Cape Peninsula University of Technology

Bioremediation of Polycyclic Aromatic Hydrocarbons (PAHs) in water using indigenous microbes of Diep- and Plankenburg Rivers, Western Cape, South Africa.

By

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DECLARATION

I, declare that the contents of this dissertation represent my own unaided work, and that the dissertation has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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ABSTRACT

This study was conducted to investigate the occurrence of PAH degrading microorganisms in two river systems in the Western Cape, South Africa, and their ability to degrade two PAH compounds (acenaphthene and fluorene). A total of 19 bacterial isolates were obtained from the Diep- and Plankenburg Rivers. These microorganisms were first identified phenotypically on various selective and general media (such as nutrient agar, Eosine Methylene Blue and Mannitol Salts Agar), followed by staining and biochemical testing, followed by molecular identification using 16S rRNA and PCR. The isolates were then tested for acenaphthene and fluorene degradation first at flask scale and then in a Stirred Tank Bioreactor at varying temperatures (25ºC, 30ºC, 35ºC, 37ºC, 38ºC, 40ºC and 45ºC). All experiments were run without the addition of supplements, bulking agents, biosurfactants or any other form of biostimulants. Four of the 19 isolated microorganisms were identified as acenaphthene and fluorene degrading isolates. Three of the four microorganisms identified as PAH degrading isolates were Gram negative isolates. Results showed that *Raoultella ornithinolytica*, *Serratia marcescens, Bacillus megaterium* and *Aeromonas hydrophila* efficiently degraded fluorene (99.90%, 97.90%, 98.40% and 99.50%) and acenaphthene (98.60%, 95.70%, 90.20% and 99.90%) at 37ºC, 37ºC, 30ºC and 35ºC, respectively. The degradation of fluorene was found to be more efficient and rapid compared to that of acenaphthene and degradation at Stirred Tank Bioreactor scale was more efficient for all treatments. Throughout the biodegradation experiments, there was an exponential increase in microbial plate counts ranging from 5×10^4 to 9×10^8 CFU/ml. The increase in plate count was observed to correlate with the efficient degradation temperature profiles and percentages. The PAH degrading microorganisms isolated during this study significantly reduced the concentrations of acenaphthene and fluorene and can be used on a larger, commercial scale to bioremediate PAH contaminated river systems. Other factors that influence the optimal expression of biodegradative potential of microorganisms other than temperature and substrate (nutrient) availability, such as pH, moisture and salinity will be investigated in future studies, as well as the factors contributing to the higher fluorene degradation compared to acenaphthene. Furthermore, the structure and toxicity of the by-products and intermediates produced during microbial metabolism of acenaphthene and fluorene should be investigated in further studies.

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DEDICATION

This Thesis is dedicated to my loving Mother; Thank you for being my shining star!

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

GLOSSARY

Polycyclic Aromatic Hydrocarbons – PAHs

Acenaphthene-Ace

Fluorene-Flu

Poly Tetra Fluoroethylene - PTFE

Department of Water Affairs and Forestry – DWAF

South African Bureau of Standards – SABS

World Health Organisation – WHO

United States Environmental Protection Agency - US EPA

Polymerase Chain Reaction – PCR

Colony Forming Units – CFU

CHAPTER ONE

1. INTRODUCTION

1.1. Background

Water is vital to all known forms of life and has many different uses, including domestic, industrial, agricultural, recreational, electrical, as well as providing a habitat for many plants and animals. Water plays a very important role in our daily activities, which makes it imperative to ensure that water resources remain as sanitary as possible. It has however become increasingly difficult to maintain the quality of aquatic ecosystems (Palaniappan et al., 2010) in recent years. Contaminants are regularly introduced into marine environments through various natural and anthropogenic activities. Some pollutants enter river systems naturally from volcanoes, natural fires and oil seeps amongst others (Hossain et al., 2012). Human activities contribute immensely to polluting water bodies where pollutants can enter water systems from industrial effluents, power generation, municipal discharges from cities and towns, accidental and deliberate oil spills, as well as recreationally, through power and speed boating activities (Chandra and Chaudhary, 2013).

Polycyclic Aromatic Hydrocarbons (PAHs) along with other pollutants reduce the quality of water by rendering water unsafe and non-potable, as many PAHs are known carcinogens, mutagens and teratogens (Samimi et al., 2009) and are known to affect the skin as well as the immunological and hepatic systems. The importance of sustainable abatement strategies to reduce contamination therefore cannot be overemphasised, as daily human activities continue to impact the environment. Research into environmentally friendly and cost effective clean-up techniques is therefore vital, and as bioremediation has been shown to be relatively cost effective, environmentally friendly and publicly accepted, it is an appealing alternative to conventional methods.

1.2. Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are a group of persistent, semi-volatile organic pollutants that are ubiquitous in the environment and enter environmental matrices via natural and anthropogenic sources such as agricultural and industrial activities (Wick et al., 2011). They are composed of two or more fused aromatics (benzene rings) and a system of hydrophobic and lipophilic double bonds throughout their hydrocarbon rings. These groups of compounds have potential harmful effects on ecosystems as well as human health as many of them have been shown to be carcinogenic, teratogenic and mutagenic (Quinn et al., 2009).

Several approaches and strategies including physical, chemical and biological strategies have been developed, optimised and utilised to mitigate the effects of these contaminants and remediate polluted sites. Some of these conventional remediation techniques have

significant limitations such as their technological complexity, high cost and the lack of public acceptance. Most of the techniques are invasive and merely relocates the contamination problem to a different site requiring further waste management. Bioremediation has been shown to be a cost effective and environmentally friendly approach to remediate contaminated sites (Soleimani, 2012). Several bacterial [*Pseudomonas*, *Alcanivorax, Microbulbifer, Sphingomonas, Micrococcus, Cellulomonas, Dietzia, Gordonia, Marinobacter* (Wu et al., (2013)] and fungal species [*Aspergillus sp*., *Trichocladium canadense*, and *Fusarium oxysporum* (Silva et al., 2009)] capable of degrading PAH compounds have been isolated and characterised. It is however imperative to investigate the PAH degrading capability of indigenous microorganisms in various ecosystems especially those that are subject to significant point sources of pollution. This study was aimed at isolating and identifying four potential PAH degrading microorganisms from the Diep- and Plankenburg Rivers in the Western Cape, South Africa, as well as investigating their degradative potential of two PAHs (acenaphthene and fluorene) under optimum temperature conditions.

1.3. **Research objectives**

The objectives of this study are to:

1. Isolate, identify and characterise PAH degrading bacteria from the Diep and Plankenburg Rivers, Western Cape, South Africa.
2. Optimise temperature conditions for microbial metabolism of the selected PAH compounds (acenaphthene and fluorene).
3. Monitor the degradation efficiency over a stipulated period of time.
4. Compare degradation efficiencies of Flask and Stirred Tank Bioreactor scale experiments.

1.4. **Significance of the research**

This study addressed the contamination of essential resources by PAHs, which is proving to be one of the numerous environmental concerns posing major risks to human and ecological health. During this study, microorganisms with the potential to use PAHs as carbon sources were isolated from PAH contaminated areas and utilised in bioreactors to degrade two forms of PAHs. The isolation and identification of these organisms will contribute greatly to developing remediation strategies for greater varieties of PAHs. It will also contribute to the growing research into the use of bioremediation as a natural, environmentally friendly and cost-effective means of environmental clean-up. The techniques used in this study could also be optimised and adopted for large-scale, on-site use in river systems.

1.5. Delineation of the research

The study did not investigate the following:

1. The bioremediation of all known Polycyclic Aromatic Hydrocarbons. The study focused on the bioremediation of acenaphthene and fluorene which were selected as model compounds due to their relative ease of solubility in acetonitrile (organic solvent) during a pre-study survey.
2. The occurrence and biodegradative potential of fungal and algal species present in the two river systems used as case study.
3. The optimisation of pH and other factors that influence microbial capability to degrade organic compounds. The study rather focused on temperature optimisation and the capability of the bacterial species to utilise the compounds as nutrient sources.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Water

Water covers more than 70% of the Earth's surface and exists in various forms (Ramanchandra and Solanki, 2007), including in the oceans, polar ice caps, clouds, rain water, rivers, freshwater aquifers, sea ice as well as in the ground and air we breathe (Ramanchandra and Solanki, 2007).

An aquatic ecosystem is a dynamic system where the biotic and abiotic components are constantly acting and reacting upon each other bringing forth structural and functional changes (Ramanchandra and Solanki, 2007). It can be described as a group of interacting organisms dependent on one another and their water environment for nutrients and shelter (Ramanchandra and Solanki, 2007). The two main types of aquatic ecosystems are marine ecosystems such as oceans, saltmarshes and lagoons, amongst others and freshwater ecosystems such as lakes, rivers and streams, amongst others (Alexander, 1999).

Aquatic ecosystems usually contain an extensive range of life forms including bacteria, fungi, and protozoa; bottom-dwelling organisms such as insect larvae, snails and worms; free-floating microscopic plants and animals known as plankton; large plants such as cattails, bulrushes, grasses, and reeds; as well as fish, amphibians, reptiles, and birds (Samimi et al., 2009). Organisms from various ecosystems differ because of varying habitats, unique to each kind of ecosystem. Many rivers are relatively oxygen-rich and fast-flowing compared to lakes therefore, species adapted to these particular river conditions are rare or absent in the still waters of lakes and ponds (Bergkamp et al., 2000).

2.2. Environmental pollution

The quality of a particular environment largely dictates the quality of life forms in that environment (Vidali, 2001). Pollution can be defined as the introduction of elements, compounds or energy (noise, heat or light) into the environment at levels that impair its functioning or that present an unacceptable risk to humans or other targets that use or are linked to that environment (Scullion, 2006).

2.3. Water pollution

Water pollution is the contamination of natural water bodies by chemical, physical, radioactive or pathogenic microbial substances (Hogan, 2013). It is a major global problem which requires on-going evaluation and revision of water resources management policies at all levels. It has been suggested that it is the leading worldwide cause of deaths and

diseases and accounts for the deaths of more than 14,000 people daily (Pink, 2006; West, 2006).

2.4. **Persistent Organic Pollutants (POPs)**

Persistent Organic Pollutants are toxic chemical compounds that are resistant to environmental (photolytic, biological and chemical) degradation (Maliszewska-Kordybach, 1999; Ritter et al., 2007) and can bioaccumulate in the food web posing significant risks to the health of ecosystems, wildlife and humans (Adeola, 2004; Seo et al., 2009). They are semi volatile, have low water solubility, high lipid solubility and high molecular masses (Ritter et al., 1995; Chandra and Chaudhary, 2013). Those with molecular masses lower than 236 g/mol are thought to be less toxic, less recalcitrant and have more reversible effects than those with higher molecular masses (Ritter et al., 2007). They usually have one or more cyclical ring structures of either aromatic or aliphatic nature, lack polar functional groups (Chandra and Chaudhary, 2013) and are frequently halogenated, usually with chlorine. The presence of more chlorine groups in a POP structure confers higher resistance to degradation (Chandra and Chaudhary, 2013).

Most of these compounds are created in industrial processes either intentionally or as by-products (Ritter et al., 2007), as many are currently or have been used in the past as pesticides, in the production of solvents, polyvinyl chloride, and pharmaceuticals (Ritter et al., 2007). They accumulate and biomagnify in food chains through fish, predatory birds as well as mammals and therefore, enter the human system through diets (Skoglund et al., 1996; Herbert et al., 1997).

They have numerous adverse effects on wildlife and humans (Damastra, 2002; Chandra and Chaudhary, 2013), have been shown to cause diseases in birds, marine mammals (Jones and De Voogt, 1999) as well as human beings and can induce neurobehavioural defects, disrupt endocrine, reproductive and immune function amongst other lethal effects (El-Shahawi et al., 2010). The compounds that make up POPs are also classified as PBTs (Persistent, Bioaccumulative and Toxic) or TOMPs (Toxic Organic Micro Pollutants) and are generally divided into three broad categories including Pesticides, such as Dichlorodiphenyltrichloroethane (DDT), chlordane, toxaphene, mirex, aldrin, dieldrin, endrin and heptachlor. Industrial chemicals, Polychlorinated biphenyls (PCBs), hexachlorobenzene by-products and contaminants, such as dioxins and furans and Polycyclic Aromatic Hydrocarbons (PAHs), certain brominated flame-retardants, as well as some organometallic compounds such as tributyltin (TBT) (Ritter et al., 2007).

2.5. **Properties of Polycyclic Aromatic Hydrocarbons**

Polycyclic Aromatic Hydrocarbons are a class of organic compounds containing two or more fused benzene rings with various structural configurations (Prabhu and Phale, 2003), where the rings could be in linear, angular or clustered arrangements (Lundestedt, 2003). PAHs are lipophilic, have low vapour pressure, low water solubility and high melting and boiling points (Skupinska et al., 2004). They contain only carbon and hydrogen atoms and in addition, nitrogen, sulphur and oxygen atoms may readily substitute in the benzene ring to form heterocyclic aromatic compounds, which are usually grouped with the PAHs (Lundestedt, 2003). Also, PAHs substituted with alkyl groups are normally found together with the PAHs in environmental matrices (Lundestedt, 2003, Nkansah, 2012).

According to the International Union of Pure and Applied Chemistry (IUPAC) (2006), the simplest PAHs are phenanthrene and anthracene, which both contain three fused aromatic rings (Buha, 2011). Polycyclic Aromatic Hydrocarbons are classified as low molecular weight (LMW) if they have two or three fused rings or high molecular weight (HMW) if they have four or more fused rings (Wick et al., 2011). Low Molecular Weight PAHs are more susceptible to degradation and volatilisation compared to the HMW PAHs (Harvey, 1998). As molecular weight increases, hydrophobicity/lipophilicity increases, water solubility decreases, vapour pressure decreases, and the compound will have a more recalcitrant structure (Maliszewska-Kordybach, 1999; Luch, 2005). The average half-life of the tricyclic phenanthrene ranges from 16 to 126 days in soil, whereas for the five ringed HMW PAH benzo(a)pyrene, the half-life may range from 229 to 1500 days (Sojinu et al., 2011). High molecular weight PAHs persist in the environment because of low water solubility, low volatility, resistance to leaching, and their recalcitrant nature (Wild and Jones, 1995; Jones et al., 1996).

Alkyl (CH_2- group) substitution of the aromatic ring results in an overall decrease in the PAH solubility, although there are some exceptions to this rule such as benzo(a)anthracene which is less soluble than either methyl or ethylbenzo(a)anthracene (Luch, 2005). Molecules with a linear arrangement tend to be less soluble than angular or perifused molecules. For instance, anthracene is less soluble than phenanthrene, and naphthalene is less soluble than chrysene or benzo(a)anthracene (Okere and Semple, 2012). The solubility of PAHs in water is enhanced three to four fold by a rise in temperature from 5ºC to 30ºC. Dissolved and colloidal organic fractions also enhance the solubility of PAHs which are incorporated into micelles (Luch, 2005). The United States Environmental Protection Agency (US EPA, 1991) has designated 32 PAH compounds as priority pollutants (Luch, 2005) including the HMW PAHs (benzo(a)pyrene, dibenz(a,h)anthracene and indeno(1,2,3-cd)pyrene, amongst others) and the LMW PAHs (naphthalene, acenaphthene, fluorene and phenanthrene, amongst others) which are often monitored for measurement in environmental samples. Some US

EPA priority PAH structures are shown in Fig. 1 while Table 1 shows the properties of some of the compounds.

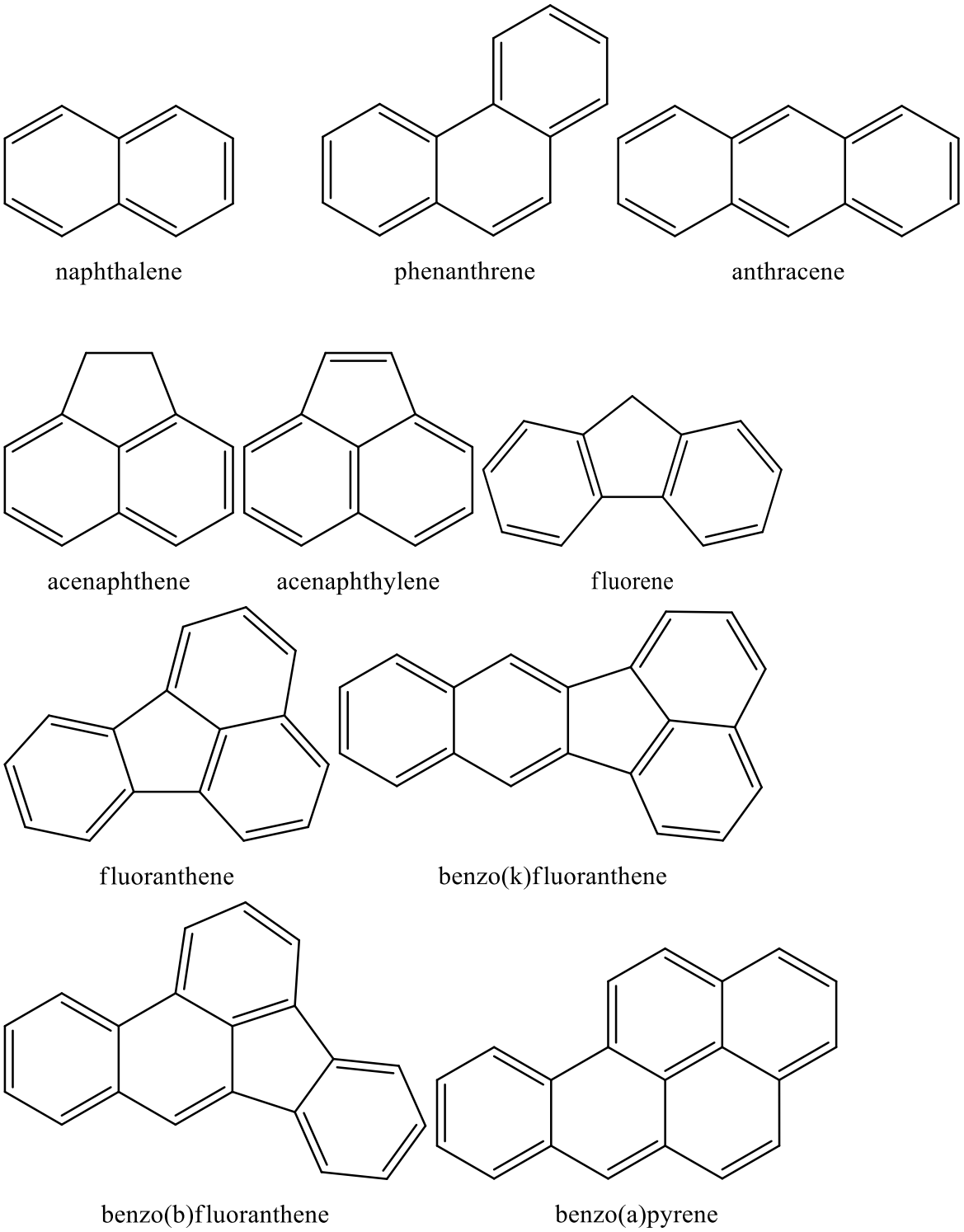


Figure 1: Chemical structures of selected US EPA priority PAHs (Cheremisinoff and Davletshin, 2010; Ukiwe et al., 2013).

Table 1: Properties of some US EPA priority PAHs (Okere and Semple, 2012).

PAH	Molecular weight	Melting point (ºC)	Water solubility	Log Kow
Naphthalene	128.2	79-82	320	3.5
Acenaphthene	152.2	95	5.3	3.95
Acenaphtylene	152.2	72-82	3.93	3.94
Fluorene	166.2	115-116	1.85	4.28
Phenanthrene	178.2	99	1.24	5.62
Anthracene	178.2	218	0.64	5.33
Fluoranthene	202.3	110	0.25	4.62
Pyrene	202.3	156	0.14	4.47
Benzo(a)anthracene	228.3	158	0.01	5.30
Chrysene	228.3	255	0.002	5.30
Benzo(b)fluoranthene	252.3	168	-	5.74
Benzo(K)fluoranthene	252.31	215	-	6.06
Benzo(a)pyrene	252.3	179	0.0038	5.74
Benzo(g,h,i)perylene	276.3	273	0.00026	6.20
Dibenzo(a,h)anthracene	278.35	262	0.0005	6.84
Indeno(1,2,3-c,d) pyrene	276.3	163	Insoluble	6.20

Keynote: Log Kow: Octanol-water partition co-efficient.

2.6. **Sources and Occurrence of PAHs**

Polycyclic Aromatic Hydrocarbons are products of pyrolysis of organic material (Wild and Jones, 1995; Lenicek et al., 1997) and are formed by the incomplete combustion of coal, oil, garbage, agricultural wastes and other organic substances such as wood, meat and tobacco (Sowa, 2011). Other sources include volcanoes, bush and prairie fires (Zhang and Tao, 2009; Sojinu et al., 2011) as well as exhaust fumes from vehicles and other engines. The effects of PAHs in the environment and the routes of wildlife or human exposure are influenced by the environmental medium (air, food, water or soil) in which the PAHs reside (Arey and Atkinson, 2003). They have been found occurring in air, soil, surface water, groundwater as well as sediments.

2.6.1. **Sources and occurrence in soil**

Polycyclic Aromatic Hydrocarbons enter the soil through atmospheric deposition from long range transport, industrial contamination (aluminium and coke production, petrochemical processes, wood preservation, rubber tyre and cement manufacturing as well as many other industrial processes), waste incineration and bush fires (Wick et al., 2011).

Several researchers have observed greater amounts of PAHs in urban soils as they are more exposed to the PAHs produced by both stationary (power plants, industries, and residential heating) and mobile sources (traffic emissions, and road by-products such as

wearing of tyres and asphalt constituents) (Kamaljit et al., 2010). Maisto et al. (2006) reported that total PAHs were 2 - 20 times greater in the urban areas of Naples (Italy) than the park soils that were 12 km away. Also, a study in Ontario (Canada) by Concord Scientific Corporation and Beak Consultants (1992) showed that a higher concentration of PAHs was associated with locations close to airports, highways, rail stations and heavy industries. In New Orleans, Wang et al. (2008) observed the highest amounts of PAHs in soils close to the roads (7,189 µg/kg), as compared to open spaces that were 10 m away from the roads (2,404 µg/kg). Similar results were shown by Wilcke (2000) who reported that PAH levels declined exponentially with an increase in distance from the roads due to the reduced vehicular emissions.

2.6.2. **Sources and occurrence in aquatic ecosystems**

Concentrations of PAHs in the aquatic environment are generally highest in sediment, intermediate in biota and lowest in the water column [Canadian Council of Ministers of the Environment (CCME), 1992]. They generally accumulate in sediments because they tend to adsorb to particulate matter that settle at the bottom of aquatic ecosystems (Juhasz and Naidu, 2000; Perelo, 2010) and are often encountered in more significant concentrations in water bodies close to point sources of contamination such as industries [World Health Organisation (WHO), 2003].

The PAH compounds that are most frequently detected at relatively high concentrations in water include: acenaphthene, fluorene, phenanthrene, acenaphthylene, benzo(a)anthracene and benzo(k)fluoranthene while the most common group of molecules found occurring in sediments are the four ring PAHs (Dhananjayan et al., 2012). Studies have shown that four ring PAHs such as chrysene were the most dominant of the PAH distributions in sediments from San Francisco Bay (Pereira et al., 1996) as well as in the Kor River (Iran), where the four ringed PAH fluoranthene was the most dominant in sediment samples (Kafilzadeh et al., 2011). The PAH composition in the sediments reflect the source from which the PAHs are derived as shown in studies performed by Simpson et al. (1998) and Sojinu et al. (2010). Lower molecular weight PAHs are usually formed from petrogenic sources while pyrolysis of fossil materials yields HMW PAH assemblages (Helfrich and Armstrong, 1986). A study by Sojinu et al. (2010) in oil exploration areas of The Niger Delta (Nigeria) showed that the PAHs occurring in most of the sediment samples are of petrogenic origin.

2.6.3. **Sources and occurrence in surface waters**

PAHs enter surface waters mainly via atmospheric particulate matter deposition (including wet and dry deposition of particles and vapours), runoff from polluted ground sources, urban

runoff, municipal wastewater discharges, industrial effluents, oil spills and seeps (Latimar and Zheng, 2003; Yanyangwu, 2012).

Atmospheric deposition is considered to be an important input of PAHs to surface waters where 10% - 80% of PAH inputs to the world's oceans is estimated to be from atmospheric sources (Motelay-massei et al., 2006). Rainwater has been shown to contain many organic compounds including PAHs, where the concentration of PAHs in rainfall can sometimes be much higher than in the receiving water body (Manoli and Samara, 1999b).

A significant amount of PAHs carried to surface waters by sewers comes from urban runoff. Urban runoff consists of the storm water from impervious areas, such as roads, motorways, paved parking lots and roofs, as well as pervious areas (including and not exclusive to gardens, unpaved parking areas and construction sites). As a consequence, urban runoff is usually PAH laden which is invariably deposited onto surfaces, as well as mobile-related PAHs from gasoline and oil drips or spills, exhaust products, tyre particles, and bitumen from road surfaces (Bomboi and Hernandez, 1991). Studies have revealed that PAH concentrations in urban runoff are five to ten times higher during autumn and winter, due to increased vehicular activities during this time, coupled with the use of heating systems (Bomboi and Hernandez, 1991). The relative contribution of urban runoff to receiving waters is site-specific and dependent on the relative magnitudes of the wet and dry weather discharges.

Municipal and domestic wastewaters also release PAHs into surface waters. Concentrations of total PAHs in raw municipal wastewaters have been found to vary significantly, depending on the amount of industrial effluents possibly co-treated with domestic wastewaters. Treated wastewaters usually contain PAHs at much lower concentrations due to their removal by adsorption on particles, biodegradation or volatilisation (Manoli and Samara, 1999a).

2.6.4. **Sources and occurrence in groundwater**

PAHs in groundwater may originate from polluted surface water bodies, irrigation with contaminated water, leachates from solid waste disposal sites or contaminated soil (Manoli and Samara, 1999a). Groundwater concentrations of carcinogenic PAHs reported for US groundwater ranged from 0.2 ng/l to 6.9 ng/l, while the corresponding concentrations in surface waters were between 0.1 ng/l and 800 ng/l and most frequently between 2 and 50 ng/l (Nigam and Singh, 2011). Acenaphthene (0.88317 mg/l), acenaphthylene (0.18837 mg/l), naphthalene (0.52510 mg/l), fluorene (0.20438 mg/l), phenanthrene (0.26732 mg/l) and anthracene (0.25084 mg/l) were detected in the groundwater sources of a coastal settlement near the Port Harcourt refinery company situated at Okrika Mainland, Port Harcourt (Nigeria) (Okoli et al., 2011). However, it has been reported that PAH

concentrations in near surface groundwater may increase after periods of rain, indicating that a quick transfer from rainwater into groundwater is possible (Manoli and Samara, 1999b).

2.6.5. **Sources and occurrence in drinking water**

The presence of PAHs in drinking water may be attributed to raw water sources from surface or groundwater or to the use of coal tar-coated pipes in public water supply systems (Vega et al., 2011). It has been shown that chlorination of drinking water may lead to the formation of oxygenated and chlorinated PAHs, which are more toxic than the parent PAH compounds (Shiraishi et al., 1985; Manoli and Samara, 1999a). According to the WHO, results obtained from a survey conducted to establish guidelines for the assessment of drinking water quality revealed elevated concentrations of PAHs (predominantly fluoranthene, phenanthrene, benzo(b)fluoranthene, pyrene, indeno(1,2,3-cd)pyrene) in rainwater and especially in snow and fog (WHO, 1997). This is probably a result of the adsorption of the compounds to air particulate matter, which is finely dispersed into the water during wet deposition (WHO, 2003).

2.6.6. **Sources and occurrence in food**

PAHs enter plants essentially through atmospheric deposition on grains, fruits and vegetables especially those with broad leaves such as spinach (Fismes et al., 2002) and via uptake from contaminated soil and groundwater (Fismes et al., 2002). Vegetation in urban areas especially those close to roads and industries have been reported to have higher doses of PAHs (Gomes et al., 2013). Grazing cattle and poultry which may ingest particulate matter from soil are susceptible to contamination by PAHs adsorbed to particles (Scientific Committee on Food, European Commission, 2002). Aquatic foods such as fish, mussels, shellfish and shrimp are contaminated through absorption of contaminated fluvial and marine waters (Mackay and Fraser, 2000; Menichini and Bocca, 2003). The extent of accumulation and retention of PAHs in marine organisms are influenced by several factors and mechanisms such as physicochemical, organismal physiology, the available fraction of the PAH compound that can be readily absorbed by the organism, the distribution and profile of PAH compounds in the aquatic ecosystem, their uptake and partitioning in different tissues, their rates of elimination as well as their potential for persistence in varying species (Meador et al., 1995).

2.7. **Effects of PAHs**

Polycyclic Aromatic Hydrocarbons are of great environmental and health concern as they are recalcitrant, bioaccumulative and toxic to living organisms and ecosystems (Kweon et al., 2011; Hajisamoh, 2013). The toxicity of PAHs depends on a number of factors including the

species, route of exposure and molecular structure (Ramesh et al., 2004). The uptake of high doses of persistent molecules can have hazardous effects on flora and, through the food chain, on fauna and human health (Langenbach, 2013). Humans are exposed to PAHs through inhalation, ingestion of contaminated food and water and through dermal contact (Sowa, 2011).

2.7.1. **Human health effects**

The effects of PAHs on human health depend mainly on the duration and rate of exposure as well as the concentration and the innate toxicity of the individual PAH (Buha, 2011). Factors such as pre-existing health status and age can also influence the effects that human exposure to PAHs induces (Buha, 2011). It is often difficult to ascribe exclusive health effects in epidemiological studies to specific PAHs because exposure mostly occurs to a combination of different PAHs [Agency for Toxic Substances & Disease Registry (ATSDR), 2011]. However, some studies have shown that exposure to certain individual PAHs over extended periods at elevated concentrations can induce deleterious effects (Langenbach, 2013).

Naphthalene, a common micropollutant in potable water (Sudip et al., 2002), binds covalently to molecules in liver, kidney and lung tissues, thereby enhancing its toxicity. It is also known to cause haemolytic anaemia and eye defects [International Programme on Chemical Safety (IPCS), 2000] as well as acting as an inhibitor of mitochondrial respiration (Falahatpisheh et al., 2001). In cases of acute exposure especially in the case of occupational exposure, signs and symptoms such as nausea, vomiting, abdominal pain, diarrhoea, headache, confusion, profuse sweating, fever, tachycardia, tachypnoea and agitation may be induced (IPCS, 2000). Acute dermal exposure to naphthalene has been associated with mild irritation and in some sensitive individuals may cause dermatitis [Centres for Disease Control and Prevention (CDC), 2009]. Naphthalene is not believed to cause cancer in humans but has been shown to induce tumours in laboratory mice and other animals (IPCS, 2000). Ingestion of naphthalene is not a common route of exposure; however the effects observed are similar to those seen following acute inhalation, with the likely complication of abdominal pain (IPCS, 2000).

Phenanthrene, a major constituent of urban air pollution, has been shown to be mutagenic and impair immune function (CDC, 2009). It is a weak inducer of sister chromatid exchanges, a potent inhibitor of gap junctional intercellular communication (Weis et al., 1998) and is a known photosensitizer of skin as well as being a mild allergen (Mastrangela et al., 1999; Sudip et al., 2002). Acenaphthene has been proven to have harmful effects on skin, body fluids and immune system of animals after both short and long-term exposure (ATSDR, 1995) while studies have also shown that fluorene induces skin and eye irritation

and is a potential carcinogen (New Jersey Department of Health and Senior Services, 1999). Benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene, benzo(k)fluranthene, dibenz(a,h)anthracene and indeno(1,2,3-c,d)pyrene has been shown to be carcinogenic (Sram et al., 1999; Liu et al., 2001).

2.7.2. **Effects on aquatic ecosystem**

Many studies have examined the effects of PAHs on aquatic organisms (Varanasi et al., 1989; Harvey, 1997). They have been shown to be toxic to a variety of aquatic organisms; acting as carcinogens, DNA mutagens, and endocrine disruptors (Pittinger et al., 1987; Hellou et al., 2006). Adverse biological effects associated with PAHs in sediments include decreased benthic invertebrate abundance, distorted diversity and growth as well as physiological and behavioural changes (Liu et al., 2013).

In aquatic mammals and fish, the immunotoxic effects of PAHs have been widely demonstrated (Weeks and Warimer, 1984; Hellou et al., 2006). Exposure to PAHs has been reported to affect egg production in fish and alter normal fish development. Cytogenetic toxicity was also observed in fish larvae exposed as eggs, to low concentrations of petrogenic source PAHs (Carls et al., 1999). Similarly, classical cytogenetic techniques showed significant genetic toxicity in herring larvae, in association with the Exxon Valdez spill in Alaska, the effects of which were correlated with levels of PAHs found in mussels in the area (Hose and Brown, 1998). Biochemical defects have been observed in fish in coastal waters, lakes and rivers in a number of countries (Payne et al., 2003). Alteration of phase I and to a lesser extent phase II enzymes which play a major role in detoxification and other biochemical processes have also been observed (Hose and Brown, 1998). Changes in hormones, energy reserves and serum enzymes in fish exposed to PAHs have occasionally been reported. Results obtained from experimental studies with fish chronically exposed to sediments contaminated with PAHs of petrogenic or pyrolytic sources as well as industrial formulations such as creosote, indicate that PAHs are a likely cause of some of the pathological defects found in fish in highly contaminated environments (Payne et al., 2003). An unusually high prevalence of oral, dermal, and hepatic neoplasms have been observed in bottom-dwelling fish from polluted sediments containing grossly elevated PAH levels (Couch and Harshbarger, 1985).

Anthracene, which is known to produce highly cytotoxic by-products in some organisms when they are simultaneously exposed to the chemical compound and ultraviolet light damaged the gills of fish upon exposure to low levels of PAHs (Olaniran et al., 2013).

Kim et al. (2007) found that adult Pacific oysters (*Crassostrea gigas*) had marked decreased productivity when exposed to even low concentrations of PAHs. A study by Martineau et al. (2002) in St Lawrence Estuary, Quebec (Canada) on SLE Beluga (*Delphinapterus leucas*)

suggests a correlation between the incidence of cancer in these species and the presence of PAHs in their habitat produced by local aluminium smelters. Aluminium smelting can lead to PAH pollution because the reducing agent used in the process is commonly a source of carbon such as coke or charcoal (Habashi, 2003).

Stream biota, such as insect larvae and crustaceans, are also affected adversely, resulting in reduced species diversity in affected areas (Beasley and Kneale, 2002). A study by Ikenaka et al. (2013) demonstrated that B(a)P notably induced a decrease in zooplankton abundance on the zooplankton community.

Researchers have expressed concerns that PAHs and other toxic components in oil could wipe out generations of some species (Mascarelli, 2010). Microorganisms live in areas of delicate balance, which can be disrupted by episodes of pollution (Beazley et al., 2012) causing drastic changes to microbial communities in ecosystems. Selective pressure has been seen to be placed on communities of organisms after major pollution incidents such as oil spills (Beazley et al., 2012). Post spill samples after the Deep water Horizon oil spill in the Gulf of Mexico near Mississipi (United States) contained mostly oil-degrading organisms resulting from a drastic decrease in diversity. Selective pressure placed upon communities of organisms by the large increase of petroleum and other petroleum derivatives selects for the survival of organisms that can use petroleum and derivatives as energy, electron, and or carbon sources (Beazley et al., 2012).

2.8. **Persistence of PAHs in the environment**

Polycyclic Aromatic Hydrocarbons in the environment are usually subjected to processes such as volatilization, photo-oxidation, chemical oxidation, adsorption onto organic matter and leaching, as well as microbial degradation responsible for PAH losses (Wild and Jones, 1995; Pantsyrnayaa et al., 2011). In aquatic systems the environmental fate of PAHs is influenced by dissolution, adsorption onto suspended solids and subsequent sedimentation, biotic and abiotic degradation as well as uptake and accumulation by aquatic organisms (Pantsyrnayaa et al., 2011). PAHs escape degradation and persist in environmental matrices for long periods because of a variety of factors including chemical structure, environmental conditions, the concentration and dispersion of the PAH as well as the bioavailability of the contaminant (Bamforth and Singleton, 2005).

Generally, the higher the molecular weight of a PAH molecule, the higher the hydrophobicity and toxicity, and the longer the environmental persistence of such a molecule (Cerniglia, 1992; Bamforth and Singleton, 2005). The age of the contaminant in the sediment or soil also has an effect on the biodegradability of the PAH molecule (Hatzinger and Alexander, 1995). A study using phenanthrene as a model PAH showed that phenanthrene

mineralisation and therefore biodegradability was significantly reduced with time of ageing of the contaminant (Hatzinger and Alexander, 1995; Rhodes et al., 2008).

The occurrence of PAHs with co-contaminants such as hydrocarbons, phenols, BTEX compounds and heavy metals is another factor that can prolong their residence time in the environment (Bamforth and Singleton, 2005). Aliphatic hydrocarbons and BTEX compounds are readily biodegradable by the indigenous microbial community, at a rate relative to the more complex chemical structures of the PAHs. This results in the depletion of available oxygen in the surrounding environment and the onset of anaerobicity (Bamforth and Singleton, 2005). Though it has recently been concluded that there is a real potential for the biodegradation of PAHs in the absence of molecular oxygen, details regarding the efficiency and scale of PAH degradation in anaerobic environments are still comparatively limited, with rates of anaerobic organic matter oxidation up to an order of magnitude less than those under aerobic conditions. In addition, there is the possibility that the presence of heavy metals in contaminated matrices could inhibit microbial growth and limit the metabolism of contaminants under anaerobic conditions (Bamforth and Singleton, 2005).

2.9. **Bioavailability of PAHs for microbial degradation**

One of the most important factors that directly influence the efficiency of biological treatment is the availability of contaminants for the degrading microorganisms (bioavailability) (Lawniczak, 2013; Olaniran et al., 2013). Bioavailability can be defined as the effect of physicochemical and microbiological factors on the rate and degree of biodegradation (Mueller et al., 1996). It is the percentage of contaminant that can be readily accessed and degraded by microorganisms (Bosma et al., 1997; Maier et al., 2000).

Generally, contaminants ‘escape’ degradation due to reasons that include: (i) contaminant toxicity to the microorganisms, (ii) preferential feeding of microorganisms on other substrates, (iii) unfavourable environmental conditions in the matrix for propagation of appropriate microorganisms and (iv) poor contaminant bioavailability to microorganisms (Castaldini, 2008). PAHs have also been shown to be stable due to their structure which consists of several double bonds (Web, 2011). They have low water solubility and studies have revealed that PAHs in the solid state are consumed by microorganisms only after they are transferred to the aqueous phase through the dissolution process (Volkering et al., 1993; Olaniran et al., 2013). Studies have also shown that the strong adsorption capacity that PAHs have for particulate matter contributes largely to the recalcitrance of PAHs, which in turn significantly reduces their bioavailability (Castaldini, 2008).

In contaminated sediments availability depends on physical factors such as grain size of the sediment, suspended particulate materials and biological factors including wildlife diversity of the aquatic ecosystem (benthic or pelagic organisms) and mode of exposure to the

contaminants. One of the best methods proposed for assessing the bioavailability of sediment-associated contaminants is to observe their accumulation in organisms (Geffard et al., 2003; Lu et al., 2006).

Several researchers have been able to biologically degrade PAHs by increasing their availability to microbial metabolism (Maier et al., 2000; Wick et al., 2011). This is usually achieved using various methods such as biostimulation, bioaugmentation, composting, land farming, use of surfactants, solvents and other solubility enhancers (Wick et al., 2011). In experimental conditions, the addition of detergents and non-ionic surfactants such as Tween-20 and Tween-80 increases the solubility and substrate availability to ligninolytic enzymes and cells (Riess et al., 2005). Various surfactants could increase the rate of anthracene, pyrene, and benzo(a)pyrene oxidation by *Bjerkandera sp.* BOS55 by two-to-five fold with a degradation efficiency of 74%, 79% and 88%, respectively (Riess et al., 2005). The stimulating effect of surfactants was found to be solely due to the increased bioavailability of PAHs, indicating that the oxidation of PAHs by the extracellular ligninolytic enzymes is limited by low compound bioavailability (Lu et al., 2006).

However, some studies have shown that the use of synthetic surfactants to clean up contaminated sites may result in the introduction of more pollutants (Wang and Brusseau, 1993; Makkar and Rockne, 2003) which suggests they might be unsafe for the environment after extensive use. The application of some surfactants might improve the solubility and desorption rate of the PAH compounds to the aqueous phase but not necessarily improve the degradation rate and efficiency (Makkar and Rockne, 2003). A study conducted by Mulder et al. (1998) showed that the introduction of hydropropyl-ß-cyclodextrin (HPCD), a well-known PAH solubility enhancer, significantly increased the solubilisation of PAHs, although it did not improve the biodegradation rate of PAHs (Mulder et al., 1998). This asserts that factors other than solubility affect degradation rates of PAHs.

The dissolution rate of PAH particles can also be improved by the hydrodynamic conditions of the system in a bioreactor. Riess et al. (2005) demonstrated that the volumetric mass transfer coefficient of PAH particles was significantly enhanced in a bioreactor. The use of glass beads in the reactor significantly increased the turbulence at the interfacial surface of solid particles, thus reducing the film thickness and enhancing the mass transfer coefficient. On the other hand, the grinding force acting on the PAH particles by the beads broke up the PAH particles, increasing the surface area of particles and improving the mass transfer coefficient. Many other researchers have also reported the effect of hydrodynamics on the mass transfer rate of PAH particles (Vinas et al., 2005; Singh, 2006).

2.10. **PAH remediation**

The remediation strategies designed to combat PAH pollution are generally classified into three main categories including physical (excavation, retrieval and off-site disposal, dredging, dry excavation of sediments, volatilisation, thermal treatment, capping technique and incineration), chemical (chemical oxidation, photocatalysis and solvent extraction, amongst others) and biological (bioremediation) (Singh, 2006).

2.10.1. **Physical treatment of PAHs**

Physical treatment systems may be used in conjunction with attenuation approaches or in the case of polluted groundwater, can take the form of permeable reactive barriers that transform pollutants into environmentally acceptable forms (Tong and Yuan, 2012).

(i) Excavation, retrieval and off-site disposal of PAHs

It usually involves pre-treatment of the contaminated media in order to meet land disposal regulations, where-after the contaminated material is removed and transported to permitted off-site treatment sites and/or disposal facilities. Cost implications brought about by factors such as the distance from the contaminated site to the nearest disposal facility, as well as the depth and composition of the media requiring excavation should be considered when selecting a disposal site. In addition, it is highly challenging and increasingly expensive to find new landfill sites for the final disposal of the contaminating material (Ruihong, 2006; Castaldini, 2008). Significant risks may also arise during handling and transport of hazardous material (Ruihong, 2006). Other limitations to be considered include, generation of undesirable emissions, and possible health risks associated with transportation of the excavated material through residential areas, while disposal options for certain wastes (mixed waste or transuranic waste) may be limited (US EPA, 1991; Pavel and Gavrilescu, 2008).

(ii) Dredging of PAHs

Dredging is one of the most expensive techniques that yield the greatest mass removal of contaminated sediments from the aquatic environment (Zeller and Cushing, 2005). It however merely relocates the contamination problem to another site as the removed sediment must be deposited elsewhere, thus requiring further waste management. Other factors to consider during dredging, include (i) the dredge may skip contaminated areas, (ii) residual contaminated sediment may mix with underlying surrounding sediments and (iii) the re-suspended material can migrate downstream with consequent effects on receiving ecosystems (Perelo, 2010).

Dredging may lead to long or short-term increases of contaminant bioavailability, facilitating pollutant entrance into food chains thereby increasing the chances of biomagnification of pollutants (Zeller and Cushing, 2005; Perelo, 2010). As dredging is also a very invasive technique, it very likely leads to the alteration or destruction of the benthic community. Furthermore, the presence of boulders and debris may limit the efficacy of the procedure thereby impeding low contaminant clean-up levels [National Research Council (NRC), 1997]. Nevertheless, dredging has been used to effectively remediate several lakes, rivers and harbours in the United States, Spain, Australia and other countries because it permanently removes the contaminated sediment (Birch and Taylor, 2002; Zeller and Cushing, 2005; Cassado-Martinez et al., 2006). Most of the removed material is usually disposed in landfills while some are put in Confined Aquatic Disposal (CAD) cells which considerably saves cost (Perelo, 2010).

(iii) Dry excavation (of sediments) of PAHs

Dry excavation differs from dredging since the removal of sediment follows a significant dewatering of the water body, which enhances access to the contaminated areas and minimises contaminant migration downstream during excavation. As with dredging, removed sediment requires subsequent treatment and/or deposition. This technique is very expensive and poses similar benefits and limitations as dredging in addition to the technical challenge of maintaining “dry” conditions (Reible et al., 2006).

(iv) Capping technique

Capping consists of covering the contaminated sediment surface with clean material, thus isolating the contaminated sediments (Perelo, 2010). Potential benefits are the reduction of contaminant water column concentrations and therefore reduced bioavailability to benthic and aquatic organisms, as well as the minimisation of downstream migration (Perelo, 2010). Applying caps to contaminated sediment is typically less expensive than dredging, with costs depending primarily on cap design.

Long-term monitoring and maintenance is required as capping is only an interim solution since the contaminants remain on site (Zeller and Cushing, 2005; Castaldini, 2008). There is also the possibility that contaminated sediments can be mixed or re-suspended during the placement of capping material (Perelo, 2010). Placement may also be challenging in deeper waters, areas with wave action, boat traffic, or large target surface areas (Perelo, 2010). Long-term risks include, the erosion of the cap and diffusion of contaminants through the cap material, disruption of groundwater seepage patterns, and the creation of flow bypassing around the caps (Reible et al., 2006). Additionally, depending on the capping material used, benthic communities may be altered (Perelo, 2010). The presence of an *in-situ* cap shifts the

deposition of labile organic matter to the newly formed cap-water interface, thus removing a source of carbon and organic substrates from the biologically active sediment, where biotransformations are most likely to occur (Himmelheber et al., 2007).

(v) Thermal treatment of PAHs

Thermal treatment generally involves the destruction or removal of contaminants through exposure to high temperature in treatment cells, combustion chambers, or other means used to contain the contaminated media during the remediation process (Pavel and Gavrilescu, 2008). It usually involves techniques such as hot gas decontamination, incineration, open burn/open detonation, pyrolysis, thermal desorption as well as *ex-situ* vitrification, amongst others (Pavel and Gavrilescu, 2008).

Thermal degradation of PAHs can occur at low, intermediate or high temperatures in the presence of additives, as well as in the presence or absence of oxygen. Low-temperature thermal processes are generally classified as operating below 177ºC, though temperatures slightly above this are acceptable (Nkansah, 2012). Uncontrolled low temperature thermal treatment of organic waste can lead to the formation of PAHs with more than four rings while degradation occurs for only those with less than four rings (Pakpahan et al., 2009). According to a study conducted on the thermal stability of benzo(a)pyrene, benzo(a)anthracene and dibenzo(a,h)anthracene at temperatures between 100ºC - 200ºC, the loss of each PAH depends on treatment time. A greater percentage of the original compounds were lost at 200ºC than at 100ºC (Chen et al., 2003). Medium or intermediate temperature thermal processes usually take place between 177ºC - 370ºC (Nkansah, 2012) and intermediate thermal treatments usually employ subcritical water for the removal and H_2O_2 for the oxidation of PAHs (Pakpahan et al., 2009).

2.10.2. **Chemical treatment of PAHs**

Chemical remediation involves the use of chemicals to extract pollutants from contaminated media. Most common methods used include solvent extraction and chemical oxidation (Riser-Roberts, 2010). Solvent extraction is particularly beneficial for the remediation of PAHs since solvents are capable of desorbing and removing recalcitrant contaminants from contaminated matrices. Chemical remediation techniques are relatively cheaper since methods are used *in-situ* thereby eliminating costs associated with transportation of contaminated samples for off-site treatment.

2.11. **Demerits of conventional PAH remediation techniques**

Conventional remediation techniques have significant limitations such as their technological complexity, high cost and the lack of public acceptance (Castaldini, 2008). Traditional methods of oil spill clean-up include the use of controlled burns, skimmers, vacuum pumping, manual mechanical clean-up, low pressure flush and the use of gelling agents, amongst others. These techniques will only remove the contaminants from the affected environment and transfer them to another location (Castaldini, 2008). A more effective approach than these traditional methods would be to adopt an environmentally sustainable technique that will either completely destroy the pollutants or transform them into harmless substances (Lundestedt, 2003; Castaldini, 2008).

2.12. **Bioremediation (Biological Treatment of Contaminants)**

Bioremediation, also known as bioreclamation or biorestoration is a pollution control strategy that uses biological systems to convert various toxic compounds into innocuous forms (Vidali, 2001). It involves the use of living organisms, primarily microorganisms, to degrade or detoxify hazardous waste into harmless substances such as carbon dioxide, water and cell biomass (Barret et al., 2010; Langenbach, 2013). In this technique, the biodegradative abilities of microorganisms are harnessed to remove or detoxify environmental pollutants (Da Silva et al., 2003; Singh and Tripahthi, 2007). The technology can adopt a natural degradation pathway or utilise recombinant organisms to use certain toxic compounds as carbon or energy sources (Lu et al., 2011). Bioremediation can occur on its own through natural attenuation (intrinsic bioremediation), but in most cases could take several years as various bioremediation strategies have been developed to promote the microbial metabolism of contaminants by adjusting several variables (Langenbach, 2013). This can be achieved by biostimulation (stimulating viable native microbial population), bioaugmentation (artificial introduction of viable populations), biosorption (dead microbial biomass), bioaccumulation (live cells), phytoremediation (plants) as well as rhizoremediation (plant and microbe interaction) (Sharma, 2012). Other examples of bioremediation technologies are land farming, bioventing, bioleaching, composting and the use of bioreactors amongst others (Vidali, 2001; Chadrankant and Shwetha, 2011). Bioremediation technologies can be applied *in-situ* or *ex-situ*, are relatively cost effective and use low-technology methods that are non-invasive and which generally have a high public acceptance (Sharma, 2012; Castaldini 2008).

The success of bioremediation is governed by three important factors, including availability of efficient degraders, accessibility of contaminants and a conducive environment (Chadrankant and Shwetha, 2011). Several life forms are known to efficiently degrade various kinds of hazardous wastes; however, microorganisms are more successfully used for

the process of bioremediation because of their ubiquitous distribution in normal and extreme environments, fast biomass growth, easy manipulation and high diversity of catabolic enzymes (Sharma, 2012; Langenbach, 2013).

2.13. **Bioaugmentation**

Bioaugmentation involves the addition of specifically formulated microorganisms or an inoculum of microorganisms with known pollutant transformation abilities to a contaminated site to reinforce natural biological processes (Tyagi et al., 2011; Sharma, 2012). It is done in conjunction with the development and monitoring of an ideal growth environment in which these selected bacteria can live and work. The basic premise for this intervention is that the metabolic capacities of the indigenous microbial community already present in the contaminated site will be enhanced by an exogenously enhanced genetic diversity, thus leading to a wider repertoire of productive biodegradation reactions (Leung, 2004; Swaranjit et al., 2003).

The most commonly adapted options for bioaugmentation include the addition of a pre-adapted pure bacterial strain (or consortium), introduction of genetically engineered microorganisms, and the incorporation of biodegradation relevant genes into a vector to be transferred by conjugation into indigenous microorganisms (El Fantroussi and Agathos, 2005; Tyagi et al., 2011).

2.14. **Biostimulation**

Many microorganisms naturally possess the ability to degrade, transform, or chelate various toxic chemicals, but these natural transformation processes are relatively slow. In order to achieve desired treatment results, environmental conditions that would permit microbial growth and activity must be created (Chandrakant and Shwetha, 2011).

Biostimulation is a technique developed to achieve optimum conditions for microbial growth within contaminated sites. It also involves stimulating the viable microbial population by adjusting water, air and nutrient supply. It involves the introduction of additional nutrients (organic or inorganic), bulking agents such as woodchips, compost and electron donors or acceptors to a contaminated site (Namkoong et al., 2002; Scow and Hicks, 2005).

2.15. **Phytoremediation**

Phytoremediation is the use of green plant-based systems to degrade, assimilate, metabolise or detoxify pollutants in contaminated soils, sediments and water. Phytoremediation is a fledgling technology intended to address a wide variety of surficial contaminants. Phytoremediation targets currently include contaminating metals, metalloids,

petroleum hydrocarbons, pesticides, explosives, chlorinated solvents, and industrial by-products (Du et al., 2011).

This technology makes use of naturally occurring processes by which plants and their microbial rhizosphere flora degrade and/or sequester organic and inorganic pollutants (Pradhan et al., 1998). Research has shown that various grasses and leguminous plants are potential candidates for phytodegradation of organics (Ukiwe et al., 2013). Some tropical plants have also been reported to show effective degradation potential due to inherent properties such as deep fibrous root system and tolerance to high hydrocarbon and low nutrient availability (Ukiwe et al., 2013). Chen et al. (2003) reported that the tall fescue grass (*Festuca arundinacea*) and switch grass (*Pannicum virgatum*) are capable of degrading about 38% of pyrene in 190 days. Other plants have also been reported to efficiently degrade PAHs. Industrial hemp (*Cannabis sativa*) was reported to degrade B(a)P and chrysene, Rye grass (*Lolium multiflorum*) and water hyacinth (*Eichhornia crassipes*) were reported to reduce about 45% of naphthalene in waste water in seven days (Ukiwe et al., 2013) and Bermuda grass (*Cynodon dactylon*) which was reported to degrade naphthalene.

2.16. **Bioremediation of PAHs**

Bioremediation of PAH waste has been extensively studied at both the laboratory and commercial levels and has also been implemented at a number of contaminated sites (Purwaningsih, 2002; Prince, 2010). The mechanisms by which microorganisms degrade PAHs include metabolism or co-metabolism, where co-metabolism has been shown to be especially relevant for the degradation of mixtures of PAHs (Prince, 2010). Polycyclic Aromatic Hydrocarbon degradation can be either aerobic or anaerobic in nature, but the aerobic pathways, their kinetics, enzymatic and genetic regulation thereof are more extensively documented (Arun et al., 2010; Wick et al., 2011).

Most biological transformations of aromatic ring structures are catalysed by mono or dioxygenases and therefore proceed only when molecular oxygen is available for ring cleavage. It may thus appear that aromatic metabolism is restricted to aerobes possessing oxygenase enzymes. Microbial transformation of aromatic compounds under denitrifying, sulphate-reducing and methanogenic conditions, however, is fundamentally different from degradation under aerobic conditions (Karthikeyan and Bhandari, 2001).

2.16.1. **Aerobic bioremediation of PAHs**

The aerobic biodegradation process also known as aerobic respiration is the breakdown of contaminants by microorganisms in the presence of oxygen (Bamforth and Singleton, 2005; Gan et al., 2009). Aerobic bacteria use oxygen as an electron acceptor to break down both the organic and inorganic matter into smaller compounds, often producing carbon dioxide

and water as the final products (Gan et al., 2009). The principal mechanism for the aerobic bacterial metabolism of PAHs is the initial oxidation of the benzene ring by the action of dioxygenase enzymes to form cis-dihydrodiols. These dihydrodiols are dehydrogenated to form dihydroxylated intermediates that may be further metabolised via dihydroxy compounds (catechols) to carbon dioxide and water (Habe and Omori, 2003; Wick et al., 2011). White rot fungi produce extracellular lignin-degrading enzymes with low substrate specificity, which makes them an ideal degrader of PAHs under aerobic conditions (Gan et al., 2009).

2.16.2. **Anaerobic bioremediation of PAHs**

PAHs commonly contaminate anaerobic environments such as aquifers (Bakermans et al., 2002) and marine sediments (Coates et al., 1997). Aerobic environments such as contaminated soils, sediments and groundwater can also develop anaerobic zones (Anderson and Lovely, 1997; Bamforth and Singleton, 2005). This is due to the organic contaminant stimulating the *in-situ* microbial community resulting in the depletion of molecular oxygen during aerobic respiration. This oxygen is not replenished at the same rate as it is depleted, resulting in the formation of anaerobic zones proximal to the contaminant source (Bamforth and Singleton, 2005).

In such cases when oxygen is absent or limited, biodegradation can occur anaerobically. Unlike aerobic biodegradation, anaerobic microorganisms uses other available substances such as nitrate, sulphate, iron, manganese and carbon dioxide as their electron acceptors to break down organic compounds into smaller constituents, often producing carbon dioxide and methane as the final products (Gan et al., 2009). Alternatively, some anaerobic microorganisms can break down organic contaminants by fermentation whereby the organic contaminants act as the electron acceptors (Gan et al., 2009; Ukiwe et al., 2013).

Primarily, anaerobic biodegradation is enforced when the degree of contamination is very high, limiting oxygen flow due to organic matter pore saturation or clogging of aggregates. This technology is a promising remediation process for accidental oil spills as well as remediation of water-submerged soil such as paddy fields and swamps. Furthermore, anaerobic biodegradation is anticipated to replace aerobic biodegradation since a large aeration area is not necessary to reduce total remediation costs. Anaerobic bioremediation may also be applied for the treatment of deep underground soil and groundwater since the process is not oxygen dependent (Prince, 2010; Karigar and Rao, 2011).

2.17. **Factors affecting bioremediation of PAHs**

The success of bioremediation depends on the microbial population involved, degree of acclimation, accessibility of contaminants, chemical structure of the compound, cellular transport properties, chemical partitioning in growth media as well as a conducive

environment for remediation (Swaranjit and Randhir, 2010). Efficiency related to the above factors is further dependent on pH, temperature, oxygen, salinity, nature and pollution history of the contaminated site, accessibility of nutrients, the occurrence of other toxic compounds (co-contamination), amongst others (Margesin and Schinner, 2001).

2.17.1. **pH**

Most important PAH degrading microorganisms perform best when pH is neutral. However, fungi are known to be more tolerant to acidic conditions (Al-Daher et al., 1998). Many sites contaminated with PAHs are however not at the optimal pH for bioremediation (Prince, 2010). Many retired gasworks sites have been used as case studies as they often contain significant quantities of demolition waste, such as concrete and brick. Leaching of this material will increase the pH of the soil, resulting in less favourable conditions for microbial metabolism (Bamforth and Singleton, 2005). In addition the oxidation and leaching of coal spoil will create an acidic environment through the release and oxidation of sulphides. As the pH of contaminated sites can often be linked to the pollutant, the indigenous microorganisms at the sites might not have the capacity to transform PAHs under acidic or alkaline conditions. Therefore, it is common practice to adjust the pH at these sites, by the addition of lime (Bamforth and Singleton, 2005).

2.17.2. **Temperature**

Generally bacterial metabolic activity and PAH biodegradation increases with increasing temperatures up to an optimum temperature reported to be around 30ºC to 40ºC (Zhang et al., 2005; Okere and Semple, 2012). Hydrocarbon utilising bacteria such as *Geobacillus, Alcanivorax and Pseudomonas sp.* can also adapt to temperature extremes to maintain metabolic activity (Kostka et al., 2011; Zhang et al., 2012). Hydrocarbon degradation has previously been reported both at temperatures close to freezing and above 30ºC (Kostka et al., 2011).

2.17.3. **Salinity**

Studies have shown that there are positive correlations between salinity and rates of mineralisation of PAHs. Hypersalinity has been reported to result in reduced microbial metabolic rates (Dupraz and Visscher, 2005). A study by Minai et al. (2012) showed that PAH degradation was more efficient in a medium containing 0% NaCl than in 5% NaCl medium.

2.18. **Biosurfactants**

Biosurfactants are a structurally diverse group of surface-active compounds synthesised by a variety of microorganisms. They are amphiphilic molecules that have both hydrophobic and hydrophilic domains and are capable of lowering the surface and interfacial tension of a growth medium (Pacwa-Plociniczak et al., 2011). They are usually used as additives to counter the low aqueous solubility of PAHs and enhance the efficiency of bioremediation (Gan et al., 2009). They are environmentally friendly, biodegradable, less toxic and non-hazardous, are highly reactive and are active at extreme temperatures, pH and salinity (Das et al., 2008; Pacwa-Plociniczak et al., 2011). Biosurfactants are categorised by their chemical composition, molecular weight, physicochemical properties, mode of action and microbial origin (Nguyen et al., 2008; Nievas et al., 2008). Based on molecular weight they are divided into low-molecular mass biosurfactants including glycolipids, phospholipids and lipopeptides and high molecular weight biosurfactants/bioemulsifiers (amphipathic polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixtures of lipopeptides, glycolipids, neutral lipids and fatty acids) (Cameotra and Bollaga, 2003; Pacwa-Plociniczak et al., 2011). Biosurfactants are applied in a variety of ways; the molecules may either be added externally (influent, spraying or injection) or produced on site which seems especially promising in case of *in-situ* treatment. In the latter case, the production of biosurfactants may be obtained by bioaugmentation with appropriate microorganisms since autochthonous microorganisms do not usually exhibit satisfactory efficiency (Lawniczak et al., 2013).

Biosurfactants facilitate the transport of hydrophobic contaminants into the aqueous phase through specific interactions resulting in solubilisation thereby increasing their bioavailability which potentially makes them more susceptible to biodegradation (Maier and Soberón-Chávez 2000; Costa et al., 2011). They enhance hydrocarbon biodegradation by two mechanisms (Pacwa-Plociniczak et al., 2011). The first involves the increase of substrate availability for microorganisms, while the other involves interaction with the cell surface which increases the hydrophobicity of the surface, allowing hydrophobic substrates to associate more easily with bacterial cells (Mulligan and Gibbs, 2004). By reducing surface and interfacial tensions, biosurfactants increase the surface areas of insoluble compounds leading to increased mobility and bioavailability of hydrocarbons. The capability of biosurfactants and biosurfactant producing bacterial strains to enhance availability of organic contaminants and biodegradation rates, have been reported by many authors (Deziel et al., 1996; Rahman et al., 2003; Inakollu et al., 2004). Obayori et al. (2009) investigated the biodegradative properties of biosurfactant produced by *Pseudomonas sp*. LP1 strain on crude oil and diesel. The results obtained confirmed the ability of strain LP1 to metabolise the hydrocarbon components of crude and diesel oil. It was reported that 92.34%

degradation of crude oil and 95.29% removal of diesel oil was achieved during the investigation. Biodegradative properties of biosurfactant producing *Brevibacterium sp.* PDM-3 strain were tested by Reddy et al. (2010). The study showed that this strain could degrade 93.92% of phenanthrene and is capable of degrading other PAHs such as anthracene and fluorene. Other microorganisms such as *Bacillus subtilis, Pseudomonas aeruginosa* and *Torulopsis bombicola* have been reported to produce surfactants such as surfactin, rhamnolipid and sophorolipid capable of improving PAH bioremediation (Kuyukina et al., 2005; Cottin and Merlin, 2007).

2.19. **Bacterial degradation of PAHs**

Bacterial species are actively involved in the degradation of organic pollutants from contaminated sites. These organisms belong to a number of genera including; *Pseudomonas*, *Alcanivorax, Microbulbifer, Sphingomonas, Micrococcus, Cellulomonas, Dietzia, Gordonia, Marinobacter Mycobacterium, Haemophilus, Rhodococcus, Paenibacillus Bacillus, Aeromonas, Burkholderia, Xanthomonas, Micrococcus, Arthrobacter, Acinetobacter, Alcanivorax , Corynebacterium* and *Enterobacter* (Bayoumi, 2009; Wu et al., 2013).

Many studies have revealed that several bacterial species can utilise naphthalene as a sole source of carbon and energy. These belong to the genera *Alcaligenes, Burkholderia*, *Mycobacterium*, *Polaromonas, Pseudomonas, Ralstonia, Rhodococcus, Sphingomonas*, *Streptomyces* (Seo et al., 2009) as well as *Bacillus firmus*-APIS272, *Pseudomonas alcaligenes-DAFS311* and *Bacillus subtilis-SBS526* (Bayoumi, 2009). Anthracene has been reported to be completely mineralised by *Bacillus firmus-APIS272*, *Bacillus subtilis-SBS526*, *Bacillus licheniformis*, *Burkholderia cepacia-DAFS11, Pseudomonas alcaligenes-DAFS311*, *Sphingomonas sp., Nocardia sp., Beijerinckia sp., Rhodococcus sp.* and *Mycobacterium sp.* (Bayoumi, 2009; Mrozik et al., 2009). Various *Mycobacterium, Brevibacterium, Sphingomonas*, *Rhodotorula, Aeromonas Acidovorax, Arthrobacter* and *Comamonas species* have been reported to metabolise phenanthrene (Romero et al., 1998; Mrozik et al., 2009). A study by Bayoumi (2009) showed that *Bacillus subtilis-SBS526*, *Micrococcus lylae–SBS661* are able to mineralise acenaphthene. Species of *Arthrobacter*, *Brevibacterium, Burkholderia*, *Mycobacterium, Pseudomonas* and *Sphingomonas* have been reported to degrade fluorene (Seo et al., 2009). *Mycobacterium* has been extensively studied and is well-known to mineralise high molecular weight PAHs such as fluoranthene, pyrene, and benzo(a)pyrene (Seo et al., 2009). Strains in the genera *Burkholderia*, *Pasteurella, Rhodococcus, Sphingomonas*, and *Stenotrophomonas* have been isolated to degrade fluoranthene, using it as a sole carbon and energy source (Mrozik et al., 2009; Seo et al., 2009). *Rhodococcus sp., Bacillus cereus, Burkholderia cepacia, Cycloclasticus sp*., *Pseudomonas fluorescens, Pseudomonas stutzeri, Sphingomonas sp.*, *Sphingomonas*

paucimobilis and *Stenotrophomonas maltophilia* are all efficient pyrene degraders (Seo et al., 2009). Rehmann et al. (1998) isolated a *Mycobacterium spp*. strain KR2 from a gaswork site which was able to utilise pyrene as sole carbon and energy source. The isolate metabolised up to 60% of the pyrene within eight days at 20ºC. *Pseudomonas, Agrobacterium, Bacillus*, *Burkholderia, Sphingomona*s, *Rhodococcus*, *Mycobacterium* as well as mixed culture of *Pseudomonas* and *Flavobacterium* species have all been reported to efficiently degrade B(a)P (Bhatnagar and Kumari, 2013). *Bacillus firmus* has been reported to completely degrade benzo(b)fluoranthene, dibenzo(a,h) anthracene and indeno (1,2,3-c,d) pyrene (Bayoumi, 2009).

2.20. **Fungal degradation of PAHs**

Fungal species are tolerant to high concentrations of recalcitrant compounds and are able to flourish in extreme conditions such as at high temperature and under low pH conditions, which makes them highly proficient PAH degraders (Anastasi et al., 2013; Ding et al., 2013). Furthermore, the fact that fungi form large, branching mycelia makes it possible for them to grow and distribute through a solid matrix to degrade PAHs within contaminated areas (*in-situ*) by virtue of secreting extracellular enzymes or by sequestration of PAHs (Silva et al., 2009; Bhattacharya et al., 2012). In addition to biodegradation and mineralisation of PAHs, fungal species are capable of adsorbing PAHs onto their hydrophobic cell wall (Tekere et al., 2005). Due to the irregular structure of lignin, lignolytic fungi produce extracellular enzymes with very low substrate specificity, making them capable of degrading a wide array of pollutants (Juckpecha et al., 2012).

Silva et al. (2009) reported that LMW PAHs (2 - 3 rings) were found to be degraded most efficiently by *Aspergillus sp*., *Trichocladium canadense* and *Fusarium oxysporum* while for HMW PAHs (4 - 7 rings) maximum degradation has been observed by *Trichocladium canadense*, *Aspergillus sp*., *Verticillium sp.* and *Achremonium sp*. The study also proved that fungi had a great capability to degrade a broad range of PAHs under low-oxygen conditions. A study by Bhattacharya et al. (2012) showed that *Pleurotus ostreatus* was able to degrade B(a)P and that *Phanerocheate chrysosporium* showed significant biosorption and biodegradation of phenanthrene.

2.21. **Algal degradation of PAHs**

Prokaryotic and eukaryotic photoautotrophic marine algae (i.e. cyanobacteria, green algae, and diatoms) are known to metabolise naphthalene (Haritash and Kaushik, 2009; Dwivedi, 2012) while Benzo(a)Pyrene is known to be transformed to diols and quinones by marine algae in a period of five to six days (Dwivedi, 2012). *Selenastrum capricornutum,*

Scenedesmus obliquus, Dunaliella sp., Chlamydomonas sp. have all been reported to efficiently degrade PAHs (Semple et al., 1999).

2.22. **Genetically Engineered Microorganisms**

Adverse environmental conditions or other unfavourable conditions might inhibit the survival of an efficient natural degrader in a natural ecosystem. Consequently, it might show less efficiency in comparison with laboratory conditions (Bustamante et al., 2011). It is possible that by enhancing the enzymatic activity of biochemical pathways using genetic engineering (resulting in higher expression of key enzymes), improved degradation of many persistent compounds (including PAHs) that are abundant in the environment could be achieved (Samanta et al., 2002).

2.23. **Bioreactors**

A bioreactor is a manufactured or engineered vessel in which a chemical process is carried out which involves organisms or biochemically active substances derived from such organisms (Atanu et al., 2011). This process can either be aerobic or anaerobic (Decker and Reski, 2008). These bioreactors are commonly cylindrical, ranging in size from litres to cubic metres and are often made of stainless steel or glass (Decker and Reski, 2008). A bioreactor may also refer to a device or system meant to grow cells or tissues in the context of cell culture (Transfiguracion et al., 2011). Bioreactors have proven to be effective in remediating soil, and in some cases water, polluted with fuel hydrocarbons (oil, gasoline, diesel) and organics (Das and Chandran, 2011). Bioreactor design is dependent on the contaminant to be remediated, the media that is contaminated as well as cost constraints (IUPAC, 2006). The microorganisms responsible for pollutant degradation are usually bacteria but can also be fungi. Usually, bioreactor operation relies on the use of native microflora already existing in the polluted media. However, whenever desirable indigenous microflora is scarce or weak or with no apparent capability of degrading the target compounds, researchers inoculate the reactors with an enriched or acclimated consortia or strains (more commonly consortia) in the form of bioaugmentation (Lu et al., 2011). The introduction of specialised biomass may permit increased biodegradation of target pollutants as well as a more effective detoxification of the solid matrix, which also significantly saves time (Robles-González et al., 2008).

One of the distinct advantages of bioreactors is the ability to manipulate, monitor and control environmental and operational variables to maximise biodegradation potential. Variables such as pH, temperature, nutrient levels, microbial activity, dissolved oxygen (in the case of aerobic reactors) are usually manipulated thus optimising contaminant degradation (Fulekar and Geetha, 2008; Robles-González et al., 2008).

2.24. **Biofilms**

Biofilms are microbial colonies which form when single microorganisms attach and aggregate on a hydrated surface and undergo a "lifestyle switch," giving up life as a single cell to live on a surface in an adhesive cell matrix with other microorganisms (Lemon et al., 2007). They are usually resistant to antimicrobial agents and studies have revealed that cells within a particular biofilm are usually of diverse community properties. Biofilms are capable of attachment to living and non-living surfaces, generate medical problems, alter industrial processes but more importantly, play an essential role in environmental clean-up.

Biofilm systems are especially suitable for the treatment of recalcitrant compounds because of their high microbial biomass and ability to immobilise compounds (Singh et al., 2006). According to these authors, bioremediation is also facilitated by enhanced gene transfer among biofilm organisms and by the increased bioavailability of pollutants for degradation as a result of bacterial chemotaxis. Strategies for improving bioremediation efficiency include genetic engineering to improve strains and chemotactic ability, the use of mixed population biofilms and optimisation of physicochemical conditions.

Biofilm-mediated bioremediation presents a proficient and safer alternative to bioremediation because cells in a biofilm have a better chance of adaptation and survival (especially during periods of stress) as they are protected within the matrix (Decho, 2000). Due to the close, mutually beneficial physical and physiological interactions among organisms in biofilms, the ability to utilise pollutants as carbon source by microorganisms is improved and this improves the efficiency of biodegradation (Singh et al., 2006).

2.25. **Diep River (34.0342ºS 18.4644ºE)**

The Diep River is located in the South Western Cape Region, North of Cape Town. According to Coastal and Environmental consulting (2011), the catchment of the Diep River has a relatively flat topography which makes it suitable for agricultural- and industrial activities as well as increased urbanisation. The Diep River and particularly its lower reaches have therefore been significantly modified over the past few centuries.

Over the years there have been significant changes to the volumes and quality of water and associated sediments flowing into the Diep River Estuary. Physical developments, such as roads and bridges has altered the functioning of the River as a completely natural system, and is presently seriously degraded (Whitfield et al., 2012). The estuary however, remains highly valuable from both a conservation and socio-economic perspective (C.A.P.E. Estuaries Programme, 2011).

2.26. **Plankenburg River (33.9167ºS 18.8500ºE)**

The Plankenburg River is approximately 10 km long and services various activities in the residential, industrial and agricultural sectors. The river system runs through the town of Stellenbosch (Western Cape Province), which is well known for its many wine estates. Adjacent to the town is the informal housing settlement of Kayamandi, which comprises a population of more than 24 645 people (Rock, 2011). Due to a lack of adequate sanitation and waste removal facilities in the informal settlement, as well as poor management and disposal of sewage, the storm water drainage pipes leading directly to the river are often used as a means of disposal of human and animal waste (Paulse et al., 2009). This pollutes the water body and renders it unsafe for domestic, agricultural, sports and recreational purposes. Also, the close proximity of various industries to the Plankenburg River predisposes it to both organic and inorganic contamination.

Polycyclic Aromatic Hydrocarbons have been detected in several South African Rivers such as, Vaal Triangle Area River where ten PAH compounds were detected (0.0538 - 0.4072 mg/l) (Moja et al., 2013), Thohoyandou, Limpopo Province where six PAH compounds were detected and quantified (0.1 - 9870 µ/l) (Nekhavhambe et al., 2014), as well as in Centurion Lake (30.3 - 213.8 ng/l), Hartebeesport Dam (21.4 - 615.7 ng/l) and the Jukskei Hennops Rivers in Johannesburg (61 - 45281 µg/kg) (Sibiya et al., 2013).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Sampling Site Identification

Three sampling points were identified along each of the two rivers studied. Along the Plankenburg River; Points A - C represents (A) an agricultural farming and residential area, and (B) a substation in industrial area and the (C) informal settlement of Kayamandi respectively. For the Diep River, Points D - F represents (D) the Zoarvlei nature reserve (industrial as well as residential), (E) the Theo Marias Sports club (industrial and residential area) and (F) the Rietvlei boating club respectively as shown in Fig. 2 below.

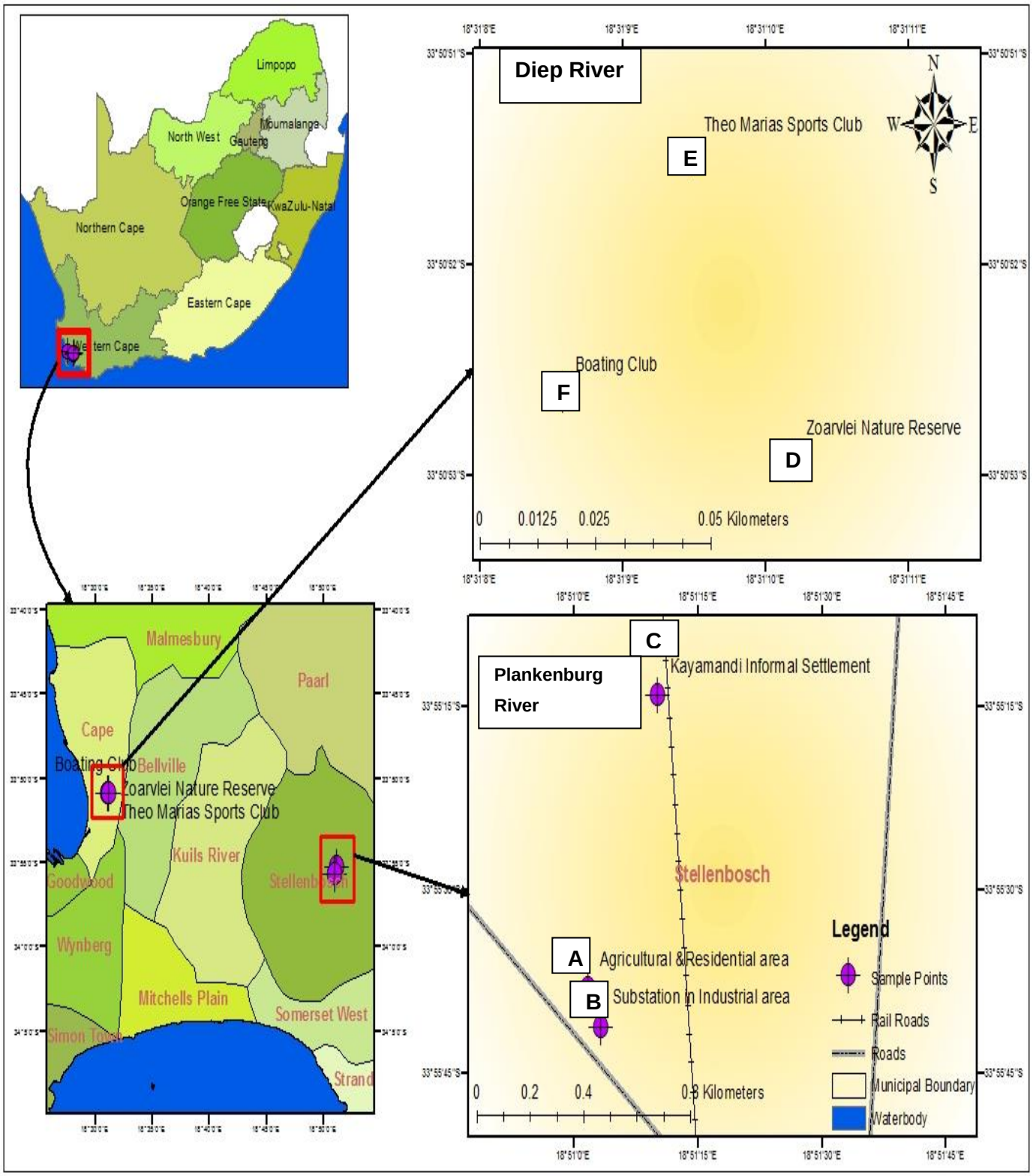


Figure 2: Map of the Diep- and Plankenburg Rivers showing locations of sampling sites (agricultural farming and residential area, substation in industrial area, informal settlement of Kayamandi, Zoarvlei nature reserve, Theo Marias sportsclub and Rietvlei boating club).

3.2. **Sampling**

Water and sediment samples were collected once a month over a one year period. Water samples were collected in sterile amber bottles, while sediment samples were collected using a sterile Ekman grab, placed in polypropylene containers and wrapped in aluminium foil. Both water and sediment samples were kept on ice at 4ºC during transportation to the laboratory for analyses. During sampling, physicochemical parameters such as temperature, pH, conductivity, Total Dissolved Solids and Salinity were measured and recorded using a handheld PCS teslr 35 multi-parameter gauge (Wirsam, SA). All samples were analysed in not more than 90 hours after collection.

3.3. **Determination of the presence and concentration of acenaphthene and fluorene in the River systems**

Acenaphthene and Fluorene were extracted from the water and sediment samples with 50 ml of a 4:1 mixture of n-hexane and dichloromethane (DCM) (Guerin, 1999) in 500 ml separating funnels. The flask was left for 15 minutes at room temperature to allow for equilibration and efficient phase separation. This procedure was repeated thrice to ensure good PAH recovery (above 70%). The extracts were combined and dried in a water bath at 35ºC under a stream of nitrogen. The dried samples were reconstituted to 2 ml with n-hexane.

The extracts were cleaned using Solid Phase Extraction (SPE) technique. The SPE glass tube frits were conditioned by eluting each one with 10 ml DCM, and 20 ml n-hexane at a flow rate of 1 ml/min. Each PAH concentrate extract was then loaded on the SPE/PTFE frits tubes and eluted with 70 ml of n-hexane. The eluates were dried in a water bath under a nitrogen stream. The resulting residues were re-dissolved in 3 ml methanol and dried again in a water bath under a nitrogen stream. Each of the dried concentrates was dissolved in 1 ml of n-hexane and filtered through 0.45 µm Millipore acrodisc membrane filters (Olatunji et al., 2014). The samples were run on GC/FID (Gas Chromatograph/ Flame Ionization Detection).

3.4. **Isolation and Identification of bacterial species from the Diep and Plankenburg River systems using conventional techniques**

To obtain pure isolated cultures, standard microbiological techniques including serial dilution, plating and culturing were employed. Various general, selective and differential media including Nutrient agar, MacConkey agar (Merck, Germany) Mannitol Salt agar, Eosine Methylene Blue (EMB) agar, *Pseudomonas* isolation agar base (Oxoid, England), *Aeromonas* isolation agar and Glutamate Starch Phenol Red (GSP) agar (Fluka, India) were used in order to obtain as many isolates as possible. Phenotypic identification techniques

such as staining and biochemical tests were conducted for 'tentative' identification of the isolates (see Table 5). Gram reaction, endospore staining, motility, methyl-red, citrate, catalase, oxidase, Voges-Proskauer, urease production and indole test were all carried out. Gram and endospore staining were done using conventional microscopy techniques (Harley, 2014). Methyl-red test was done by inoculating tryptone broth with the bacterial cultures and the set-up was incubated at 37ºC for two days. One dropfull of methyl-red was added to the broth and the colour change, was observed (McDevitt, 2009). For the citrate test, the test culture was streaked across the surface of Simmons citrate medium slants. The test tubes were then incubated at 37ºC for 24 hours, after which growth characteristics on the slant surface were observed. The catalase test was done by adding one drop of 3% H_2O_2 to a colony (on a microscope slide), followed by observing for bubbling (Harvey, 2014). The oxidase test was done using the filter paper test method, where a small piece of filter paper is soaked in 1% Kovacs oxidase reagent and allowed to dry. A well isolated colony from a fresh culture is rubbed onto the treated filter paper and colour changes were observed and recorded [American Society for Microbiology (ASM), 2013]. For the Voges-Proskauer, Barritts reagents A and B were used. MR-VP broth was inoculated with pure cultures of the test organisms (the use of heavy inoculum was avoided). The MR-VP broth tubes were incubated at 37ºC for 48 h, after which results were observed and recorded (McDevitt, 2009). For the urease test, Christensen's urea agar slants were inoculated with a heavy inoculum from an 18-hour culture across the surface of the slants and incubated with loosened caps at 35ºC. The slants were observed for colour change after six hours, 24 h and daily after that for the next six days and recorded (Brink, 2010). Bacterial cultures were inoculated in tryptone broth and incubated at 37ºC for 48 hours for the indole test. After 48 hours, 1 ml of Kovac's reagent was added to the tubes, shaked gently and observed for a red colour ring around the interface between the broth and the alcohol reagent (Harvey, 2014). Representations of some of the biochemical test set-ups are presented in Appendix III. Isolates were then selected on the basis of morphology; colour, cell shape and size, pigmentation and Gram reaction.

3.5. **Molecular Identification of bacterial isolates obtained from Diep and Plankenburg Rivers**

To further identify the isolated bacterial strains, using the ZR Fungal/Bacterial DNA kit™ (Zymo Research), DNA was extracted from the bacterial cultures. The concentrated DNA samples were amplified by Polymerase Chain Reaction (PCR) using a thermal cycler (Mastercycler® personal, Eppendorf AG, Germany). The 16S target region was amplified using the universal primers 27F (5I-AGAGTTTGATCMTGGCTCAG-3I) and 1492R (5I-CGGTTACCTTACGACTT-3I) (Lane et al., 1991; Turner et al., 1999). The PCR reactions

include an initial denaturation step at 94ºC for 10 minutes, followed by 30 cycles of denaturation at 95ºC for 15 seconds, primer annealing at 60ºC for 1 minute and primer extension at 72ºC for 1 minute. In the final step, the samples were incubated at 72ºC for 10 minutes. PCR amplification was verified by gel electrophoresis, performed in a horisontal submarine apparatus with 1% agarose gel and TAE as the tank buffer. Electrophoresis was carried out for 2 hours at 50 V and the gel visualized in an UV illuminator. The PCR amplified DNA was purified and sequenced in the forward and reverse directions on the ABI PRISM^{TM} 3500 analyser. The nucleotide sequences obtained were analysed using CLC main workbench 7 followed by a BLAST (Basic Local Alignment Search Tool) search provided by NCBI (National Centre for Biotechnology Information) (www.ncbi.nlm.nih.gov) and identified. The consensus sequences in FASTA format were deposited in the NCBI database and accession numbers were assigned to each isolate.

To generate phylogenetic relationships (trees), the nucleotide sequences of the isolated species were aligned by Mafft version 7. The aligned sequences were imported into the Molecular Evolutionary Genetics Analysis (MEGA) package version 6, where phylogenetic analyses were conducted using the neighbor-joining (NJ) method. The pairwise deletion option was used for handling alignment gaps. The evolutionary distances were computed using Kimura 2-parameter method while the strength of the branches were calculated with 1000 Bootstrap replicates.

3.6. **Identification of potential PAH-degrading bacterial species using temperature optimisation screening**

Each isolate designated ‘1’, ‘2’, ‘3’, ‘4’, ‘5’, ‘6’, ‘7’, ‘8’, ‘9’, ‘10’, ‘11’, ‘12’, ‘13’, ‘14’, ‘15’, ‘16’, ’17’, ‘18’ and ‘19’ was screened to determine which members of the bacterial species isolated are potential PAH degraders as well as their optimum PAH degrading temperature (see Tables 4-6). In simulated experiments, 114 flasks each containing 80 ml sterile distilled water, 10 ml of the analyte [acenaphthene, fluorene (Sigma Aldrich, Germany)] in solution and 10 ml of bacterial culture, were used as starter medium. The isolates were cultured in Tryptone broth overnight and each PAH compound was dissolved first in 30% acetonitrile because of PAH poor aqueous solubility and subsequently taken into solution by gently shaking in amber bottles in a shaking incubator for three days at 70 rpm. The experiment was carried out in a shaking incubator over a period of four weeks at 25ºC, 30ºC, 35ºC, 37ºC, 38ºC, 40ºC and 45ºC (Gan et al., 2009; Amenu, 2014) and 150 rpm. Bacterial culturing was done daily in order to assess which organisms can withstand the PAH compounds and which isolates can utilise them as carbon sources. The culturing was done by taking 1 ml of the sample as rapidly as possible, diluting serially ten-fold and subsequently plating on media plates. Plate count, morphology, motility, Gram reaction and biochemical tests were

all monitored throughout the experiments. The degradation potential of the identified isolates was determined using HPLC coupled with a dual wavelength absorbance detector (Dodor et al., 2004). Only isolates that could degrade up to 75% acenaphthene and fluorene were selected for subsequent degradation studies.

3.7. **Degradation study**

The microorganisms that successfully degraded acenaphthene and fluorene up to 75% and above from the temperature optimisation experiments were selected as potential PAH degraders and used for degradation experiments at both Flask and Stirred Tank Bioreactor scale. Natural conditions were mimicked, by not adding supplementary nutrients and the system was maintained in the most cost-effective manner as possible. Samples were obtained daily to monitor bacterial growth, changes in morphology, Gram reaction and the number of cells was counted using plate counts. The degree of degradation was assessed using HPLC equipped with a dual wavelength absorbance detector; the mobile phase was acetonitrile, the flow rate of the mobile phase was maintained at 1 ml/min, standard solutions of the compounds were used as reference/control, the samples were injected one after the other and the utilisation rates of the compounds were calculated based on the peak area per cent and retention time. The ratio of the amount of substrate degraded in test reactors to the amount of the substrate recovered in the control reactors was also calculated to determine the extent of degradation. Polypropylene filters were used to purify samples prior to running on the HPLC. Draw time was approximately one minute, as the sample collection was done as rapidly as possible to avoid disrupting the experiment. All experiments were run in triplicate.

3.7.1. **Flask Scale Degradation**

The methodology adopted for the flask scale experiment was similar to the temperature optimisation screening protocols. The experiment was repeated for the selected PAH degrading microorganisms and the biodegradative potential of single species were compared with consortium (cocktail) flasks. Solutions containing 10% analyte, 10 ml overnight culture and made up to a final volume of 100 ml with sterile distilled water was placed in 36 separate conical flasks. Prior to inoculating with overnight cultures, the PAHs were dissolved in acetonitrile (30%) and taken into solution. For each compound, there were two flasks containing the consortium (a combination of all isolates), as well as a flask that was not inoculated and designated as sterile control for each compound. The sterile control was to account for PAH losses due to other factors apart from biological such as photooxidation and volatilisation amongst others and was also used to compare degradative capabilities of the isolated cultures. The experiment was carried out in a shaking incubator

which was run at 25ºC, 30ºC, 35ºC, 37ºC, 38ºC, 40ºC and 45ºC at 150 rpm for 14 days (the 14 day period was selected because the PAH degrading organisms all degraded the compounds within that time range at the temperature optimisation screening scale). All flasks were capped with cotton wool and covered with aluminium foil to minimise losses due to photooxidation.

3.7.2. **Stirred Tank Bioreactor scale**

A total of nine reactors were used during this experiment. The reactors were amber coloured glass containers with a working volume of 1 L (Glasschem, Stellenbosch, South Africa). All the reactors were equipped with over-head stirrers with flat-blade radial turbine impellers and were all run at 150 rpm. The flange of each reactor had five openings which were all capped with polypropylene plastic caps and fit to the reactor vessel by a wire spring. Each reactor was run for a total of four weeks with the same content as used in the flask scale reactors. Temperatures were maintained at optimum for each sample obtained from flask scale experiments using a hot water bath equipped with a thermometer to ensure temperature accuracy. One of the nine stirred tank bioreactors was left uninoculated and served as the sterile control, while the other eight were inoculated with ‘1’, ‘2’, ‘12’, ‘13’ and the cocktail (‘1’, ‘2’, ‘12’ and ‘13’), respectively.

3.8. **Data Analysis**

All data obtained from this study were analysed by an SPSS statistical package using repeated measures ANOVA. The means and standard deviations of triplicate treatments were also calculated and Microsoft EXCEL software was used to illustrate graphs.

CHAPTER FOUR

4. RESULTS AND DISCUSSION

4.1. Physicochemical parameters and microbial numbers

The rivers investigated serve multiple functions such as domestic, recreational and agricultural purposes (Paulse et al., 2009) and the South African Bureau of Standards (SABS, 1984) as well as the Department of Water Affairs and Forestry (DWAF, 1996 a-c) have stipulated guidelines for faecal coliform counts to safeguard these natural water sources. The SABS (1984) and DWAF (1996a) have stipulated that levels of *E.coli* in natural water sources such as rivers should not exceed 2 000 microorganisms / 100 ml and 130 microorganisms / 100 ml respectively because counts above these levels may lead to waterborne infections.

During the summer months [December (month 12) to March (month 3)], microbial growth was more abundant compared to the colder months. Most of the results obtained from the Plankenburg River on average exceeded the stipulated acceptable limits (Figs. 3 and 4 and appendix 1). Generally, microbial counts obtained from sediment samples were higher than in water samples (Figs. 3 and 4). This could be attributed to the sediment matrix providing increased attachment sites for the colonisation and proliferation of bacteria (Fischer et al., 2003).

The typical range of Colony Forming Units (CFU) on plates cultured from both surface water and sediment during the summer months was 4.4×10^4 - 2.88×10^9 whereas on a typical cold sampling day there were fewer cells (between 0.6×10^3 - 8.1×10^8) as shown in Figs. 3 and 4 as well as in Appendix 1.

Microbial counts ranging between 0.6×10^3 - 4.5×10^7 (CFU/ml) were recorded from surface water at Site A (agricultural farming and residential area) while counts ranging between 1.3×10^7 and 6.6×10^7 (CFU/ml) were recorded from the sediment samples at this site. Furthermore, isolate 16, which had been isolated from agricultural wastes (Gupta et al., 2012; Pulikotil-Anthony et al., 2014) was recorded at numbers ranging between 1.0×10^4 to 3.2×10^7 CFU/ml (surface water) and 1.9×10^7 to 3.8×10^7 CFU/ml (sediment) at this site (Plankenburg Site A). The presence of this microorganism suggests that agricultural wastes are introduced into the river system from the surrounding agricultural industries. Downstream from this site, the river services an agricultural area which contributes enormously to the international wine industry and thus the economy of South Africa. According to DWAF, river water used for irrigation, such as the Plankenburg River, should not exceed 1 000 microorganisms / 100 ml (*E. coli*) in the water source. High counts recorded at this site [2.9×10^7 CFU/ml 'MA' (appendix 1) at peak summer] therefore could not only cause disease in

humans (DWAF, 1996c), but also have major effects on the economy and international relationships of the country.

For site B (a substation in Plankenburg industrial area), microbial counts ranged between 0.9 x 10^6 and 5.1 x 10^7 CFU/ml (surface water) and 5.5 x 10^3 – 5.9 x 10^8 CFU/ml (sediment) (Figs. 3 and 4). Isolates '10' (1.1 x 10^3 to 3.8 x 10^7; 5.4 x 10^4 to 7.1 x 10^7), '19' (3.2 x10^7 to 5.1 x 10^7; 5.6 x 10^7 to 1.02 x 10^8) and '8' (2.0 x 10^6 to 4.8 x 10^6; 3.3 x 10^7 to 5.9 x 10^7) (CFU/ml from surface water and sediments, respectively), which have been previously isolated from soil and dairy wastes (Patel et al., 2010) were detected at site B. There are several industries around this sampling point, and this data therefore suggest the release of industrial wastes from the industries in this vicinity into the river system (Fig. 2).

The highest microbial load (3.1 x 10^5 to 6.9 x 10^8; 1.1 x 10^6 to 2.88 x 10^9 from surface water and sediment, respectively) as well as microbial activity (Figs. 3 and 4) was recorded from site C, which is situated in close proximity to the Informal settlement of Kayamandi. This might be due to inadequate sanitation and waste removal facilities in the informal settlements, as well as poor management and disposal of sewage where human and animal wastes are directly discharged into river systems via storm water drainage pipes (Paulse et al., 2012). The dense vegetation on the river banks at this site could possibly also contribute to slower water flow, thereby promoting microbial growth and increased numbers (Fischer et al., 2003).

Various members of the *Enterobacteriaceae* such as *Raoultella, Serratia, Klebsiella, Citrobacter and Enterobacter* were detected at all sampling points along the Plankenburg River. This corresponds to results obtained by Paulse et al. (2009) who studied microbial contamination along the Plankenburg River and found high levels of faecal contamination at these sites.

Microbial counts observed along the Diep River, are also represented in Figs. 3 and 4 as well as in appendix 1. The CFU obtained from sediments and surface water samples at sites D, E and F ranged between 0.4 x 10^3 and 2.12 x10^8 (Figs. 3 and 4). The highest microbial load (2.12 x 10^8 CFU/ml) was recorded at site F (Rietvlei boating club) while the highest microbial activity was recorded at site E (Theo Marias sportsclub (Figs. 3 and 4). Isolates '15' and '14' which are halophilic microorganisms were detected at site E (Theo Marias sportsclub). The average salinity values recorded at this site ranged between 883 - 903 ppt (Table 2). These were the highest salinity values recorded from sampling points along the river systems studied, but the occurrence of these halotolerant microorganisms might also be due to the input from the Lagoon and ocean which is only a few kilometers away from this site (Haskins, 2014).

The average physicochemical parameter values obtained during winter and summer sampling occasions are shown in Table 2.

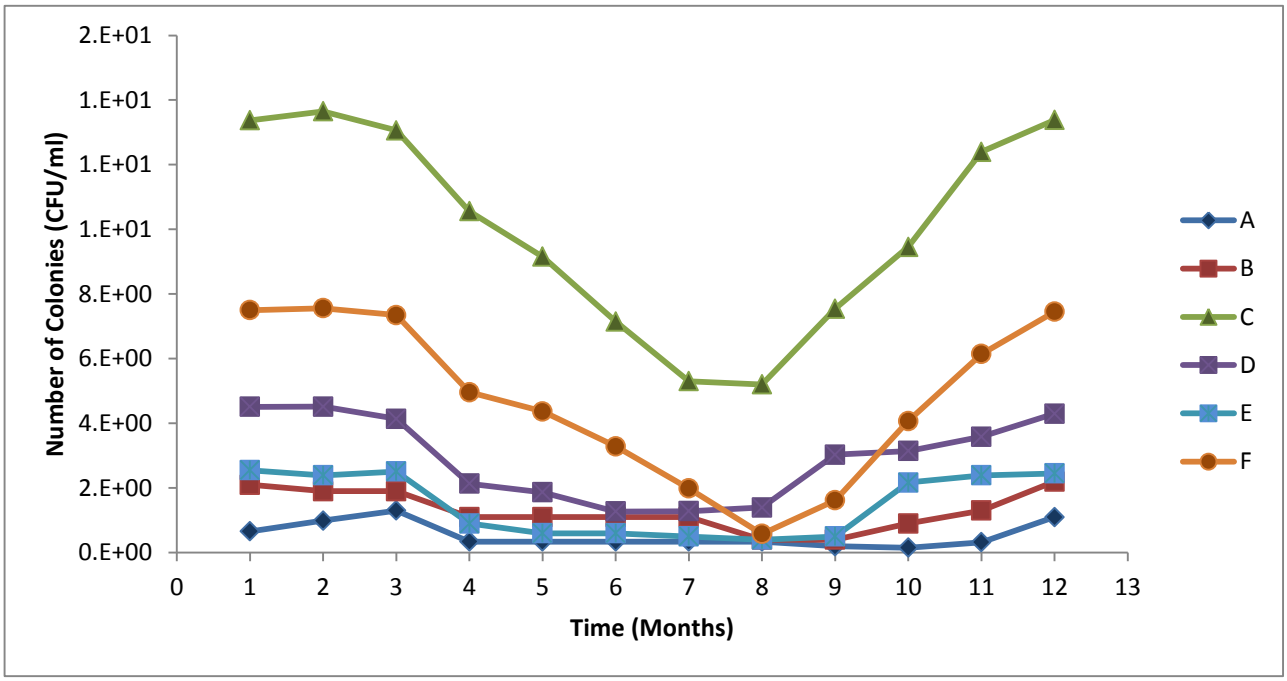


Figure 3: Comparison of microbial activity in surface water along the sampling sites of the Plankenburg (A - Agricultural farming and residential area; B - A substation in industrial area; C - The informal settlement of Kayamandi), and the Diep [(D - The Zoarvlei nature reserve (industrial as well as residential); E - The Theo Marias Sports club (industrial and residential area); F - The Rietvlei boating club] Rivers.

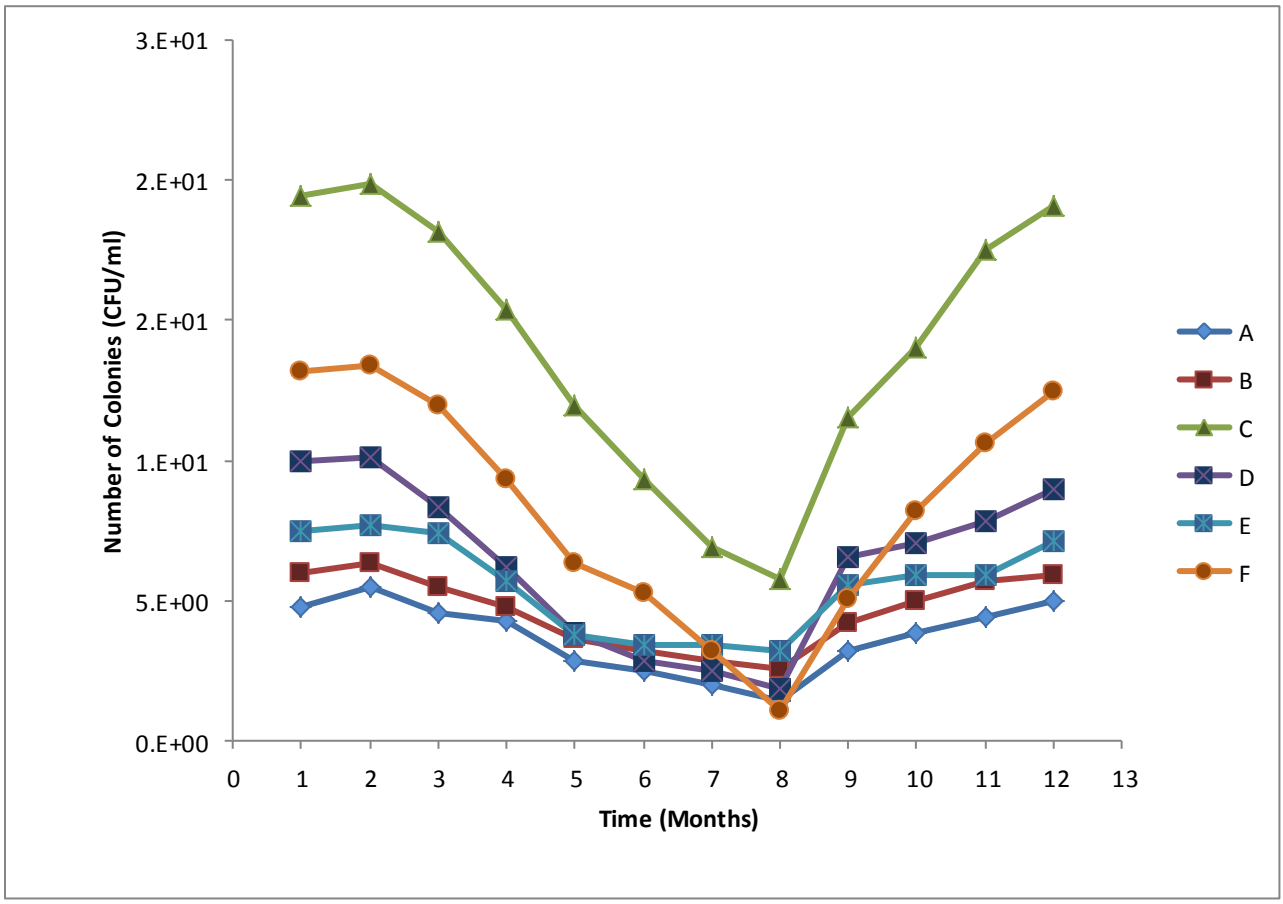


Figure 4: Comparison of microbial activity in sediment along the sampling sites of the Plankenburg (A - Agricultural farming and residential area; B - A substation in industrial area; C - The informal settlement of Kayamandi), and the Diep [(D - The Zoarvlei nature reserve (industrial as well as residential); E - The Theo Marias Sports club (industrial and residential area); F - The Rietvlei boating club] Rivers.

Table 2: Average Physicochemical parameters of River systems

Sampling points	Temperature Min - Max(^{0}C)	pH	Conductivity (S/m)	TDS (ppm)	Salinity (ppt)
A	11.8	6.7	449	318	178
	22.9	5.8	740	371	288
B	12.1	6.9	668	474	309
	22.3	6.1	711	355	356
C	12.1	7.2	708	502	327
	23.7	6.8	749	374	288
D	11.4	7.3	715	508	330
	27.8	8.0	761	383	330
E	13.5	7.1	444	210	883
	28.8	7.9	751	373	903
F	13.9	7.0	589	257	196
	28.5	7.8	773	399	236

Key: TDS: Total dissolved solids, A - agricultural farming and residential area, B - substation in industrial area, C - informal settlement of Kayamandi (on the Plankenburg River). D - Zoarvlei nature reserve, E - Theo Marias sportsclub, F - boating club (on The Diep River course).

4.2. **PAHs in the River systems**

The two PAHs investigated in this study were detected at varying concentrations in the collected sediment and water samples. They were detected at more elevated concentrations in sediment samples than in surface water samples (Table 3). This could be attributed to the capacity of PAH compounds to adsorb onto particulate matter (Wick et al., 2011). Higher concentrations of the compounds were detected during the winter months (May to September) compared to the summer sampling times (December to March). This trend is comparable with results obtained by Zhang and Tao (2009) who reported higher PAH occurrence in winter compared to summer time in Beijing, China. This suggests that the most important source of PAH compounds into these river systems might be atmospheric deposition, because during winter there is increased vehicular activity and other fossil fuel combustion activities (the use of heating systems) which significantly deposits PAH compounds into environmental matrices (Liang et al., 2008; Zhang et al., 2013). Higher concentrations of PAHs in winter can also be due to lower microbial activity at lower temperatures, and subsequent reduced degradative potential in winter months. The most contaminated site was Site F (Rietvlei boating club) with an average of 0.80 and 0.90 ppm acenaphthene and fluorene detected from sediments respectively during winter months and 0.6 and 0.7 ppm detected during summer sampling months. Therefore, in addition to atmospheric deposition, boating activities (boat emissions is a significant petrogenic PAH source), could also contribute to the input of the PAHs in the river.

Table 3: Acenaphthene and Fluorene concentrations detected at the sampling sites along the Diep- and Plankenburg Rivers.

Sampling		**Sampling points**						
Season	**matrix**	**Point A (ppm)**	**Point B (ppm)**	**Point C (ppm)**	**Point D (ppm)**	**Point E (ppm)**	**Point F (ppm)**	**PAH**
Winter	Water	ND	0.004	0.0008	ND	ND	0.004	Ace
		0.0009	0.20	0.0007	ND	0.0006	0.004	Flu
	Sediment	0.20	0.40	0.10	ND	0.07	0.80	Ace
		0.60	0.90	0.60	0.40	0.80	0.90	Flu
Summer	Water	ND	0.0006	ND	ND	ND	0.0006	Ace
		ND	0.003	ND	ND	0.0009	0.005	Flu
	Sediment	0.04	0.20	0.03	ND	0.004	0.6	Ace
		0.04	0.70	0.002	ND	0.004	0.7	Flu

Key: Point A – agricultural farming and residential area, Point B - substation in industrial area, Point C - informal settlement of Kayamandi (all on the Plankenburg River). Point D - Zoarvlei nature reserve, E - Theo Marias sports club, F - boating club (on the Diep River course), ND: Not Detected, Ace - acenaphthene, Flu – fluorine.

4.3. **Bacterial isolates identified from the Diep and Plankenburg River systems using conventional techniques.**

A total of 19 bacterial isolates were obtained from the sampling points along the Diep- and Plankenburg River systems (surface water and sediments). Their morphological and phenotypic characteristics are shown in Tables 4 and 5, respectively. All isolates grew on selective media ordinarily designed for them by the manufacturers, except certain members of *Enterobacteriaceae* such as isolates designated as ‘1’, ‘4’, ‘5’, ‘7’ and ‘18’ which were successfully cultured on *Pseudomonas* Agar base (Table 4), a culture medium which is otherwise selective *for Pseudomonas spp* generally and certain members of *Burkholderia spp.* (Thermo Scientific and Acumedia, 2008; De Jonghe et al., 2011). These isolates grew on *Pseudomonas* agar base supplemented with C-F-C (Cetrimide, Fucidin, and Cephaloridine) and *Pseudomonas* agar base supplemented with CN (Cetrimide and Sodium Nalidixate). The reason for this aberration is that the growth on CN or C-F-C medium is usually limited to *Pseudomonas spp*. but some members of the family *Enterobacteriaceae* may also be present (Thermo Scientific and Acumedia, 2008; Marta, 2011). However, other members of the *Enterobacteriaceae* family such as isolate 6 did not grow on this medium (Table 4).

Table 4: Morphological characteristics of the bacterial isolates obtained from the Diep- and Plankenburg Rivers when cultured on different growth media.

Isolate	NA	AIA	PAB	GSP	BA	MAC	EMB	MSA
1 DR	Glistening, moist, Cream, round, elevated.	Small, yellow colonies, changes media colour from green to yellow	Round, cream colonies	Small, Purple, round, elevated	Cream, round, elevated	Round, pink colonies	Large, grey, moist colonies	NG
2 DB	Large, round, white, vacuolated appearance,	NG	NG	NG	Round, smooth, cream coloured colonies	NG	Cream, mucoid colonies with golden-yellow edges and clearing zone surrounding the colonies	NG
3 PA	Large, smooth, with flat edges, elevated and colonies surrounded by bluish-green colouration	NG	Round, blue-green colonies	Round, light gray colonies	Round colonies producing metallic sheen with blue pigmentation (β-hemolytic colonies)	Pale yellow colonies	Round, pinkish colonies	NG
4 LC	Glistening, moist, round colonies	NG	Round, cream colonies	Tiny, purple, round elevated	Cream, round, elevated	Round, pink colonies	NG	NG
5 LP	Glistening, moist, colonies	NG	Round, cream, colonies	NG	Round, off-white (gamma-hemolytic colonies)	Round, pink colonies	Brown, dark-centred mucoid colonies	NG
6 MA	Smooth, translucent, small, entire, circular colonies	NG	NG	Round, small, opaque colonies (No starch hydrolysis)	Large, grey, moist colonies	Round, red colonies	Dark, round, blue-black colonies with a metallic green sheen	NG
7 DL	Round, non-pigmented, flat with irregular edges	NG	Round, flat, cream colonies	NG	Large, grey, moist colonies	Red, mucoid, circular colonies	Large, pink, mucoid colonies	NG
8PY	Large, circular beige colonies	NG	NG	NG	NG	Circular, Deep pink colonies	NG	Golden-orange, circular colonies
9 PR	Circular, convex, smooth, slightly opaque with entire margins	NG	NG	NG	Circular, convex colonies surrounded by a clear zone	Tiny, circular, brown colonies	Large, round colonies with a blue grey centre	NG
10 XP	Circular, beige colonies	NG	NG	NG	NG	Circular, Deep pink colonies	NG	Golden-orange, circular

								colonies
11 RC	Round, irregular, rugose, cream coloured colonies	NG	NG	NG	Round, cream coloured colonies	NG	Cream coloured, circular colonies with undulate margins	NG
12 SE	Red, round, small, elevated colonies	NG	NG	NG	Medium sized, buff coloured colonies	Colourless, translucent colonies	Red-pigmented, circular colonies	NG
13 AH	Large, Flat, Round, Cream coloured	Dark green opaque colonies with dark centres	NG	Yellow colonies, starch hydrolysis (media colour change from red to yellow)	small, round, smooth, colonies	Pink colonies	NG	NG
14 BA	Cream coloured, flat and circular colonies with undulate margins	NG	NG	NG	Spreading, grey colonies	NG	NG	NG
15 TA	Round, irregular, rugose, cream coloured colonies	NG	NG	NG	Beige, round elevated colonies	Round, pink colonies	NG	NG
16 QO	Irregular, dull, cream coloured colonies	NG	NG	NG	NG	Round, pink colonies	NG	NG
17 ST	Circular, slightly convex, pale orange pigmented colonies	NG	NG	NG	Non haemolytic, bright white, creamy colonies	NG	NG	Round, tiny colonies with mannitol fermentation observed (yellow medium)
18 KP	Smooth, low convex, translucent, grey, with a shiny surface and entire edge	NG	Mucoid, cream coloured, round colonies	NG	Cream, round elevated colonies	Large, round, pink, moist colonies	Large, grey, moist colonies	NG
19 DG	Round, yellow-orange pigmented colonies	NG	NG	NG	Orange-yellow pigmented colonies	NG	NG	NG

Key: NA: Nutrient Agar, AIA: *Aeromonas* Isolation Agar, PAB: *Pseudomonas* Agar Base, GSP: GSP Agar, BA: Blood Agar, MAC: MacConkey Agar, EMB: Eosine, Methylene Blue Agar, MSA: Mannitol Salt Agar NG – No growth.

Table 5: Phenotypic characterisation of the bacterial isolates obtained from the Diep- and Plankenburg River systems.

Biochemical and Gram Stains	**Test Organisms**																		
Name of Test	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Gram reaction	–	+	–	–	–	–	–	+	–	+	+	–	–	+	+	+	+	–	+
Endospore	–	+	–	–	–	–	–	+	–	–	+	–	–	+	+	+	–	–	–
Motility	–	+	+	–	–	+	+	–	–	–	+	+	+	+	+	+	–	+	–
Methyl-red	+	–	–	+	–	+	–	–	–	+	+	–	+	–	–	–	+/-	+	–
Citrate (Simmons)	+	+	+	+	+	–	+	+/-	+	–	+	+	+	+	+	+	+	+	+/-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	–	+	+	–	–	–	–	+/-	–	–	+/-	–	+	+	+/-	+	–	–	–
Voges-Proskauer	+	–	–	+	+	–	+	–	–	–	+	+	+	+	+	–	+/-	–	–
Urease production	+	–	+/-	+	+	–	+/-	–	–	–	+	–	–	+	–	–	+	+	–
Indole production	+	–	–	–	+	+	–	–	–	+/-	+/-	–	+	–	–	–	–	–	–

Key: (-): Negative, (+): Positive, (+/-): Variable

4.4. **Molecular Identification of bacterial isolates obtained from Diep- and Plankenburg Rivers.**

The BLAST Identification results shown in Table 6 were obtained from the DNA sequences queried on the NCBI database. The DNA sequences queried corresponded with the named biological sequences within the NCBI database. However, some microorganisms could not be identified conclusively and require further investigation. These isolates were only successfully identified up to the genus level, so their particular species were not conclusively determined. The evolutionary relatedness of all isolated microorganisms is shown in Fig. 5. All members of *Enterobacteriaceae*, except *Citrobacter freundii* are closely clustered (Fig. 5). The position at which *Citrobacter freundii* is situated on the tree is not totally consistent with classical taxonomy, but similar results were obtained by Dauga (2002) who also reported conflicting evolutionary relationships for *Citrobacter freundii*. The study compared phylogenetic relationships generated from *gyrB* and 16S rRNA sequences and concluded that trees based on *gyrB* are more reliable at determining relationships than trees based on 16S rRNA. This study used 16S rRNA sequences to generate phylogenetic relationships and

hence, this might be responsible for the unusual location of *C. freundii* on the phylogenetic tree.

Table 6: Molecular (BLAST) identity of the bacterial species isolated from the Diep- and Plankenburg Rivers and their respective assigned accession numbers.

Isolate	**BLAST ID**	**Accession numbers**
1	*Raoultella ornithinolytica*	KT239136
2	*Bacillus megaterium*	KT239138
3	*Pseudomonas aeruginosa*	KT282224
4	*Raoultella planticola*	KT282225
5	*Klebsiella oxytoca*	KT282226
6	*Escherichia coli*	KT282227
7	*Enterobacter cloacae*	KT282228
8	*Exiguobacterium acetylicum*	KT282229
9	*Acinetobacter sp.*	KT282230
10	*Exiguobacterium sp.*	KT726926
11	*Bacillus sp.*	KT282232
12	*Serratia marcescens*	KT239137
13	*Aeromonas hydrophila*	KT239139
14	*Bacillus aryabhattai*	KT726927
15	*Bacillus aquimaris*	KT884112
16	*Bacillus marisflavi*	KT884111
17	*Staphylococcus saprophyticus*	KT346363
18	*Citrobacter freundii*	KT315777
19	*Exiguobacterium undae*	KU179094

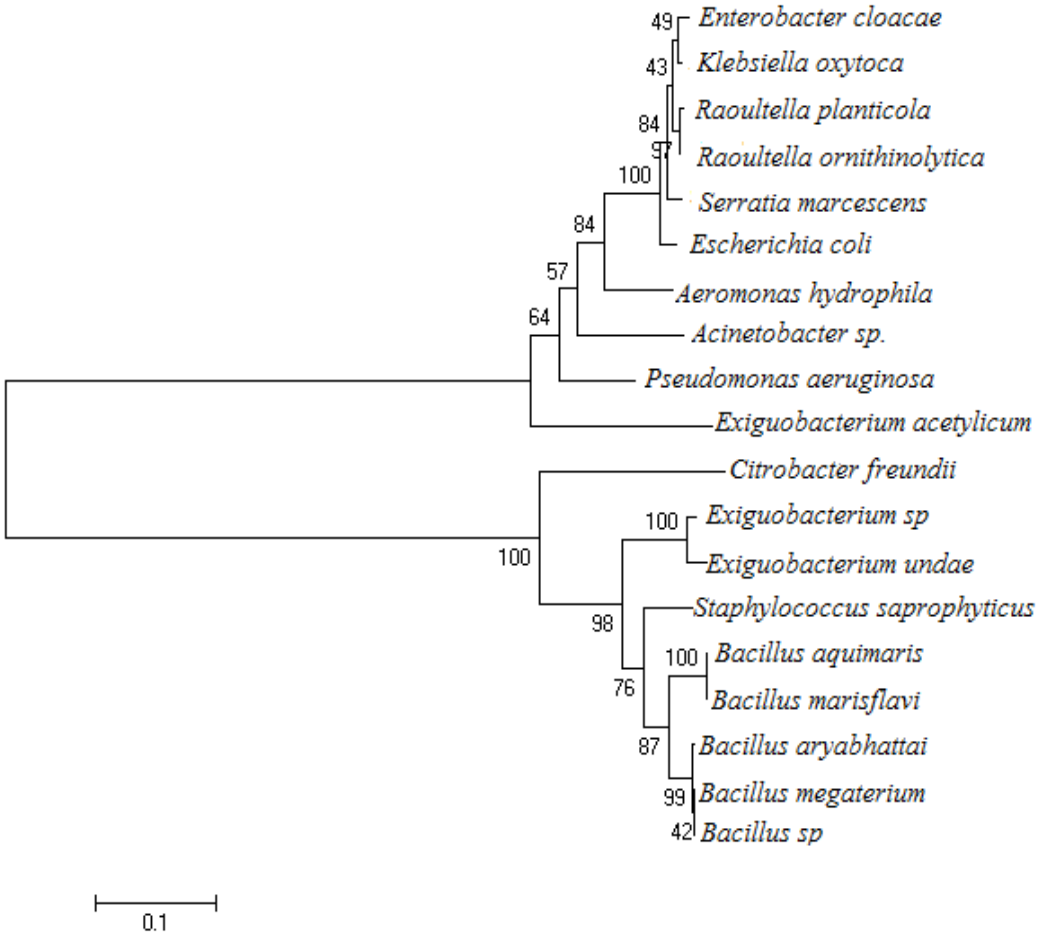


Figure 5: Neighbour-joining phylogenetic tree obtained from 16S rRNA gene sequences of all microorganisms isolated from the Diep- and Plankenburg River systems.

4.5. **Identification of potential PAH-degrading bacterial species using temperature optimisation screening**

After all the isolated species were screened, four of the isolates successfully degraded acenaphthene and fluorene above 75% and were selected for further degradation studies. The microorganisms selected were: *Raoultella ornithinolytica, Serratia marcescens, Bacillus megaterium and Aeromonas hydrophila.* Of the four selected microorganisms, three (*R. ornithinolytica, S. marcescens and A. hydrophila*) are Gram negative microorganisms. The reason why there are more Gram negative microorganisms able to 'pass' the temperature optimisation screening compared to Gram positive microorganisms is because of the thicker peptidoglycan wall that Gram positive bacteria possess. The peptidoglycan wall absorbs the contaminants (PAHs) and the bacterial cell becomes over-burdened, thus killing the cell (Silhavy et al., 2010). The percentage degradation recorded by all isolated bacterial species at the temperature optimisation screening stage is shown in Table 7. Certain isolates such

as *Bacillus sp., B. aryabhattai, B. marisflavi and C. freundii* successfully degraded up to and above 75% fluorene, but did not degrade acenaphthene accordingly (Table 7) and thus were not suitable candidates for degradation studies. The reason for this trend could not be verified as both compounds have the same number of rings and similar configuration (Fig. 1). Some bacterial species could degrade neither compound and some could not withstand the stress induced by the exposure to the compounds. This was deduced from the plate count, Gram reactions and biochemical tests monitoring during the experiments. *Klebsiella oxytoca, E. coli, E. cloacae and S. saprophyticus* did not grow on culture media plates for the duration of the experiment. For species that successfully degraded the compounds, an increase in number of colonies was observed exponentially throughout the period of the experiment. This continued well until after the microorganisms had degraded a significant portion of the compounds, after which, a decline in colony growth was observed (Figs. 6 - 14).

Table 7: Percentage degradation (%) achieved by bacterial isolates at the temperature (^{o}C) optimisation screening scale.

Isolate	Percentage degradation (%) / temperature (^{o}C)							Analyte
	25	**30**	**35**	**37**	**38**	**40**	**45**	
R. ornithinolytica	55.20	92.00	95.00	95.00	98.50	73.20	62.90	Ace
	91.20	90.00	96.50	99.00	99.60	91.60	91.60	Flu
B. megaterium	64.80	88.40	73.20	62.80	62.80	61.50	60.90	Ace
	92.30	95.40	94.60	92.30	92.30	71.40	93.80	Flu
P. aeruginosa	12.02	25.00	38.56	46.89	45.23	10.00	10.00	Ace
	66.00	68.00	70.00	70.90	70.90	55.00	47.00	Flu
R. planticola	ND	ND	ND	ND	ND	ND	ND	Ace
	ND	ND	ND	5.01	3.02	3.02	ND	Flu
K. oxytoca	ND	ND	ND	ND	ND	ND	ND	Ace
	ND	ND	ND	ND	ND	ND	ND	Flu
E. coli	ND	ND	ND	ND	ND	ND	ND	Ace
	ND	ND	ND	ND	ND	ND	ND	Flu
E. cloacae	ND	ND	ND	ND	ND	ND	ND	Ace
	ND	ND	ND	ND	ND	ND	ND	Flu
E. acetylicum	ND	ND	ND	11.09	18.66	ND	55.62	Ace
	45.00	69.58	69.71	72.89	72.00	71.00	71.00	Flu
Acinetobacter sp.	ND	ND	ND	ND	ND	ND	ND	Ace
	ND	ND	19.28	56.32	ND	ND	ND	Flu
Exiguobacterium sp.	52.00	12.00	54.00	55.00	66.21	55.89	64.00	Ace
	66.09	68.21	60.23	60.23	ND	63.20	70.23	Flu
Bacillus sp.	31.20	45.09	16.30	ND	ND	ND	ND	Ace
	88.90	93.20	91.02	87.74	25.32	18.00	07.00	Flu
Serratia marcescens	15.00	5.80	77.30	91.90	91.70	75.40	73.70	Ace
	62.30	92.30	95.40	97.90	97.90	73.40	23.20	Flu
Aeromonas hydrophila	75.40	99.40	99.50	99.20	89.88	62.80	55.20	Ace
	74.10	95.80	99.23	99.80	74.40	64.60	57.30	Flu
Bacillus aryabhattai	ND	ND	ND	ND	ND	ND	ND	Ace
	88.00	99.00	91.00	88.32	84.20	63.20	ND	Flu
Bacillus aquimaris	ND	ND	ND	ND	ND	ND	ND	Ace
	ND	ND	ND	ND	ND	ND	ND	Flu
Bacillus marisflavi	ND	ND	ND	ND	ND	ND	ND	Ace
	78.00	98.00	90.00	72.00	71.00	13.20	05.78	Flu
S. saprophyticus	ND	ND	ND	ND	ND	ND	ND	Ace
	ND	ND	ND	ND	ND	ND	ND	Flu
Citrobacter freundii	ND	ND	ND	ND	ND	ND	ND	Ace
	ND	77.00	79.30	94.32	88.56	45.23	ND	Flu
E. undae	13.02	ND	ND	ND	12.01	36.01	61.32	Ace
	70.00	69.00	58.00	23.00	73.00	73.89	74.23	Flu

Key: Ace: acenaphthene, Fluo: fluorene, ND: no degradation

4.6. **Degradation efficiencies**

4.6.1. **Flask Scale**

For all flask scale degradation experiments, the optimum temperature ranged between 30ºC and 38ºC (Table 8). These results are comparable to those obtained by Antizar-Ladislao et al. (2008) and Moscoso et al. (2012). For most experiments, there was a sharp decline in degradation efficiency at 40ºC and 45ºC (Table 8), which could be attributed to the increase

in temperatures, coupled with a reduction in oxygen in the reactors at higher temperature profiles (Vinas et al., 2005). For *Raoultella ornithinolytica* and *Serratia marcescens* on acenaphthene and fluorene, respectively, the most efficient degradation was observed at 37ºC with a mean percentage degradation of 97.80% and 99.90% achieved by *R. ornithinolytica* and degradation percentages of 91.90% and 97.90% achieved for *S. marcescens* (Table 8). This might be due to the fact that both organisms grow optimally at 37ºC (Abdou 2003; Ferrario et al., 2013). Both organisms also showed good degradation efficiencies at 38ºC, which are also comparable to results obtained by Antizar-Ladislao et al. (2008) and Gan et al. (2009). *Bacillus megaterium* efficiently degraded both compounds at 30ºC with mean degradation percentages of 88.40% and 95.40% for acenaphthene and fluorene, respectively (Table 8). This trend could also be attributed to optimum growth temperature for *B. megaterium* as shown by Logan and De Vos (2009) who compared growth temperature between 3ºC to 45ºC and concluded that 30ºC was the optimum temperature for growth of the organism. For *Aeromonas hydrophila*, optimum degradation was observed at 35ºC with percentage degradation of 99.50% and 99.10% achieved for acenapthene and fluorene, respectively. At temperature values of 30ºC, 35ºC and 37ºC, efficient degradation was achieved for acenaphthene (99.40%, 99.50%, 99.20%) and fluorene (95.20%, 99.10%, 98.50%) respectively (Table 8).

Fluorene degradation was generally more efficient and more rapid than acenaphthene degradation (Table 8) as evidenced by the higher degradation percentages obtained over a wider range of temperatures. *Raoultella ornithinolytica* degraded above 91% of fluorene at all temperature values tested (25ºC - 45ºC). This is more efficient compared to acenaphthene degradation at sub-optimal temperatures (55.20%, 73.20%, 62.90% at 25ºC, 40ºC and 45ºC, respectively). *Bacillus megaterium* also degraded above 92% at all temperature profiles except at 40ºC where 71.40% degradation was achieved. These values are better compared to 88.40% achieved at optimum temperature and between 73.20% and 64.80% achieved at sub-optimal temperatures during acenaphthene degradation studies. For most treatments, more than half of the compound had been degraded by the fifth day.

The results obtained showed that for the pure strain experiments, *Aeromonas hydrophila* degraded acenaphthene most efficiently at 99.50%, while *Raoultella ornithinolytica* degraded fluorene most efficiently at 99.90%. The poorest degradation was recorded in reactor B (acenaphthene and *B. megaterium*) for which only 88.40% degradation percentage was achieved at optimum temperature (Table 8). After the first few days of the experiment biodegradation profiles became static in reactor B. Factors influencing this occurrence could include (i) a decrease in the bioavailability of the compound; (ii) accumulation of toxic metabolites; or (iii) the enrichment of more recalcitrant compounds (Vinas et al., 2005). For the cocktail experiments, a slight improvement in degradation efficiency was observed for

both compounds, especially fluorene with degradation percentages within the range of 98.60% and 99.50% recorded at all temperature profiles. This could be due to multiple, co-operative metabolic capacities which could in turn improve the efficiency of the bioremediation processes (Janbandhu and Fulekar, 2011). At temperatures higher than 60ºC the isolates could not survive and thus degradation percentages significantly declined.

Table 8: Mean biodegradation values of acenaphthene and fluorene recorded at flask scale after 14 days.

Flasks	Temperature (^{0}C)	Initial concentration (ppm)	Residual concentration (ppm)	SD	Percentage degradation (%)
A (Ace + *R. ornithinolytica*)	25.00	50.00	22.38	0.54	55.20
	30.00	50.00	3.72	0.80	92.60
	35.00	50.00	2.31	1.50	95.40
	37.00	50.00	1.09	0.20	97.80
	38.00	50.00	0.21	0.30	95.80
	40.00	50.00	13.41	1.23	73.20
	45.00	50.00	18.56	0.90	62.90
B (Ace + *S. marcescens*)	25.00	50.00	27.49	0.02	45.00
	30.00	50.00	22.38	0.89	55.20
	35.00	50.00	11.33	0.96	77.30
	37.00	50.00	4.00	1.63	91.90
	38.00	50.00	4.14	0.64	91.70
	40.00	50.00	12.29	0.45	75.40
	45.00	50.00	13.16	1.19	73.70
C (Ace + *B. megaterium)*	25.00	50.00	17.58	0.63	64.80
	30.00	50.00	5.82	0.32	88.40
	35.00	50.00	13.41	0.74	73.20
	37.00	50.00	18.62	1.19	62.80
	38.00	50.00	18.57	1.60	62.80
	40.00	50.00	19.25	3.56	61.50
	45.00	50.00	19.50	3.36	60.90
D (Ace + *A. hydrophila)*	25.00	50.00	12.29	3.98	75.40
	30.00	50.00	0.28	0.23	99.40
	35.00	50.00	0.24	0.06	99.50
	37.00	50.00	0.42	1.75	99.20
	38.00	50.00	5.06	1.97	89.88
	40.00	50.00	18.62	2.67	62.80
	45.00	50.00	22.38	0.35	55.20
(Ace + cocktail)	25.00	50.00	13.10	0.03	73.80
	30.00	50.00	0.28	0.65	99.40
	35.00	50.00	0.30	0.54	99.40
	37.00	50.00	0.42	0.34	99.20
	38.00	50.00	0.02	0.74	99.90
	40.00	50.00	0.42	0.56	91.50
	45.00	50.00	4.78	0.21	90.40
J (fluorene + *R. ornithinolytica)*	25.00	50.00	4.22	1.23	91.60
	30.00	50.00	1.96	1.12	96.10
	35.00	50.00	0.75	1.69	98.50
	37.00	50.00	0.02	0.65	99.90
	38.00	50.00	0.18	0.36	99.60
	40.00	50.00	4.22	0.39	91.60
	45.00	50.00	4.33	0.98	91.60

K (fluorene + *S. marcescens*)	25.00	50.00	18.87	0.36	62.30
	30.00	50.00	3.65	0.21	92.30
	35.00	50.00	2.28	0.14	95.40
	37.00	50.00	1.06	0.47	97.90
	38.00	50.00	1.07	0.13	97.70
	40.00	50.00	13.29	0.36	73.40
	45.00	50.00	38.40	0.97	23.20
L (fluorene + *B. megaterium*)	25.00	50.00	2.68	1.16	94.60
	30.00	50.00	2.28	1.23	95.40
	35.00	50.00	3.85	4.56	92.30
	37.00	50.00	3.87	4.41	92.30
	38.00	50.00	3.87	2.13	92.30
	40.00	50.00	14.2	0.12	71.40
	45.00	50.00	3.08	0.96	93.80
M (fluorene +*A. hydrophila*)	25.00	50.00	12.70	1.10	74.40
	30.00	50.00	2.39	0.36	95.20
	35.00	50.00	0.44	0.21	99.10
	37.00	50.00	0.73	0.15	98.50
	38.00	50.00	12.79	0.92	74.40
	40.00	50.00	14.71	0.89	64.60
	45.00	50.00	21.36	6.39	57.30
N (fluorene + cocktail)	25.00	50.00	0.69	1.69	98.60
	30.00	50.00	0.25	2.36	99.50
	35.00	50.00	0.31	3.35	99.40
	37.00	50.00	0.29	2.13	99.40
	38.00	50.00	0.30	0.36	99.40
	40.00	50.00	0.25	0.36	99.50
	45.00	50.00	0.25	0.38	99.50

Key: Ace: acenaphthene, Cocktail: *R. ornithinolytica*, *S. marcescens*, *B. megaterium* *A. hydrophila*

4.6.2 **Stirred Tank Bioreactor Scale (STR)**

At the Stirred Tank Bioreactor scale mean percentage degradation achieved ranged between 90.20% and 99.90% for all experiments (Table 9). *R. ornithinolytica, S. marcescens, B. megaterium, A. hydrophila* and the cocktail showed a 98.60%, 95.70%, 90.20%, 99.90% and 99.60% degradation efficiency for acenaphthene and a 99.90%, 97.90%, 98.40%, 99.50% and 99.20% degradation efficiency for fluorene. The improved efficiency at STR scale compared to flask scale could be attributed to improved oxygenation and mixing afforded the system by the overhead stirrers and impellers as opposed to the gentle agitation available in the shaking incubator at the flask scale. Agitation has been shown to significantly improve the dissolution rate of PAH particles as PAH biodegradation is known to be inhibited by slow dissolution rates thus contributing to increased efficiency of STRs (Ruihong, 2006). A study by Vinas et al. (2005) showed a 12% increase in degradation efficiency rates with improved agitation and stirring compared to gentle agitation. The increased dissolution rate is influenced by two factors both of which enhance the volumetric mass transfer co-efficient. These factors include reduced film thickness due to intense turbulence generated by the agitation inside the bioreactor and the increased interfacial

surface area of the PAH particles resulting from collisions between particles and particles as well as between particles and the impeller (Ruihong, 2006).

Table 9: Mean biodegradation percentages of acenaphthene and fluorene by the PAH degrading microorganisms at the Stirred Tank Bioreactor scale.

Reactor	Initial concentration (ppm)	Residual concentration (ppm)	SD	Percentage degradation (%)
O (Ace + *R. ornithinolytica*)	50.00	0.68	0.01	98.60
P (Ace + *S. marcescens*)	50.00	2.15	0.30	95.70
Q (Ace + *B. megaterium*)	50.00	4.91	0.90	90.20
R (Ace + *A. hydrophila*)	50.00	0.03	0.06	99.90
S (ace + cocktail)	50.00	0.15	0.93	99.60
T (fluorene + *R. ornithinolytica*)	50.00	0.02	0.09	99.90
U (fluorene + *S. marcescens*)	50.00	0.99	0.36	97.90
V (fluorene + *B. megaterium)*	50.00	0.82	0.23	98.40
W (fluorene +*A. hydrophila)*	50.00	0.24	0.36	99.50
X (fluorene + cocktail)	50.00	0.39	1.6	99.20

Key: Ace - acenaphthene, Cocktail: *Raoultella ornithinolytica*, *Serratia marcescens*, *Bacillus megaterium* and *Aeromonas hydrophila.*

4.7 **Microbial Cell Count during and after degradation**

During *R. ornitinolytica* treatment of acenaphthene, at optimal temperature range (30ºC - 38ºC) there was an increase in cell count from $5x10^4$ CFU/ml to $7x10^8$ CFU/ml by the seventh day of the experiment (Fig. 6). However, by the 10^{th} day of the experiment, there was a drastic decrease in cell counts to around $5x10^3$ CFU/ml. At 25ºC, 40ºC and 45ºC there was an increase in cell counts from $5x10^4$ CFU/ml to $5x10^5$ CFU/ml, $6x10^7$ CFU/ml, $5x10^7$ CFU/ml, respectively by day five (Fig. 6). For fluorene, at 25ºC, 30ºC, 35ºC, 37ºC, 38ºC, 40ºC and 45ºC, there was an increase in cell count from $5x10^3$ CFU/ml to $6x10^5$ CFU/ml, $7x10^7$ CFU/ml, $9x10^8$ CFU/ml, $9x10^8$ CFU/ml, $9x10^8$ CFU/ml, $8x10^8$ CFU/ml and $7x10^8$ CFU/ml, respectively by the seventh day of the experiment (Fig. 7).

For *Bacillus megaterium* an increase was observed in plate count from $6x10^4$ CFU/ml to $7x10^5$ CFU/ml at 30ºC during acenaphthene treatment by the fourth day of the experiment (Fig. 8). At all other temperature profiles, there was no significant increase in plate count. For fluorene however, at 25ºC, 30ºC, 35ºC, 37ºC, 38ºC, 40ºC and 45ºC there was an increase in plate counts from $6x10^4$ CFU/ml to $9x10^5$ CFU/ml, $9x10^8$ CFU/ml, $8x10^7$ CFU/ml, $8x10^7$ CFU/ml, $8x10^5$ CFU/ml, $7x10^4$ CFU/ml and $7x10^4$ CFU/ml, respectively after 7 days (Fig. 9).

There was an increase in average plate count ranging from $4x10^5$ CFU/ml to $6x10^8$ CFU/ml after seven days for *S. marcescens* degradation experiments within the optimum temperature range for acenaphthene (35ºC - 38ºC) and fluorene (30ºC - 38ºC). At 25ºC, 40ºC and 45ºC there was no significant increase in plate count for both compounds (Figs. 10 and 11).

At Stirred Tank Bioreactor scale, an increase was also observed in plate count ranging from $4x10^4$ CFU/ml to $5x10^6$ CFU/ml to $8x10^9$ CFU/ml to $9x10^9$ CFU/ml by the seventh day for all experiments (Fig. 14), this trend is comparable with results obtained by Moscoso et al. (2012). The reason for the increase in plate count is explained by Moscoso et al. (2012), whose study revealed that the increase in plate count relates to rapid cell proliferation. The study revealed that the increase in plate count during biodegradation is due to rapid proliferation of cells because of the supply of adequate carbon sources. However, by the end of the experiments, there was either a drastic decline in cell growth or the microorganisms were dead evidenced by no cell growth on culture media plates. This was attributed to the possibility that the microorganisms had utilised all the contaminants (serving as carbon sources) and hence, stopped replicating abundantly (Cho et al., 2002). Apart from substrate depletion, another plausible explanation for the drastic decline in microbial numbers is that toxic intermediates and by-products such as salicylate as well as oxy-PAHs; including PAH-ketones, quinones and coumarins (Bamforth and Singleton, 2005; Mrozik et al., 2003) might have been produced and accumulated in the reactors thereby causing the death of the cells.

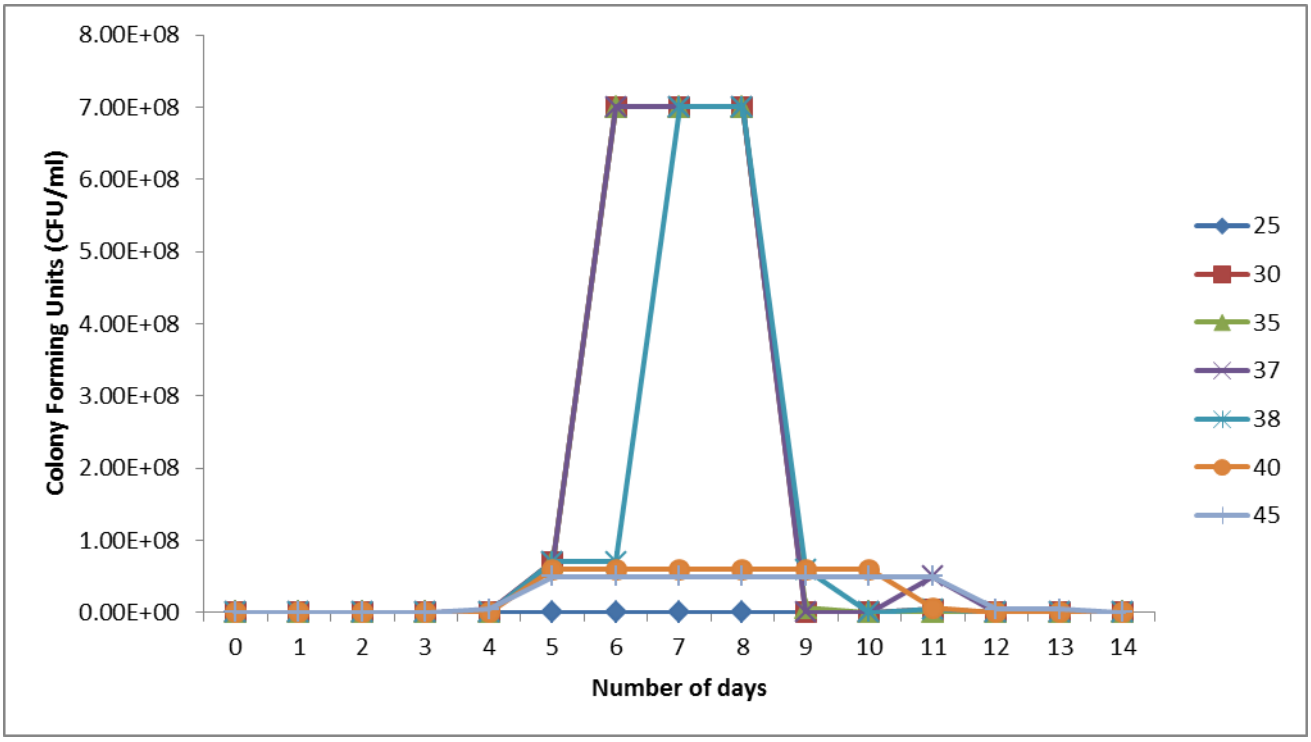


Figure 6: Microbial plate counts from first to fourteenth day during *Raoultella ornithinolytica* acenaphthene degradation experiments at 25ºC, 30ºC, 35ºC, 37ºC, 38ºC, 40ºC and 45ºC.

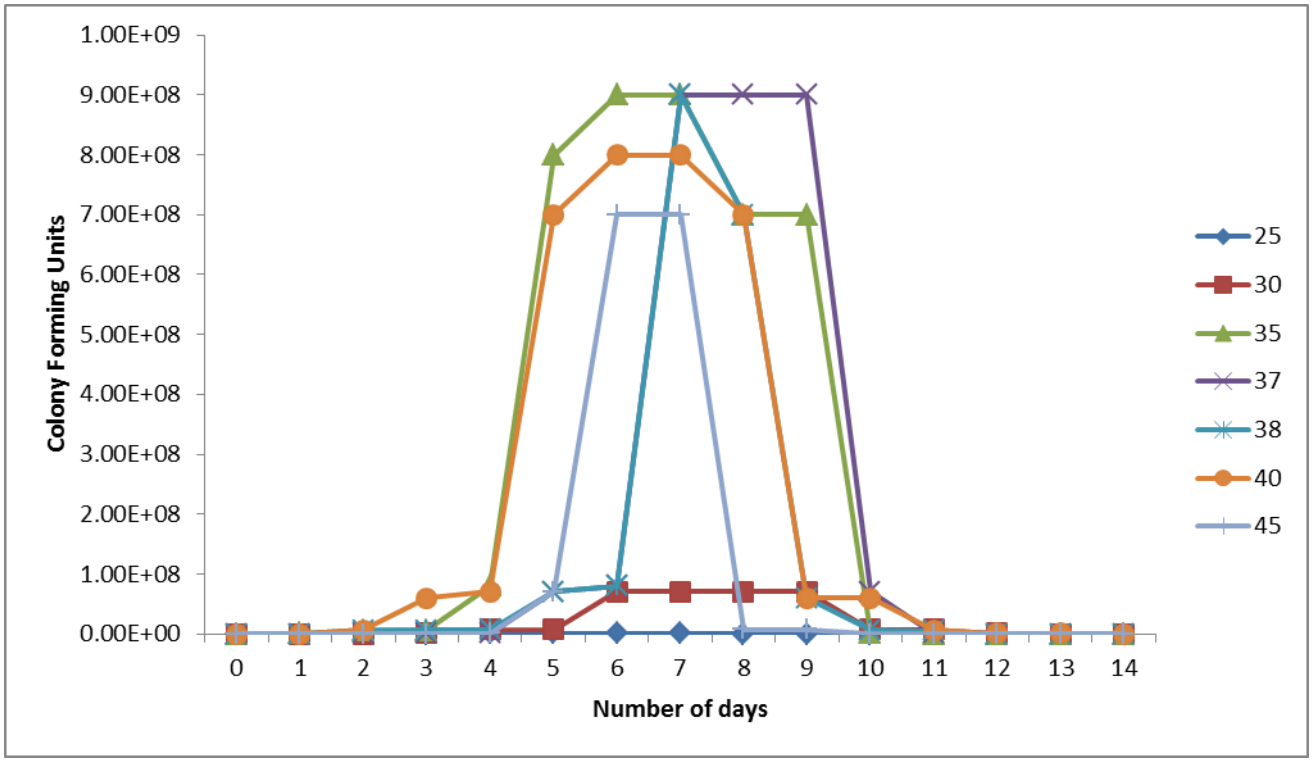


Figure 7: Microbial plate counts from first to fourteenth day during *R. ornithinolytica* fluorene degradation experiments at 25ºC, 30ºC, 35ºC, 37ºC, 38ºC, 40ºC and 45ºC.

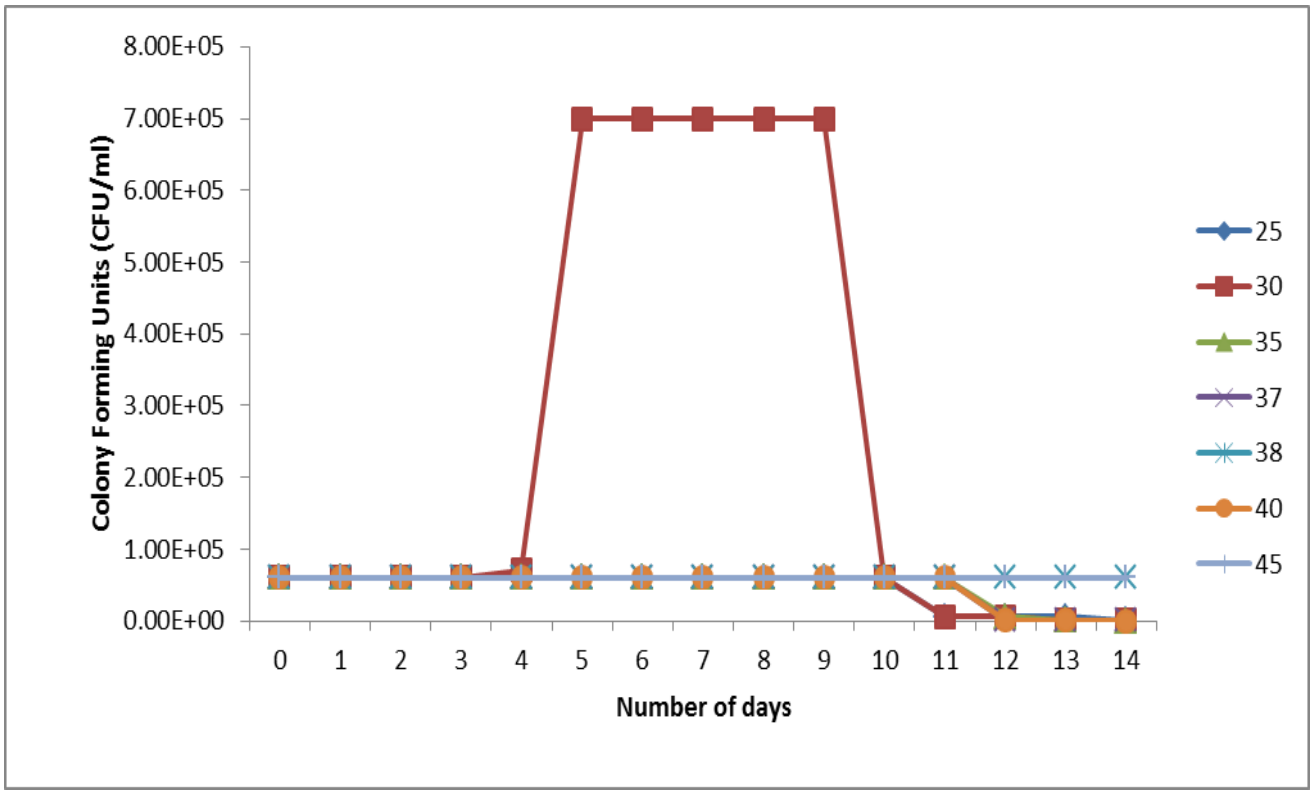


Figure 8: Microbial plate counts from first to fourteenth day during *B. megaterium* acenaphthene degradation experiments at 25ºC, 30ºC, 35ºC, 37ºC, 38ºC, 40ºC and 45ºC.

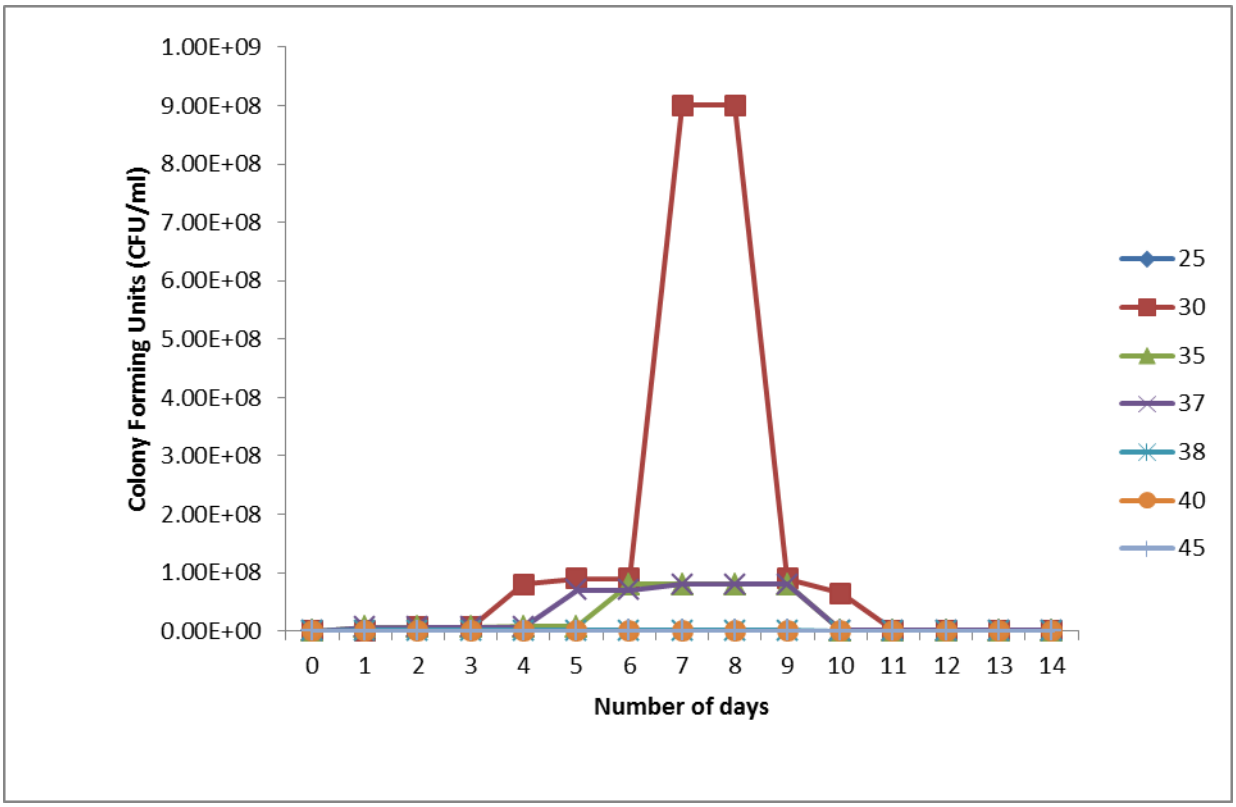


Figure 9: Microbial plate counts from first to fourteenth day during *B. megaterium* fluorene degradation experiments at 25ºC, 30ºC, 35ºC, 37ºC, 38ºC, 40ºC and 45ºC.

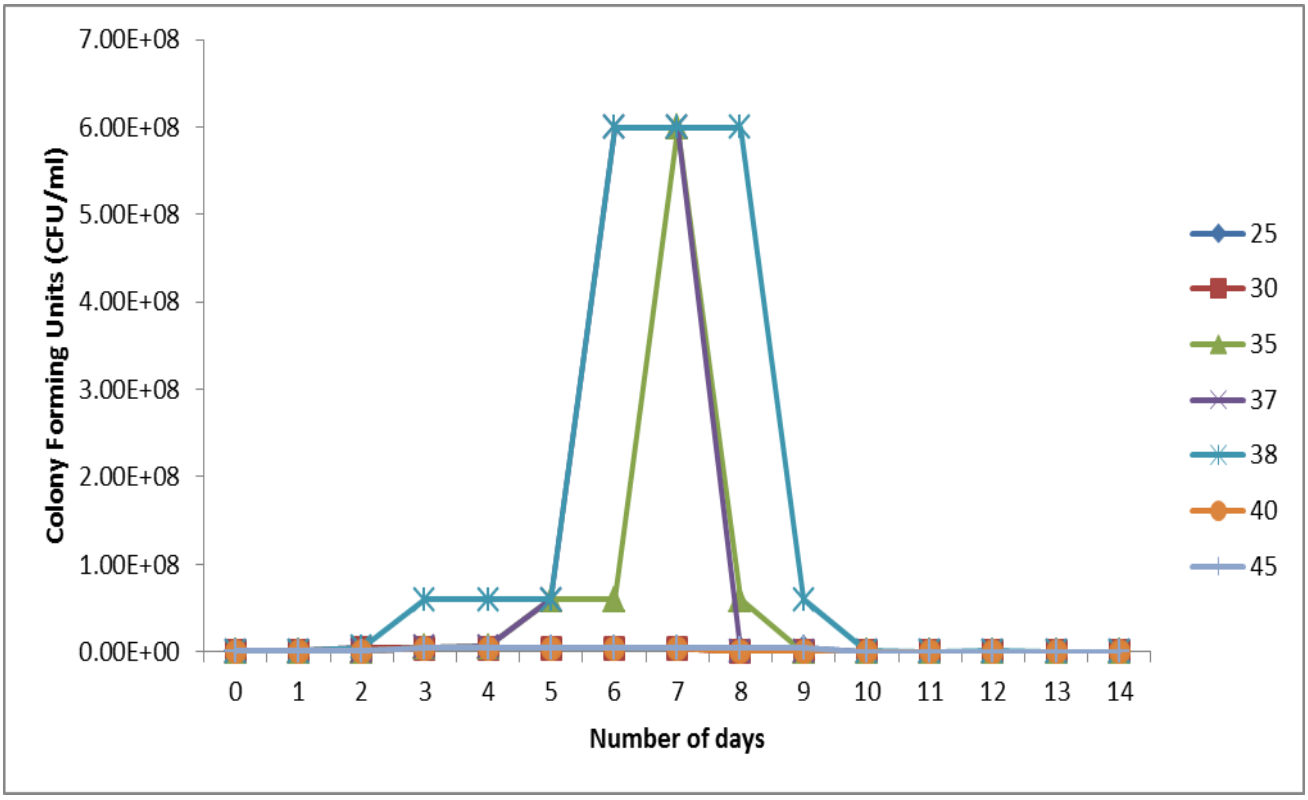


Figure 10: Microbial plate counts from first to fourteenth day during *S. marcescens* acenaphthene degradation experiments at 25ºC, 30ºC, 35ºC, 37ºC, 38ºC, 40ºC and 45ºC.

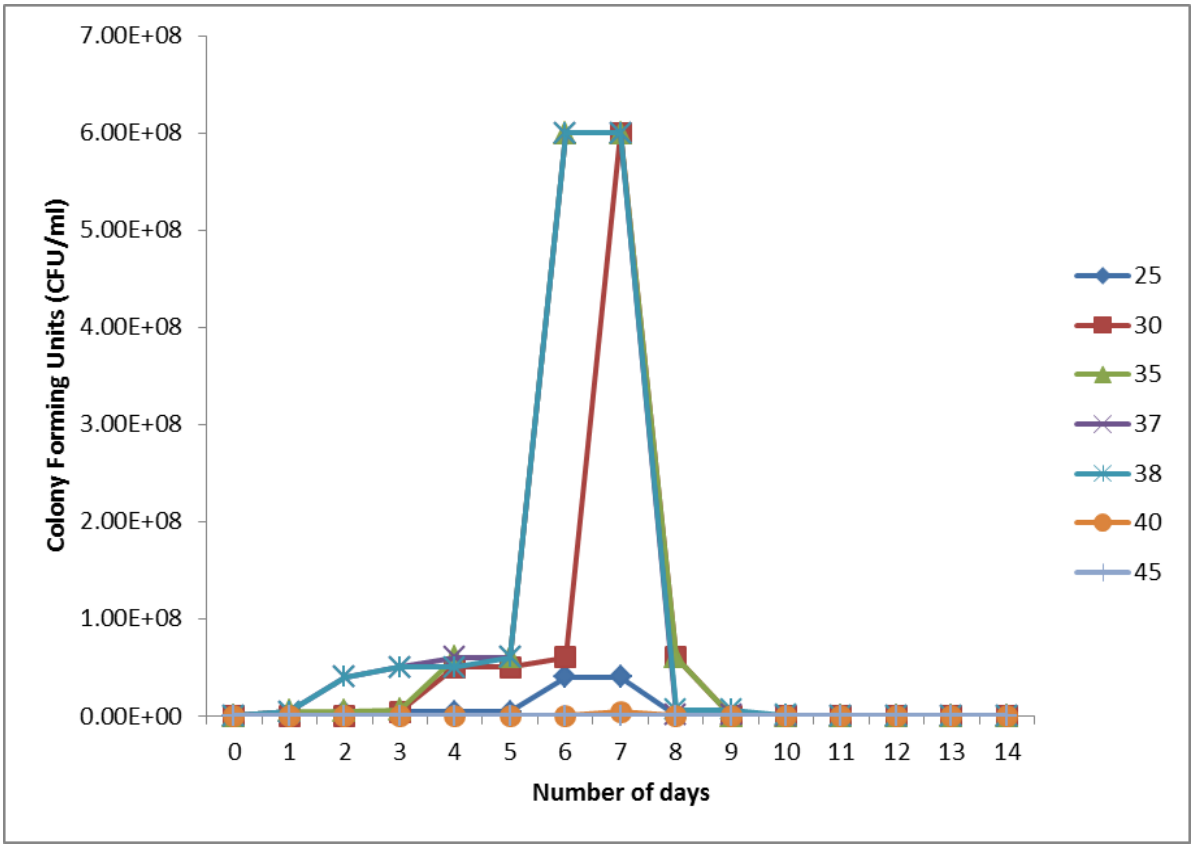


Figure 11: Microbial plate counts from first to fourteenth day during *S. marcescens* fluorene degradation experiments at 25ºC, 30ºC, 35ºC, 37ºC, 38ºC, 40ºC and 45ºC.

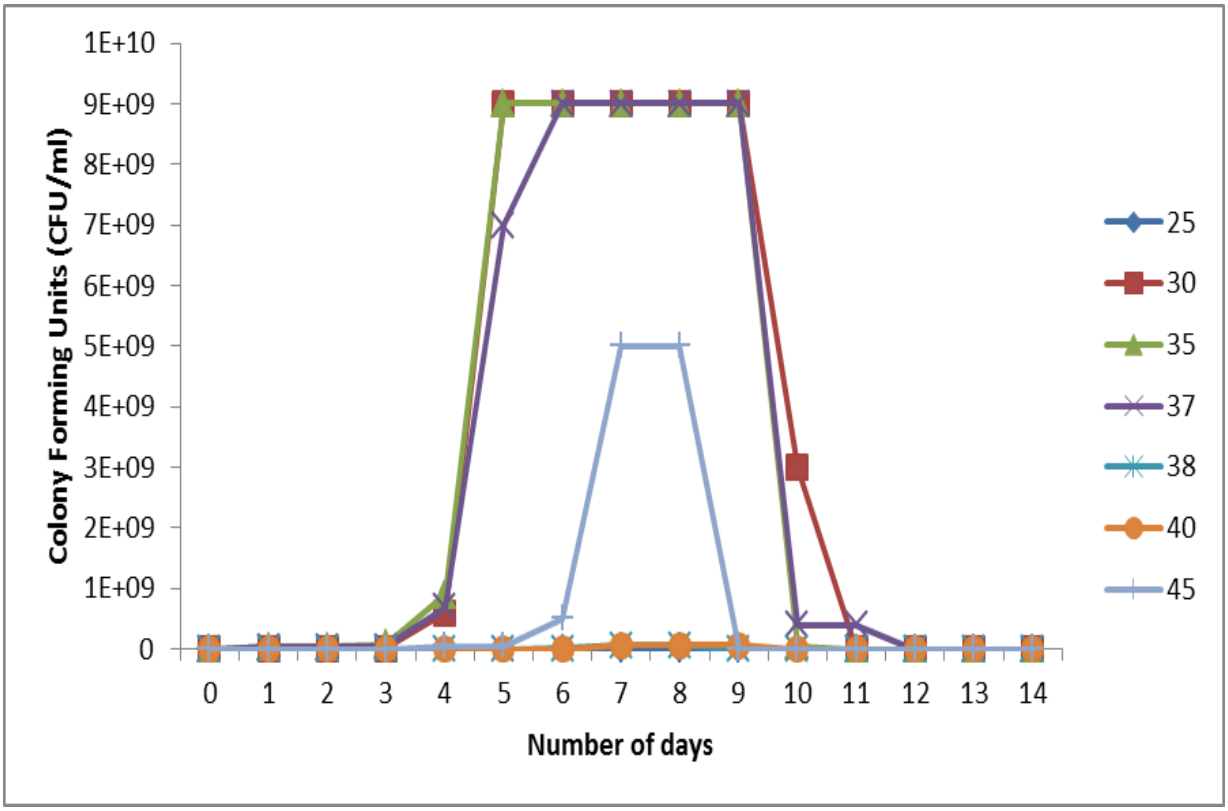


Figure 12: Microbial plate counts from first to fourteenth day during *A. hydrophila* acenaphthene degradation experiments at 25ºC, 30ºC, 35ºC, 37ºC, 38ºC, 40ºC and 45ºC.

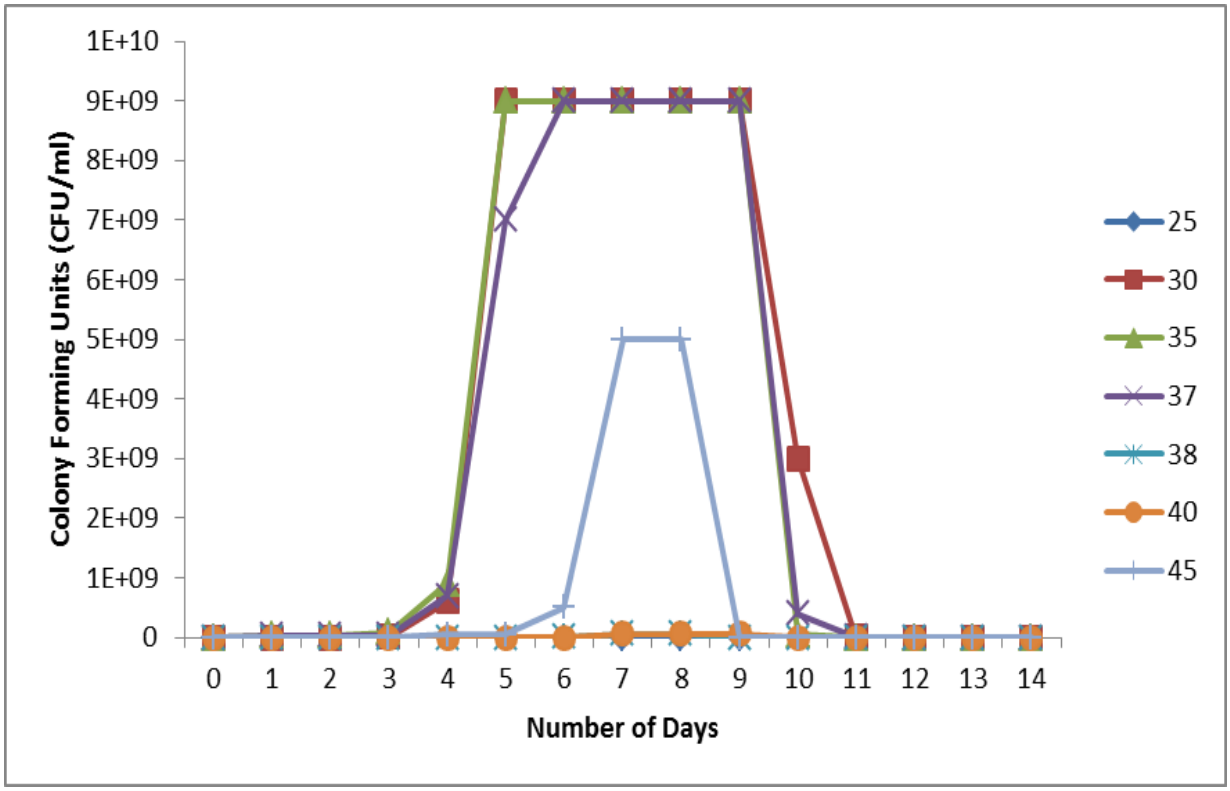


Figure 13: Microbial plate counts from first to fourteenth day during *A.hydrophila* fluorene degradation experiments 25ºC, 30ºC, 35ºC, 37ºC, 38ºC, 40ºC and 45ºC.

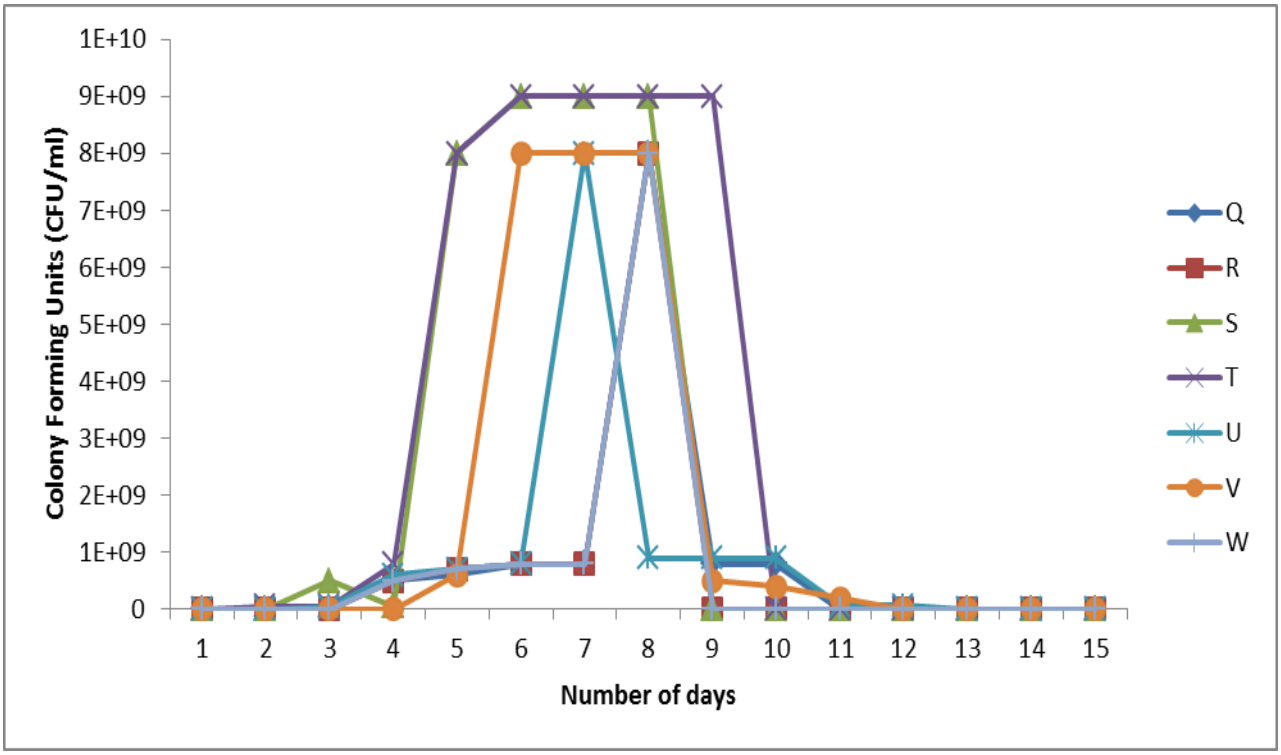


Figure 14: Microbial plate counts from first to fourteenth day during all Stirred Tank Bioreactor degradation experiments. **Legend**: O- Acenaphthene + *R. ornithinolytica*; P-Acenaphthene + *S. marcescens;* Q - Acenaphthene + *B. megaterium*; R -Acenaphthene + *A. hydrophila*; S- Ace cocktail; T -Fluorene + *R. ornithinolytica*; U- Fluorene + *S. marcescens*; V-Fluorene + *B. megaterium*; W- Fluorene + *A. hydrophila*; X -Fluorene Cocktail.

CHAPTER FIVE

5. CONCLUSION

The Plankenburg River was observed to be heavily contaminated with pathogens as microbial counts obtained from all sampling sites along this river system notably exceeded the recommended maximum limits. Although the Diep River was observed to be less contaminated compared to the Plankenburg River, microbial counts obtained from the Diep River also exceeded the recommended maximum limits of 2 000 microorganisms/ 100 ml of river water.

The indigenous microorganisms used in this study (*Raoultella ornithinolytica*, *Serratia marcescens*, *Bacillus megaterium* and *Aeromonas hydrophila*) have great hydrocarbonoclastic potential since natural attenuation occurred without any nutrient supplementation or any other sort of biostimulation. They cannot be described as obligate hydrocarbonoclastic organisms (OHCBs) since they were successfully cultured on undefined growth media.

The temperature conditions under which the microorganisms were isolated are not most suitable for the biodegradative potential of the isolates to be optimally expressed. This is shown by the varying optimum temperature profiles relevant to the flask scale experiments (none of which corresponds with the temperature conditions at sampling time). Each microorganism investigated had a particular optimum temperature requirement which directly influenced treatment efficiency of the PAH compounds. However, for the likely adoption of these microorganisms (and implicated temperature protocols) for restoration of PAH contaminated river systems, the temperature of river systems cannot be controlled in order to achieve successful bioremediation, but bioremediation efforts can be made during warmer months when the average temperature ranges between 30ºC and 38ºC, when optimal biodegradative potential of selected microorganisms can be fully harnessed.
Under appropriate temperature conditions, the organisms studied can utilise acenaphthene and fluorene as carbon or energy sources and therefore could be capable of efficiently remediating PAH polluted environments, as evidenced by the increase in cell numbers recorded during bioremediation studies.

Raoultella ornithinolytica, S. marcescens and B. megaterium can be used on a larger, commercial scale to restore polluted aquatic ecosystems. However, *A. hydrophila* has been shown to cause diseases in commercially important aquatic species such as fish, and

therefore, due to safety reasons, cannot be used on a larger, commercial scale to replenish PAH contaminated river systems.

5.1. **Recommendation**

Adequate waste removal and sanitary facilities should be installed, maintained and monitored in the Informal settlement of Kayamandi in order to avoid continued contamination of the Plankenburg River. These actions are imperative to prevent epidemic outbreak and other potential health crises. The hydrocarbonoclastic microorganisms identified in this study could potentially be utilised in the remediation of PAH polluted river systems on a commercial scale. In addition, industries in the vicinity of the river systems can use these microorganisms to pre-treat their waste/effluent prior to release into the environment or waste disposal systems. Furthermore, it would be a more efficient, cost-effective and environmentally friendly approach to pre-treat effluents and waste waters compared to many other available technologies, such as activated sludge systems, desalination, distillation, dark fermentation and wet oxidation, amongst others.

Attempts can be made to use the identified hydrocarbonoclastic microorganisms for degradation of other Polycyclic Aromatic Hydrocarbon compounds. The biological and physicochemical factors responsible for the more proficient degradation of fluorene compared to acenaphthene should also be explored. Additionally, the structure and toxicity of the by-products and intermediates produced during the microbial metabolism of acenaphthene and fluorene should be investigated in future studies.

6. **BIBLIOGRAPHY/REFERENCES**

Abdou, A. M. 2003. Purification and partial characterization of psychrotrophic *Serratia marcescens* lipase. *Journal of Dairy Science*, 86(1):127-32.

Adeola, F. O. 2004. Boon or Bane? The Environmental and Health Impacts of Persistent Organic Pollutants (POPs). *Human Ecology Review*, 11(1):27-35.

Agency for Toxic Substances and Disease Registry (ATSDR). 1995. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 1995. [accessed 22 October, 2014]. Toxicological Profile for Polycyclic Aromatic Hydrocarbons Available: http://www.atsdr.cdc.gov/toxprofiles/tp69.html. Accessed 02/06/14.

Agency for Toxic Substances and Disease Registry (ATSDR). 2011. Toxic Substances - Polycyclic Aromatic Hydrocarbons (PAHs)." ATSDR Home.Web. 25 Apr. http://www.atsdr.cdc.gov/substances/toxsubstance.asp?toxid=25. Accessed 06/10/2014.

Al-Daher, R., Al-Awadhi, N. & El-Nawawy, A. 1998. Bioremediation of damaged desert environment using the windrow soil pile system in Kuwait. *Environment International*, 24: 175–180.

Alexander, M. 1999. Biodegradation and Bioremediation. 2nd Ed. Academic Press: San Diego, USA pp. 325–327.

Amenu, D. 2014. Isolation of Poly Aromatic Hydrocarbons (PAHs) degrading bacteria. *Landmark Research Journals*, 1(1):1-3.

American Society for Microbiology, 2013. Biochemical Test Protocols. *ASM Microbe Library*, pp 1-4.

Anastasi, A., Tigini, V. & Varese, G. C. 2013. The bioremediation potential of different ecophysiological groups of fungi. Goltapeh, E. M., Danesh, Y. R. & Varma, A. (eds.), *Fungi as bioremediators*; *Soil Biology,* Springer, Berlin, ISBN: 978-3-642-33810-6. pp 29-49.

Anderson, R. T. & Lovley, D. R. 1997. Ecology and biogeochemistry of *in-situ* groundwater remediation. *Advances in Microbial Ecology*, 15:289-350.

Antizar-Ladislao, B., Spanova, K., Beck, A. J. & Russell, N. J. 2008. Microbial community structure changes during bioremediation of PAHs in an aged coal-tar contaminated soil by in-vessel composting. *International Biodeterioration & Biodegradation*, 61(4):357-364.

Arey, J. & Atkinson, R. 2003. Photochemical reactions of PAHs in the atmosphere. In: PAHs: An Ecotoxicological Perspective (Douben PET, ed). West Sussex, UK: Wiley, pp 47-63.

Arun, K., Ashok, M. & Rajesh, S. 2010. Crude oil PAH constitution, degradation pathway and associated bioremediation microflora: an overview. *International Journal of Environmental Sciences*, 1(7):11-22.

Atanu, K. J., Mahalakshmi, D. K. & Sridhari, G. 2011. Integrated application of bioprocess engineering and biotechniques for quality & bulk drug manufacturing. *Journal of Bioequivalence and bioavailability*, 11:3-11.

Bamforth, S. M. & Singleton, I. 2005. Bioremediation of Polycyclic Aromatic Hydrocarbons: current knowledge and future directions. *Journal of Chemical Technology and Biotechnology*, 80:723-736.

Bakermans, C., Hohnstock-Ashe, A. M., Padmanabhan, S., Padmanabhan, P. & Madsen, E. L. 2002. Geochemical and physiological evidence for mixed aerobic and anaerobic field biodegradation of coal tar waste by subsurface microbial communities. *Microbial Ecology*, 44:107-117.

Barret, M., Carrère, H., Delgadillo, L. & Patureau, D. 2010. PAH fate during the anaerobic digestion of contaminated sludge: Do bioavailability and/or co-metabolism limit their biodegradation? *Water Research*, 44(13):3797-3806.

Bayoumi, R. A. 2009. Bacterial bioremediation of Polycyclic Aromatic Hydrocarbons in heavy oil contaminated soil. *Journal of Applied Science Research*, 5(2):197-211.

Beasley, G. & Kneale, P. 2002. Reviewing the impact of metals and PAHs on macro-invertebrates in urban watercourses. *Progress in Physical Geography*, 26(2):236-270.

Beazley, M., Martinez, R., Rajan, S., Powell, J., Piceno, Y., Tom, L., Anderson, G., Hazen, T., Nostrand, J., Zhou, J., Mortazavi, B. & Sobecky, P. 2012. Microbial community analysis of a coastal salt marsh affected by the Deepwater Horizon Oil Spill. *Plos One*, 7(7):1-13.

Bergkamp, G., McCartney, M., Dugan. P., McNeely, J. & Acreman, M., 2000. Dams, ecosystem functions and environmental restoration. Thematic Review II.1, prepared as an input to the World Commision on Dams, Cape Town, www.dams.org. Accessed 02/06/2013.

Bhattacharya, S., Angayarkanni, J., Das, A. & Palaniswamy, M. 2012. Mycoremediation of Benzo(a)Pyrene by *Pleurotus ostreatus* isolated from Wayanad district in Kerala, India *International Journal of Pharmacy and Biological Science*, 2(2):84-93.

Bhatnagar, S. & Kumari, R. 2013. Bioremediation: A sustainable tool for environmental management; A review. *Annual Review & Research in Biology*, 3(4):974-993.

Birch, G. F. & Taylor, S. E. 2002. Assessment of possible sediment toxicity of contaminated sediments in Port Jackson, Sydney, Australia. *Hydrobiologia*, 472:19-27.

Bomboi, M. T. & Hernandez, A. 1991. Hydrocarbons in urban runoff: Their contribution to the wastewaters. *Water Research*, 25:557-565.

Bosma, T. N., Middeldorp, P. J., Schraa, G. & Zehnder, A. J. 1997. Mass transfer limitation of biotransformation: quantifying bioavailability. *Environmental Science & Technology*, 31(1):248-252.

Brink, B. 2010. Urease Test Protocol*. American Society for Microbiology (ASM Microbe Library)* pp 1-5.

Buha, A. 2011. Polycyclic Aromatic Hydrocarbons *Toxipedia*, 1:12-16.

Bustamante, M. A., Mier, M. V., Estrada, J. A. & Domiguez, C. D. 2011. Nitrogen and potassium variation on contaminant removal for a vertical subsurface flow lab scale constructed wetland. *Bioresource Technology*, 102(17):7745-7754.

Cameotra, S. S. & Bollaga, J. 2003. Biosurfactant-enhanced bioremediation of Polycyclic Aromatic Hydrocarbons. *Critical Reviews in Environmental Science and Technology*, 33(2): 111-126.

Canadian Council of Ministers of the Environment (CCME). 1999. A protocol for the derivation of ecological effects-based and human health-based soil quality criteria for contaminated sites, Ottawa. CCME subcommittee on Environmental quality criteria for contaminated sites, Environment Canada, pp 123-133.

Canadian Council of Ministers of the Environment (CCME). 2008. Canadian Soil Quality Guidelines: Carcinogenic and Other Polycyclic Aromatic Hydrocarbons (PAHs) (Environmental and Human Health Effects). Canadian Council of Ministers of the Environment, Ottawa, Canada.

C.A.P.E. Estuaries Programme. 2011. Estuary management plan for the Diep Estuary. Document prepared by *Coastal & Environmental Consulting,* pp 6-18.

Carls, M. G., Rice, S. D. & Hose, J. E. 1999. Sensitivity of fish embryos to weathered crude oil: Low level exposure during incubation causes malformations, genetic damage, and mortality in larval Pacific herring (*Clupea pallasi*). *Environmental Toxicology and Chemistry*, 18:481-493.

Castaldini, F. 2008. Bioremediation of PAHs – limitations and solutions. Universita Di Bologna Alma Mater Digital Library, pp 230-256.

Cassado-Martinez, M. C., Buceta, J. L., Belzunce, L. J. & DelValls, T. A. 2006. Using sediment quality guidelines for dredged material management in commercial ports from Spain. *Environment International*, 32(3):388-396.

Centers for Disease Control and Prevention (CDC). 2009. Fourth National Report on Human Exposure to Environmental Chemicals. pp 434-456.

Cerniglia, C. E. 1992. Biodegradation of Polycyclic Aromatic Hydrocarbons. *Biodegradation*, 3:351-368.

Chandra, R. & Chaudhary, S. 2013. Persistent Organic Pollutants in environment and their health hazards. *International Journal of Bioassays*, 2(9):1232-1238.

Chadrankant, S. K. & Shwetha, S. R. 2011. Role of Microbial Enzymes in the Bioremediation of Pollutants: A Review. *Enzyme Research*, 11:1-11.

Chen, Y. C., Banks, M. K. & Schwab, A. P. 2003. Pyrene degradation in the rhizosphere of tall fescue (*Festuca arundinacea*) and switchgrass (*Panicum virgatum*). *Environmental Science and Technology,* 37:5778-5782.

Cheremisinoff, N. P. & Davletshin, A. 2010. Emergency response management of offshore oil spills: Guidelines for Emergency Responders, 2:25-36 ISBN-13: 978-0470927120.

Cho, H., Kim, Y., Lim, H., Cho, J., Kang, J. W. & Kim, K. S. 2002. Oxidation of Polycyclic Aromatic Hydrocarbons by ozone in the presence of sand. *Water Science and Technology*, 43(5):349-356.

Coates, J. D., Woodard, J., Allen, J., Philip, P. & Lovely, D. R. 1997. Anaerobic degradation of Polycyclic Aromatic Hydrocarbons and Alkanes in petroleum-contaminated marine harbour sediments. *Applied Environmental Microbiology*, 63:3589-3593.

Coastal and Environmental Consulting (Lynn Jackson, Julian Conrad & Marillie Carstens). 2011. Estuary Management Plan for the Diep Estuary. C.A.P.E. Estuaries Programme, pp 11-18.

Concord Scientific and Beak Consultants. 1992. Polycyclic Aromatic Hydrocarbons: sources, fate and levels in air, water, soil, sediments, sludge and food in Ontario / prepared for Hazardous Contaminants Branch, *Environment Ontario* ISBN Number 0772992819, pp 25-56.

Costa, P. M., Caeiro, S., Lobo, J., Martins, M., Ferreira, A. M., Caetano, M., Vale, C., DelValls, T. Á. & Costa, M. H. 2011. Estuarine ecological risk based on hepatic histopathological indices from laboratory and *in-situ* tested fish. *Marine Pollution Bulletin*, 62: 55-65.

Cottin, N. C. & Merlin, G. 2007. Study of pyrene biodegradation capacity in two types of solid media. *Science of the Total Environment*, 380:116-123.

Couch, J. A. & Harshbarger, J. C. 1985. Effects of carcinogenic agents on aquatic animals: An environmental and experimental overview. *Journal of Environmental Science and Health*: *Environmental Health and Carcinogenesis Reviews*, 3(1):63-105.

Damstra, T. 2002. Potential effects of certain Persistent Organic Pollutants and endocrine disrupting chemicals on the health of children. *Journal of Toxicology-Clinical Toxicology*, 40: 457-465.

Da Silva, M., Cerniglia, C. E., Pothuluri, J. V., Canhos, V. P. & Espoisto, E. 2003. Screening Filamentous Fungi Isolated From Estuarine Sediments for Ability to Oxidize Polycyclic Aromatic Hydrocarbons. *World Journal of Microbiology & Biotechnology*, 19:399-405.

Das, P., Mukherjee, S. & Sen, R. 2008. Improved bioavailability and biodegradation of a model Poly Aromatic Hydrocarbon by a biosurfactant producing bacterium of marine origin. *Chemosphere*, 72:1229-1234.

Das, N. & Chandran, P. 2011. Microbial degradation of petroleum hydrocarbon contaminants: An Overview. *Biotechnology Research International*, 94:1-13.

Dauga, C. 2002. Evolution of the gyrB gene and the molecular phylogeny of *Enterobacteriaceae*: a model molecule for molecular systematic studies. *International Journal of Systematic and Evolutionary Microbiology*, 52:531-547.

Decho, A. W. 2000. Microbial biofilms in intertidal systems: an overview *Continental Shelf Research*, 20:1257-1273.

Decker, E. L. & Reski, R. 2008. Current achievements in the production of complex biopharmaceuticals with moss bioreactor. *Bioprocess and Biosystems Engineering*, 31:3-9.

De Jonghe, V., Coorevits, A., Van Hoorde, K., Messens, W., Van Landschoot, A., De Vos, P. & Heyndrickx, M. 2011. Influence of storage conditions on the growth of *Pseudomonas Species* in refrigerated raw milk. *Applied and Environmental Microbiology*, 77(2):460-470.

Deziel, E., Paquette, G., Villemur, R., Lépine, F. & Bisaillon, J. G. 1996. Biosurfactant production by a soil *Pseudomonas* strain growing on Polycyclic Aromatic Hydrocarbons. *Applied Environmental Microbiology*, 62:1908-1912.

Dhananjayan, V., Muralidharan, S. & Peter, V. R. 2012. Occurrence and distribution of Polycyclic Aromatic Hydrocarbons in water and sediment collected along the Harbour Line, Mumbai, India. *International Journal of Oceanography*, 40:1-7.

Ding, J., Chen, B. & Zhu, L. 2013. Biosorption and biodegradation of Polycyclic Aromatic Hydrocarbons by *Phanerochaete chrysosporium* in aqueous solution. *Environmental Chemistry*, 58(6):613-621.

Dodor, D. E., Hwang, H. M. & Ekunwe, S. I. 2004. Oxidation of anthracene and benzo(a)pyrene by immobilized laccase from *Trametes versicolor*. *Enzyme and Microbial Technology*, 35(2-3):210-217.

Dupraz, C. & Visscher, P. T. 2005. Microbial lithification in marine stromatolites and hypersaline mats. *Trends in Microbiology*, 13(9):429-438.

Du, W., Sun, Y., Cao, L., Huang, J., Ji, R., Wang, X., Wu, J., Zhu, J. & Guo, H. 2011. Environmental fate of phenanthrene in lysimeter planted with wheat and rice in rotation. *Journal of Hazardous Materials*, 188:408-413.

Department of Water Affairs and Forestry (DWAF). 1996a. South African water quality for fresh water (2nd edition). Domestic water use. Environmental Services, CSIR, Pretoria, South Africa, 2:12-37.

Department of Water Affairs and Forestry (DWAF). 1996b. South African water quality for fresh water (2nd edition). Domestic water use. Environmental Services, CSIR, Pretoria, South Africa, 1:77-87.

Department of Water Affairs and Forestry (DWAF). 1996c. South African water quality for fresh water (2nd edition). Agricultural water use. Environmental Services, CSIR, Pretoria, South Africa, 4:70-71.

Dwivedi, S. 2012. Bioremediation of heavy metal by algae: current and future perspective. *Journal of Advanced Laboratory Research in Biology*, 3(3):195-199.

El Fantroussi, S. & Agathos, S. 2005. Is bioaugmentation a feasible strategy for pollutant removal and site remediation? *Current Opinion in Microbiology*, 8:268-275.

El-Shahawi, M. S., Hamza, A., Bashammakh, A. S. & Al-Saggaf, W. T. 2010. An overview on the accumulation, distribution, transformations, toxicity and analytical methods for the monitoring of persistent organic pollutants. *Talanta*, 80(5):1587-1597.

Falahatpisheh, M. H., Donnelly, K. C. & Ramos, K. S. 2001. Antagonistic interactions among nephrotoxic Polycyclic Aromatic Hydrocarbons. *Journal of toxicology and Environmental Health*, 62(7):543-60.

Ferrario, C., Ricci, G., Borgo, F., Rollando, A. & Fortina, M. G. 2013. Genetic investigation within *Lactococcus garvieae* revealed two genomic lineages. *FEMS Microbiology Letters*, 332:153-161.

Fischer, H., Sukhodolov, A., Wilczek, S. & Engelhardt, C. 2003. Effects of flow dynamics and sediment movement on microbial activity in a lowland river. *River Research & Applications*, 19:473-482.

Fismes, J., Perrin-Ganier, C., Empereur-Bissonnet, P. & Morel, J. L. 2002. *Journal of Environmental Quality*, 31(5):1649-1656.

Fulekar, M. H. & Geetha, M. 2008. Bioremediation of chlorpyrifos in surface soil treatment unit using microbial consortium. *Canadian Journal of Pure & Applied Sciences*, 2(2):267-273.

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):532-549.

Geffard, O., Geffard, A., His, E. & Budzinski, H. 2003. Assessment of the bioavailability and toxicity of sediment-associated Polycyclic Aromatic Hydrocarbons and heavy metals applied to *Crassostrea gigas* embryos and larvae. *Marine Pollution Bulletin*, 46(4):481-490.

Gomes, A., Santos, C., Almeida, J., Elias, M. & Roseiro, L. C. 2013. Effect of fat content, casing type and smoking procedures on PAHs contents of Portuguese traditional dry fermented sausages. *Food and Chemical Toxicology*, 58:369-374.

Guerin, T. F. 1999. The extraction of aged Polycyclic Aromatic Hydrocarbon (PAH) residues from clay soil using sonification and soxhlet extraction procedure: A comparative study. *Journal of Environmental Monitoring*, 1:63-67.

Gupta, K., Chatterjee, C. & Gupta, B. 2012. Isolation and characterisation of heavy metal tolerant Gram-positive bacteria with bioremedial properties from municipal waste rich soil of Kestopur canal (Kolkata), West Bengal, India. *Biologia*, 67(5):827-836.

Habe, H. & Omori, T. 2003. Genetics of Polycyclic Aromatic Hydrocarbon metabolism in diverse aerobic bacteria. *Bioscience Biotechnology Biochemistry*, 67(2):225-243.

Habashi, F. 2003. Extractive metallurgy of aluminum, in Handbook of Aluminum: Alloy production and materials manufacturing. G. E. Totten and D. S. MacKenzie, (Eds.), First ed: Marcel Dekker, Volume 2 pp. 1-45.

Hajisamoh, A. 2013. Pollution Levels of 16 Priority PAHs in the Major Rivers of Southern Thailand. *Journal of Chemistry*, 2(1):7-11.

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

Harley, J. P. 2014. laboratoryResource Guide:Laboratory Exercises in Microbiology. University of Kentucky. McgrawHill, US pp 8-91.

Harvey, R. G. 1997. Polycyclic Aromatic Hydrocarbons. Wiley-VCH, Inc., New York, pp 667-670.

Harvey, R. G. 1998. Environmental chemistry of PAHs. In: A.H. Neilson (ed.) The handbook of environmental chemistry: PAHs and related compounds. Springer, New York, NY, pp1-54.

Haskins, C. 2014. Rietvlei Mac water quality report. Transport for Cape Town (TCT). pp. 12-16.

Hatzinger, P. B. & Alexander, M. 1995. Effect of aging on chemicals in soil on their biodegradability and extractability. *Environmental Science & Technology*, 29:537-545.

Helfrich, J. & Armstrong, D. E. 1986. Polycyclic Aromatic Hydrocarbons in sediments of the Southern basin of Lake Michigan. *Journal of Great Lakes Research,* 12 (3):192-199.

Hellou, J., Leonard, J., Collier, T. K. & Ariese, F. 2006. Assessing PAH exposure in feral finfish from the Northwest Atlantic. *Marine Pollution Bulletin*, 52(4):433-441.

Herbert, C. E., Shutt, J. L. & Norstrom, R. J. 1997. Dietary changes cause temporal fluctuations in polychlorinated biphenyl levels in herring gull eggs from Lake Ontario. *Environmental Science & Technology*, 31:1012-1017.

Himmelheber, D. W. Pennell, K. D. & Hughes, J. B. 2007. Natural attenuation processes during *in-situ* capping. *Environmental Science & Technology*, 41:5306-5313.

Hogan, C. M. 2013. Water Pollution. *The Encyclopedia of the Earth*. Assessed 12-09-2013 pp. 12-36.

Hose, J. E. & Brown, E. D. 1998. Field applications of the piscine anaphase aberration test: lessons from the Exxon Valdez oil spill. *Mutation Research*, 399:167-178.

Hossain, M. M., Nazmul-Islam, K. M. & Rahman, I. M. 2012. An Overview of the Persistent Organic Pollutants in the Freshwater System. *Ecological Water Quality – Water Treatment and Reuse*, 24:455-470.

Ikenaka, Y., Sakamoto, M., Nagata, T., Takahashi, H., Miyabara, Y., Hanazato, T., Ishizuka, M., Isobe, T., Kim, J. W. & Chang, K. H. 2013. Effects of Polycyclic Aromatic Hydrocarbons (PAHs) on an aquatic ecosystem: acute toxicity and community-level toxic impact tests of benzo(a)pyrene using lake zooplankton community. *Journal of Toxicological Sciences*, 38(1):131-136.

Inakollu, S., Hung, H. & Shreve, G. S. 2004. Biosurfactant enhancement of microbial degradation of various structural classes of hydrocarbon in mixed waste systems. *Environmental Engineering Science*, 21:463-469.

International Programme on Chemical Safety (IPCS). 2000. Human exposure assessment; Environmental Health Criteria, 214, pp 44-46.

International Union of Pure and Applied Chemistry (IUPAC). 2006. Compendium of Chemical Terminology, 'Bioreactor'. 2nd edition, pp 106-158.

Janbandhu, A. & Fulekar, M. H. 2011. Biodegradation of Phenanthrene using adapted microbial consortium isolated from petrochemical contaminated environment. *Journal of Hazardous Materials*, 187:333-340.

Jones, K. C. & De Voogt, P. 1999. Persistent organic pollutants (POPs) state of the science. *Environmental Pollution*, 100(1-3):209-221.

Jones, K. C., Alcock, R. E., Johnson, D. L. Northcott, G. L. Semple, K. T. & Woolgar, J. G. 1996. Organic chemicals in contaminated land: analysis, significance and research priorities. *Land Contamination & Reclamation*, 4:189-197.

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):57-88.

Juckpecha, K., Pinyakonga, O. & Rerngsamrana, P. 2012. Degradation of Polycyclic Aromatic Hydrocarbons by newly isolated *Curvularia sp.* F18, *Lentinus sp.* S5 and *Phanerochaete sp.* T20. *Science Asia*, 38:147-156.

Kafilzadeh, F., Shiva, A. H. & Malekpour, R. 2011. Determination of Polycyclic Aromatic Hydrocarbons (PAHs) in water and sediments of the Kor River, Iran. *Middle-East Journal of Scientific Research*, 10(1):1-7.

Kamaljit, B., Gurpal, S. T., Tait, C. & Lena, M. 2010. Polycyclic Aromatic Hydrocarbons in urban soils of different land uses in Miami, Florida. *Soil & Sediment Contamination*, 19:231-243.

Karigar, C. S. & Rao, S. S. 2011. Role of microbial enzymes in the bioremediation of Pollutants: A Review. *Enzyme Research*, 1:1-11.

Karthikeyan, R. & Bhandari, A. 2001. Anaerobic biotransformation of aromatic and Polycyclic Aromatic Hydrocarbons in soil microcosms: A Review. *Journal of Hazardous Substance Research*, 3:3-19.

Kim, S. J., Kweon, O., Jones, R. C., Freeman, J. P., Edmondson, R. D. & Cerniglia, C. E. 2007. Complete and integrated pyrene degradation pathway in *Mycobacterium vanbaalenii* PYR-1 based on systems biology*. Journal of Bacteriology*, 189:464-472.

Kostka, J. E., Prakash, O., Overholt, W. A., Green, S. J., Freyer, G. Carion, A., Delgardio, J., Norton, N., Hazen, T. C. & Huettel, M. 2011. Hydrocarbon-degrading bacteria and the bacterial community response in Gulf of Mexico Beach Sands Impacted by the Deepwater Horizon Oil Spill. *Applied and Environmental Microbiology*, 77(22):7962-7974.

Kuyukina, M. S., Ivshina, I. B., Makarov, S. O., Litvinenko, L. V., Cunningham, C. J. & Philip, C. J. 2005. Effect of biosurfactants on crude oil desorption and mobilisation in a soil system. *Environment International*, 31:155-161.

Kweon, O., Kim, S. J., Holland, R. D., Chen, H., Kim, D. W., Gao, Y., Yu, L. R., Baek, S., Baek, D. H. & Ahn, H. 2011. Polycyclic Aromatic Hydrocarbon metabolic network in *Mycobacterium vanbaalenii* PYR-1. *Journal of Bacteriology*, 193:4326-4337.

Lane, D. J. 1991. 16S/23S rRNA sequencing. In: Nucleic acid techniques in bacterial systematics. Stackebrandt, E., and Goodfellow, M. (eds.), John Wiley and Sons, New York, NY, pp 115-175.

Langenbach, T. 2013. Persistence and Bioaccumulation of Persistent Organic Pollutants (POPs). Agricultural and Biological Sciences; Applied Bioremediation- Active and Passive approaches. Yogesh B. Patil and Prakash Rao (eds) ISBN 978-953-51-1200-6, pp 223-229.

Latimer, J. S. & Zheng, J. 2003. The sources, transport and fate of PAHs in the marine environment. In: PAHs: An ecotoxicology perspective Douben P.E. (ed.), John Wiley and Sons, West Sussex, UK, pp 9-33.

Lawniczak, L., Marecik, R. & Chrzanowski, L. 2013. Contributions of biosurfactants to natural or induced bioremediation. *Applied Microbiology & Biotechnology*, 97:2327-2339.

Lemon, K. P., Higgins, D. E. & Kolter, R. 2007. Flagella-mediated motility is critical for *Listeria monocytogenes* biofilm formation. *Journal of Bacteriology*, 189:4418-4424.

Lenicek, J., Sekyra, M., Pandey, P., Citkova, M., Be-Nes, I., Novotna, J., Kociano, S., Helaskova, J. & Simonova, A. 1997. Polycyclic Aromatic Hydrocarbons at "Program Teplice" sites in the Czech Republic. *Toxicological & Environmental Chemistry*, 58:25-35.

Leung, M. 2004. Bioremediation: techniques for cleaning up a mess, *Journal of Biotechnology*, 2:18-22.

Liang, Y., Fung, P. K., Tse, M. F, Hong, H. C. & Wong, M. H. 2008. Sources and seasonal variation of PAHs in the sediments of drinking water reservoirs in Hong Kong and the Dongjiang River (China). *Environmental Monitoring and Assessment*, 146(1-3):41-50.

Liu, K., Han, W., Pan, W. & Riley, J. T. 2001. Polycyclic Aromatic Hydrocarbon (PAH) emissions from a coal fired pilot FBC system. *Journal of Hazardous Materials*, 84:175-188.

Liu, J., Zhang, L., Winteroth, L., Garcia, M., Weiman, S., Wong, J., Sunwoo, J. & Nadeou, K. 2013. Epigenetically Mediated Pathogenic Effects of Phenanthrene on Regulatory T Cells. *Journal of Toxicology*, 12:104-116.

Logan, N. A. & De Vos, P. 2009. Genus *Bacillus* Cohn 1872 In: Vos, P.D. Garrity, G., Jones, D, Krieg, N. R., Ludwig, W. Rainey, F. A., Schleifer, K. H & Whitman, W.B. (Eds.), Bergey's manual of systematic bacteriology, *The Firmucutes*, 3:21-127.

Luch, A. 2005. The carcinogenic effects of Polycyclic Aromatic Hydrocarbons. London: Imperial College Press. ISBN 1-86094-417-5, pp 223-300.

Lu, X., Reible, D. D. & Fleeger, J. W. 2006. Bioavailability of Polycyclic Aromatic Hydrocarbons in field-contaminated Anacostia River (Washington, DC) sediment. *Environmental Toxicology and Chemistry*, 25(11):2869-2874.

Lu, J. C., Li, Z. T., Hussain, K. & Yang, G. K. 2011. Bioremediation: The New Directions of Oil Spill Cleanup. *Middle-East Journal of Scientific Research*, 7(5):738-740.

Lundestedt, S. 2003. Analysis of PAHs and their transformation products in contaminated soil and remedial processes. Department of Environmental Chemistry, Umea University. Umea, Sweden. ISBN 9173054526, 817-901.

Mackay, D. & Fraser, A. 2000. Bioaccumulation of Persistent Organic Chemicals: Mechanisms and models. *Environmental Pollution*, 110:375-391.

Maier, R. M., Pepper, I. L. & Gerba, C. P. 2000. Environmental Microbiology. Academic Press, New York, 112-132.

Maier, R. M. & Soberón-Chávez, G. 2000. *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. *Applied Microbiology & Biotechnology*, 54:625-633.

Maisto, G., De Nicola, F., Iovieno, P., Prati, M. V. & Alfani, A. 2006. PAHs and trace elements in volcanic urban and natural soils. *Geoderma*, 136:20-27.

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):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):131-136.

Manoli, E. & Samara, C. 1999a. Occurrence and mass balance of Polycyclic Aromatic Hydrocarbons in the Thessaloniki sewage treatment plant. *Journal of Environmental Quality*, 28:176-186.

Manoli, E. & Samara, C. 1999b. Polycyclic Aromatic Hydrocarbons in natural waters: sources, occurrence and analysis. TRAC *Trends in Analytical Chemistry*, 18(6):417-428.

Margesin, R. & Schinner, F. 2001. Bioremediation (natural attenuation and biostimulation) of diesel-oil-contaminated soil in Alpine glacier skiing area. *Applied and Environmental Microbiology*, 67(7):3127-3133.

Marta, D. 2011. Molecular monitoring of meat spoiling *Pseudomonas species* and analysis of *Staphylococcal* enterotoxin expression and formation. Doctoral thesis. Department of Microbiology and Biotechnology, Faculty of food science. Corvinus University of Budapest.pp 11-22.

Martineau, D., Lemberger, K., Dallaire, A., Labelle, P., Lipscomb, T., Michel, P. & Mikaelian, I. 2002. Cancer in Wildlife, a Case Study: Beluga from the St. Lawrence Estuary, Québec, Canada. *Environmental Health Perspectives*, 110:285-292.

Mascarelli, A. 2010. Deepwater Horizon: After the oil. *Nature News*, 467:22-24.

Mastrangela, G., Fadda, E. & Marzia, V. 1999. Polycyclic Aromatic Hydrocarbons and cancer in man. *Environmental Health Perspectives*, 104:1166–1170.

McDevitt, S. 2009. Methyl Red and Voges-Proskauer Test Protocols*. American Society for Microbiology (ASM Microbe Library)* pp 1-3.

Meador, J. P., Stein, J. E. Reichert, W. L. & Varanasi, U. 1995. Bioaccumulation of Polycyclic Aromatic Hydrocarbons by marine organisms. *Reviews of Environmental Contamination and Toxicology*, 143:79-165.

Menichini, E. & Bocca, B. 2003. In: Caballero, B., Trugo, L. C & Finglas, P. M. (eds.), Encyclopaedia of Food Sciences and Nutrition, Academic Press, Amsterdam, Netherlands, pp 4616-4618.

Minai, T. D., Minoui, S. & Herfatmanesh, A. 2012. Effect of salinity on biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs) of heavy crude oil in soil. *Bulletin of Environmental Contamination and Toxicology*, 82:179-184.

Moscoso, F., Tejiz, I., Deive, F. J. & Sanroman, M. A. 2012. Efficient PAHs biodegradation by a bacterial consortium at flask and bioreactor scale. *Bioresource Technology*, 119:270-276.

Moja, S. J., Mtunzi, F. & Madlanga, X. 2013. Determination of Polycyclic Aromatic Hydrocarbons (PAHs) in river water samples from the Vaal Triangle area in South Africa. *Journal of Environmental Science & Health Part A: Toxic/ Hazardous Substances & Environmental Engineering*, 48(8):847-854.

Motelay-Massei, A., Garban, B., Tiphagne-larcher, K., Chevreuil, M. & Ollivon, D. 2006. Mass balance for Polycyclic Aromatic Hydrocarbons in the urban watershed of Le Havre (France): Transport and fate of PAHs from the atmosphere to the outlet. *Water Research*, 40(10):3-11.

Mrozik, A., Piotrowska-Seget, S. & Labuzek, S. 2003. Bacterial degradation and Bioremediation of Polycyclic Aromatic Hydrocarbons. *Polish Journal of Environmental Studies*, 12(1):15-25.

Mrozik, A., Piotrowska-Seget, Z. & Labuzek, S. 2009. Bacterial degradation and bioremediation of Polycyclic Aromatic Hydrocarbons. *Polish Journal of Environmental studies*, 12(1):15-25.

Mueller, J. G., Cerniglia, C. E. & Pritchard, P. H. 1996. Bioremediation of environments contaminated by Polycyclic Aromatic Hydrocarbons. In: Bioremediation: principles and applications, Crawford, R.L and Crawford, D.L. (eds.), Cambridge University Press, Idaho, pp 125-194.

Mulder, H., Breure, A. M., Van Honschooten, D., Grotenhuis, J. T., Van Andel, J. G. & Rulkens, W. H. 1998. Effect of biofilm formation by *Pseudomonas* 8909N on the bioavailability of solid naphthalene. *Applied Microbiology& Biotechnology*, 50:277-283.

Mulligan, C. N. & Gibbs, B. F. 2004. Types, production and applications of biosurfactant products. *Indian National Science Academy*, 1:31-55.

Namkoong, W., Hwang, E. Y., Park, J. S. & Choi, J. Y. 2002. Bioremediation of diesel contaminated soil with composting. *Environmental pollution*, 119:23-31.

National Research Council (NRC). 1997. Contaminated sediments in ports and waterways; cleanup strategies and technologies. National Academy Press, Washington DC. pp 22-38.

Nekhavhambe, T. J., Ree, T. V. & 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*, 40:1816-7950.

New Jersey Department of Health and Senior Services. 1999. Hazardous Substance Fact Sheet. CAS number 86-73-7.

Nguyen, T. T., Youssef, N. H., McInerney, M. J. & Sabatini, D. A. 2008. Rhamnolipid biosurfactant mixtures for environmental remediation. *Water Research*, 42:1735-1743.

Nievas, M. L., Commendatore, M. G., Estevas, J. L. & Bucalá, V. 2008. Biodegradation pattern of hydrocarbons from a fuel oil-type complex residue by an emulsifier-producing microbial consortium. *Journal of Hazardous Materials*, 154:96-104.

Nigam, P. S. & Singh, A. 2011. Production of liquid biofuels from renewable resources. *Progress in Energy and Combustion Science*, 37(1):52-68.

Nkansah, M. A. 2012. Environmental Remediation: removal of Polycyclic Aromatic Hydrocarbons. Ph.D thesis, Department of Chemistry, Faculty of Mathematics and Natural Sciences. University of Bergen, Norway, pp 22-31.

Obayori, O. S., Ilori, M. O., Adebusoye, S. A., Oyetibo, G. O., Omotayo, A. E. & Amund, O. O. 2009. Degradation of hydrocarbons and biosurfactant production by *Pseudomonas sp.* strain LP1. *World Journal* of *Microbiology & Biotechnology*, 25:1615-1623.

Okere, U. V. & Semple, K. T. 2012. Biodegradation of PAHs in 'Pristine' soils from different climatic regions. *Journal of Bioremediation & Biodegradation*, 1(6):1-11.

Okoli, C. G., Ogbuagu, D. H., Madu, G. S. & Njoku-Tony, R. F. 2011. Proximal input of Polynuclear Aromatic Hydrocarbons (PAHs) in groundwater sources of Okrika Mainland, Nigeria. *Scientific Research*, 2(6):848-854.

Olaniran, A., Balgobind, A. & Pillay, B. 2013. Bioavailability of heavy metals in soil: Impact on microbial biodegradation of organic compounds and possible improvement strategies. *International Journal of Molecular Science*, 14(5):10197-10228.

Olatunji, O. S., Fatoki, O. S., Opeolu, B. O. & Xhimba, B. J. 2014. Determination of Polycyclic Aromatic Hydrocarbons [PAHs] in processed meat products using gas chromatography – Flame ionization detector. *Food Chemistry*, 156:296-300.

Pacwa-Plociniczak, M., Plaza, G. A., Piotrowska-Seget, Z. & Cameotra, S. S. 2011. Environmental applications of biosurfactants: Recent advances. *International Journal of Molecular science*, 12(1):633-654.

Pakpahan, E. N., Isa, M. H., Kutty, S. R. & Malakahmad, A. 2009. Effect of temperature on the formation and degradation of Polycyclic Aromatic Hydrocarbons. Proceedings of the International Conference on Emerging Technologies in Environmental Science and Engineering, Aligarh Muslim University, Aligarh, India, pp 569-574.

Palaniappan, M., Gleick, P. H., Allen, L., Cohen, M. J., Christian-Smith, J. & Smith, C. 2010. Clearing the waters: A focus on water quality solutions. *United Nations Environment Programme (UNEP),* ISBN: 978-92-807-3074-6, pp 3-16.

Pantsyrnayaa, T., Blancharda, F., Delaunaya, S., Goergena, J. L., Guédona, E., Gusevab, E. & Boudrant, J. 2011. Effect of surfactants, dispersion and temperature on solubility and biodegradation of phenanthrene in aqueous media. *Chemosphere*, 83(1):29-33.

Patel, A. K., Ahire, J. J., Pawar, S. P., Chaudhari, B. L., Shouche, Y. S. & Chincholkar, S. B. 2010. Evaluation of probiotic characteristics of siderophoregenic *Bacillus spp*. isolated from dairy waste. *Applied Biochemistry and Biotechnology*, 160:140-155.

Paulse, A. N., Jackson, V. A. & Khan, W. 2009. Comparison of microbial contamination at various sites along the Plankenburg and Diep Rivers, Western Cape, South Africa. *Water SA*, 35(4):469-478.

Paulse, A. N., Jackson, V. A., Khan, S. & Khan, W. 2012. Isolation and identification of bacterial pollutants from the Berg and Plankenburg Rivers in the Western Cape, South Africa. *Water SA*, 38(5):819-824.

Pavel, L. V. & Gavrilescu, M. 2008. Overview of *ex-situ* decontamination techniques for soil cleanup. *Environmental Engineering & Management Journal*, 7(6):815-834.

Payne, J. F., Mathieu, A. & Collier, T. K. 2003. Ecotoxicological studies focusing on marine and freshwater fish. In: Dauben, P.E. (Ed.), PAHs: An Ecotoxicological Perspective. Wiley, West Sussex, UK, pp 191-224.

Pereira, W. E., Hostettler, F. D. & Rapp, J. B. 1996. Distributions and fate of chlorinated pesticides, biomarkers and Polycyclic Aromatic Hydrocarbons in sediments along a contamination gradient from a point-source in San Francisco Bay, California. *Marine Environmental Research*, 41(3):299-314.

Perelo, L. W. 2010. Review: *In-situ* and bioremediation of organic pollutants in aquatic sediments. *Journal of Hazardous Materials*, 177(1-3):81-89.

Pink, D. H. 2006. Wall Street Bear Discussion Board Investing in Tomorrow's Liquid Gold. *Yahoo.* http://finance.yahoo.com/.Accessed 12-09-2014.

Pittinger, C. A., Buikema, A. L. & Falkinham, J. O. 1987. *In-situ* variations in oyster mutagenicity and tissue concentrations of Polycyclic Aromatic Hydrocarbons. *Environmental Toxicology and Chemistry*, 6:51-60.

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

Pradhan, S. P., Conrad, J. R., Paterek, J. R. & Srivastava, V. J. 1998. Potential of phytoremediation for treatment of PAHs in soil at MGP sites. *Journal of Soil Contamination*, 7:467-480.

Prince, R. C. 2010. Bioremediation of marine oil spills. *Handbook of Hydrocarbon and Lipid Microbiology*, 6: 2617-2630.

Pulikotil-Anthony, K. J., Murugan, M. & Gurunathan, S. 2014. Biosynthesis of silver nanoparticles from the culture supernatant of *Bacillus marisflavi* and their potential antibacterial activity. *Journal of Industrial and Engineering Chemistry*, 20(4):1505-1510.

Purwaningsih, I. S., Hill, G. A. & Headley, J. V. 2002. Air stripping and dissolution rates of aromatic hydrocarbon particles in a bioreactor. *Chemical Engineering Communications*, 189(2):268-283.

Quinn, L., Pieters, R., Nieuwoudt, C., Borgen, A., Kylin, H. & Bouwman, H. 2009. Distribution profiles of selected organic pollutants in soils and sediments of industrial, residential and agricultural areas of South Africa. *Journal of Environmental Monitoring*, 9(11):1647-1657.

Rahman, K. S., Rahman, T. J., Lakshmanaperumalsamy, P., Marchant, R. & Banat, I. M. 2003. The potential of bacterial isolates for emulsification with range of hydrocarbons. *Acta Biotechnologica*, 4:335-345.

Ramanchandra, T. V. & Solanki, M. 2007. Ecological assessment of lentic water bodies of Bangalore. ENVIS Technical Report 25, CES, IISc, Bangalore. pp 2-9.

Ramesh, A., Walker, S. A., Hood, D. B., Guillien, M. D., Schneider, K. & Weyhand, E. H. 2004. Bioavailability and risk assessment of orally ingested Polycyclic Aromatic Hydrocarbons. *International Journal of Toxicology*, 23(5):301-333.

Reddy, M. S., Naresh, B., Leela, T., Prashanthi, M., Madhusudhan, N. C., Dhanasri, G. & Devi, P. 2010. Biodegradation of phenanthrene with biosurfactant production by a new strain of *Brevibacillus species. Bioresource Technology*, 101:7980-7983.

Rehmann, K., Noll, H. P. & Steiberg, C. E. 1998. Pyrene degradation by *Mycobacterium sp.* Strain KR2. *Chemosphere*, 36(14):2977-2992.

Reible, D. D., Lambert, D., Constant, D. W., Mutch, R. D. & Zhu, Y. 2006. Active capping demonstration in the Anacostia River, Washington, DC. *Remediation*, 17:39-53.

Rhodes, A., Carlin, A. & Semple, T. 2008. Impact of black carbon in the extraction and mineralisation of phenanthrene in soil. *Environmental Science and Technology*, 42(3):740-745.

Riess, R., Nemati, M., Headley, J. & Hill, G. 2005. Improved mass transfer and biodegradation rates of naphthalene particles using a novel bead mill bioreactor. *Journal of Chemical Technology and Biotechnology*, 80(6):662-668.

Riser-Roberts, E. 2010. Remediation of petroleum contaminated soils. Biological, physical and chemical processes. Lewis Publishers. ISBN 0-87371858-5. pp 11-23.

Ritter, L., Solomon, K. R., Forget, J., Stemeroff, M. & O´Leary, C. 1995. Persistent Organic Pollutants. An assessment report on: DDT, aldrin, dieldrin, endrin, chlordane, heptachlor, hexachlorobenzene, mirex, toxaphene, polychlorinated biphenyls, dioxins and furans, document prepared for the International Programme on Chemical Safety (IPCS) within the

framework of the Inter-Organization Programme for the Sound Management of Chemicals (IOMC) pp 22-48.

Ritter, L., Solomon, K. R., Forget, J., Stemeroff, M. & O'Leary, C. 2007. Persistent Organic Pollutants. United Nations Environment Programme. http://www.chem.unep.ch/pops/ritter/en/ritteren.pdf. Accessed 06-09-2014.

Robles-González, I. V., Fava, F. & Poggi-Varaldo, H. M. 2008. A review on slurry bioreactors for bioremediation of soils and sediments. *Microbial Cell Factories*, 7:5-15.

Rock, D. 2011. The location shall be called Kaya Mandi: A history of Kaya Mandi (MA). Masters degree thesis: Stellenbosch University. pp 16-19.

Romero, M. C., Cazau, M. C., Giorgieri, S. & Arambari, A. M. 1998. Phenanthrene degradation by microorganisms isolated from a contaminated stream. *Environmental Pollution*, 101:335-359.

Ruihong, Y. 2006. Bioremediation of Polycyclic Aromatic Hydrocarbon (PAH)-contaminated soils in a roller baffled bioreactor. A thesis submitted to the College of Graduate Studies and Research, Department of Chemical Engineering University of Saskatchewan Saskatoon, Saskatchewan, pp 12-26.

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

Samimi, S. V., Rad, R. A & Ghanizadeh, F. 2009. Polycyclic Aromatic Hydrocarbon contamination levels in collected samples from vicinity of a highway. *Iranian Journal of Environmental Health Science & Engineering*, 6:47-52.

Scientific Committee on Food, European Commission. 2002. Opinion of the Scientific Committee on Food on the risks to human health of Polycyclic Aromatic Hydrocarbons in food. http://ec.europa.eu/food/fs/sc/scf/out153_en.pdf. Accessed 26/02/2015.

Scow, K. M. & Hicks, K. A. 2005. Natural attenuation and enhanced bioremediation of organic contaminants in groundwater. *Current Opinion in Biotechnology*, 16(3):246-53.

Scullion, J. 2006. Remediating polluted soils. *Natural Sciences*, 93:51-65.

Semple, K. T., Cain, R. B. & Schimdt, S. 1999. Biodegradation of aromatic compounds by microalgae. *FEMS Microbiology Letters*, 170:291-300.

Seo, J., Young, S. K. & Qing, X. L. 2009. Bacterial degradation of aromatic compounds. *International Journal of Environmental Research and Public Health*, 6(1):278-309.

Sharma, S. 2012. Bioremediation: features, strategies and applications. *Asian Journal of Pharmacy and Life Science*, 2(2):202-213.

Shiraishi, N., Pilkington, N. H., Otsuki, A. & Fuwa, K. 1985. Occurrence of chlorinated Polynuclear Aromatic Hydrocarbons in tap water. *Environmental Science and Technology*, 19:585-590.

Sibiya, P., Chimuka, L., Cukrowska, E. & Tutu, H. 2013. Development and application of microwave assisted extraction (MAE) for the extraction of five Polycyclic Aromatic Hydrocarbons in sediment samples in Johannesburg area, South Africa. *Environmental Monitoring and Assessment*, 185(7):5537-5550.

Silhavy, T. J., Kahne, D. & Walker, S. 2010. The bacterial cell envelope. *Cold Spring Harbor Perspectives in Biology,* doi:10.1101/cshperspectives.org Accessed 28/08/2015.

Silva, I. S., Grossman, M. & Durranta, L. R. 2009. Degradation of Polycyclic Aromatic Hydrocarbons (2-7 rings) under microaerobic and very-low-oxygen conditions by soil fungi. *International Biodeterioration & Biodegradation*, 63(2):224-229.

Simpson, C. D., Harrington, C. F., Cullen, W. R., Bright, D. A. & Reimer, K. J. 1998. Polycyclic Aromatic Hydrocarbon contamination in marine sediments near Kitimat, British Columbia. Environmental Science & Technology, 32(21):3266-3272.

Singh, H. 2006. Fungal Metabolism of Polycyclic Aromatic Hydrocarbons. In: Singh, H (ed) mycoremediation; fungal bioremediation. John Wiley and sons, New Jersey, pp 283-356.

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

Singh, S. N. & Tripathi, R. D. 2007. Environmental bioremediation technologies. *Earth Sciences & Geography*, 13:106-125.

Skoglund, T. S., Pascher, R. & Berthold, C. H. 1996. Heterogeneity in the columnar number of neurons in different neocortical areas in the rat. *Neuroscience Letters*, 208:97-100.

Skupinska, K., Mislewicz, I. & Kasprzycka-Guttman, T. 2004. Polycyclic Aromatic Hydrocarbons: Environmental appearance and impact on living organisms. *Acta poloniae Pharmaceutica-Drug Research*, 61(3):233-240.

Soleimani, M. 2012. Bioremediation: an environmental friendly solution for oil contaminated soils. *Journal of bioremediation and biodegradation*, 3(109):2155-6199.

Sojinu, O. S., Sonibare, O. O. & Zeng, E. Y. 2011. Concentrations of Polycyclic Aromatic Hydrocarbons in soils of a mangrove forest affected by a forest fire. *Toxicological & Environmental Chemistry*, 93(3):450-461.

Sowa, D. 2011. *Sphingomonas spp*: Agents of bioremediation and pathogenesis. *Microbe wiki,* 3:1-5.

Sram, R. J., Binková, B., Rössner, P., Rubeš, J., Topinka, J. & Dejmeka, J. 1999. Adverse reproductive outcomes from exposure to environmental mutagens. *Mutation Research*, 428: 203-215.

Sudip, K. S., Om, V. S. & Rakesh, K. J. 2002. Polycyclic Aromatic Hydrocarbons: environmental pollution and bioremediation. *Trends in Biotechnology*, 20:243-248.

Swaranjit, S. C. & Randhir, S. M. 2010. Biosurfactant-enhanced bioremediation of hydrophobic pollutants. *Pure and Applied Chemistry*, 82(1):97-116.

Tekere, M., Read, J. & Mattiasson, B. 2005. Polycyclic Aromatic Hydrocarbon biodegradation in extracellular fluids and static batch cultures of selected sub-tropical white rot fungi. *Journal of Biotechnology*, 115:367-77.

Thermo Scientific and Acumedia. 2008. Oxoid microbiology products, *Pseudomonas* Isolation Agar Base *Manufacturing Quality Standards Catalogues*. www.sigmaaldrich.com/Accessed 02-03-2014. pp 1-3.

Tong, M. & Yuan, S. 2012. Physiochemical technologies for hydrocarbon remediation and disposal: A review. *Journal of Hazardous Materials*, 14:229-230.

Transfiguracion, J., Mena, J. A., Aucoin, M. G. & Kamen, A. A. 2011. Development and validation of a HPLC method for the quantification of *Baculovirus* particles. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 879:61-68.

Turner, S., Pryer, K. M., Miao, V. P. & Palmer, J. D. 1999. Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. *Journal of Eukaryotic Microbiology*, 46:327-338.

Tyagi, M., De Fonseca, M. M. & De Carvalho, M. 2011. Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. *Biodegradation*, 22:231-241.

Ukiwe, L. N., Egereonu, U. U., Njoku, P. C., Nwoko, C. I., Jude, I. & Allinor, J. I. 2013. Polycyclic Aromatic Hydrocarbons degradation techniques: A review. *International Journal of Chemistry*, 5(4):43-55.

United States Environmental Protection Agency (US EPA), 1991. Drinking water criteria for Polycyclic Aromatic Hydrocarbons (PAHs). Prepared by office of Health and Environmental Assessment for office of water, EPA Washington DC, ECA0-CIN-D010. pp 12-22.

Varanasi, U., Reichert, W. L. & Stein, J. E. 1989. ^{32}P-Postlabeling analysis of DNA adducts in liver of wild English sole (*Parophrys vetulus*) and winter flounder *(Pseudopleuronectes americanus*). *Cancer Research*, 49:11-71.

Vega, S., Gutierrez, R., Ortiz, R., Schettino, B., Ramirez, M. & Perez, J. 2011. Hydrocarbons derived from petroleum in bottled drinking water from Mexico City. *Bulletin of Environmental Contamination and Toxicology*, 86:632-636.

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

Vinas, M., Sabate, J., Espuny, M. & Solanas, A. 2005. Bacterial community dynamics and Polycyclic Aromatic Hydrocarbon degradation during bioremediation of heavily creosote-contaminated soil. *Applied and Environmental Microbiology*, 71(11):7008-7018.

Volkering, F., Breure, A. M. & Van Andel, J. G. 1993. Effect of microorganisms on the bioavailability and biodegradation of crystalline naphthalene. *Applied Microbiology and Biotechnology*, 40:535-540.

Wang, X. & Brusseau, M. L. 1993. Solubulisation of some low polarity organic compounds by hydroxypropyl-β-cyclodextrin. Environmental Science & Technology, 24(7):1086-1089.

Wang, G., Zhang, Q., Ma, P. & Rowden, J. 2008. Sources and distribution of Polycyclic Aromatic Hydrocarbons in urban soils. Case studies of Detroit and New Orleans. *Soil and Sediment Contamination*, 17(6):547-563.

Web, 2011. About Coal Tar and Polycyclic Aromatic Hydrocarbons (PAHs). The truth about refined coal tar based pavement sealers and PAHs. <http://www.truthaboutcoaltar.com/aboutcoaltar. Accessed 02-09-2014.

Weeks, B. A. & Warimer, J. E. 1984. Effects of toxic chemicals on macrophage phagocytosis in two estuarine fishes. *Marine Environmental Research*, 14:327-335.

Weis, L. M., Rummel, A. M., Masten, S. J., Trosko, J. E. & Upham, B. L. 1998. Bay or bay like regions of Polycyclic Aromatic Hydrocarbons were potent inhibitors of gap junctional intercellular communication. *Environmental Health Perspectives*, 106 (1):17-22.

West, L. 2006. World Water Day: A Billion People Worldwide Lack Safe Drinking Water. *Environmental Issues*, pp. 12-18.

Whitfield, A. K., Bate, B. C., Adams, J. B., Cowley, P. D., Froneman, P. W., Gama, P. T., Strydon, N. A., Taljaard, S. & Theron, A. K. 2012. Review of the ecology and management of temporarily open/closed estuaries in South Africa, with particular emphasis on River flow and mouth state as primary drivers of these systems. *African Journal of Marine Science*, 34(2): 163-180.

Wick, A. F., Haus, N. W., Sukkariyah, B. F., Hearing, K. C. & Lee Daniels, W. 2011. Remediation of PAH-contaminated soils and sediments: A literature review. Department of Crop and Soil Environmental Sciences Blacksburg, VA 24061 Virginia Polytechnic Institute and State University.

Wilcke, W. 2000. Polycyclic Aromatic Hydrocarbons (PAHs) in soil. *Journal of Plant Nutrition & Soil Science*, 163:229-248.

Wild, S. R. & Jones, K. C. 1995. Polynuclear Aromatic Hydrocarbons in the United Kingdom environment: a preliminary source inventory and budget. *Environmental Pollution*, 88(1):91-108.

World Health Organisation (WHO). 1997. Guidelines for drinking water quality Addendum to Vol. 2. Health criteria and other supporting information. pp. 12-26.

World Health Organisation (WHO). 2003. Polynuclear Aromatic Hydrocarbons in Drinking-water Background document for development of WHO Guidelines for Drinking-water Quality Originally published in Guidelines for drinking-water quality, 2nd ed. Addendum to Vol. 2. Health criteria and other supporting information. World Health Organization, Geneva.

Wu, M., Chen, L., Tian, Y., Dinga, Y. & Dick, W. A. 2013. Degradation of Polycyclic Aromatic Hydrocarbons by microbial consortia enriched from three soils using two different culture media. *Environmental Pollution*, 178:152-158.

Yanyangwu, D. 2012. Anaerobic biodegradation of PAHs in River sediments under denitrifying conditions - Another choice? *Yanyangwu wordpress.com* Accessed 04-08-2013.

Zeller, C. & Cushing, B. 2005. Panel discussion: remedy effectiveness: what works, what doesn't. *Integrated Environmental Assessment and Management*, 2:75-79.

Zhang, X. L., Tao, S., Liu, W. X., Yang, Y., Zuo, Q. & Liu, S. Z. 2005. Source diagnostics of Polycyclic Aromatic Hydrocarbons based on species ratios: a multimedia approach. *Environmental Science & Technology*, 39:9109-9114.

Zhang, Y. & Tao, S. 2009. Global atmospheric emission inventory of Polycyclic Aromatic Hydrocarbons (PAHs) for 2004. *Atmospheric Environment*, 43:812-819.

Zhang, J., Zhang, X., Liu, J., Li, R. & Shen, B. 2012. Isolation of a thermophilic bacterium, *Geobacillus sp*. SH-1, capable of degrading aliphatic hydrocarbons and naphthalene simultaneously, and identification of its naphthalene degrading pathway. *Bioresource Technology,* 124:83-89.

Zhang, H., Sun, L., Sun, T., Li, H. & Luo, Q. 2013. Spatial distribution and seasonal variation of Polycyclic Aromatic Hydrocarbons (PAHs) contaminations in surface water from the Hun River, Northeast China. *Environmental Monitoring and Assessment*; 185(2):1451-1462.

7. APPENDICES

7.1. Appendix I: Bacterial species recorded at sampling points along the Diep- and Plankenburg Rivers, Western Cape, South Africa, and the mean plate count numbers obtained during summer and winter sampling time.

		Mean summer microbial counts (CFU/ml)				Mean winter microbial counts (CFU/ml)			
Site	**Isolated microorganism**	**SW**	**SDEV (+\-)**	**SD**	**SDEV (+\-)**	**SW**	**SDEV (+\-)**	**SD**	**SDEV (+\-)**
A	*Raoultella ornithinolytica*	3.1×10^{7}	1.3×10^{4}	6.1×10^{7}	1.0×10^{2}	0.9×10^{7}	6.0×10^{0}	1.3×10^{7}	6.1×10^{3}
	Bacillus megaterium	3.5×10^{7}	2.0×10^{2}	4.9×10^{7}	2.4×10^{1}	1.3×10^{6}	1.3×10^{4}	2.1×10^{7}	2.1×10^{0}
	Pseudomonas aeruginosa	3.7×10^{7}	1.3×10^{1}	6.6×10^{7}	3.4×10^{3}	1.2×10^{4}	1.1×10^{4}	1.3×10^{7}	4.0×10^{0}
	Escherichia coli	2.9×10^{7}	4.0×10^{0}	2.9×10^{7}	2.7×10^{1}	2.5×10^{7}	1.9×10^{4}	2.7×10^{7}	0.7×10^{1}
	Acinetobacter sp.	0.9×10^{7}	1.1×10^{4}	6.2×10^{7}	2.1×10^{3}	1.3×10^{4}	3.3×10^{1}	1.3×10^{7}	4.2×10^{3}
	Bacillus sp	1.9×10^{7}	1.3×10^{1}	TNTC	ND	4.9×10^{3}	2.1×10^{4}	6.1×10^{7}	1.1×10^{0}
	Serratia marcescens	4.5×10^{7}	6.0×10^{1}	6.1×10^{7}	4.2×10^{1}	2.1×10^{3}	1.3×10^{2}	3.3×10^{7}	3.0×10^{2}
	Aeromonas hydrophila	0.6×10^{7}	1.0×10^{3}	3.9×10^{7}	1.8×10^{3}	0.6×10^{3}	2.8×10^{3}	1.5×10^{7}	2.2×10^{5}
	Bacillus marisflavi	3.2×10^{7}	1.1×10^{4}	3.8×10^{7}	1.5×10^{4}	1.0×10^{4}	1.1×10^{1}	1.9×10^{7}	1.6×10^{1}
	S. saprophyticus	3.5×10^{5}	6.3×10^{2}	3.3×10^{7}	2.7×10^{1}	2.2×10^{3}	3.3×10^{0}	2.5×10^{7}	1.4×10^{2}
B	*Raoultella ornithinolytica*	4.4×10^{4}	4.6×10^{2}	7.1×10^{6}	2.8×10^{2}	1.2×10^{4}	27×10^{1}	4.2×10^{5}	3.4×10^{0}
	Bacillus megaterium	2.3×10^{6}	2.4×10^{3}	6.9×10^{7}	3.3×10^{3}	1.1×10^{6}	3.3×10^{4}	4.3×10^{4}	2.9×10^{1}
	Pseudomonas aeruginosa	2.1×10^{5}	1.0×10^{3}	6.4×10^{5}	3.6×10^{1}	2.3×10^{4}	1.3×10^{2}	5.5×10^{3}	3.1×10^{4}
	Escherichia coli	1.9×10^{7}	1.3×10^{2}	5.9×10^{8}	2.9×10^{3}	0.9×10^{3}	2.0×10^{2}	3.3×10^{4}	1.4×10^{0}
	Exiguobacterium acetylicum	4.8×10^{6}	1.1×10^{4}	5.9×10^{7}	3.6×10^{4}	2.0×10^{6}	1.8×10^{3}	3.3×10^{7}	1.2×10^{0}
	Exiguobacterium sp.	3.8×10^{7}	1.1×10^{4}	7.1×10^{7}	2.9×10^{1}	1.1×10^{3}	2.0×10^{2}	5.4×10^{4}	2.2×10^{4}
	Serratia marcescens	4.7×10^{5}	6.3×10^{2}	7.1×10^{7}	3.8×10^{2}	3.4×10^{3}	1.1×10^{4}	4.6×10^{5}	3.1×10^{2}
	Aeromonas hydrophila	5.7×10^{4}	1.0×10^{3}	5.9×10^{6}	2.9×10^{2}	1.9×10^{4}	8.1×10^{4}	2.3×10^{5}	2.2×10^{5}
	S. saprophyticus	3.7×10^{6}	1.6×10^{1}	4.2×10^{5}	7.0×10^{2}	1.1×10^{7}	3.3×10^{2}	1.9×10^{4}	5.1×10^{2}
	Exiguobacterium undae	5.1×10^{7}	3.4×10^{0}	10.2×10^{7}	3.3×10^{2}	3.2×10^{7}	2.9×10^{2}	5.6×10^{7}	1.1×10^{3}
	Citrobacter freundii	4.8×10^{6}	2.0×10^{2}	9.2×10^{5}	2.6×10^{2}	3.3×10^{5}	3.3×10^{2}	2.2×10^{6}	4.1×10^{2}
C	*Raoultella ornithinolytica*	9.8×10^{7}	3.1×10^{4}	26.6×10^{7}	2.1×10^{4}	3.1×10^{5}	1.7×10^{4}	7.7×10^{6}	3.3×10^{1}
	Bacillus megaterium	8.6×10^{7}	2.7×10^{1}	2.88×10^{9}	1.9×10^{1}	8.5×10^{7}	1.2×10^{3}	2.3×10^{7}	2.2×10^{4}
	Pseudomonas aeruginosa	9.5×10^{6}	4.2×10^{1}	2.26×10^{7}	1.2×10^{0}	7.3×10^{6}	2.3×10^{6}	1.3×10^{8}	1.7×10^{2}
	Raoultella planticola	7.5×10^{7}	2.3×10^{2}	1.96×10^{8}	2.3×10^{2}	2.36×10^{6}	2.0×10^{2}	1.2×10^{8}	1.1×10^{3}
	Klebsiella oxytoca	6.4×10^{6}	1.6×10^{2}	2.12×10^{8}	3.1×10^{5}	7.8×10^{7}	1.8×10^{2}	1.1×10^{6}	1.3×10^{4}
	Escherichia coli	3.6×10^{6}	2.1×10^{3}	13.6×10^{8}	2.2×10^{2}	10.6×10^{7}	3.3×10^{1}	5.6×10^{7}	1.2×10^{5}
	Enterobacter cloacae	6.2×10^{7}	1.1×10^{4}	19.9×10^{7}	1.0×10^{3}	6.9×10^{6}	4.2×10^{2}	2.3×10^{8}	1.0×10^{1}
	Acinetobacter sp.	5.9×10^{7}	6.0×10^{0}	10.9×10^{7}	1.1×10^{3}	3.9×10^{6}	3.6×10^{2}	9.0×10^{6}	1.0×10^{1}
	Serratia marcescens	6.9×10^{8}	2.0×10^{2}	17.9×10^{7}	1.0×10^{3}	2.3×10^{7}	3.1×10^{5}	8.1×10^{8}	1.3×10^{2}
	Bacillus sp.	TNTC	ND	TNTC	ND	TNTC	4.2×10^{1}	9.1×10^{7}	ND
	Aeromonas hydrophila	5.2×10^{6}	3.1×10^{2}	2.13×10^{8}	2.1×10^{2}	6.5×10^{7}	1.6×10^{2}	7.1×10^{8}	5.0×10^{1}
D	*Pseudomonas aeruginosa*	7.9×10^{6}	1.6×10^{2}	7.9×10^{6}	2.6×10^{1}	3.3×10^{4}	2.8×10^{2}	6.1×10^{5}	3.7×10^{1}
	Escherichia coli	3.9×10^{5}	2.3×10^{4}	3.6×10^{7}	3.1×10^{3}	1.3×10^{3}	3.3×10^{5}	2.2×10^{6}	4.2×10^{0}
	Bacillus aquimaris	9.1×10^{7}	1.1×10^{3}	1.37×10^{6}	4.0×10^{3}	2.6×10^{3}	2.5×10^{2}	0.36×10^{4}	1.6×10^{0}
	Aeromonas hydrophila	2.12×10^{7}	2.3×10^{4}	1.0×10^{7}	2.6×10^{2}	2.3×10^{5}	2.1×10^{4}	1.52×10^{7}	2.4×10^{3}
E	*Pseudomonas aeruginosa*	1.3×10^{6}	1.1×10^{2}	11.8×10^{7}	2.1×10^{0}	7.2×10^{4}	1.6×10^{4}	3.1×10^{5}	1.8×10^{2}
	Escherichia coli	3.8×10^{7}	2.3×10^{4}	3.6×10^{7}	3.6×10^{1}	2.2×10^{6}	2.3×10^{2}	2.9×10^{6}	1.1×10^{0}
	Acinetobacter sp.	3.4×10^{7}	5.1×10^{0}	2.9×10^{6}	2.6×10^{2}	1.4×10^{7}	4.5×10^{2}	2.3×10^{7}	2.5×10^{1}
	Aeromonas hydrophila	3.1×10^{7}	2.3×10^{1}	12.3×10^{6}	3.1×10^{3}	2.6×10^{7}	1.7×10^{1}	1.3×10^{4}	2.6×10^{0}
	Bacillus aryabhattai	1.9×10^{7}	7.1×10^{4}	2.2×10^{7}	2.6×10^{2}	0.6×10^{4}	8.4×10^{1}	2.3×10^{5}	3.4×10^{2}
	Bacillus aquimaris	7.1×10^{4}	2.3×10^{2}	3.9×10^{6}	2.1×10^{1}	0.4×10^{3}	2.3×10^{2}	2.2×10^{4}	2.7×10^{1}
	Enterobacter cloacae	6.6×10^{7}	4.1×10^{2}	8.1×10^{7}	1.3×10^{2}	4.4×10^{7}	2.1×10^{2}	6.1×10^{7}	1.1×10^{2}
F	*Escherichia coli*	8.8×10^{7}	2.2×10^{2}	2.12×10^{8}	2.3×10^{4}	3.2×10^{7}	1.3×10^{3}	6.7×10^{7}	1.7×10^{0}

	Enterobacter cloacae	6.8×10^{6}	2.8×10^{1}	1.65×10^{7}	1.0×10^{0}	2.4×10^{4}	2.8×10^{4}	2.6×10^{7}	1.2×10^{4}
	Acinetobacter sp.	3.4×10^{5}	3.1×10^{4}	6.6×10^{6}	1.9×10^{2}	1.1×10^{3}	5.6×10^{2}	2.9×10^{5}	2.8×10^{1}
	Serratia marcescens	7.8×10^{4}	4.0×10^{1}	5.6×10^{6}	3.8×10^{3}	1.8×10^{4}	2.9×10^{3}	6.1×10^{3}	1.4×10^{1}
	Aeromonas hydrophila	7.9×10^{5}	2.7×10^{4}	5.4×10^{7}	5.1×10^{1}	2.6×10^{5}	4.2×10^{2}	4.2×10^{6}	1.5×10^{3}

Key: SW: surface water, SD: sediment, TNTC: too numerous to count, SDEV: standard deviation, ND: not determined

Appendix II

7.2. Consensus Sequences for all isolated PAH degrading Microorganisms

TTAAGCTACCTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGG
CCCGGGAACGTATTCACCGTAGCATTCTGATCTACGATTACTAGCGATTCCGACTTCATG
GAGTCGAGTTGCAGACTCCAATCCGGACTACGACATACTTTATGAGGTCCGCTTGCTCTC
GCGAGGTCGCTTCTCTTTGTATATGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGG
CCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGA
GTTCCCGRCCGRAYCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACC
CAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAG
GCACCAAAGCATCTCTGCTAAGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTT
GCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTT
TTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACT
CCTCAAGGGAACAACCTCCAAGTCGACATCGTTTACAGCGTGGACTACCAGGGTATCTAA
TCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTT
CGCCACCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCC
CCTCTACAAGACTCWAGCCTGCCAGTTTCRRATGCAGTTCCCAGGTTGAGCCCGGGGATT
TCACATCYGACTTRACAGACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGC
TTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGAG
TAACGTCAATCRMYAAGGTTATTAACCTTAWYGCCTTCCTCCTCGCTGAAAGTACTTTAC
AACCCGAAGGCCTTCTTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCA
ATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTG
GTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGAGCCATTACCCCACCTACTAG
CTAATCCCATCTGGGCACATCTGATGGCATGAGGCCCGAAGGTCCCCCACTTTGGTCTTG
CGACGTTATGCGGTATTAGCTACCGTTTCCAGTAGTTATCCCCCTCCATCAGGCAGTTTC
CCAGACATTACTCACCCGTCCGCCGCTCGTCACCCGAGAGCAAGCTCTCTGTGCTACCG

Consensus sequence for *R. ornithinolytica*

AAGTGGGTAAGCGCCCTCCCGAAGGTTAAGCTACCTACTTCTTTTGCAACCCACTCCCAT
GGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTAGCATTCTGATCTAC
GATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACG
TACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATACGCCATTGTAGC
ACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCA
GTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGAACCGCTGGCAACAAAGGATAAGG
GTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGC
AGCACCTGTCTCAGAGTTCCCGAAGGCACCAAWCCATCTCTGSWAAGTTCTCTGGATGTC
AAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGC
GGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGATT
TAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAATCGACATCGTTTA
CAGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGT
CAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTC
ACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCTAGCTTGCCAGTTTCAAATGC
AGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAACAAACCGCCTGCGTGCGCTT
TACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGG
AGTTAGCCGGTGCTTCTTCTGCGAGTAACGTCAATTGATGARCGTATTAAGYTCACCACC
TTCCTCCTCGCTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATGGCT
GCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGA
CCGTGTCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTA
GGTGAGCCATTACCCCACCTACTAGCTAATCCCATCTGGGCACATCTGATGGCAAGAGGC
CCGAAGGTCCCCCTCTTTGGTCTTGCGACGTTATGCGGTATTAGCTACCGTTTCCAGTAG
TTATCCCCCTCCATCAGGCAGTTTCCCAGACATTACTCACCCGTCCGCCGCTCGTCACCC
RGGGAGCAAGCTCCCCTGTGCTACCGCTCGACT

Consensus sequence for *S. marcescens*

CTCCTTACGGTTACTCCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGT
GTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTC
CAGCTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAATGGTTTTATGGGATTG
GCTTGACCTCGCGGTCTTGCAGCCCTTTGTACCATCCATTGTAGCACGTGTGTAGCCCAG
GTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAG
TCACCTTAGAGTGCCCAACTAAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGG
GACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTG
TCCCCCGAAGGGGAACGCTCTATCTCTAGAGTTGTCAGAGGATGTCAAGACCTGGTAAGG
TTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAAT
TCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTG
CAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTAC
CAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTACAGACCA
AAAAGCCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTG
GAATTCCGCTTTTCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCACGGTTG
AGCCGTGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGCGCGCTTTACGCCCAATAAT
TCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGC
TTTCTGGTTAGGTACCGTCAAGGTACGAGCAGTTACTCTCGTACTTGTTCTTCCCTAACA
ACAGAGTTTTACGACCCGAAAGCCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTC
GTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTC
CCAGTGTGGCCGATCACCCTCTCAGGTCGGCTATGCATCGTTGCCTTGGTGAGCCGTTAC
CTCACCAACTAGCTAATGCACCGCGGGCCCATCTGTAAGTGATAGCCGAAACCATCTTTC
AATCATCTCCCATGAAGGAGAAGATCCTATCCGGTATTAGCTTCGGTTTCCCGAAGTTAT
CCCAGTCTTACAGGCAGGTTGCCCACGTGTTACTCACCCGTCCGCCGCTAACGTCATAGA
AGCAAGCTTCTAATCAGTTCGCTCGAC

Consensus sequence for *B. megaterium*

GAAGGTTAAGCTATCTACTTCTGGTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTA
CAAGGCCCGGGAACGTATTCACCGCAACATTCTGATTTGCGATTACTAGCGATTCCGACT
TCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGCGCTTTTTGGGATTCGCTCA
CTATCGCTAGCTTGCAGCCCTCTGTACGCGCCATTGTAGCACGTGTGTAGCCCTGGCCGT
AAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCC
CTTGAGTTCCCACCATTACGTGCTGGCAACAAAGGACAGGGGTTGCGCTCGTTGCGGGAC
TTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTTCTGATTC
CCGAAGGCACTCCCGTATCTCTACAGGATTCCAGACATGTCAAGGCCAGGTAAGGTTCTT
CGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATT
TGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGATTTAACGCGTTAGCTCCGGAA
GCCACGTCTCAAGGACACAGCCTCCAAATCGACATCGTTTACGGCGTGGACTACCAGGGT
ATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGC
CGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTC
TACCCCCCTCTACAAGACTCTAGCTGGACAGTTTTAAATGCAATTCCCAGGTTGAGCCCG
GGGCTTTCACATCTAACTTATCCAACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGAT
TAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTC
TGCGAGTAACGTCACAGCCAGCAGGTATTAGCTACTGACCTTTCCTCCTCGCTGAAAGTG
CTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATGGCTGCATCAGGGTTTCCCCCAT
TGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTG
TGGCTGATCATCCTCTCAGACCAGCTAGGGATCGTCGCCTTGGTGAGCCATTACCTCACC
AACTAGCTAATCCCACCTGGGCATATCCAATCGCGCAAGGCCCKAAGGTCCCCTGCTTTC
CCCCGTAGGGCGTATGCGGTATTAGCAGTCGTTTCCAACTGTTATCCCCCTCGACTGGGC
AATTTCCCAGGCATTACTCACCCGTCCGCCGCTCGCCGGCAAAAGTAGCAAGCTACTTTC
CCGCTGCCGC

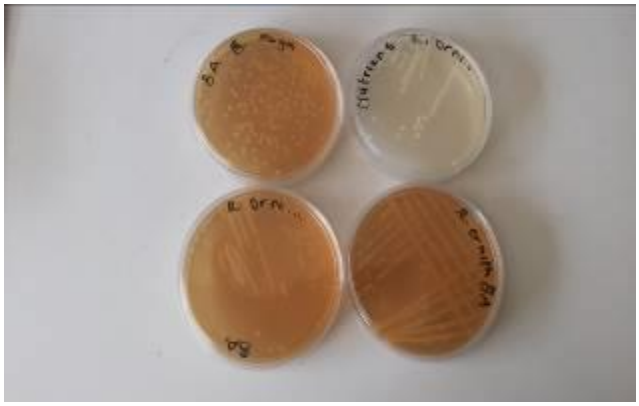
Consensus sequence for *A. hydrophila*

Appendix III

7.3. **Photographs**

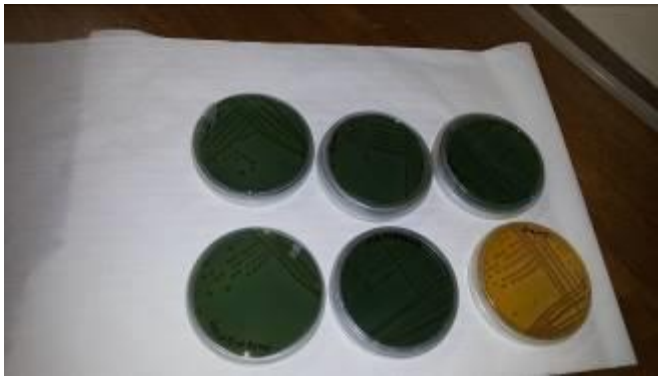
Agricultural farming area on Plankenburg River

Point E (Theo Marias Sports Club)



***B. megaterium* and *R. ornithinolytica* on BA and NA**

***Aeromonas hydrophila* on AIA**



Aeromonas hydrophila

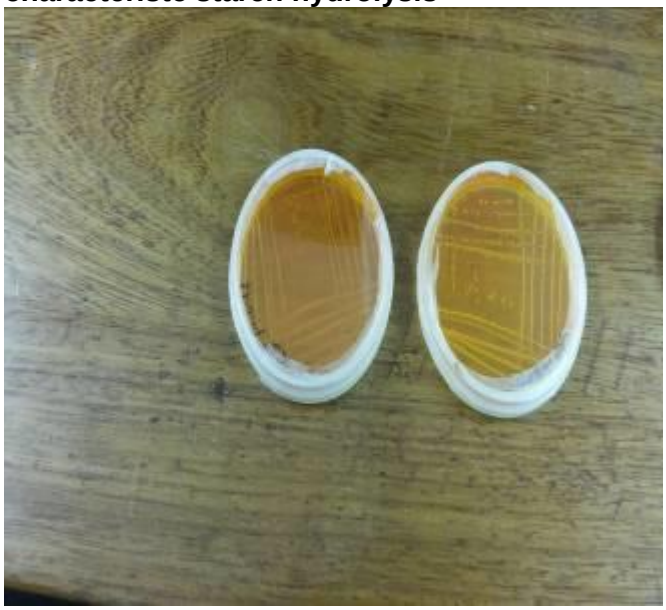
***Aeromonas hydrophila* on AIA after acenaphthene degradation.**

Spread plate (Informal settlement)

Streak plates

***Raoutella ornithinolytica* on GSP (left) *Aeromonas hydrophila* on GSP with the characteristc starch hydrolysis**

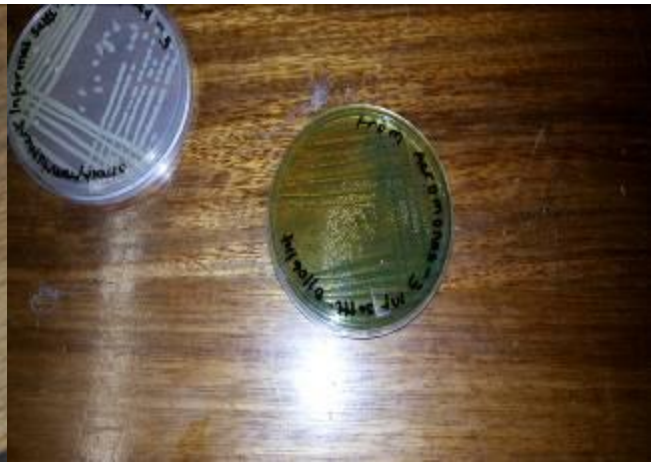
***Aeromonas hydrophila* on GSP agar (left) and on NA (right)**



***Aeromonas hydrophila* on GSP agar**

***Aeromonas hydrophila* on NA**

***Raoutella ornithinolytica on* AIA**



***Raoutella ornithinolytica on* AIA**

Media for biochemical testing

Biochemical tests setup

Flask scale degradation in shaking incubator

Bioreactor flange