. The results from Figure indicates, both cultures can degrade fluoranthene effectively. Figure 5-1 indicates, on day seven of incubation, 97.5% of fluoranthene was degraded, using a mixed culture and 85.6% of fluoranthene was degraded using pseudomonas aeruginosa. The mixed culture had a high removal efficiency and was used for further experiments.

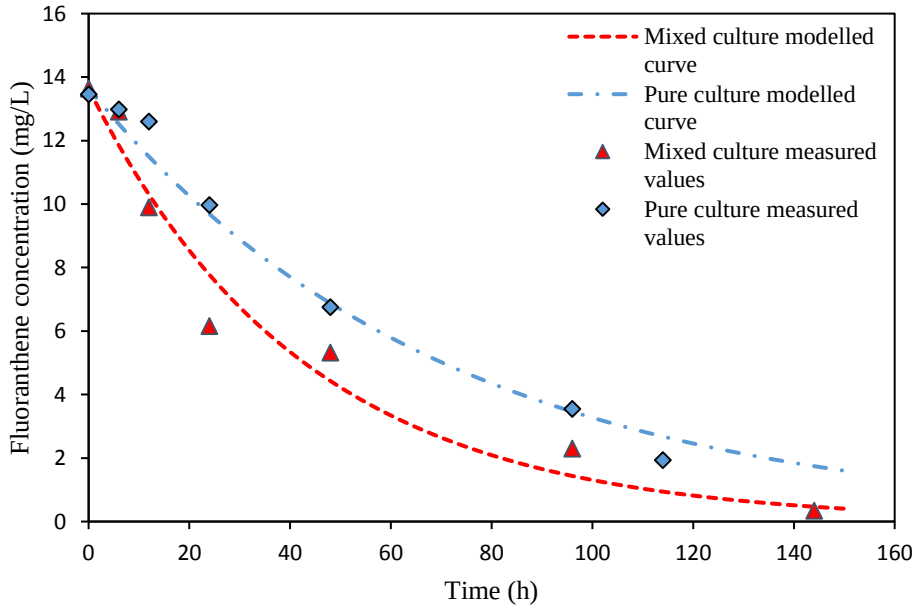


Figure 5-1: Fluoranthene degradation using pseudomonas aeruginosa (blue) and fluoranthene degradation using mixed culture (red)

5.2 Batch Studies Results

5.2.1 Fluoranthene Degradation Batch Studies

Four sets of flasks were used in batch fluoranthene degradation experiments. Experiments were performed in duplicates and an average was recorded. The first two sets were prepared, using various concentrations of fluoranthene, dissolved in biosurfactants; 100mL of MSM inoculated with +/- 5% wet culture biomass was added to these concentrations in a 250mL Erlenmeyer flask. The final concentration of fluoranthene in the flasks were 90 and 250 mg/L respectively. The third set was prepared adding 20mg of solid fluoranthene directly to 100mL of MSM inoculated with and incubated with +/- 5% wet culture biomass in a 250mL Erlenmeyer flask, composing a final concentration of 200 mg/L. The last set was prepared as a control, adding dissolved fluoranthene of 100mL of MSM with no culture inoculated in a flask. The flasks were incubated and samples were drawn from the flasks periodically (0 to 23 d), measuring the remaining fluoranthene concentration in the flasks. Figure indicates the degradation of different initial concentration in 250mL Erlenmeyer flasks.

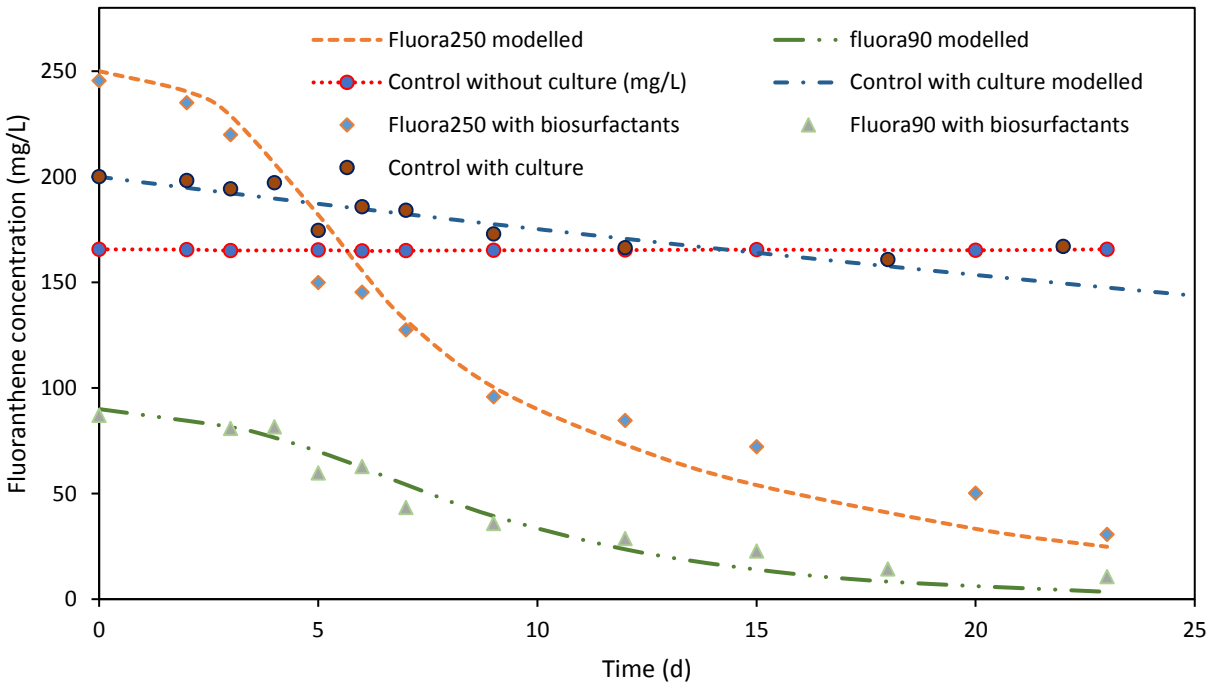


Figure 5-2: fluoranthene degradation in batch studies

Figure 5-2 indicates fluoranthene degradation results at day three of incubation with 6.3% removed from the flask containing 90 mg/L of fluoranthene, and 8.44% removed from the flask containing 250mg/L. At day six there was a 27.9% and 37.8% removal and at day 23 there was an 87.8% and 90.1% removal respectively. It is observed that the degradation rate of fluoranthene in a solution with high initial concentration is significantly high in the first six days of incubation. This observation is attributed to the high biomass concentration (high concentration of substrate yields high biomass concentration) in the solution presented in Figure 5-4, resulting in a faster removal rate of fluoranthene. Low substrate concentration yields to low biomass concentration in solution since microorganism use substrate as source of food and energy, resulting in slightly slower removal rates of fluoranthene. A degradation of fluoranthene in a batch without biosurfactants, was reported to be 16.5%. In a control batch flask, no removal of fluoranthene was observed.

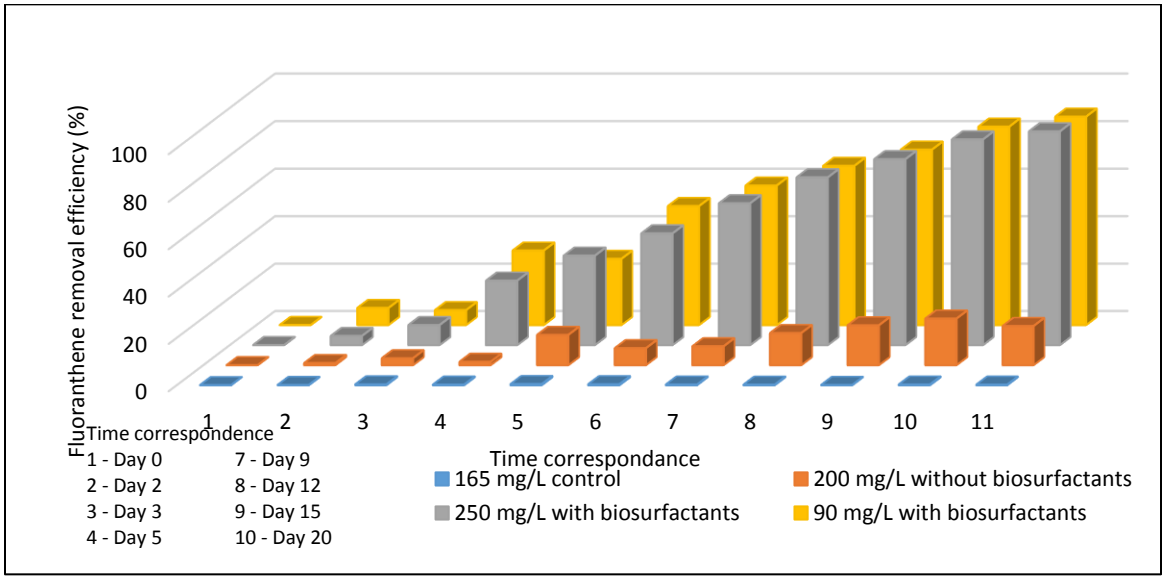


Figure 5-3: Batch fluoranthene removal efficiency in terms of percentage.

The fluoranthene batch experimental data were fitted in to the Monod model using the equations presented in section 3.12.2 and the graphs are presented in Figure 5-2. The substrate utilisation rates (μ_{max}) and half saturation coefficient (K_s) were determined using parameter estimation technique on the AQUASIM software package (Reichert, 1998).

Table 5-1: Fluoranthene kinetics parameter estimations from aquasim simulations

Parameters	250 mg/L with biosurfactants	90 mg/L with biosurfactants
μ_{max} (h^{-1})	0.2946	0.236
K_S (mg/L)	991.84	992
CHI (x^2)	2213	588.299

Table 5-1 indicates the results for μ_{max} and $K_{s,}$ after the fitting of experimental data are presented. The degradation rate coefficients for the batch with 250 mg/L and 90 mg/L are 0.295 d^{-1} and 0.236 d^{-1}. This confirms that substrate was removed slightly faster in a batch with a higher initial fluoranthene concentration. In a batch without biosurfactants, a rate of 0.011 d^{-1} was calculated. The lower removal rate is due to the unavailability of the fluoranthene, since it was added to the flask in a solid form.

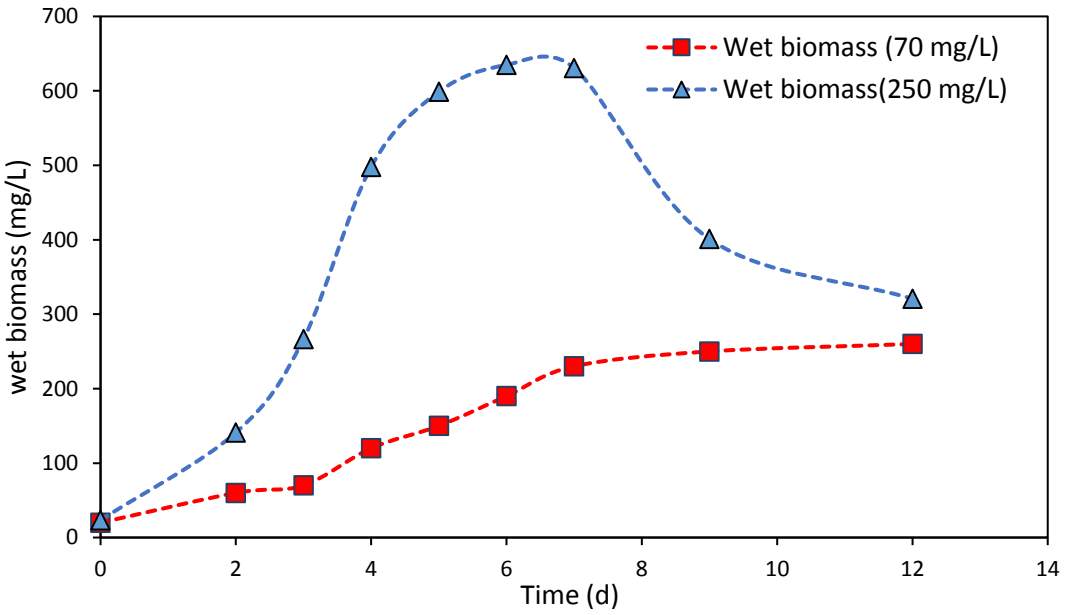


Figure 5-4: Biomass growth in for 250 mg/L batch (triangle) and 90 mg/L batch (square)

5.2.2 Triphenylene Degradation Batch Studies

Triphenylene, a High Molecular Weight Polycyclic Aromatic Hydrocarbon with a lower solubility compared with fluoranthene, was used to evaluate if they can be degraded successfully, using a microbial consortium. Various concentrations of triphenylene dissolved in biosurfactants (20 and 62 mg/L) were used in degradation experiments. Each of these concentrations was added to three

100mL of MSM inoculated with approximately 5% wet culture biomass in a 250 Ml Erlenmeyer flask and incubated. Samples were drawn from the flasks periodically (0 to 22 d) to measure the remaining triphenylene concentration in the flasks. Similar observations to fluoranthene degradation study were made when evaluating the results from two different batch experiments presented in Figure 5-5.

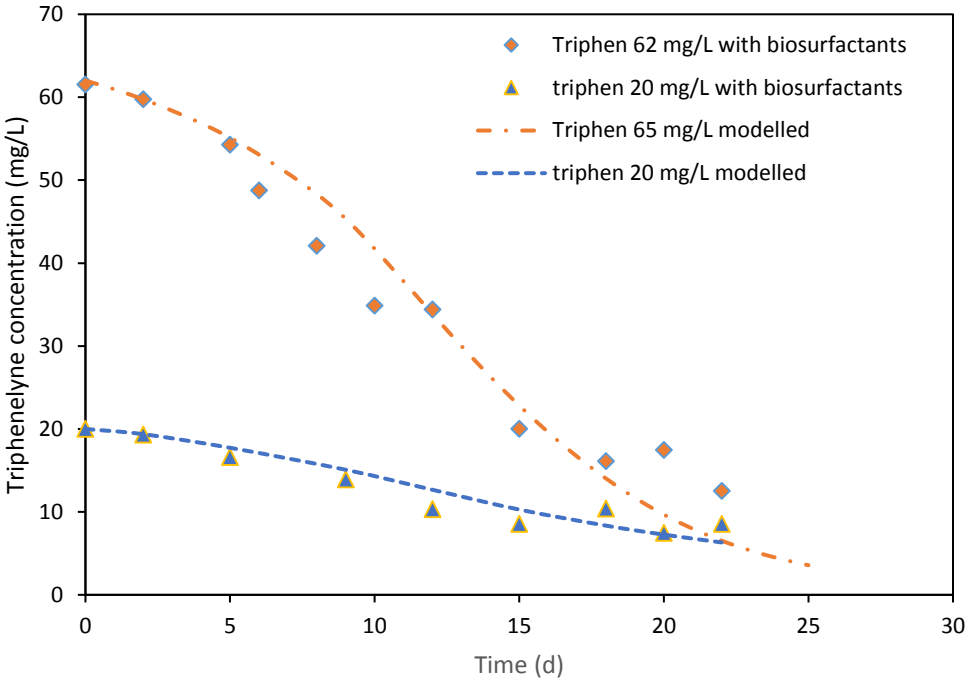


Figure 5-5: Triphenylene degradation in batch results

After five days of incubation, it is observed that the flask with high initial concentration (62 mg/L) had 11.77% triphenylene removed and the flask with the lowest initial concentration (20 mg/L) had 16.78% triphenylene removed. On day 15 there was 67.44% and 52.24% triphenylene removed from the flasks respectively. After 22 d of incubation there was 79.6% triphenylene removal from the flask with 62 mg/L initial concentration and 62.62% triphenylene removed from the flask with 20 mg/L initial concentration. Compared with the degradation of fluoranthene, removal rate of triphenylene was slower; on day 12, fluoranthene indicated a higher removal percentage than triphenylene.

The Triphenylene batch experimental data were also fitted into the Monod model and the results are presented in Figure 5-5. The substrate utilisation rates (μ_{max}) and half saturation coefficient

(K_s) were then determined, using parameter estimation techniques on the AQUASIM software package (Reichert, 1998). The results are presented in Table 5-2 below.

Table 5-2: Triphenylene kinetics parameter estimations from aquasim simulations

Parameters	62 mg/L with biosurfactants (equation 3)	20 mg/L with biosurfactants (equation 3)
μ_{max} (h^{-1})	0.116	0.128
K_S (mg/L)	234	451
CHI (x^2)	224.14	20.2

The degradation rate coefficients for the batch with 62 mg/L and 20 mg/L are 0.116 d^{-1} and 0.128 d^{-1}. This confirms that substrate removal rate for fluoranthene was higher than the substrate removal rate of triphenylene.

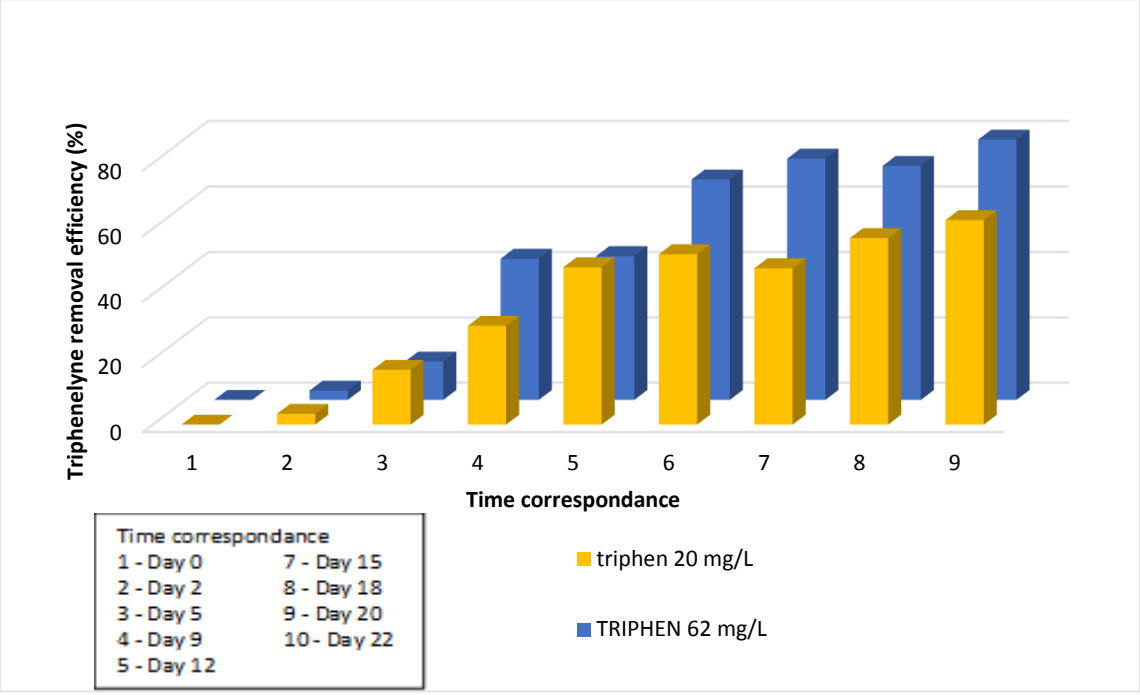


Figure 5-6: Batch triphenylene removal efficiency in terms of percentage.

5.3 Continuous Flow Kinetics

5.3.1 Tracer Profile

Tracer studies were conducted to evaluate the performance of the CSTR system and the distribution of Sodium compound as an illustrate substrate from one reactor compartment to another. This was performed with the aim to investigate if the measured values would resemble a pattern similar to that of a modelled curve from Aquasim software. The curve was simulated using the same conditions the reactor held during the tracer study experimental run. The inflow to the reactor at a rate of 0.8 L/h had an initial sodium concentration of 340 mg/L. Samples were taken from various compartments periodically for 7 d. The results in Figure 5-7 indicate a very close relationship between the modelled curve and the measured values. The modelled curve cuts through the measured values and the measured values trend is at a proximity with the modelled curve. This observation then used modelled curves to evaluate the degradation of PAHs with continuous flow.

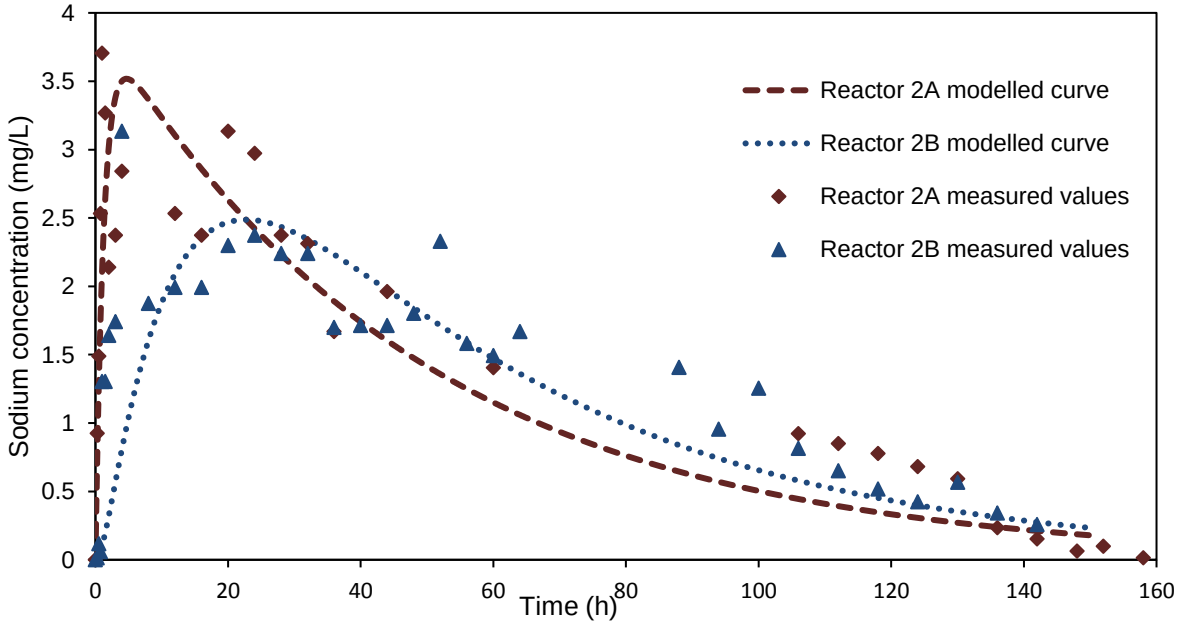


Figure 5-7: Tracer studies results (dotted points) with simulated curves simulating the same conditions

5.3.2 Fluoranthene Degradation in Feed-Batch CSTR using Culture Consortia

A preliminary run was performed using a feed-batch system instead of feeding the dissolved PAHs from reactor Tank 1 to test-run the performance of the reactor and the behaviour of fluoranthene

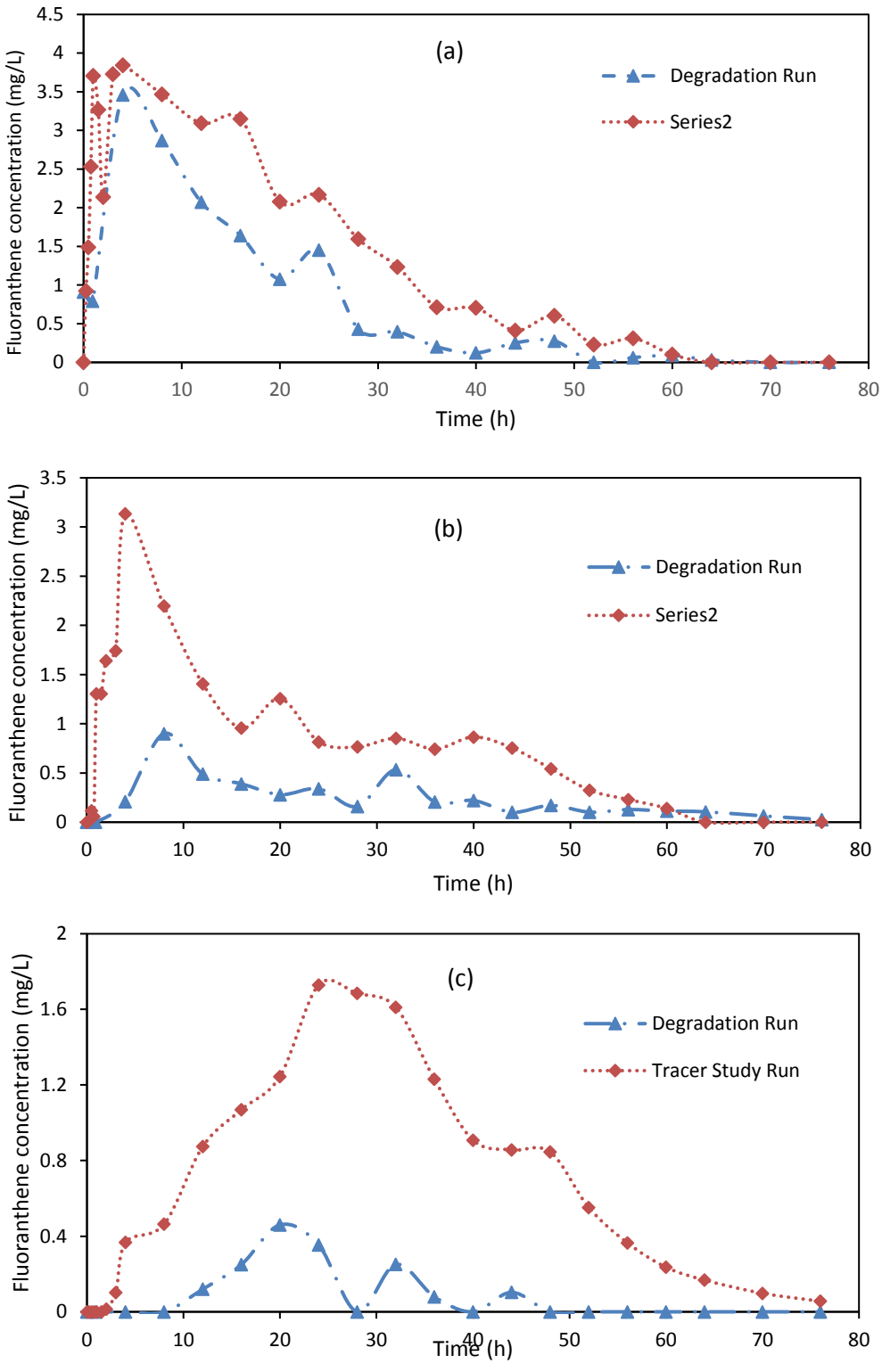


Figure 5-8: Three stage system preliminary CSTR fluoranthene degradation results. (Top: Reactor 2 in-tank results. Centre: Reactor 2 effluent results. Bottom: Biofilm effluent)

in the reactor. Dissolved fluoranthene with concentration of 463.89 mg/L was used for the feed, fed for 4 h. The results in Figure 5-8 indicates that there is degradation of fluoranthene in the system when comparing the tracer studies run results and the degradation run results. The graphs do not show smooth curves, due to factors, such as human errors and instrumental errors.

Figure 5-8 presents results, indicating degradation in the system. This was proved by the gap between the tracer study data (red line) and the degradation data (blue line) indicating that fluoranthene was removed from the water during the system's operation. From figure 5-8(a), it was observed that during the first eight hours of the system's run, the tracer and degradation study data points are very close to each other. This indicates that during the first eight hours of the run, there were close to zero fluoranthene degradation in the system. This is attributed by introducing the toxic fluoranthene into the reactor. Microorganisms adapted to its presence and use it as their source of carbon and energy. After the microorganisms adapted, they could use fluoranthene as their carbon and energy source and broke it down using their metabolism, resulting in its degradation. The appearance of a gap between the two trends after 8 h of run validated that the microorganism could degrade fluoranthene. The highest fluoranthene concentration inside Reactor 2A compartment was 3.46 mg/L; the tracer study compound concentration was 3.84 mg/L, indicating degradation success.

Figure 5-8(b) indicates the results from Reactor 2 effluent. The results indicate that the highest effluent from the compartment was 0.9 mg/L with an average effluent of 0.45 mg/L. The gap between the tracer and degradation study runs, still indicates degradation of fluoranthene in the system.

Figure 5-8(c) indicates the results of fluoranthene degradation in the biofilm tank compartment. The highest influent concentration from Reactor 2 into the biofilm tank was 0.9 mg/L and the highest effluent from the biofilm tank was 0.46 mg/L with an average effluent of 0.28 mg/L. The fluoranthene removal in the system was calculated, considering the highest concentration inside the Reactor 2A compartment and the highest effluent from the biofilm tank. Fluoranthene removal from the aqueous solution in the system was 86.7%

5.3.3 Fluoranthene Degradation in a CSTR System

5.3.3.1 Reactor 2A degradation study

Prior to the experimental run, microbial consortia were grown for 3 d in the system. At the initiation of the experiment, the microorganism population was huge. Fluoranthene, dissolved in the Reactor 1 compartment, was pumped into the Reactor 2A compartment. A timer was set to pump for 30 min and stopped for 30 min to add dissolved fluoranthene into Reactor 2A at a rate of 0.691 L/h for a period of 36 h. During the first 36 h of the run, limited fluoranthene removal was achieved from the Reactor 2A compartment, compared to the difference between the two trends after 30 h. When fluoranthene was first pumped into the reactor, the microorganisms responded to their introduction and had to adapt, using the fluoranthene as their substrate. Thereafter the removal percentage of fluoranthene increased, signifying that the microorganisms could adapt and use fluoranthene as their substrate, leading to its degradation through their metabolism. Figure 5-9 observes a difference between the measured values and the modelled curve (abiotic system), attributed by the presence of fluoranthene degrading microorganism. The gap between the two trends indicates fluoranthene removal from Reactor 2A, using the area under the tracer curve and the measured value curve, indicating total fluoranthene removed from the Reactor 2A compartment. From annexure IV, tracer curve area (a) is 5141 mm^2 and measured values curve (b) is 2010.5 mm^2; 60.89% of fluoranthene was removed from compartment 2A.

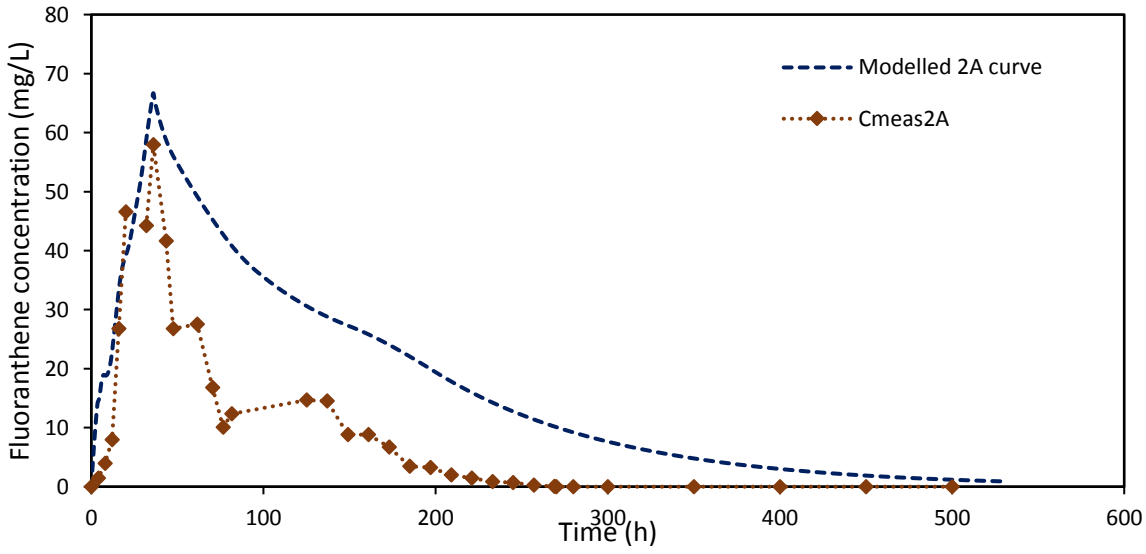


Figure 5-9: Reactor 2A effluent

5.3.3.2 Reactor 2B degradation study

Figure 5-10 indicates a difference between the measured values and the modelled curve, attributed by the presence of fluoranthene degrading microorganisms. It was also observed from the graph that the measured values trend has a close relationship to the modelled curve. The area under the tracer curve and the measured value curve was used to calculate the total fluoranthene removed from the Reactor 2B compartment. From annexure IV, tracer curve area (c) is 5317 mm^2 and measured values curve (d) is 1594 mm^2; 70.02% of fluoranthene was removed from compartment 2A

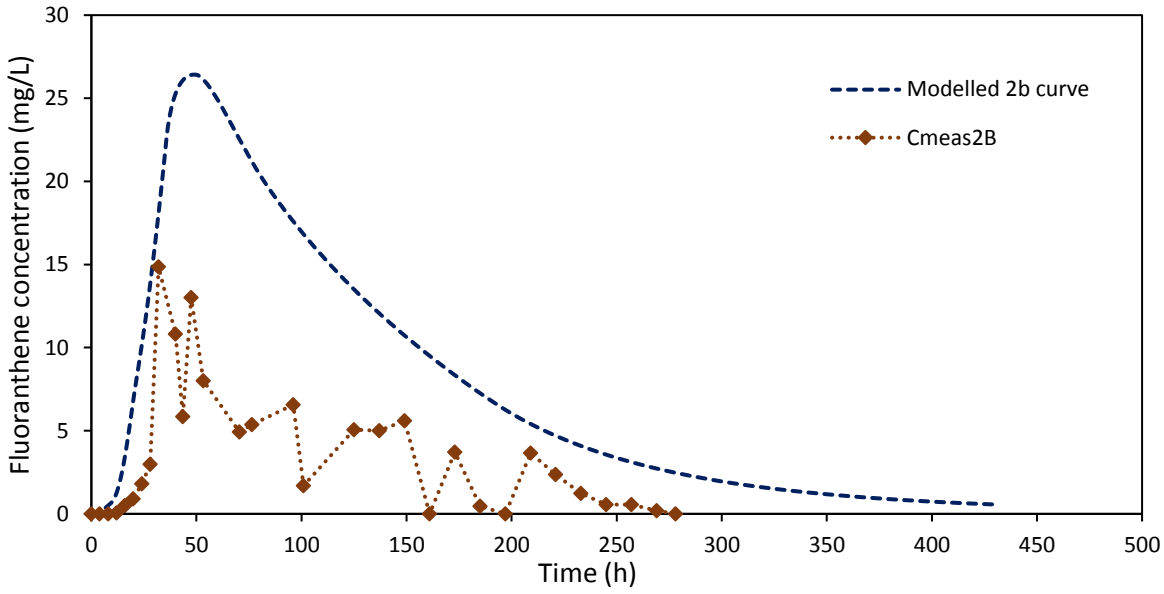


Figure 5-10: Reactor 2B effluent

5.3.3.3 Biofilm reactor degradation study

Initial concentration of incoming flow to biofilm reactor compartment was 14.86 mg/L and the highest effluent from this compartment was 4.60 mg/L. Using the area under the tracer curve and the measured value curve, totalling fluoranthene removed from the biofilm tank compartment. From annexure IV, tracer curve area (e) is 4147.5 mm^2 and measured values curve (f) is 947 mm^2; 77.17% of fluoranthene was removed from the biofilm compartment.

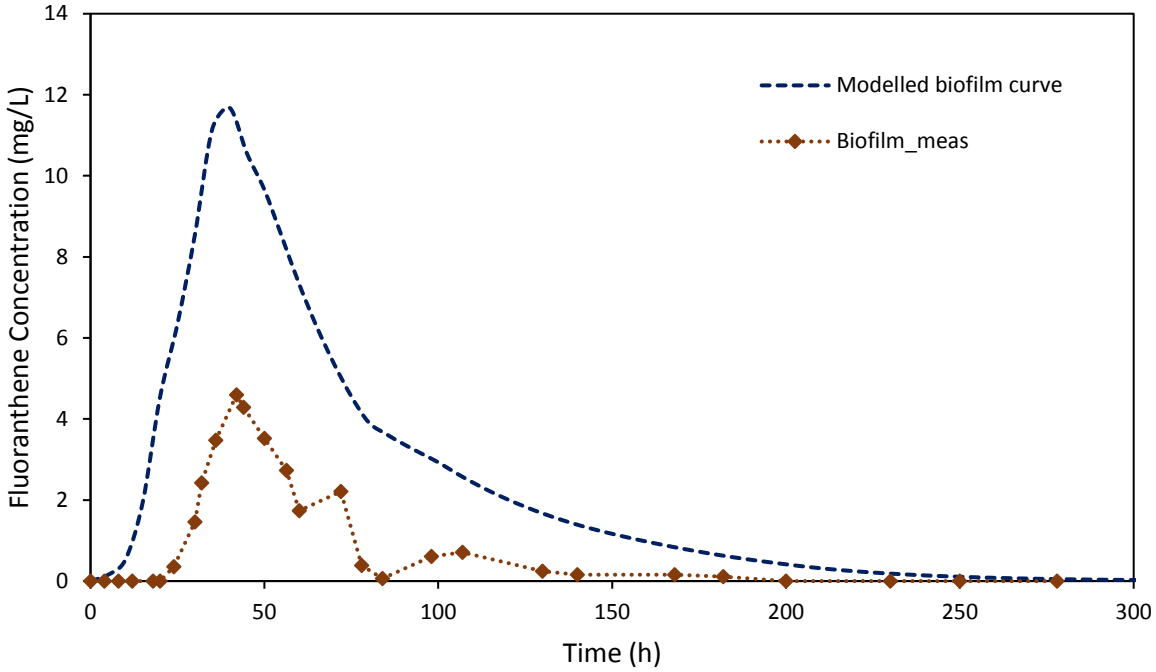


Figure 5-11: Biofilm reactor tank effluent

5.3.4 Biofilm Mass Balance

The degradation of fluoranthene was analysed by conducting a mass balance. The theoretical degradation of fluoranthene representing 100% removal of fluoranthene is plotted with the actual measured fluoranthene degradation in Figure 5-12. The closeness of the two plots signified a high degradation in the system. In phase I on the graph, the very close relationship between the two plots indicated, nearly all the fluoranthene in the influent was degraded. From phase II the system was decreasing in removal percentage, reflected by the shift of the measured data curve from the theoretical curve. Phase III reflects a system failure on the degradation of fluoranthene, indicated by an almost horizontal line on the measured data curve. This is attributed by the system being overloaded with the fluoranthene, leading to a decrease in removal percentage. In phase IV, the parallel line of the measured data to the theoretical curve, reflected that the system recovered, indicating a high fluoranthene removal efficiency.

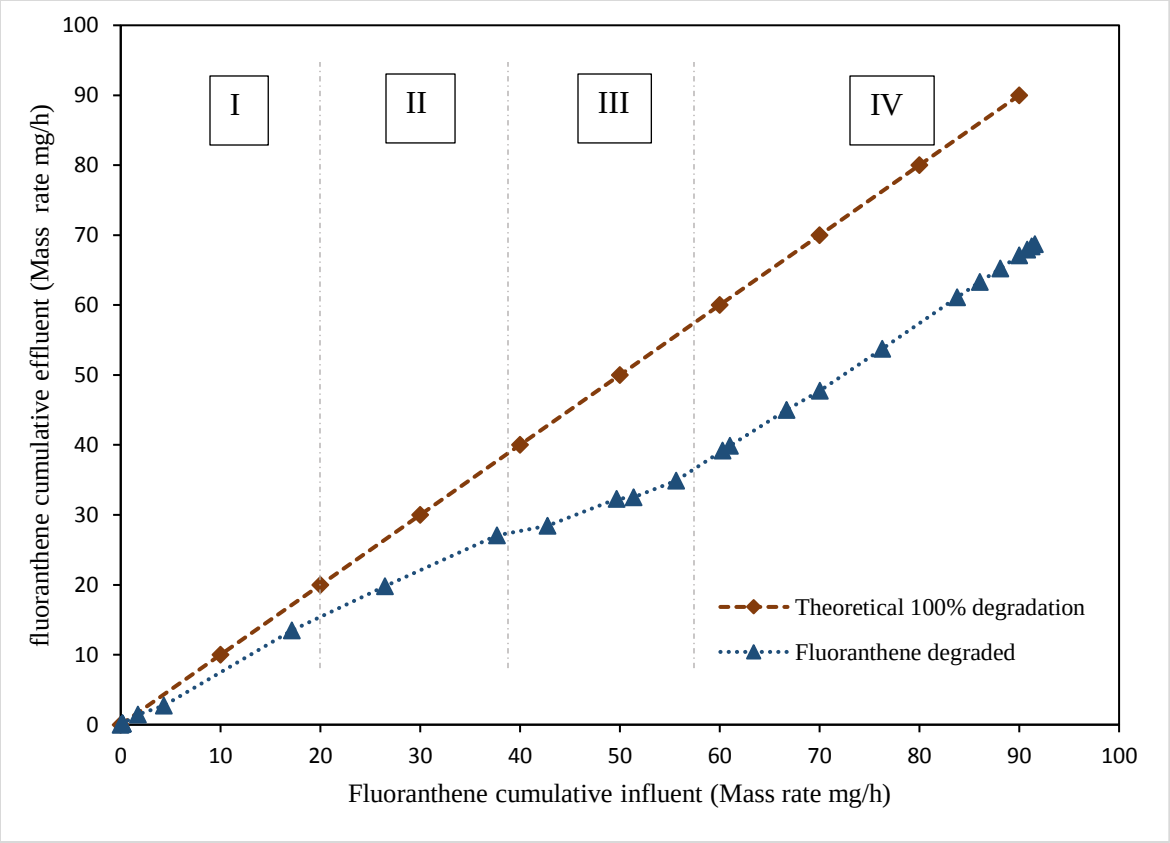


Figure 5-12: Cumulative fluoranthene influent to the biofilm tank and cumulative effluent from the biofilm tank

5.4 Summary

Pseudomonas aeruginosa was isolated from the engine oil contaminated soil and microbial consortia could degrade fluoranthene in batch studies during preliminary experiments. Although both cultures could degrade fluoranthene, microbial consortia proved to hold a higher removal rate than single culture pseudomonas aeruginosa. Results from batch experimental data using only microbial consortia, indicated that in a system with higher initial concentration of both fluaoranthene and triphenylene, there was a higher removal of the pollutants within the first three days of incubation. Fluoranthene was successfully dissolved and degraded in a continuous stirred tank bio-reactor. The performance percentage of fluoranthene removal per stage, were 70.87%, 77.94% and 88.55% for Reactor 2A, Reactor 2B and biofilm tank compartments, respectively. Examining each individual reactor compartment performance indicated: 60.89% of Reactor 2A

fluoranthene influent was degraded, 70.02% of Reactor 2B fluoranthene influent was degraded and 77.17% of biofilm tank fluoranthene influent was degraded. This indicates that the biofilm tank compartment had the best performance in fluoranthene removal efficiency.

CHAPTER 6: ADVANCED CONTINUOUS FLOW REACTOR KINETICS

6.1 Kinetic Studies Theory

6.1.1 Stirred Tank Bioreactor Model Assumption

To model the kinetics of fluoranthene kinetics in the stirred tank reactor compartment 2 a model described in section 3.12.2 was used. The concentrations of fluoranthene in the reactor did not cause any measurable growth, and therefore the first order substrate utilisation rate with biomass as a constant catalyst was used to describe the kinetics using equation:

$$\frac{dC}{dt} = -k_d\, CX \qquad [6\text{-}1]$$

Where variables are:
C (mg/L) = substrate concentration
X (mg carbon/L) = biomass concentration
t (h) = time
k_d (mg substrate/ mg carbon/h) = the maximum substrate utilisation rate per unit biomass

6.1.2 Biofilm Tank Model Assumptions

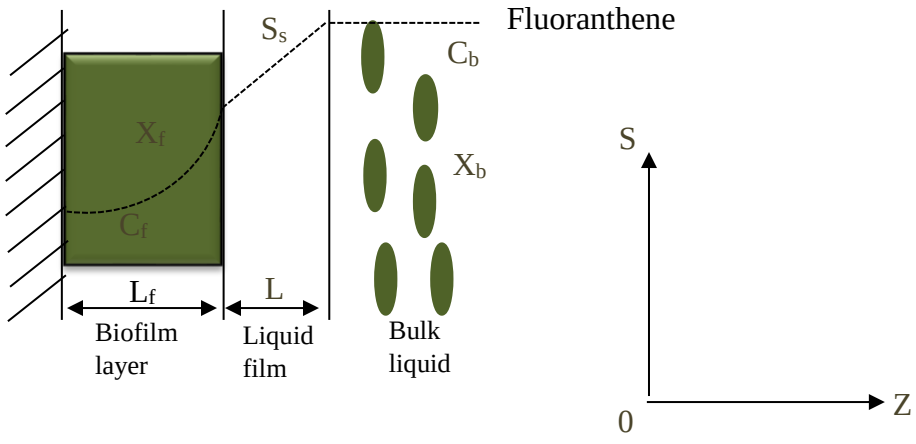


Figure 6-1: Substrate concentration profile in the biofilm (Lin *et al*. 2009)

Figure above indicates a concentration profile (hypothetical) of fluoranthene biodegradation in the bulk liquid, liquid film and biofilm. To model the kinetics of fluoranthene degradation, adopted from Lin *et al*., (2009), the following assumptions are made:

- Biofilm is homogeneous
- The density of biofilm remains constant
- The increase in the biofilm thickness is due to microbial growth and the anaerobic microflora from suspended growth is negligible
- The flow pattern of the liquid is not influenced by biofilm growth
- The substrate is transported from bulk liquid - liquid film - biofilm in the Z direction by molecular diffusion

Based on the assumption above, the balance for fluoranthene in the biofilm can be expressed by the following equation (6):

$$\frac{dC_f}{dt} = D_f \frac{d^2 C_f}{dZ_f^2} - \frac{k_f C_f}{(K_{Sf} + C_f)} X_f \qquad [6\text{-}2]$$

Where C_f is the concentration of fluoranthene in the biofilm; X_f is the biofilm density of fluoranthene degrading cells; D_f is the diffusion coefficient for fluoranthene in the biofilm; Z_f is the radical distance in biofilm (L); k_f is the monod maximum specific utilisation rate of fluoranthene in the biofilm, K_{sf} is the monod half-velocity coefficient of fluoranthene in the biofilm.

The balance for fluoranthene in the bulk liquid can be described by the following equation:

$$\frac{dC_b}{dt} = \frac{Q}{V\varepsilon}(C_{b0} - C_b) - k_f(C_b - C_S)\frac{A}{V\varepsilon} - \frac{k_d C}{(K_{Sb} + C_b)} X_f \qquad [6\text{-}3]$$

Where C_b is the concentration of fluoranthene in the bulk liquid; C_s is the fluoranthene concentration in the liquid-biofilm interface; X_f is the biofilm density of fluoranthene degrading cells; Q is the influent flow rate; V is the working volume of the reactor; ε is the reactor porosity; A is the total surface area of the media; k_d is the monod maximum specific utilisation rate of fluoranthene in the bulk liquid; K_{sb} is the monod half-velocity coefficient of fluoranthene in the bulk liquid.

6.2 Continuous Flow Kinetics Studies

6.2.1 Continuous Stirred Tanks Bioreactors Kinetic Studies

During the continuous flow experiments, the biomass in Reactor 2A and 2B was considered constants and the first order substrate utilisation rate with biomass as a constant catalyst was used to describe the kinetics. To determine the kinetics in reactor compartments 2A and 2B, first order substrate utilisation rate with biomass as a constant catalyst was used.

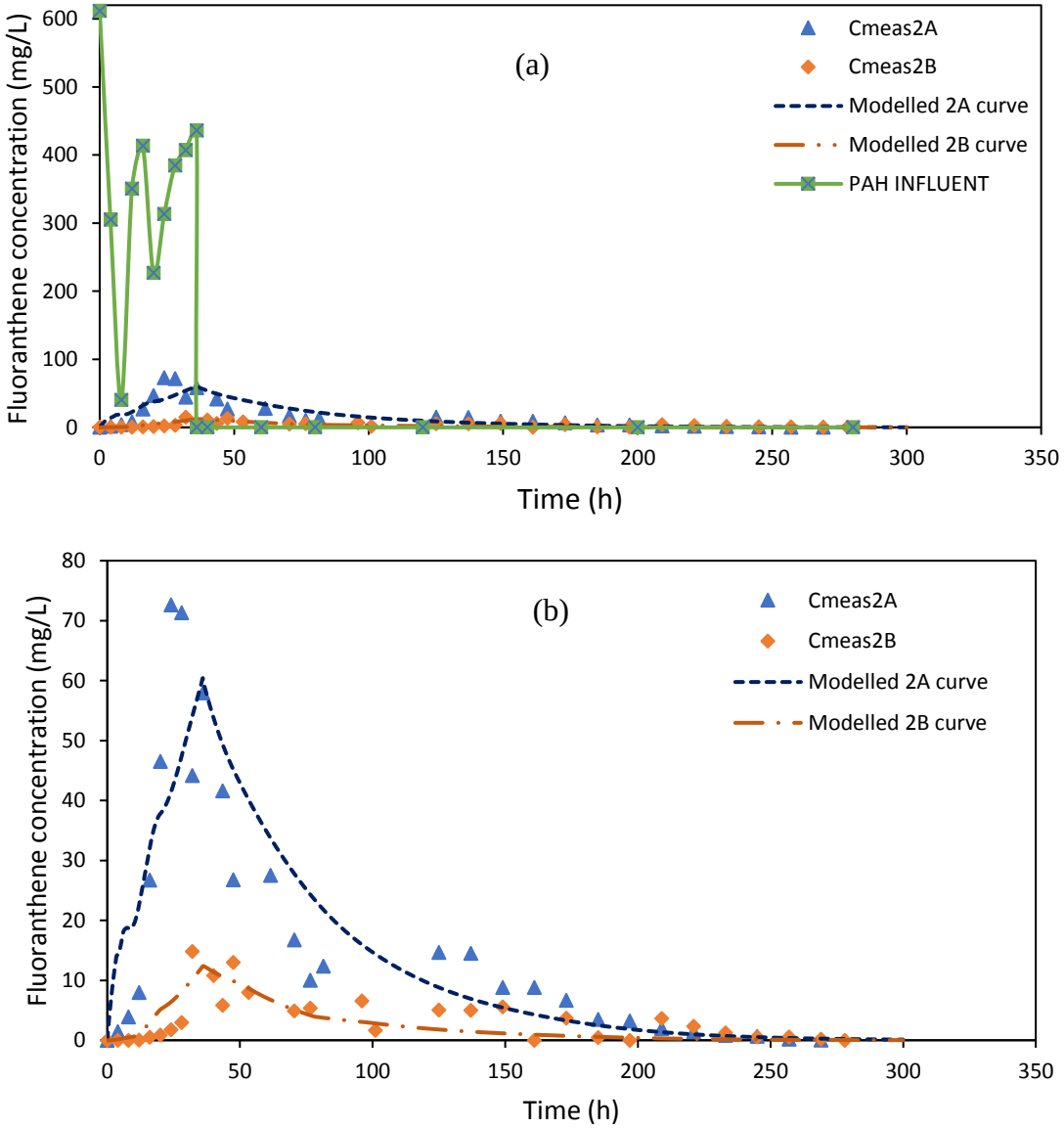


Figure 6-2: Model fits for fluoranthene degradation in the continuous flow system from reactor compartment 2A and reactor compartment 2B. (a) Represents the model fits including the influent data from reactor 2A. (b) Is a downscaled figure for the model fits.

Figure 6-2 presents the experimental data fits on to the model and the parameter estimations for both compartment 2A and compartment 2B are presented in Table 6-1 respectively.

Table 6-1: Fluoranthene continuous flow kinetics parameter estimations from aquasim simulations

Parameters	Compartment 2A	Compartment 2B
k_d (h^{-1})	0.00032	0.0054
CHI (x^2)	584.5	557.31

In an ideal reactor, a perfectly mixed continuous tank reactor is modelled. In reality, ideal systems rarely exist and there is often an observation of various behaviour from the expected exemplar or idealised reactor. Three concepts with an effect resulting in a non-ideal model includes: the distribution of residence time in the system, the quality of mixing and the model used to describe the system.

In a system with inadequate distribution of residence time, the model may indicate that underestimating or overestimating the real measured values, due to the inaccuracy in the residence time caused by factors, such as flow rate variations during the experimental runs, might have happened and were not recorded. In the current study, flow rates were measured frequently and every time a new flow rate was recorded, revealed variations throughout every single experimental run. From figure 6-2(b), during the first ± 100 h Reactor 2A model mostly overestimated the real measured concentration of fluoranthene in the system. This could be due to the lower inflow rates observed during the experimental runs, due to some of the flow blockages in the tubes (clips were used to slow down the flow rate and sometimes they would clog the tubes at the clipping points). The increase in the retention/residential time in the system, allowed longer contact time between the fluoranthene compounds and the biomass, resulting in more fluoranthene degradation. After 100 h of the experimental run, the model underestimated the concentration of fluoranthene in the system. There was almost no blockage of the tubes to decrease the flow.

In systems with lower or insufficient mixing, the mass transfer boundary layer thickness influence was larger than the specific reaction rate at cell surfaces. The removal rate of the compound would then decrease due to the slow transportation of the compound from the bulk liquid to the cell. If the mixing was great, the transfer boundary layer would be eliminated, resulting in the mass transport resistance elimination. Reactor 2A, had powerful stirrers which had the mass transport

resistance eliminated. In Reactor 2B, the system was regarded as a plug flow system, due to the lack of stirring in the reactor. This means that there was a mass transport resistance involved, reflected on Figure 6-2(b), by the underestimation of fluoranthene concentration in the system on most of the modelling of the Reactor 2B data.

6.3 Biofilm Kinetic Study

6.3.1. Mass Balance for Substrate in the Bulk Liquid

A general description of conversion on a completely mixed liquid phase of a biofilm reactor at steady state is based on a mass balance for the component of interest (fluoranthene). For fluoranthene consumed in the biofilm reactor, the steady state mass balance can be represented as follows (IWA Task Group on Biofilm Modeling 2006):

$$Q(C_{in} - C_{eff}) = j_f \times A_f \qquad [6\text{-}4]$$

Where Q is the flow rate, C_{in} is the influent concentration, C_{eff} is the is the liquid bulk concentration, j_f is the flux at the biofilm surface and A_f is the available biofilm surface area.

In a shallow biofilm system, it can be assumed that the bulk liquid is perfectly mixed and then the rate r_B is the same in the whole bulk volume and concentration in the bulk (C_B) and effluent concentration (C_{eff}) is the same.

6.3.2. Mass Balance for Substrate in the Biofilm

In the biofilm, fluoranthene is transported by diffusion due to its gradient in the z-direction (distance inside biofilm) and it is consumed by the biomass present in the biofilm. For a homogeneous shallow biofilm in a steady state, it can be expressed by (IWA Task Group on Biofilm Modeling 2006):

$$D_i \frac{d^2 C_f}{dX^2} + r_i = 0 \qquad [6\text{-}5]$$

Differential equation 9 can be solved analytically only for first or zero order reaction kinetics of biological processes. The substrate volumetric conversion rate r_i is (IWA Task Group on Biofilm Modeling 2006):

$$r_i = q_{max} \times \frac{C_f}{C_f + K_i} \times X \qquad [6\text{-}6]$$

Where q_{max} is the maximum specific substrate conversion rate, C_f is the fluoranthene concentration at the biofilm matrix, K_i is the affinity constant of substrate and X is the biomass of the biofilm. However, at high substrate concentration in the ($C_i >> K_i$) the reaction can be considered zero order while at low concentration ($C_i << K_i$) the rate can be considered first order and thus solved analytically. Using the AQUASIM model, q_{max} and K_i were estimated using the parameter estimation technique when the measured biofilm effluent was fitted into a model simulated using the substrate volumetric conversion rate equation 10. q_{max} and K_i were estimated to be 1.353 h^{-1} and 30.8998 mg/L respectively. It was then concluded that $C_i << K_i$ and thus first order kinetics was used to evaluate the flux of fluoranthene onto a shallow biofilm.

$$j_f = \frac{q_{max} \times L_f \times X \times C_{Lf}}{K_i} \times \alpha \qquad [6\text{-}7]$$

Where $\alpha = \frac{tanh\beta}{\beta}$; $\beta = \sqrt{\frac{q_{max} \times X \times L_f}{D_i \times K_i}}$

A study was done by Wicke et al (2008) to evaluate the diffusion of fluoranthene into biofilm matrix under different temperatures, it was concluded that D_i for fluoranthene under a temperature of 25 ^oC (room temperature), which was the condition for the current study, was 0.009 m^2/h (Wicke et al. 2008).

The following parameters were used to determine the flux of fluoranthene into a biofilm:

Table 6-2: Parameters used for mass balance calculations

parameters	values	origin
q_{max} (h^{-1})	1.353	Estimated in AQUASIM using parameter estimation technique
K_i (mg/L)	30.899	Estimated in AQUASIM using parameter estimation technique
D_i (m^2/h)	0.009	Literature review (Wicke et al. 2008)
X (mg/L)	230	Measured from the experiments
Q (mg/m^3)	8.63×10^{-4}	Measured from the experiments
L_f (m)	0.0005	Literature review (Cui & Kim 2013)

Table 6-3: Flux determination and comparison

Time	Biofilm_influent (mg/L)	Biofilm_effluent (mg/L)	j_i_1 (mg/m^2.h) Equation 11	J_i_2(mg/m^2.h) Equation 8
0	0.000	0.000	0.000	0.000
4	0.000	0.000	0.000	0.000
8	0.000	0.000	0.000	0.000
12	0.050	0.000	0.000	0.001
20	0.900	0.000	0.000	0.001
24	1.803	0.352	0.002	0.002
32	14.86	2.422	0.011	0.016
42	5.860	4.598	0.021	0.002
50	8.012	3.524	0.016	0.006
72	4.920	2.208	0.010	0.003
78	5.372	0.384	0.002	0.006
98	6.562	0.610	0.003	0.008
107	1.693	0.714	0.003	0.001
130	5.003	0.246	0.001	0.006
140	5.610	0.154	0.001	0.007
168	3.714	0.158	0.001	0.005
182	0.456	0.109	0.001	0.001
200	3.647	0.000	0.000	0.005
230	1.230	0.000	0.000	0.002
250	0.562	0.000	0.000	0.001
278	0.200	0.000	0.000	0.000
AVERAGE	3.355	0.737	0.003	0.003

After calculating both the flux from the mass balance for fluoranthene in the bulk liquid and the mass balance for substrate in the biofilm (Table 6-3) it can be concluded that the maximum specific substrate conversion rate (q_{max}) estimated was correct. This is due to the closeness of both the flux from the mass balance for fluoranthene in the bulk liquid and the mass balance for substrate in the biofilm which are 0.00334 mg/m^2.h and 0.00329 mg/m^2.h respectively.

CHAPTER 7: CONCLUSION AND RECOMMENDATIONS

7.1. Conclusion

The study consisted of two sets of experiment, namely: batch experiments conducted using Erlenmeyer flasks and a continuous flow experiment using CSTR – fixed film with continuous flow configurations.

During the batch studies, Pseudomonas aeruginosa isolates were sourced from an engine oil contaminated soil. The isolates were tested, establishing if they can produce biosurfactants using a drop-collapse, oil displacement and E24% methods. Isolated strains, providing positive results were selected and used for further experiments. Isolated strains providing negative results were considered to have zero potential in producing biosurfactants and was discarded. The positive strains were preserved in a glycerol solution and stored at -70^{o}C for further use in the batch and continuous experiments.

Biosurfactants were produced first in batch experiments, determining the optimal conditions in which there ia greater production. Three methods with various nutrient contents were used and the most effective method in production was determined to be Method 3, which had olive oil as carbon source and $NaNO_3$ as nitrogen source. This method was then indicated for biosurfactant production in Reactor 1 for the continuous flow experiment. Physical characteristics of biosurfactants were reported as surface tension of the medium reduction from 55 to 27.82 mN/m, emulsification index of 64.29% and foaming appearance during the production. Chemical characterisation conducted using the Thin Layer Chromatography (TLC) and Fourier Transform Infrared Spectroscopy (FTIR), suggested that the biosurfactants produced are Lipepotides.

A comparison between the isolated from contaminated soil and a mixed culture obtained from a study previously conducted at the University of Pretoria, was made to observe the microorganisms with greater potential, to degrade fluoranthene. The mixed culture indicated more degradation capacity than the pure isolates of Pseudomonas aeruginosa; the mixed culture had less biosurfactant production than that of Pseudomonas aeruginosa.

Biosurfactants were used to dissolve fluoranthene and triphenylene in batch system and only fluoranthene in the continuous flow system. It was observed from the batch experiments that fluoranthene had more dissolution factors than that of triphenylene, which is attributed by fluoranthene aqueous solubility being higher than that of triphenylene. Fluoranthene was also successfully dissolved in Reactor 1 of the continuous flow system.

Biosurfactants were used to dissolve fluoranthene and triphenylene in batch system and only fluoranthene in the continuous flow system. It was observed from the batch experiments that fluoranthene had more dissolution factor than that of triphenylene, which is attributed by the fact that fluoranthene aqueous solubility is higher than that of triphenylene. Fluoranthene was also successfully dissolved in reactor 1 of the continuous flow system.

Triphenylene degradation using the mixed culture in a batch experiments achieved 79.6% and 62.62% removal after 22 d of incubation with 62 mg/L and 20 mg/L initial triphenylene concentrations respectively. In batch system, fluoranthene degradation achieved 90.1% and 87.8% removal with 250 mg/L and 70 mg/L fluoranthene initial concentrations respectively. During the continuous; 60.89% degradation of influent fluoranthene into Reactor 2A was achieved, 70.02% degradation of influent fluoranthene into Reactor 2B was achieved and 77.17% degradation of the influent fluoranthene into the biofilm tank was achieved. The continuous flow system results indicated that the biofilm tank had the highest fluoranthene removal efficiency.

The findings of this study can add a significant value to the wastewater treatment plants that have a challenge in removing PAHs from the wastewaters rich in PAHs. The process developed in this study, is an open system that can be easily adapted and implemented in wastewater treatment and improve the wastewater effluents quality.

7.2. Recommendations

Recommendations indicate, to further study the pathway breakdown of the fluoranthene to evaluate and ensure that the by-products produced during the degradation process are less harmful and not more toxic compounds. For the current study, only few runs were conducted in the reactor and there was no determination of a breakpoint fluoranthene concentration (a concentration which will overload the system and fail). This could be a future study case where the highest fluoranthene

concentration of the reactor without failing, can be determined. An intensive study on the biofilm process can also be conducted with multiple runs of the experiments to determine more parameters to define the biofilm kinetic processes.

APPENDIX A

1. Fluoranthene Batch Simulation Data

```
************************************************************************

AQUASIM Version 2.0 (win/mfc) - Listing of System Definition

************************************************************************

Date and time of listing:  05/07/2017  10:01:23




************************************************************************
Variables
************************************************************************
C:              Description:          Fluoranthene concentration
                Type:                 Dyn. Volume State Var.
                Unit:                 mg/L
                Relative Accuracy:    1e-006
                Absolute Accuracy:    1e-006
------------------------------------------------------------------------
Cin:            Description:          Initial concentration of fluoranthe
                                      ne in batch
                Type:                 Formula Variable
                Unit:                 mg/L
                Expression:           250
------------------------------------------------------------------------
C_measured:     Description:          measured values from batch experime
                                      nts
                Type:                 Real List Variable
                Unit:                 mg/L
                Argument:             t
                Standard Deviations:  global
                Rel. Stand. Deviat.:  0
                Abs. Stand. Deviat.:  1
                Minimum:              0
                Maximum:              1e+009
                Interpolation Method: linear interpolation
                Sensitivity Analysis: inactive
                Real Data Pairs (9 pairs):
                   0               245.352
                   2               191.968
                   3               168.758
                   4               118.303
                   5               65.486
                   6               46.297
                   7               20.398
                   9               13.825
                   12              5.542
```

```
--------------------------------------------------------------------------
Ks:             Description:          Half-saturation constant
                Type:                 Constant Variable
                Unit:                 mg/L
                Value:                769.85367
                Standard Deviation:   1
                Minimum:              0
                Maximum:              1000
                Sensitivity Analysis: active
                Parameter Estimation: active
--------------------------------------------------------------------------
KsX:            Description:
                Type:                 Constant Variable
                Unit:
                Value:                20
                Standard Deviation:   1
                Minimum:              0
                Maximum:              100
                Sensitivity Analysis: inactive
                Parameter Estimation: inactive
--------------------------------------------------------------------------
t:              Description:          Time
                Type:                 Program Variable
                Unit:                 d
                Reference to:         Time
--------------------------------------------------------------------------
u_max:          Description:          Maximum substrate utilisation rate
                Type:                 Constant Variable
                Unit:                 1/d
                Value:                0.93222971
                Standard Deviation:   1
                Minimum:              0
                Maximum:              100
                Sensitivity Analysis: active
                Parameter Estimation: active
--------------------------------------------------------------------------
u_maxX:         Description:
                Type:                 Constant Variable
                Unit:
                Value:                0.8
                Standard Deviation:   1
                Minimum:              0
                Maximum:              100
                Sensitivity Analysis: inactive
                Parameter Estimation: inactive
--------------------------------------------------------------------------
X:              Description:          BIOMASS
                Type:                 Formula Variable
                Unit:                 mg/L
                Expression:           0
--------------------------------------------------------------------------
X_meas:         Description:          Biomass concentration
                Type:                 Real List Variable
                Unit:                 mg/L
```

```
                Argument:             t
                Standard Deviations:  global
                Rel. Stand. Deviat.:  0
                Abs. Stand. Deviat.:  1
                Minimum:              0
                Maximum:              1e+009
                Interpolation Method: linear interpolation
                Sensitivity Analysis: inactive
                Real Data Pairs (9 pairs):
                   0                23.4
                   2                141.03
                   3                266.59
                   4                497.98
                   5                598.754
                   6                634.785
                   7                630.748
                   9                400.958
                   12               320.781
------------------------------------------------------------------------
Y:              Description:          Yield coefficient
                Type:                 Constant Variable
                Unit:
                Value:                1
                Standard Deviation:   1
                Minimum:              0
                Maximum:              100
                Sensitivity Analysis: inactive
                Parameter Estimation: inactive
************************************************************************




************************************************************************
Processes
************************************************************************
biomass_growth:Description:          biomass growth
                Type:                 Dynamic Process
                Rate:                 (Y*u_maxX*C)*X_meas/(KsX+C)
                Stoichiometry:
                  Variable : Stoichiometric Coefficient
                  C : 1
------------------------------------------------------------------------
degradation:    Description:          Biological degradation rate of PAH
                Type:                 Dynamic Process
                Rate:                 ((u_max*C)/(Ks+C))*X_meas
                Stoichiometry:
                  Variable : Stoichiometric Coefficient
                  C : -1
************************************************************************
```

```
************************************************************************
Compartments
************************************************************************
Batch:          Description:          Batch flask
                Type:                 Mixed Reactor Compartment
                Compartment Index:    0
                Active Variables:     C
                Active Processes:     degradation
                Initial Conditions:
                  Variable(Zone) : Initial Condition
                  C(Bulk Volume) : Cin
                Inflow:               0
                Loadings:
                Volume:               1
                Accuracies:
                  Rel. Acc. Q:        0.001
                  Abs. Acc. Q:        0.001
                  Rel. Acc. V:        0.001
                  Abs. Acc. V:        0.001
------------------------------------------------------------------------
BIOMASS:        Description:
                Type:                 Mixed Reactor Compartment
                Compartment Index:    0
                Active Variables:     X
                Active Processes:     biomass_growth
                Initial Conditions:
                  Variable(Zone) : Initial Condition
                  X_meas(Bulk Volume) : 0
                Inflow:               0
                Loadings:
                Volume:               1
                Accuracies:
                  Rel. Acc. Q:        0.001
                  Abs. Acc. Q:        0.001
                  Rel. Acc. V:        0.001
                  Abs. Acc. V:        0.001
************************************************************************




************************************************************************
Definitions of Calculations
************************************************************************
calc1:          Description:
                Calculation Number:   0
                Initial Time:         0
                Initial State:        given, made consistent
                Step Size:            0.1
                Num. Steps:           150
                Status:               active for simulation
```

```
                                        inactive for sensitivity analysis
************************************************************************



************************************************************************
Definitions of Parameter Estimation Calculations
************************************************************************
fit1:          Description:
               Calculation Number:   0
               Initial Time:         0
               Initial State:        given, made consistent
               Status:               active
               Fit Targets:
                 Data : Variable (Compartment,Zone,Time/Space)
                 C_measured : C (Batch,Bulk Volume,0)
------------------------------------------------------------------------
fit2:          Description:
               Calculation Number:   0
               Initial Time:         0
               Initial State:        given, made consistent
               Status:               inactive
               Fit Targets:
                 Data : Variable (Compartment,Zone,Time/Space)
                 X_meas : X (BIOMASS,Bulk Volume,0)
************************************************************************



************************************************************************
Plot Definitions
************************************************************************
Fluoranthene_250:
               Description:          Degradation of fluoranthene in batc
                                     h experiment
               Abscissa:             Time
               Title:                Fluoranthene degradation in batch e
                                     xperiments
               Abscissa Label:       Time (d)
               Ordinate Label:       PAH concentration (mg/L)
               Curves:
                 Type : Variable [CalcNum,Comp.,Zone,Time/Space]
                 Value : C_measured [0,Batch,Bulk Volume,0]
                 Value : C [0,Batch,Bulk Volume,0]
************************************************************************
```

```
**************************************************************************
Calculation Parameters
**************************************************************************
Numerical Parameters:    Maximum Int. Step Size:  1
                         Maximum Integrat. Order: 5
                         Number of Codiagonals:   1000
                         Maximum Number of Steps: 1000
--------------------------------------------------------------------------
                         Fit Method:              simplex
                         Max. Number of Iterat.:  100
**************************************************************************



**************************************************************************
Calculated States
**************************************************************************
Calc. Num.  Num. States  Comments
0           151          Range of Times: 0 - 15
**************************************************************************
```

2. Triphenylene Batch Simulation Data

```
**************************************************************************

AQUASIM Version 2.0 (win/mfc) - Listing of System Definition

**************************************************************************

Date and time of listing:  07/06/2017  10:44:21



**************************************************************************
Variables
**************************************************************************
C:              Description:          Triphenylene concentration
                Type:                 Dyn. Volume State Var.
                Unit:                 mg/L
                Relative Accuracy:    1e-006
                Absolute Accuracy:    1e-006
--------------------------------------------------------------------------
Cin:            Description:          Initial concentration of Triphenyle
                                      ne in batch
                Type:                 Formula Variable
                Unit:                 mg/L
                Expression:           70
--------------------------------------------------------------------------
C_measured:     Description:          measured values from batch experime
                                      nts
```

```
                Type:                   Real List Variable
                Unit:                   mg/L
                Argument:               t
                Standard Deviations:    global
                Rel. Stand. Deviat.:    0
                Abs. Stand. Deviat.:    1
                Minimum:                0
                Maximum:                1e+009
                Interpolation Method:   linear interpolation
                Sensitivity Analysis:   inactive
                Real Data Pairs (9 pairs):
                   0                    61.551
                   1                    70.151
                   2                    35.112
                   4                    25.906
                   6                    19.418
                   7                    15.039
                   9                    13.134
                   10                   7.486
                   12                   6.55
-------------------------------------------------------------------------
Ks:             Description:            Half-saturation constant
                Type:                   Constant Variable
                Unit:                   1/d
                Value:                  217.78026
                Standard Deviation:     1
                Minimum:                0
                Maximum:                500
                Sensitivity Analysis:   active
                Parameter Estimation:   active
-------------------------------------------------------------------------
t:              Description:            Time
                Type:                   Program Variable
                Unit:                   d
                Reference to:           Time
-------------------------------------------------------------------------
u_max:          Description:            Maximum substrate utilisation rate
                Type:                   Constant Variable
                Unit:                   1/d
                Value:                  0.21652997
                Standard Deviation:     1
                Minimum:                0
                Maximum:                100
                Sensitivity Analysis:   active
                Parameter Estimation:   inactive
-------------------------------------------------------------------------
X:              Description:            Biomass concentration
                Type:                   Real List Variable
                Unit:                   mg/L
                Argument:               t
                Standard Deviations:    global
                Rel. Stand. Deviat.:    0
                Abs. Stand. Deviat.:    1
                Minimum:                0
```

```
                Maximum:              1e+009
                Interpolation Method: linear interpolation
                Sensitivity Analysis: inactive
                Real Data Pairs (9 pairs):
                   0                  20
                   2                  60
                   3                  70
                   4                  120
                   5                  150
                   6                  190
                   7                  230
                   9                  250
                   12                 260
*************************************************************************



*************************************************************************
Processes
*************************************************************************
degradation:    Description:          Biological degradation rate of PAH
                Type:                 Dynamic Process
                Rate:                 (u_max*C)
                Stoichiometry:
                  Variable : Stoichiometric Coefficient
                  C : -1
*************************************************************************



*************************************************************************
Compartments
*************************************************************************
Batch:          Description:          Batch flask
                Type:                 Mixed Reactor Compartment
                Compartment Index:    0
                Active Variables:     C
                Active Processes:     degradation
                Initial Conditions:
                  Variable(Zone) : Initial Condition
                  C(Bulk Volume) : Cin
                Inflow:               0
                Loadings:
                Volume:               1
                Accuracies:
                  Rel. Acc. Q:        0.001
                  Abs. Acc. Q:        0.001
                  Rel. Acc. V:        0.001
                  Abs. Acc. V:        0.001
*************************************************************************
```

```
**************************************************************************
Definitions of Calculations
**************************************************************************
calc1:          Description:
                Calculation Number:   0
                Initial Time:         0
                Initial State:        given, made consistent
                Step Size:            0.1
                Num. Steps:           150
                Status:               active for simulation
                                      inactive for sensitivity analysis
**************************************************************************
```

```
**************************************************************************
Definitions of Parameter Estimation Calculations
**************************************************************************
fit1:           Description:
                Calculation Number:   0
                Initial Time:         0
                Initial State:        given, made consistent
                Status:               active
                Fit Targets:
                  Data : Variable (Compartment,Zone,Time/Space)
                  C_measured : C (Batch,Bulk Volume,0)
**************************************************************************
```

```
**************************************************************************
Plot Definitions
**************************************************************************
Triphenylene_70:
                Description:          Degradation of Triphenylene in batc
                                      h experiment
                Abscissa:             Time
                Title:                Triphenylene degradation in batch e
                                      xperiments
                Abscissa Label:       Time (d)
                Ordinate Label:       PAH concentration (mg/L)
                Curves:
                  Type : Variable [CalcNum,Comp.,Zone,Time/Space]
                  Value : C [0,Batch,Bulk Volume,0]
                  Value : C_measured [0,Batch,Bulk Volume,0]
```

```
************************************************************************
```

```
************************************************************************
Calculation Parameters
************************************************************************
Numerical Parameters:    Maximum Int. Step Size:  1
                         Maximum Integrat. Order: 5
                         Number of Codiagonals:   1000
                         Maximum Number of Steps: 1000
------------------------------------------------------------------------
                         Fit Method:              simplex
                         Max. Number of Iterat.:  100
************************************************************************
```

```
************************************************************************
Calculated States
************************************************************************
Calc. Num.  Num. States  Comments
0           151          Range of Times: 0 - 15
************************************************************************
```

3. Fluoranthene CSTR Simulation Data

```
************************************************************************

AQUASIM Version 2.0 (win/mfc) - Listing of System Definition

************************************************************************

Date and time of listing:  08/30/2017  10:49:13
```

```
************************************************************************
Variables
************************************************************************
C:              Description:          Subtrate (Fluoranthene)
                Type:                 Dyn. Volume State Var.
                Unit:                 mg/L
                Relative Accuracy:    1e-006
                Absolute Accuracy:    1e-006
```

```
--------------------------------------------------------------------------
Cin1:          Description:          Initial Concentration
               Type:                 Real List Variable
               Unit:                 mg/L
               Argument:             t
               Standard Deviations:  global
               Rel. Stand. Deviat.:  0
               Abs. Stand. Deviat.:  1
               Minimum:              0
               Maximum:              1e+009
               Interpolation Method: linear interpolation
               Sensitivity Analysis: inactive
               Real Data Pairs (17 pairs):
                  0               611.79
                  4               305.11
                  8               40.199
                  12              350.42
                  16              413.313
                     .               .
                     .               .
                  60              0
                  80              0
                  120             0
                  200             0
                  280             0
--------------------------------------------------------------------------
Cin2:          Description:          2A influent
               Type:                 Real List Variable
               Unit:                 mg/L
               Argument:             t
               Standard Deviations:  global
               Rel. Stand. Deviat.:  0
               Abs. Stand. Deviat.:  1
               Minimum:              0
               Maximum:              1e+009
               Interpolation Method: linear interpolation
               Sensitivity Analysis: inactive
               Real Data Pairs (601 pairs):
                  0               0
                  0.5             2.646
                  1               5.034
                  1.5             7.263
                  2               9.242
                     .               .
                     .               .
                  298             0.1219
                  298.5           0.1202
                  299             0.1186
                  299.5           0.1169
                  300             0.1153
--------------------------------------------------------------------------
C_2Ameas:      Description:          measures effluent concentration fro
                                     m reactor 2A
               Type:                 Real List Variable
```

```
                Unit:                  mg/L
                Argument:              t
                Standard Deviations:   global
                Rel. Stand. Deviat.:   0
                Abs. Stand. Deviat.:   1
                Minimum:               0
                Maximum:               1e+009
                Interpolation Method:  linear interpolation
                Sensitivity Analysis:  inactive
                Real Data Pairs (29 pairs):
                   0                   0
                   4                   1.456
                   8                   3.917
                   12                  7.98
                   16                  26.75
                      .                   .
                      .                   .
                   221                 1.464
                   233                 0.849
                   245                 0.683
                   257                 0.235
                   269                 0.063
-------------------------------------------------------------------------------
C_2Bmeas:       Description:           measures effluent concentration fro
                                       m reactor 2A
                Type:                  Real List Variable
                Unit:                  mg/L
                Argument:              t
                Standard Deviations:   global
                Rel. Stand. Deviat.:   0
                Abs. Stand. Deviat.:   1
                Minimum:               0
                Maximum:               1e+009
                Interpolation Method:  linear interpolation
                Sensitivity Analysis:  inactive
                Real Data Pairs (31 pairs):
                   0                   0
                   4                   0
                   8                   0
                   12                  0.05
                   16                  0.5
                      .                   .
                      .                   .
                   233                 1.23
                   245                 0.562
                   257                 0.552
                   269                 0.2
                   278                 0
-------------------------------------------------------------------------------
D:              Description:           Diffusion coeff
                Type:                  Constant Variable
                Unit:                  m^2/h
                Value:                 20
                Standard Deviation:    1
```

```
               Minimum:              0
               Maximum:              100
               Sensitivity Analysis: inactive
               Parameter Estimation: inactive
-------------------------------------------------------------------------
k1:            Description:          subtrate removal coefficient in rea
                                     ctor 2A
               Type:                 Constant Variable
               Unit:                 1/h
               Value:                0.00032762996
               Standard Deviation:   1
               Minimum:              0
               Maximum:              100
               Sensitivity Analysis: inactive
               Parameter Estimation: inactive
-------------------------------------------------------------------------
k2:            Description:          subtrate removal coefficient in rea
                                     ctor 2B
               Type:                 Constant Variable
               Unit:                 1/h
               Value:                0.0054
               Standard Deviation:   1
               Minimum:              0
               Maximum:              100
               Sensitivity Analysis: inactive
               Parameter Estimation: active
-------------------------------------------------------------------------
Q:             Description:          Flow rate
               Type:                 Formula Variable
               Unit:                 L/h
               Expression:           0.863
-------------------------------------------------------------------------
Q2:            Description:          PAH flow into reactor 2B
               Type:                 Real List Variable
               Unit:                 L/h
               Argument:             t
               Standard Deviations:  global
               Rel. Stand. Deviat.:  0
               Abs. Stand. Deviat.:  1
               Minimum:              0
               Maximum:              1e+009
               Interpolation Method: linear interpolation
               Sensitivity Analysis: inactive
               Real Data Pairs (11 pairs):
                  0              0
                  0.5            0
                  10             0.2
                  20             0.9
                  36             1.4
                  50             1.5
                  78             1.1
                  98             1.3
                  137.5          1.4
                  196.5          1.6
```

```
                  290             1.5
-------------------------------------------------------------------------
Q_2A:          Description:
               Type:                  Formula Variable
               Unit:                  L/h
               Expression:            Q_pah+Q_biomass
-------------------------------------------------------------------------
Q_biomass:     Description:           BIOMASS flow into reactor 2A
               Type:                  Real List Variable
               Unit:                  L/h
               Argument:              t
               Standard Deviations:   global
               Rel. Stand. Deviat.:   0
               Abs. Stand. Deviat.:   1
               Minimum:               0
               Maximum:               1e+009
               Interpolation Method: linear interpolation
               Sensitivity Analysis: inactive
               Real Data Pairs (17 pairs):
                  0               0.961
                  4               0.661
                  8               0.961
                  12              0.961
                  16              0.961
                     .               .
                     .               .
                  78              0.361
                  96              0.261
                  150             0.161
                  200             0.361
                  250             0.361
-------------------------------------------------------------------------
Q_pah:         Description:           PAH flow into reactor 2A
               Type:                  Real List Variable
               Unit:                  L/h
               Argument:              t
               Standard Deviations:   global
               Rel. Stand. Deviat.:   0
               Abs. Stand. Deviat.:   1
               Minimum:               0
               Maximum:               1e+009
               Interpolation Method: linear interpolation
               Sensitivity Analysis: inactive
               Real Data Pairs (79 pairs):
                  0               0.691
                  0.5             0
                  1               0.691
                  1.5             0
                  2               0.691
                     .               .
                     .               .
                  90              0
                  150             0
                  190             0
```

```
                  220             0
                  278             0
--------------------------------------------------------------------------
t:              Description:          Time
                Type:                 Program Variable
                Unit:                 h
                Reference to:         Time
--------------------------------------------------------------------------
X:              Description:
                Type:                 Dyn. Volume State Var.
                Unit:                 mg/L
                Relative Accuracy:    1e-006
                Absolute Accuracy:    1e-006
--------------------------------------------------------------------------
X2:             Description:
                Type:                 Formula Variable
                Unit:                 mg/L
                Expression:           20
--------------------------------------------------------------------------
X_meas:         Description:          Constant biomass
                Type:                 Real List Variable
                Unit:                 mg/L
                Argument:             t
                Standard Deviations:  global
                Rel. Stand. Deviat.:  0
                Abs. Stand. Deviat.:  1
                Minimum:              0
                Maximum:              1e+009
                Interpolation Method: linear interpolation
                Sensitivity Analysis: inactive
                Real Data Pairs (37 pairs):
                   0               60
                   4               60
                   8               60
                   12              60
                   16              60
                      .               .
                      .               .
                   221             60
                   233             60
                   245             60
                   257             60
                   269             60
**************************************************************************
```

```
**************************************************************************
Processes
**************************************************************************
proc1:          Description:          Fluoranthene degradation rate from
                                      reactor 2A
```

```
                Type:                   Dynamic Process
                Rate:                   k1*C*X
                Stoichiometry:
                  Variable : Stoichiometric Coefficient
                  C : -1
-------------------------------------------------------------------------
proc2:          Description:            Fluoranthene degradation rate from
                                        reactor 2A
                Type:                   Dynamic Process
                Rate:                   k2*C*X2
                Stoichiometry:
                  Variable : Stoichiometric Coefficient
                  C : -1
*************************************************************************




*************************************************************************
Compartments
*************************************************************************
biomass:        Description:
                Type:                   Mixed Reactor Compartment
                Compartment Index:      0
                Active Variables:       X_meas
                Active Processes:
                Initial Conditions:
                Inflow:                 0
                Loadings:
                Volume:                 269
                Accuracies:
                  Rel. Acc. Q:          0.001
                  Abs. Acc. Q:          0.001
                  Rel. Acc. V:          0.001
                  Abs. Acc. V:          0.001
-------------------------------------------------------------------------
comp2:          Description:
                Type:                   Mixed Reactor Compartment
                Compartment Index:      0
                Active Variables:       C, X
                Active Processes:       proc1
                Initial Conditions:
                  Variable(Zone) : Initial Condition
                  C(Bulk Volume) : 0
                Inflow:                 Q_2A
                Loadings:
                  Variable : Loading
                  C : Q_pah*Cin1
                  X : Q_biomass*X_meas
                Volume:                 38.7
                Accuracies:
                  Rel. Acc. Q:          0.001
                  Abs. Acc. Q:          0.001
```

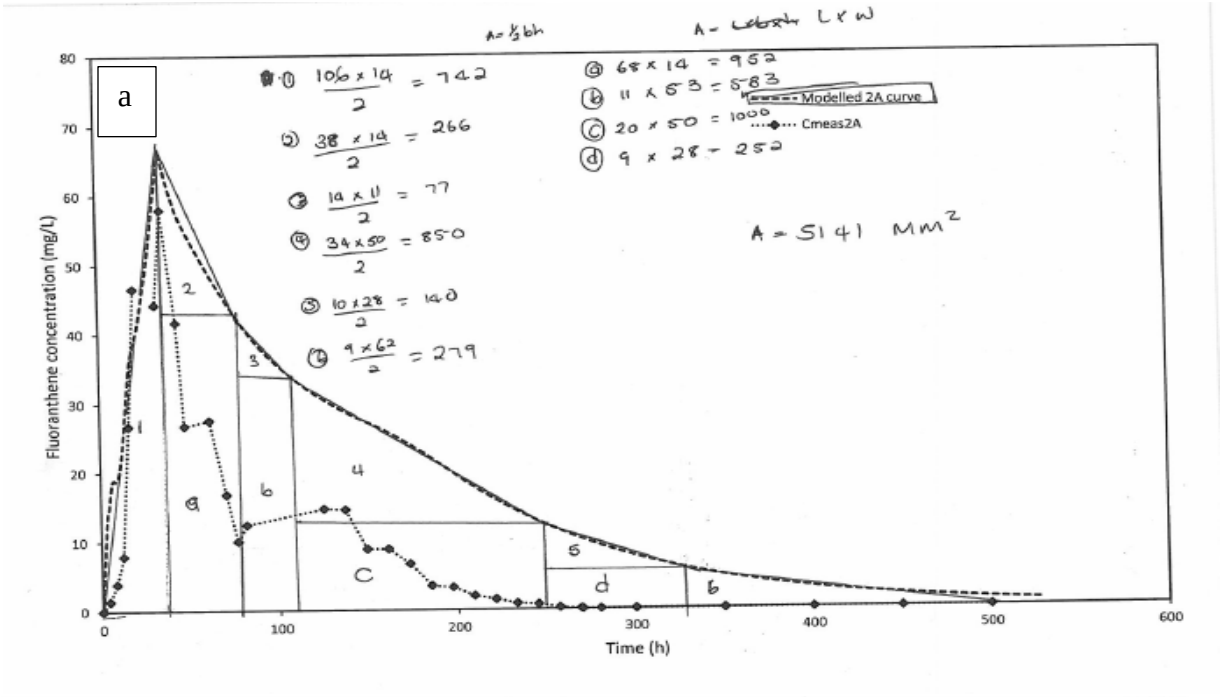
```
                Rel. Acc. V:        0.001
                Abs. Acc. V:        0.001
-------------------------------------------------------------------------
COMP3:          Description:        plug flow system
                Type:               Advective-Diffusive Comp.
                Compartment Index:  0
                Active Variables:   C
                Active Processes:   proc2
                Initial Conditions:
                Inflow:             Q2
                Loadings:
                  Variable : Loading
                  C : Q2*Cin2
                Lateral Inflow:     0
                Start Coordinate:   0
                End Coordinate:     30
                Cross Section:      1
                Glob. Diffusivity:  D
                Num. of Grid Pts:   105 (low resolution)
                Accuracies:
                  Rel. Acc. Q:      0.001
                  Abs. Acc. Q:      1e-006
                  Rel. Acc. D:      1e-006
                  Abs. Acc. D:      1e-006
-------------------------------------------------------------------------
comp3:          Description:
                Type:               Mixed Reactor Compartment
                Compartment Index:  0
                Active Variables:   C
                Active Processes:   proc2
                Initial Conditions:
                  Variable(Zone) : Initial Condition
                  C(Bulk Volume) : 0
                Inflow:             0
                Loadings:
                Volume:             9
                Accuracies:
                  Rel. Acc. Q:      0.001
                  Abs. Acc. Q:      0.001
                  Rel. Acc. V:      0.001
                  Abs. Acc. V:      0.001
-------------------------------------------------------------------------
dissolution:    Description:
                Type:               Mixed Reactor Compartment
                Compartment Index:  0
                Active Variables:   C
                Active Processes:
                Initial Conditions:
                  Variable(Zone) : Initial Condition
                  C(Bulk Volume) : Cin1
                Inflow:             0
                Loadings:
                Volume:             29.12
                Accuracies:
```

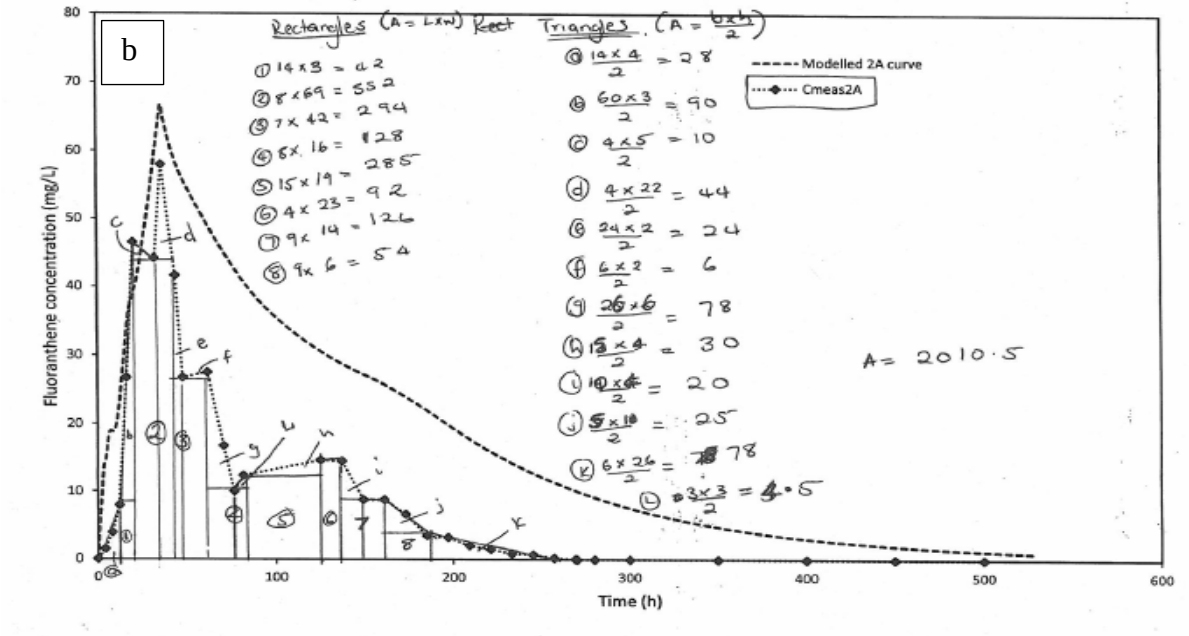
```
                Rel. Acc. Q:        0.001
                Abs. Acc. Q:        0.001
                Rel. Acc. V:        0.001
                Abs. Acc. V:        0.001
**************************************************************************



**************************************************************************
Links
**************************************************************************
link1:          Type:               Advective Link
                Link Index:         1
                Compartment In:     dissolution
                Connection In:      Outflow
                Compartment Out:    comp2
                Connection Out:     Inflow
                Bifurcations:
--------------------------------------------------------------------------
link3:          Type:               Advective Link
                Link Index:         1
                Compartment In:     biomass
                Connection In:      Outflow
                Compartment Out:    comp2
                Connection Out:     Inflow
                Bifurcations:
**************************************************************************



**************************************************************************
Definitions of Calculations
**************************************************************************
calc1:          Description:
                Calculation Number: 0
                Initial Time:       0
                Initial State:      given, made consistent
                Step Size:          0.5
                Num. Steps:         600
                Status:             active for simulation
                                    inactive for sensitivity analysis
**************************************************************************



**************************************************************************
Definitions of Parameter Estimation Calculations
**************************************************************************
```

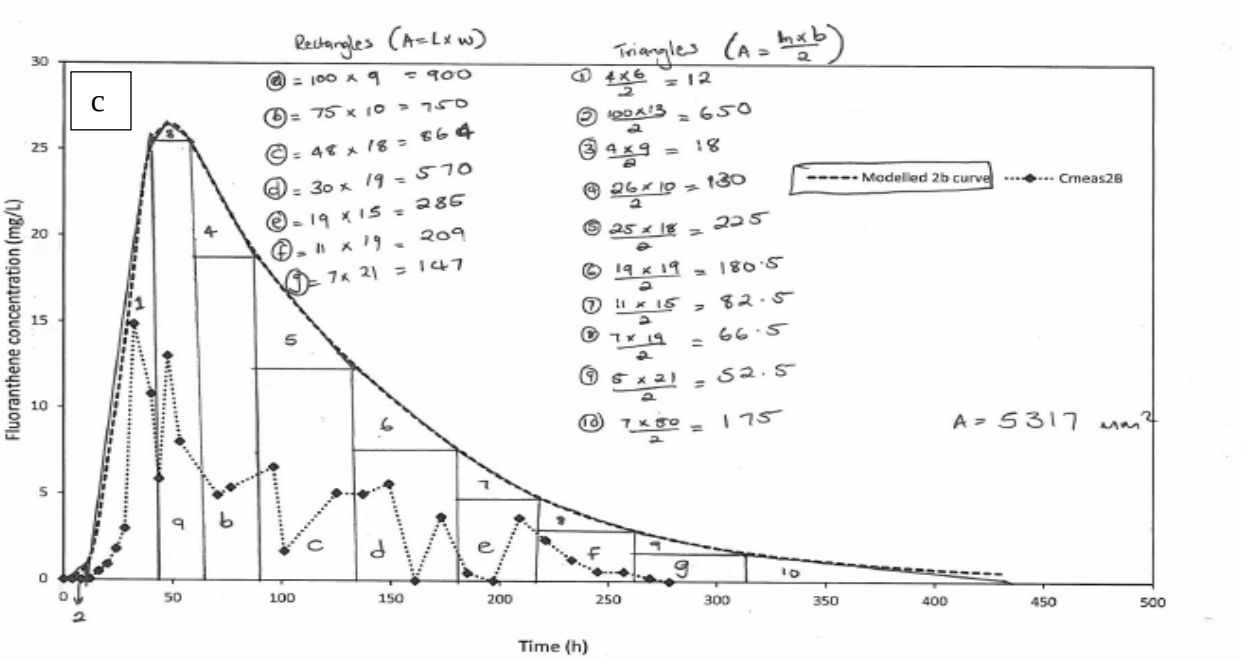
```
fit1:          Description:
               Calculation Number:   0
               Initial Time:         0
               Initial State:        given, made consistent
               Status:               inactive
               Fit Targets:
                 Data : Variable (Compartment,Zone,Time/Space)
                 C_2Ameas : C (comp2,Bulk Volume,0)
-------------------------------------------------------------------------
fit2:          Description:
               Calculation Number:   0
               Initial Time:         0
               Initial State:        given, made consistent
               Status:               active
               Fit Targets:
                 Data : Variable (Compartment,Zone,Time/Space)
                 C_2Bmeas : C (COMP3,Water Body,0)
*************************************************************************




*************************************************************************
Plot Definitions
*************************************************************************
Fluoranthene_reactor2A:
               Description:          Reactor 2A
               Abscissa:             Time
               Title:                Reactor 2A effluent
               Abscissa Label:       Time (h)
               Ordinate Label:       Fluorathnene concentration
               Curves:
                 Type : Variable [CalcNum,Comp.,Zone,Time/Space]
                 Value : C [0,comp2,Bulk Volume,0]
                 Value : C_2Ameas [0,comp2,Bulk Volume,0]
                 Value : C [0,comp3,Bulk Volume,0]
-------------------------------------------------------------------------
Fluoranthene_reactor2B:
               Description:          Reactor 2B
               Abscissa:             Time
               Title:                Reactor 2B effluent
               Abscissa Label:       Time (h)
               Ordinate Label:       Fluorathnene concentration
               Curves:
                 Type : Variable [CalcNum,Comp.,Zone,Time/Space]
                 Value : C [0,comp3,Bulk Volume,0]
                 Value : C_2Bmeas [0,comp3,Bulk Volume,0]
-------------------------------------------------------------------------
plot5:         Description:
               Abscissa:             Time
               Title:
               Abscissa Label:
               Ordinate Label:
```

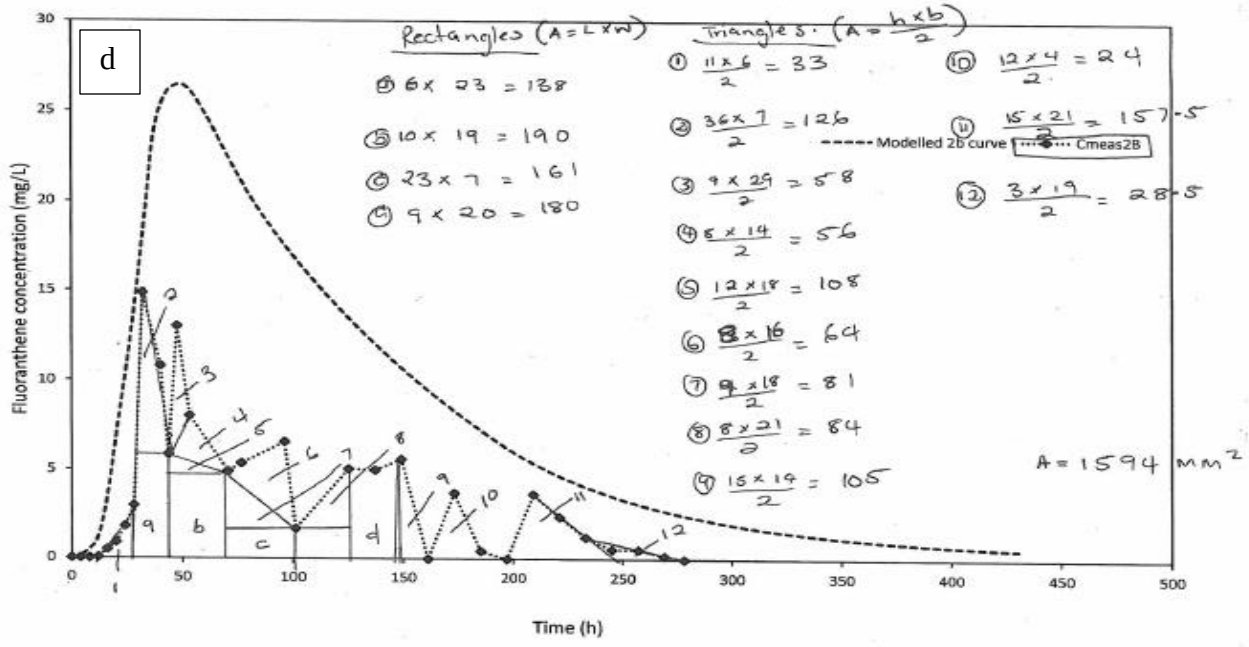
```
               Curves:
                 Type : Variable [CalcNum,Comp.,Zone,Time/Space]
                 Value : C [0,COMP3,Water Body,0]
                 Value : C_2Bmeas [0,COMP3,Water Body,0]
-------------------------------------------------------------------------
plot6:         Description:
               Abscissa:             Time
               Title:
               Abscissa Label:       t
               Ordinate Label:       f
               Curves:
                 Type : Variable [CalcNum,Comp.,Zone,Time/Space]
                 Value : C [0,comp2,Bulk Volume,0]
                 Value : C [0,COMP3,Water Body,0]
                 Value : C_2Ameas [0,comp2,Bulk Volume,0]
                 Value : C_2Bmeas [0,COMP3,Water Body,0]
*************************************************************************




*************************************************************************
Calculation Parameters
*************************************************************************
Numerical Parameters:    Maximum Int. Step Size:  1
                         Maximum Integrat. Order: 5
                         Number of Codiagonals:   1000
                         Maximum Number of Steps: 1000
-------------------------------------------------------------------------
                         Fit Method:              simplex
                         Max. Number of Iterat.:  100
*************************************************************************




*************************************************************************
Calculated States
*************************************************************************
Calc. Num.  Num. States  Comments
0           601          Range of Times: 0 - 300
*************************************************************************
```

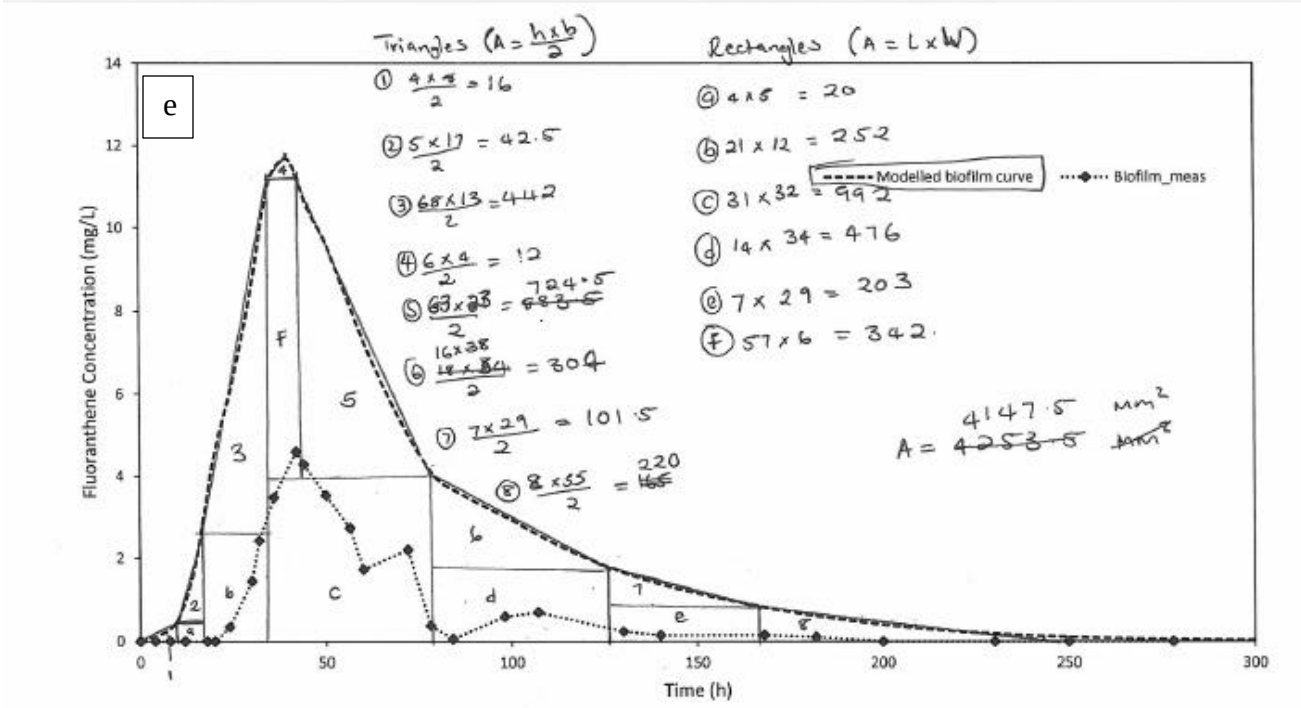
APPENDIX B: Curve Area Calculations

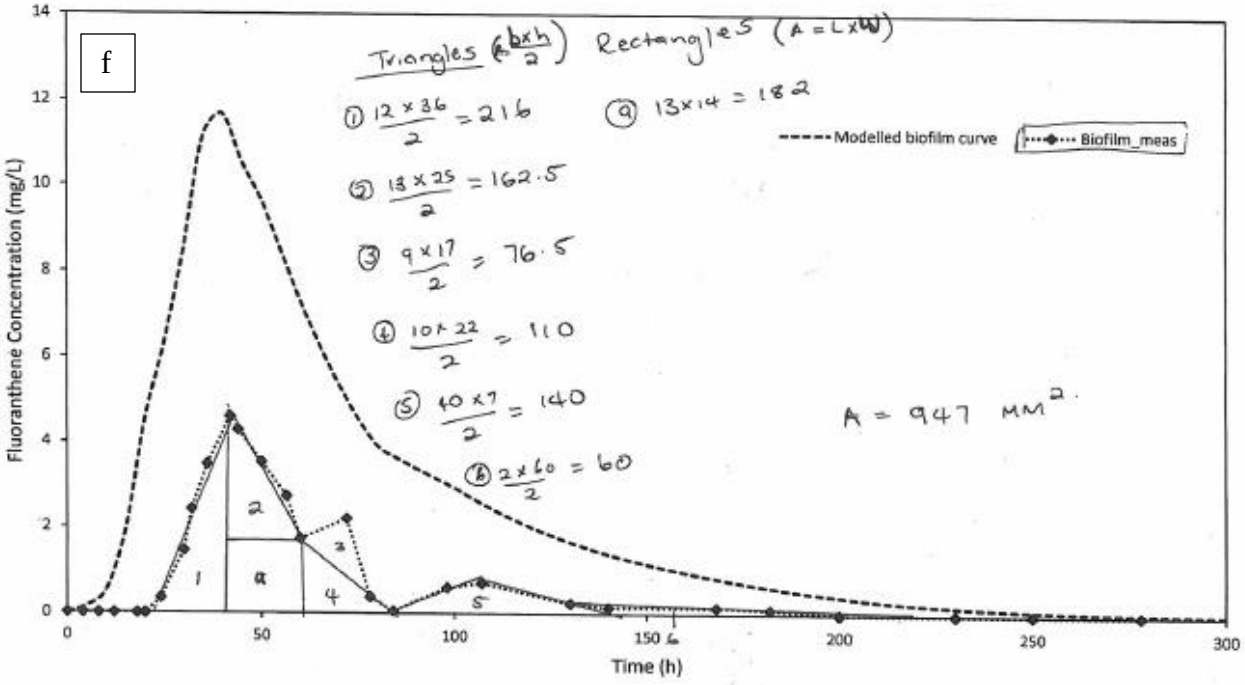





c
Rectangles (A=L x w)
Triangles (A = b x h / 2)
Modelled 2b curve
Cmeas2B
A = 5317 mm²
Fluoranthene concentration (mg/L)
Time (h)


d
Rectangles (A=L x w)
Triangles (A = b x h / 2)
Modelled 2b curve
Cmeas2B
A = 1594 mm²
Fluoranthene concentration (mg/L)
Time (h)


e
Triangles (A = h x b / 2)
Rectangles (A = L x W)
① 4×8/2 = 16
② 5×17/2 = 42.5
③ 68×13/2 = 442
④ 6×4/2 = 12
⑤ 724.5
⑥ 16×38/2 = 304
⑦ 7×29/2 = 101.5
⑧ 8×55/2 = 220
ⓐ 4×5 = 20
ⓑ 21×12 = 252
ⓒ 31×32 = 992
ⓓ 14×34 = 476
ⓔ 7×29 = 203
Ⓕ 57×6 = 342
A = 4147.5 mm²
Modelled biofilm curve
Biofilm_meas
Fluoranthene Concentration (mg/L)
Time (h)


f
Triangles (b×h/2)
Rectangles (A = L×W)
① 12×36/2 = 216
② 13×25/2 = 162.5
③ 9×17/2 = 76.5
④ 10×22/2 = 110
⑤ 40×7/2 = 140
⑥ 2×60/2 = 60
⑦ 13×14 = 182
A = 947 mm².
Modelled biofilm curve
Biofilm_meas
Fluoranthene Concentration (mg/L)
Time (h)

Bibliography:

Abdel-Shafy, H.I. & Mansour, M.S.M., 2016. A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation. *Egyptian Journal of Petroleum*, 25(1), pp.107–123. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1110062114200237.

Abdulsalam, S. et al., 2011. Comparison of biostimulation and bioaugmentation for remediation of soil contaminated with spent motor oil. *Int. J. Environ. Sci. Tech*, 8(1), pp.187–194.

Adams, G.O. et al., 2015. Bioremediation, Biostimulation and Bioaugmention: A Review. *International Journal of Environmental Bioremediation & Biodegradation*, 3(1), pp.28–39. Available at: http://pubs.sciepub.com/ijebb/3/1/5.

Antizar-Ladislao, B. et al., 2007. The influence of different temperature programmes on the bioremediation of polycyclic aromatic hydrocarbons (PAHs) in a coal-tar contaminated soil by in-vessel composting. *Journal of Hazardous Materials*, 144(1–2), pp.340–347.

Atlas, R.M., 1995. Bioremediation of petroleum pollutants. *International Biodeterioration and Biodegradation*, 35(1–3), pp.317–327.

Bamforth, S.M. & Singleton, I., 2005. Bioremediation of polycyclic aromatic hydrocarbons: Current knowledge and future directions. *Journal of Chemical Technology and Biotechnology*, 80(7), pp.723–736.

Banat, I.M., 1995. Biosurfactants production and possible uses in microbial enhanced oil recovery and oil pollution remediation: A review. *Bioresource Technology*, 51(1), pp.1–12.

Benincasa, M., 2007. Rhamnolipid produced from agroindustrial wastes enhances hydrocarbon biodegradation in contaminated soil. *Current Microbiology*, 54(6), pp.445–449.

Bento, F.M. et al., 2005. Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. *Bioresource Technology*, 96(9), pp.1049–1055.

Bento, F.M. et al., 2005. Diversity of biosurfactant producing microorganisms isolated from soils contaminated with diesel oil. *Microbiological Research*, 160(3), pp.249–255.

Bezza, F.A. & Chirwa, E.M.N., 2015. Production and applications of lipopeptide biosurfactant for bioremediation and oil recovery by Bacillus subtilis CN2. *Biochemical Engineering Journal*, 101, pp.168–178. Available at: http://dx.doi.org/10.1016/j.bej.2015.05.007.

Bezza, F.A. & Nkhalambayausi Chirwa, E.M., 2016. Biosurfactant-enhanced bioremediation of aged polycyclic aromatic hydrocarbons (PAHs) in creosote contaminated soil. *Chemosphere*, 144, pp.635–644. Available at: http://dx.doi.org/10.1016/j.chemosphere.2015.08.027.

Bodour, A.A. & Miller-Maier, R.M., 1998. Application of a modified drop-collapse technique for surfactant quantitation and screening of biosurfactant-producing microorganisms. *Journal of Microbiological Methods*, 32(3), pp.273–280.

Bojes, H.K. & Pope, P.G., 2007. Characterization of EPA's 16 priority pollutant polycyclic aromatic hydrocarbons (PAHs) in tank bottom solids and associated contaminated soils at oil exploration and production sites in Texas. *Regulatory Toxicology and Pharmacology*, 47(3), pp.288–295.

Boldrin, B., Tiehm, A. & Fritzsche, C., 1993. Degradation of phenanthrene, fluorene, fluoranthene, and pyrene by a Mycobacterium sp. *Applied and Environmental Microbiology*, 59(6), pp.1927–1930.

Bustamante, M., Durán, N. & Diez, M., 2012. Biosurfactants are useful tools for the bioremediation of contaminated soil: a review. *Journal of soil science and plant …*, 12(4), pp.667–687. Available at: http://www.scielo.cl/scielo.php?script=sci_arttext&pid=S0718-95162012000400004.

Chakraborty, T., Chakraborty, I. & Ghosh, S., 2011. The methods of determination of critical micellar concentrations of the amphiphilic systems in aqueous medium. *Arabian Journal of Chemistry*, 4(3), pp.265–270. Available at: http://dx.doi.org/10.1016/j.arabjc.2010.06.045.

Chen, Y. et al., 2013. Generation and distribution of PAHs in the process of medical waste

incineration. *Waste Management*, 33(5), pp.1165–1173. Available at: http://dx.doi.org/10.1016/j.wasman.2013.01.011.

Churchill, S.A., Harper, J.P. & Churchill, P.F., 1999. Isolation and Characterization of a Mycobacterium Species Capable of Degrading Three-and Four-Ring Aromatic and Aliphatic Hydrocarbons. *Applied and Environmental Microbiology*, 65(2), pp.549–552.

Collins, J.F. et al., 1991. Risk assessment for benzo[a]pyrene. *Regulatory Toxicology and Pharmacology*, 13(2), pp.170–184.

Cui, F. & Kim, M., 2013. Use of Steady-State Bio fi lm Model to Characterize Aerobic Granular Sludge. *Environmental Science & Technology*, 47, pp.12291–12296.

Das, N. & Chandran, P., 2011. Microbial degradation of petroleum hydrocarbon contaminants: an overview. *Biotechnology research international*, 2011, p.941810.

Doong, R.A. & Lin, Y.T., 2004. Characterization and distribution of polycyclic aromatic hydrocarbon contaminations in surface sediment and water from Gao-ping River, Taiwan. *Water Research*, 38(7), pp.1733–1744.

Edokpayi, J. et al., 2016. Determination and Distribution of Polycyclic Aromatic Hydrocarbons in Rivers, Sediments and Wastewater Effluents in Vhembe District, South Africa. *International Journal of Environmental Research and Public Health*, 13(4), p.387. Available at: http://www.mdpi.com/1660-4601/13/4/387.

Fieser, L.F., 1938. Carcinogenic activity, structure, and chemical reactivity of polynuclear aromatic hydrocarbons. *Cancer Research*, 34, pp.37–124.

Flotron, V. et al., 2005. Removal of sorbed polycyclic aromatic hydrocarbons from soil, sludge and sediment samples using the Fenton's reagent process. *Chemosphere*, 59(10), pp.1427–1437.

Gan, S., Lau, E. V. & Ng, H.K., 2009. Remediation of soils contaminated with polycyclic aromatic hydrocarbons (PAHs). *Journal of Hazardous Materials*, 172(2–3), pp.532–549.

George, S. & Jayachandran, K., 2013. Production and characterization of rhamnolipid biosurfactant from waste frying coconut oil using a novel Pseudomonas aeruginosa D. *Journal of Applied Microbiology*, 114(2).

Ghosal, D. et al., 2016. Current State of Knowledge in Microbial Degradation of Polycyclic Aromatic Hydrocarbons (PAHs): A Review. *Frontiers in Microbiology*, 7(August). Available at: http://journal.frontiersin.org/Article/10.3389/fmicb.2016.01369/abstract.

Ghosh, I., Jasmine, J. & Mukherji, S., 2014. Biodegradation of pyrene by a Pseudomonas aeruginosa strain RS1 isolated from refinery sludge. *Bioresource Technology*, 166, pp.548–558. Available at: http://dx.doi.org/10.1016/j.biortech.2014.05.074.

Gong, Z. et al., 2006. Removal of polycyclic aromatic hydrocarbons from manufactured gas plant-contaminated soils using sunflower oil: Laboratory column experiments. *Chemosphere*, 62(5), pp.780–787.

Haritash, A.K. & Kaushik, C.P., 2009. Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. *Journal of Hazardous Materials*, 169(1–3), pp.1–15.

Horvath, R.S., 1972. Microbial Co-Metabolism and the Degradation of Organic Compounds in Nature. *Bacteriological Reviews*, 36(2), pp.146–155.

IWA Task Group on Biofilm Modeling, 2006. *Mathematical Modeling of Biofilms* Report No., London: IWA Publishing.

Iwamoto, T. & Nasu, M., 2001. Current bioremediation practice and perspective. *Journal of bioscience and bioengineering*, 92(1), pp.1–8.

Jonsson, S. et al., 2007. Degradation of polycyclic aromatic hydrocarbons (PAHs) in contaminated soils by Fenton's reagent: A multivariate evaluation of the importance of soil characteristics and PAH properties. *Journal of Hazardous Materials*, 149(1), pp.86–96.

Juhasz, A.L., Britz, M.L. & Stanley, G.A., 1996. Degradation of high molecular weight polycyclic aromatic hydrocarbons by Pseudomonas cepacia. *Biotechnology Letters*, 18(5), pp.577–582.

Juhasz, A.L. & Naidu, R., 2000. Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene. *International Biodeterioration & Biodegradation*, 45(1–2), pp.57–88.

Kabziński, A., 2002. Determination of polycyclic aromatic hydrocarbons in water (including drinking water) of Łódź. *Polish Journal of …*, 11(6), pp.695–706. Available at: http://6csnfn.pjoes.com/pdf/11.6/695-706.pdf.

Kanaly, R. a & Harayama, S., 2000. Biodegradation of High-Molecular-Weight Polycyclic Aromatic Hydrocarbons by Bacteria MINIREVIEW Biodegradation of High-Molecular-Weight Polycyclic Aromatic Hydrocarbons by Bacteria. , 182(8), pp.2059–2067.

Kim, K.-H. et al., 2013. A review of airborne polycyclic aromatic hydrocarbons (PAHs) and their human health effects. *Environment International*, 60(October), pp.71–80. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0160412013001633.

Knightes, C.D. & Peters, C.A., 2003. Aqueous Phase Biodegradation Kinetics of 10 PAH Compounds. *Environmental Engineering Science*, 20(3), pp.207–218.

Kosaric, N., 1992. Biosurfactants in industry. *Pure and Applied Chemistry*, 64(11), pp.1731–1737.

Kumar, M. et al., 2006a. Polycyclic Aromatic Hydrocarbon Degradation by Biosurfactant-Producing Pseudomonas sp. IR1. *Zeitschrift für Naturforschung C*, 61(3–4), pp.203–212. Available at: http://www.degruyter.com/view/j/znc.2006.61.issue-3-4/znc-2006-3-409/znc-2006-3-409.xml.

Kumar, M. et al., 2006b. Polycyclic aromatic hydrocarbon degradation by biosurfactant-producing Pseudomonas sp. IR1. *Journal of Biosciences*, 61(3–4), pp.203–212.

Launen, L.A. et al., 2002. Bioremediation of Polyaromatic Hydrocarbon- Contaminated Sediments in Aerated Bioslurry Reactors. *Bioremediation Journal*, 6(2), p.125. Available at: http://search.ebscohost.com/login.aspx?direct=true&db=a9h&AN=7073336&lang=es&site=ehost-live.

Lee, S.H. et al., 2008. Degradation of phenanthrene and pyrene in rhizosphere of grasses and legumes. *Journal of Hazardous Materials*, 153(1–2), pp.892–898.

Li, X. et al., 2016. Air pollution from polycyclic aromatic hydrocarbons generated by human activities and their health effects in China. *Journal of Cleaner Production*, 112, pp.1360–1367.

Li, X. et al., 2012. Solvent extraction for heavy crude oil removal from contaminated soils. *Chemosphere*, 88(2), pp.245–249. Available at: http://dx.doi.org/10.1016/j.chemosphere.2012.03.021.

Lin, Y. et al., 2009. Biodegradation of phenol with chromium (VI) reduction in an anaerobic fixed-biofilm process-Kinetic model and reactor ... *Journal of Hazardous Materials*, 172, pp.1394–1401.

Long, A.S., Margaret, W., et al., 2016. Oral Exposure to Commercially Available Coal Tar-Based Pavement Sealcoat Induces Murine Genetic Damage and Mutations. *Environmental and molecular mutagenesis*, 57, pp.535–545.

Long, A.S., Lemieux, C.L., et al., 2016. Tissue-specific in vivo genetic toxicity of nine polycyclic aromatic hydrocarbons assessed using the Muta^{TM}Mouse transgenic rodent assay. *Toxicology and Applied Pharmacology*, 290, pp.31–42. Available at: http://dx.doi.org/10.1016/j.taap.2015.11.010.

Mahanty, B., Pakshirajan, K. & Venkata Dasu, V., 2008. Biodegradation of pyrene by Mycobacterium frederiksbergense in a two-phase partitioning bioreactor system. *Bioresource Technology*, 99(7), pp.2694–2698.

Makkar, R.S. & Rockne, K.J., 2003. Comparison of Synthetic Surfactants and Biosurfactants in Enhancing Biodegradation of Polycyclic Aromatic Hydrocarbons. *Environmental Toxicology and Chemistry*, 22(10), p.2280. Available at: http://doi.wiley.com/10.1897/02-472.

Makkar, R.S. & Rockne, K.J., 2003. Comparison of synthetic surfactants and biosurfactants in enhancing biodegradation of polycyclic aromatic hydrocarbons. *Environmental Toxicology*

and Chemistry, 22(10), pp.2280–2292.

Maliszewska-Kordybach, B., 1999. Sources, Concentrations, Fate and Effects of Polycyclic Aromatic Hydrocarbons (PAHs) in the Environment. Part A: PAHs in Air. *Polish Journal of Environmental Studies*, 8(3), pp.131–136.

Mamba, B.B., Rietveld, L.C. & Verberk, J.Q.J.C., 2008. SA drinking water standards under the microscope. *Water Wheel*, 7(1), pp.24–27.

Manoli, E. & Samara, C., 1999. Polycyclic aromatic hydrocarbons in natural waters: Sources, occurrence and analysis. *TrAC - Trends in Analytical Chemistry*, 18(6), pp.417–428.

Manzetti, S., 2013. Polycyclic Aromatic Hydrocarbons in the Environment: Environmental Fate and Transformation. *Polycyclic Aromatic Compounds*, 33(4), pp.311–330. Available at: http://www.tandfonline.com/doi/abs/10.1080/10406638.2013.781042.

Marcoux, J. et al., 2000. Optimization of high-molecular-weight polycyclic aromatic hydrocarbons' degradation in a two-liquid-phase bioreactor. *Journal of applied microbiology*, 88, pp.655–662.

Masten, S.J. & Davies, S.H.R., 1997. Efficacy of in-situ ozonation for the remediation of PAH contaminated soils. *Journal of Contaminant Hydrology*, 28(4), pp.327–335.

Mbadinga, S.M. et al., 2011. Microbial communities involved in anaerobic degradation of alkanes. *International Biodeterioration and Biodegradation*, 65(1), pp.1–13. Available at: http://dx.doi.org/10.1016/j.ibiod.2010.11.009.

Meliani, A. & Bensoltane, A., 2014. Enhancement of Hydrocarbons Degradation by Use of Pseudomonas. *Petroleum & Environmental Biotechnology*, 5(1), pp.1–7.

Misaki, K. et al., 2016. Tumour-promoting activity of polycyclic aromatic hydrocarbons and their oxygenated or nitrated derivatives. *Mutagenesis*, 31(2), pp.205–213. Available at: https://academic.oup.com/mutage/article-lookup/doi/10.1093/mutage/gev076.

Nekhavhambe, T.J., van Ree, T. & Fatoki, O.S., 2014. Determination and distribution of

polycyclic aromatic hydrocarbons in rivers, surface runoff, and sediments in and around Thohoyandou, Limpopo Province, South Africa. *Water SA*, 40(3), pp.415–424.

Nieuwoudt, C. et al., 2011. Polycyclic Aromatic Hydrocarbons (PAHs) in Soil and Sediment from Industrial, Residential, and Agricultural Areas in Central South Africa: An Initial Assessment. *Soil and Sediment Contamination*, 20, pp.188–204. Available at: http://www.tandfonline.com/doi/abs/10.1080/15320383.2011.546443.

O'Mahony, M.M. et al., 2006. The use of ozone in the remediation of polycyclic aromatic hydrocarbon contaminated soil. *Chemosphere*, 63(2), pp.307–314.

Obayori, O.S. et al., 2008. Pyrene-degradation potentials of Pseudomonas species isolated from polluted tropical soils. *World Journal of Microbiology and Biotechnology*, 24(11), pp.2639–2646.

Okedeyi, O.O. et al., 2013. Distribution and potential sources of polycyclic aromatic hydrocarbons in soils around coal-fired power plants in South Africa. *Environmental Monitoring and Assessment*, 185(3), pp.2073–2082.

Pannu, J.K., Singh, A. & Ward, O.P., 2004. Vegetable oil as a contaminated soil remediation amendment: Application of peanut oil for extraction of polycyclic aromatic hydrocarbons from soil. *Process Biochemistry*, 39(10), pp.1211–1216.

Patowary, K., Kalita, M.C. & Deka, S., 2015. Degradation of polycyclic aromatic hydrocarbons (PAHs) employing biosurfactant producing Pseudomonas aeruginosa KS3. *Indian Journal of Biotechnology*, 14(April), pp.208–215.

Poglazova, M.N. et al., 1967. Destruction of Benzo(a) Pyrene by Soil Bacteria. *Life Sciences*, 6, pp.1053–1062.

Pornsunthorntawee, O. et al., 2008. Structural and physicochemical characterization of crude biosurfactant produced by Pseudomonas aeruginosa SP4 isolated from petroleum-contaminated soil. *Bioresource Technology*, 99(6), pp.1589–1595.

Rasamiravaka, T. et al., 2015. The formation of biofilms by pseudomonas aeruginosa: A review

of the natural and synthetic compounds interfering with control mechanisms. *BioMed Research International*, 2015.

Rodriguez-mateus, Z., Agualimpia, B. & Zafra, G., 2016. Isolation and Molecular Characterization of Microorganisms with Potential for the Degradation of Oil and Grease from Palm Oil Refinery Wastes. , 49, pp.517–522.

Sachdev, D.P. & Cameotra, S.S., 2013. Biosurfactants in agriculture. *Applied Microbiology and Biotechnology*, 97(3), pp.1005–1016.

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

Santhini, K. & Parthasarathi, R., 2014. Isolation and Screening of Biosurfactant Producing Microorganisms from Hydrocarbon Contaminated Soils from Automobile Workshop. *International Journal of Pharmaceutical & Biological Archives*, 5(2), pp.158–167.

Sato, M. et al., 2011. Investigation of polycyclic aromatic hydrocarbons (PAHs) content in bottom ashes from some Japanese waste incinerators and simple estimation of their fate in landfill. *Sustainable Environment Research*, 21(4), pp.219–227. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-84872687642&partnerID=tZOtx3y1.

Satoh, S., Mimuro, M. & Tanaka, A., 2013. Construction of a Phylogenetic Tree of Photosynthetic Prokaryotes Based on Average Similarities of Whole Genome Sequences. *PLoS ONE*, 8(7), p.e70290.

Satpute, S.K. et al., 2010. Methods for investigating biosurfactants and bioemulsifiers: a review. *Critical reviews in biotechnology*, 30(2), pp.127–144.

Schneider, J. et al., 1996. Degradation of pyrene, benz[a]anthracene, and benzo[a]pyrene by Mycobacterium sp. strain RJGII-135, isolated from a former coal gasification site. *Applied and Environmental Microbiology*, 62(1), pp.13–19.

Seo, J.S., Keum, Y.S. & Li, Q.X., 2009. Bacterial degradation of aromatic compounds. *International Journal of Environmental Research and Public Health*, 6(1), pp.278–309.

Shukla, S.K. et al., 2014. Biofilm-Mediated Bioremediation of Polycyclic Aromatic Hydrocarbons. In *Microbial Biodegradation and Bioremediation*. pp. 203–232. Available at: http://linkinghub.elsevier.com/retrieve/pii/B978012800021200008X.

Singh, A.K. & Cameotra, S.S., 2014. Influence of microbial and synthetic surfactant on the biodegradation of atrazine. *Environmental Science and Pollution Research*, 21(3), pp.2088–2097.

Singh, R., Paul, D. & Jain, R.K., 2006. Biofilms: implications in bioremediation. *Trends in Microbiology*, 14(9), pp.389–397.

Smith, M.J. et al., 2006. Effects of polycyclic aromatic hydrocarbons on germination and subsequent growth of grasses and legumes in freshly contaminated soil and soil with aged PAHs residues. *Environmental Pollution*, 141(3), pp.519–525.

Stach, J.E.M. & Burns, R.G., 2002. Enrichment versus biofilm culture: A functional and phylogenetic comparison of polycyclic aromatic hydrocarbon-degrading microbial communities. *Environmental Microbiology*, 4(3), pp.169–182.

Trummler, K., Effenberger, F. & Syldatk, C., 2003. An integrated microbial/enzymatic process for production of rhamnolipids and L-(+)-rhamnose from rapeseed oil with Pseudomonas sp. DSM 2874. *European Journal of Lipid Science and Technology*, 105(10), pp.563–571.

Ukiwe, L.N. et al., 2013. Polycyclic Aromatic Hydrocarbons Degradation Techniques: A Review. *International Journal of Chemistry*, 5(4), pp.43–55. Available at: http://www.ccsenet.org/journal/index.php/ijc/article/view/30512.

Varjani, S.J. & Upasani, V.N., 2017. A new look on factors affecting microbial degradation of petroleum hydrocarbon pollutants. *International Biodeterioration & Biodegradation*, 120, pp.71–83. Available at: http://dx.doi.org/10.1016/j.ibiod.2017.02.006.

Vidali, M., 2001. Bioremediation - An overview. *Pure Applied Chemistry*, 73(7), pp.1163–1172.

Villemur, R. et al., 2000. Two-liquid-phase slurry bioreactors to enhance the degradation of high-molecular-weight polycyclic aromatic hydrocarbons in soil. *Biotechnology Progress*,

16(6), pp.966–972.

Vital-Lopez, F.G., Reifman, J. & Wallqvist, A., 2015. Biofilm Formation Mechanisms of Pseudomonas aeruginosa Predicted via Genome-Scale Kinetic Models of Bacterial Metabolism. *PLoS Computational Biology*, 11(10), pp.1–24.

Wang, W. et al., 2010. Concentrations, sources and spatial distribution of polycyclic aromatic hydrocarbons in soils from Beijing, Tianjin and surrounding areas, North China. *Environmental Pollution*, 158(5), pp.1245–1251. Available at: http://dx.doi.org/10.1016/j.envpol.2010.01.021.

Wheatley, A.D. & Sadhra, S., 2004. Polycyclic aromatic hydrocarbons in solid residues from waste incineration. *Chemosphere*, 55(5), pp.743–749.

Whyte, L.G., Greer, C.W. & Inniss, W.E., 1996. Assessment of the biodegradation potential of psychrotrophic microorganisms. *Canadian journal of microbiology*, 42(2), pp.99–106.

Wicke, D., Böckelmann, U.T.A. & Reemtsma, T., 2008. Environmental Influences on the Partitioning and Diffusion of Hydrophobic Organic Contaminants in Microbial Biofilms. *Environmental Science & Technology*, 42(6), pp.1990–1996.

Wilson, S.C. & Jones, K.C., 1993. Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): a review. *Environmental pollution (Barking, Essex : 1987)*, 81(3), pp.229–249.

Wimpenny, J., Manz, W. & Szewzyk, U., 2000. Heterogeneity in bio ¢ lms. *FEMS Microbiology Reviews*, 24, pp.661–671.

Zhang, Y. et al., 2016. Biological impact of environmental polycyclic aromatic hydrocarbons (ePAHs) as endocrine disruptors. *Environmental Pollution*, 213, pp.809–824. Available at: http://dx.doi.org/10.1016/j.envpol.2016.03.050.

Zhang, Y. & Tao, S., 2009. Global atmospheric emission inventory of polycyclic aromatic hydrocarbons (PAHs) for 2004. *Atmospheric Environment*, 43(4), pp.812–819. Available at: http://dx.doi.org/10.1016/j.atmosenv.2008.10.050.