COMPOST BIOREMEDIATION OF OIL SLUDGE BY USING DIFFERENT MANURES UNDER LABORATORY CONDITIONS

by

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submitted in accordance with the requirements for the degree of

MASTER OF SCIENCE

in the subject

ENVIRONMENTAL SCIENCES

at the

UNIVERSITY OF SOUTH AFRICA

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CO-SUPERVISOR: Dr. M. S. THANTSHA

JUNE 2012

DECLARATION

I, ONYEDIKACHI UBANI sincerely and solemnly declare that the work:

COMPOST BIOREMEDIATION OF OIL SLUDGE BY USING DIFFERENT MANURES UNDER LABORATORY CONDITIONS is my own work and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references.

Sign……..
For student

Sign…….
Supervisor

DEDICATION

This research dissertation is dedicated to Almighty God the creator of the universe. To my darling wife, Mrs Christiana Uzoamaka Ubani. To my loving parents Snr. AP. Luke O. Ubani (aka Prime Minister) and Mother Josephine N. Ubani (Lolo). To my lovely sister, Mrs Juliana Ifeoma John and to all who believes that God is able to do all things.

ACKNOWLEDGEMENTS

Almighty father to you be all the glory, for your grace and mercy. I have nothing to give you than to say thank you Lord for being with me till date and through this journey, may your name be praised.

My sincere gratitude goes to my supervisor, Prof Harrison Ifeanyichukwu Atagana not only for initiating the project but for fatherly guidance, support and advice when I needed them most during the course of this research. Sir thank you for being there for us, I hope to work with you all the way. My special thanks goes to my Co-supervisor Dr. M. S. Thantsha of University of Pretoria for providing laboratory space and specialist guidance during the period of study.

My heartfelt gratitude goes to the National Research Foundation of South Africa (NRF) for providing the funds for this research and also bursary to support me during the research.

To Prof OR Awofolu; former COD Environmental Sciences UNISA for your kind assistance, Prof JM Mphahlele and Prof M Nindi, of Department of Chemistry UNISA for allowing me access to their laboratory, to the Department of Environmental Sciences UNISA for the opportunity to study and University of South Africa for providing the opportunity to study and also the financial support.

My special thanks to Mrs Kali of Water Lab Pretoria for your help. To Dr Rasheed Adeleke for your kind support and advice. To Dr. IP Molobela for being my sister and giving listening ear each time I call. To Renate Zipfel and Grace of FABI UP for your guidance. To Mr Chucks Moses for investing in me. Also to the entire staff of ISTE for accommodating us: Prof D. Mogari, Prof J Kriek, Dr EC Ochonogor, Dr UI Ogbonnaya, EG Makwakwa, Mrs DP Ramodipa, MM Mokobaki, JJ Dhlamini, MM Sebaiwa.

I would like to thank my brothers and sisters for their support, Mr Christopher Okpechi, Mr Samuel Okpechi, Angelina Onyeanula, my late in-law Mr Chidi John, Mr Felix Uche, Mr Stanley Uche, Barr. Emeka Opara, Mr Chijioke Nwachukwu, Mr Eric N Opah, Dennis Ikpe, I love you all.

I would not forget my beloved friends and brothers Raymond Anyasi, Emeka Enemchukwu, Samuel Umezurike, Richard Ochonogor, Mojirola Oshoba, Llehlogonolo A Mashigo, Biotumeleng, Olu Okedeyi, Rodah Mbewe, Franklin Nwankwo, Sarah, Lerato Mahanyele, Martha Mahlangu, Cornelius, Biyi Olukunle, God has used you in one way or the other to support me, thank you all, I owe you guys alot.

I gratefully want to acknowledge my spiritual parents, Pastor David and Pastor Mrs Francisca Okonkwo for your prayers, thank you for being there.

ABSTRACT

This study was conducted to measure the reduction in polycyclic aromatic hydrocarbons (PAHs) content in oil sludge by co-composting the sludge with pig, cow, horse and poultry manures under laboratory conditions. Four kilograms of soil spiked with 800g of oil sludge was co-composted differently with each manure in a ratio of 2:1 (w/w) spiked soil: manure and wood-chips in a ratio of 2:1 (w/v) spiked soil: wood-chips. Control was set up similar as the one above but without manure. Mixtures were incubated for 10 months at room temperature. Compost piles were turned weekly and moisture level was maintained at between 50% and 70%. Moisture level, pH, temperature, CO_2 evolution and oxygen consumption were measured monthly and the ash content at the end of experimentation. Bacteria capable of utilizing PAHs were isolated, purified and characterized by molecular techniques using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), amplification of the 16S rDNA gene using the specific primers (16S-P1 PCR and 16S-P2 PCR) and the amplicons were sequenced. Extent of reduction of PAHs was measured using automated soxhlet extractor with Dichloromethane as the extraction solvent coupled with gas chromatography/mass spectrometry (GC/MS). Temperature did not exceed $27.5^{O}C$ in all compost heaps, pH ranged from 5.5 to 7.8 and CO_2 evolution was highest in poultry manure at 18.78µg/dwt/day. Microbial growth and activities were enhanced. Bacteria identified were *Bacillus*, *Arthrobacter* and *Staphylococcus* species. Results from PAH measurements showed reduction between 77 and 99%. The results from the control experiments may be because it was invaded by fungi. Co-composting of spiked soils with animal manures enhanced the reduction in PAHs. Interestingly, all bacteria isolated and identified in this study were present in all treatments, including the control.

Keywords: *Bioremediation, Co-composting, Oil refinery sludge, PAHs, Bacteria spp, Animal manures, Molecular techniques.*

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CHAPTER TWO

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BTEX	Benzene, Toluene, Ethylbenzene, Xylene
C:N:P	Carbon Nitrogen Phosphorus
CADM	Concentration Acid Digestion Method
CCD	Charge Coupled Device
CCl_4	Carbon Tetrachloride
CNS	Central Nervous System
CONCAWE	Conservation of Clean Air and Water in Europe
CTAB	Cetyl trimethyl Ammonium Bromide
ddNTP's	Didoxyribonucleotide Triphosphate
DM	Dry Matter
dNTP's	Deoxyribonucleotide Triphosphate
EDTA	Ethylene Diamine Tetraacetic Acid
EPA	Environmental Protection Agency
FABI	Forest and Agricultural Biotechnology Institute
GC/MS	Gas Chromatography-Mass Spectrometry
IARC	International Agency for Research on Cancer
ICP	Inductive Coupled Plasma- Spectrophotometer
Kb	Kilobases Mass
Mg/Kg	Milligram per Kilogram
MPN	Most Probable Number
MSA	Mineral Salt Agar

MSD	Mass Spectrometry Detector
MSM	Mineral Salt Medium
NADH	Nicotinamide-Adenine-Dinucleotide Hydride
NAH7	Nicotinamide-Adenine- Hydride
NCBI	National Centre for Biotechnology Information
PAHs	Polycyclic Aromatic Hydrocarbons
PCBs	Polychlorinated Biphenyls
PCR-DGGE	Polymerease Chain Reaction Denaturing Gradient Gel Electrophoresis
PEG	Polyethylene Glycol
pH	Negative logarithm to base 10
RCRA	Restriction Conservation and Recovery Act
rDNA	ribosome Deoxyribonucleic Acid
RNA	Ribonucleic Acid
SAPIA	South African Petroleum Industry Association
SDS	Sodium Dodecyl Sulphate
SVOC'S	Semivolatile Organic Compounds
TAE	Tris Acetate Ethylene DiamineTetraacetic Acid
TE	Tris Ethylene Diamine Tetraacetic Acid
THC	Total Hydrocarbon
TPHs	Total Petroleum Hydrocarbons
UNISA	University of South Africa
USAEPA	United State of America Environmental Protection Agency
UV	UltraViolet light
VOC'S	Volatile Organic Carbons
WHC	Water Holding Capacity

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CHAPTER ONE

Introduction

1.0 Background of the study

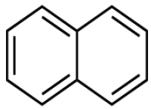
Oil sludge is a thick, viscous mixture of sediments, water, oil and high hydrocarbon concentration, encountered during crude oil refining, cleaning of oil storage vessels and waste treatment. The chemical composition of oil sludge is complex and depends on the source. Oil sludge is mainly composed of alkanes, aromatics, asphaltenes and resin (Diallo *et al.,* 2000). It has high content of aromatic hydrocarbons in the range of 1- 40 carbon atoms (US EPA, 1997).The two major sources of oil sludge are oil storage tanks and refinery-wastewater treatment plants (Shie *et al.,* 2004, Wang *et al.,* 2010). Oil sludge found in crude oil storage tanks is typically made up of sulphides, phenols, heavy metals, aliphatic and polycyclic aromatic hydrocarbons (PAHs) of 4, 5, 6 and more rings, in over 10-20 fold concentration (Li *et al.,* 1995). More than 90% of oil sludge material is composed of paraffin, asphaltenes and aromatic hydrocarbon mixtures. Paraffins are saturated hydrocarbon (alkanes) that have the general formula $C_nH_{2n}+_2$ and can either be straight chains (n-paraffins) or branched chains (isoparaffins). Asphaltenes are polycyclic aromatic clusters, substituted with varying alkyl side chain. Aromatics hydrocarbons are unsaturated ring type (complex polycyclic of three or more fused aromatic rings) compounds, which reacts readily because they have carbon atoms that are deficient in hydrogen. All aromatics hydrocarbons have at least one benzene ring as part of their molecular structure. These components are highly recalcitrant under normal conditions. Such characteristics are attributed to their strong molecular bonds, high molecular weights, hydrophobicity and relative low solubility in water.

Oil sludge has been classified by the United States Environmental Protection Agency (US EPA) as a hazardous organic complex (US EPA, 1997, Liu *et al.,* 2010). This contaminant enters the environment as a result of human activities, which includes deliberate dumping, improper treatments and management, storage, transportation and landfill disposal. This calls for concern because many of the oil sludge components have been found to be cytotoxic, mutagenic and potentially carcinogenic (Bojes and Pope, 2007). The environmental impact of oil sludge contamination includes physical and chemical alteration of natural habitats, lethal and sub-lethal toxic effects on aquatics and terrestrial ecosystem. Oil sludge contains volatile organic carbons (VOCs) and semivolatile organic carbons (SVOCs) (e.g. PAHs) which over the years have been reported as being genotoxic (Mishra *et al.,* 2001; Bach *et al.,* 2005, Bojes and Pope, 2007). They have cumulative effect on the central nervous system (CNS) leading to dizziness, tiredness loss of memory and headache, and the effect depends on duration of exposure. In severe cases, PAH metabolism in human body produces epoxide compound with mutagenic and carcinogenic properties that affects the skin, blood, immune system, liver, spleen, kidney, lungs, developing foetus, it also causes weight loss (TERA, 2008; API, 2008; Sidney, 2008, Bayoumi, 2009). However, environmental regulations in many parts of the world have stressed on the necessity to decrease emission of volatile organic compounds and polycyclic aromatic hydrocarbon (PAHs), and have placed more restriction on land disposal of oil sludge (Mahmoud, 2004). The South African petroleum industry association (SAPIA) reported that about 19.5 million tonnes of crude oil are brought into South Africa annually to feed the country's four largest refineries (Maila, 2004). These refineries stores and refine the crude oil thereby generating substantial quantity of oil sludge. To the best of my knowledge, there has not been any report on how these oil refineries treat the much anticipated oil sludge they generate neither has there been any literature

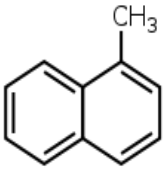
report on oil sludge treatment in South Africa. Rather, there are reports on regulations, restrictions and guidelines given to oil refineries by South African Department of Environmental Affairs, South African National Environmental Management and South African Department of Water Affairs and Forestry to manage oil sludge, control as well as monitor any air pollution from land disposal units (Quarterly Government Gazette No: 22, 2009).

1.1 Some important compounds present in oil sludge.

Some important polycyclic aromatic hydrocarbons (PAHs) of environmental concern present in oil sludge include, naphthalene, 1-methyl naphthalene, 2-methyl naphthalene, acenaphthylene, acenaphthene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, chrysene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene and indenol(1,2,3-cd)pyrene. The chemical structures of these complex ring molecules are indicated in Fig 1.0.

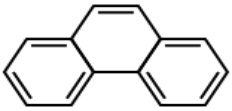


Naphthalene

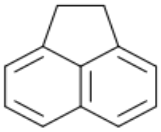


1-methyl Naphthalene

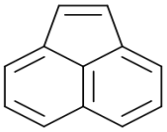
Anthracene



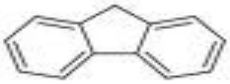
Phenanthrene



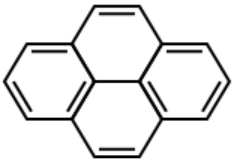
Acenaphthene



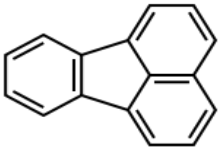
Acenaphthylene



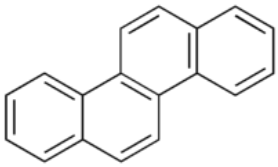
Fluorene



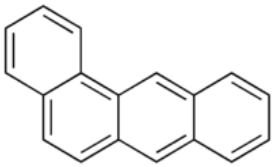
pyrene



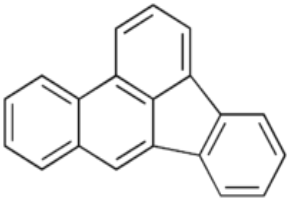
fluoranthene



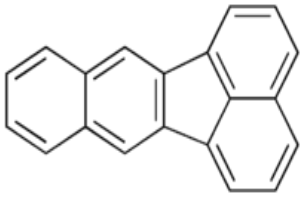
Chrysene



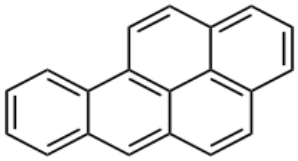
Benzo[a]anthracene



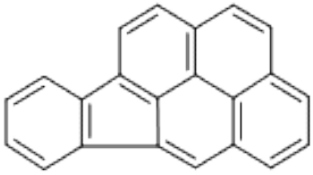
Benzo[b]fluoranthene



Benzo[k]fluoranthene



Benzo[a]pyrene



Indenol (1, 2, 3-cd) pyrene

Fig 1.0. Some polycyclic aromatic hydrocarbons (PAHs) of environmental concern present in oil-sludge.

These PAHs which may be acute hazardous organic compounds are present in substantial quantities in oil sludge and are susceptible to microbial degradation (Gibson and Subramanian, 1984; Mueller *et al.*, 1991; Field *et al.*, 1992; Sutherland *et al.*, 1995). The proposed catabolic pathways by aerobic bacteria for some of the petroleum hydrocarbons are briefly explained in pages 6, 8, 10, 11, 13, 15 and 17.

1.1.1 Naphthalene

It is an aromatic hydrocarbon, with molecular formula $C_{10}H_8$ and the structure of two fused benzene rings. Biodegradation of naphthalene involves the microbial utilisation of naphthalene as described by Gibson and Subramanian (1984) and documented in a proposed catabolic pathway as shown in Fig 1.1. (Ri-He *et al.*, 2008). The initial reaction in the bacterial oxidation

of naphthalene involves the formation of dihydrodiol intermediates. Bacteria oxidised naphthalene to D-*trans*-1, 2-dihydroxy-1, 2-dihydronaphthalene (Gibson and Subramanian, 1984). Bacteria utilise a dioxygenase reaction to initiate the degradation of naphthalene, a reaction which is further catalysed by dehydrogenase to give 1, 2-dihydroxynaphthalene (Gibson and Subramanian, 1984; Sutherland *et al.,* 1995). The bacterial naphthalene dioxygenase system is particularly useful for oxidising bi- and tri- cyclic PAH substrates, such as naphthalene, phenanthrene and anthracene. The naphthalene dioxygenase system is a multicomponent enzyme. Generally, it includes nicotinamide-adenine-dinucleotide hydride (NADH) oxidoreductase, ferredoxin and oxygenase component that contains the active site. The naphthalene catabolic gene (*nah*) of NAH7 is organised into two operons. The *nal* operon encoding the upper pathway enzymes involved in the conversion of naphthalene to salicylate. The *sal* operon encoding the lower pathway enzymes involved in the conversion of salicylate to pyruvate and acetyl Coenzyme A. The two operons are closely genetically linked to each other and to their common regulatory gene *nahR*. The enzymes involved in the conversion of naphthalene to salicylate can also degrade phenanthrene to naphthalene- 1, 2-diol (Lloyd-Jones *et al.,* 1999). Studies (e.g. Gibson and Subramanian, 1984; Sutherland et al., 1995) have shown that naphthalene can be degraded by fungi. Fungi can oxidise naphthalene to trans-2-dihydroxy-1,2-dihydronaphthalene, 1-naphthol and 2-naphthol (Gibson and Subramanian,1984).

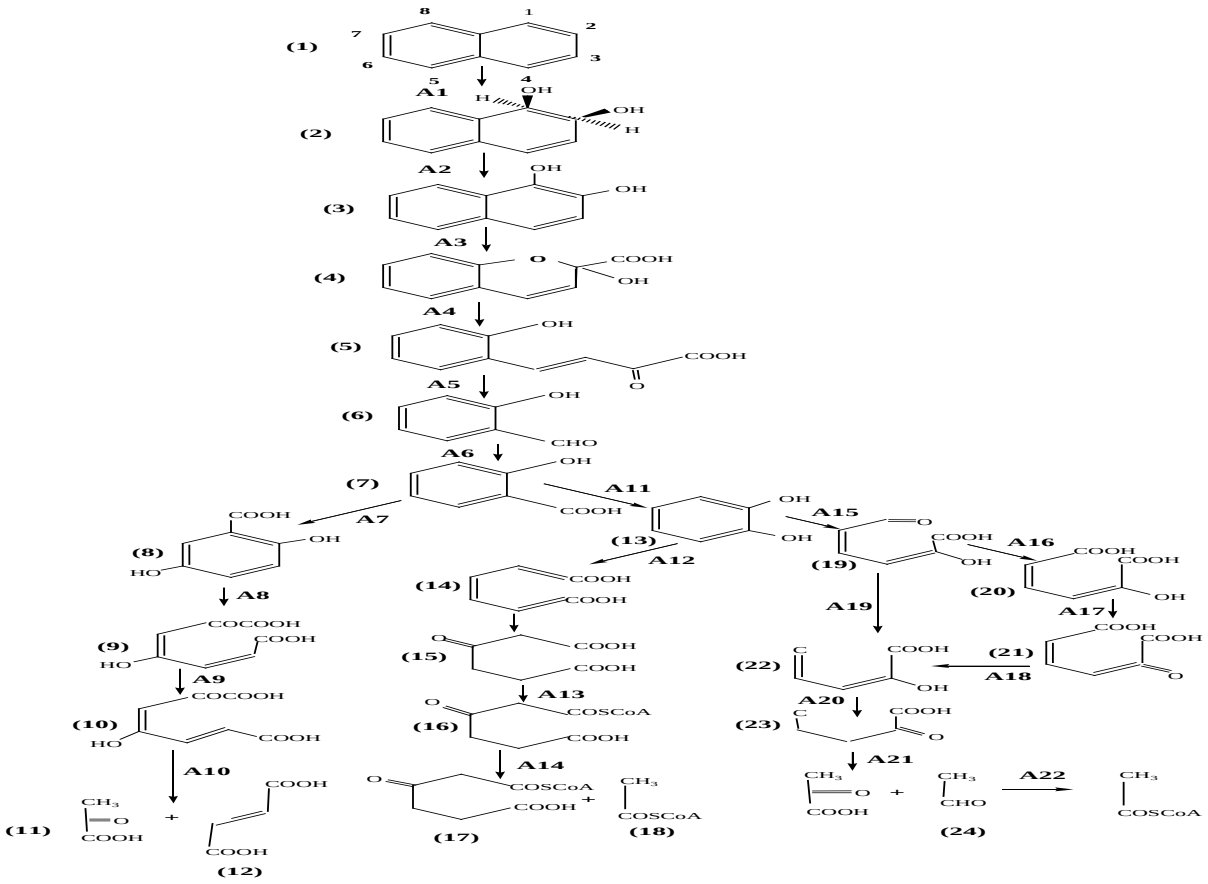


Fig. 1.1. Proposed catabolic pathways of naphthalene by aerobic bacteria. *The compounds are naphthalene (1), cis-1,2- dihydroxy-1,2-dihydronaphthalene (cis-naphthalene dihydrodiol) (2), 1,2-dihydroxynaphthalene (3), 2-hydroxy-2H-chromene-2-carboxylic acid (4), trans-o-hydroxybenzylidenepyruvic acid (5), salicylaldehyde (6), salicylic acid (7), gentisic acid (8), maleylpyruvic acid (9), fumarylpyruvic acid (10), pyruvic acid (11), fumaric acid (12), catechol (13), cis,cis-muconic acid (14), β-ketoadipic acid (15), β-ketoadipyl-CoA (16), succiny-CoA (17), acetyl-CoA (18), 2-hydroxymuconic-semialdehyde (19), 2- hydroxymuconic acid (20),4-oxalocrotonic acid (21), 2-oxo-4-pentenoic acid (22),4-hydroxy-2-oxovaleric acid (23) and acetaldehyde (24). The enzymes involved in each reaction step are naphthalene dioxygenase (NahAaAbAcAd) (step A1), cis-naphthalene dihydrodiol dehydrogenase (NahB) (A2), 1,2-dihydronaphthalene dioxygenase (NahC) (A3), 2-hydroxy-2H-chromene-2-carboxylate isomerise (NahD) (A4), trans-o-hydroxybenzylidenepyruvic hydratase-aldolase (NahE) (A5), salicylaldehyde dehydrogenase (NahF) (A6), salicylate 5-hydroxylase (NagGHAaAb) (A7), gentisate 1,2-dioxygenase (Nahl) (A8), maleylpyruvate isomerise (NagL) (A9), fumarylpyruvate*

hydrolase (NagK) (A10), salicylylate hydroxylase (NahG) (A11), catechol1,2-dioxygenase (A12), β-ketoadipate:succinyl-CoA transferase (A13), β-ketoadipyl-CoA thiolase (A14), catechol 2,3-dioxygenase (NahH) (A15), hydroxymuconic-semialdehyde dehydrogenase (Nahl) (A16), 4-oxalocrotonate isermerase (NahJ) (A17), 4-oxalocrotonate decarboxylase (NahK) (A18), hydroxymuconic-semialdehyde hydrolase (NahN) (A19), 2-oxo-4-pentenoate hydratase (NahL) (A20), 2-oxo-4-hydroxypentennoate aldolase (NahM) (A21) and acetaldehyde hydrogenase (NahO) (22) (Ri-He *et al.,* 2008).

1.1.2 Phenanthrene

Phenanthrene is a polycyclic aromatic hydrocarbon composed of three fused benzene rings. Many species of bacteria found in soil are capable of utilising phenanthrene as a growth substrate. The degradation of this compound by bacteria follows an oxidative pathway (Gibson and Subramanian, 1984; Sutherland *et al.,* 1995, Zhao *et al.,* 2009). Bacteria can oxidise phenanthrene to cis-1, 2-dihydroxy-1,2-dihydrophenanthrene, which forms 1,2-dihydrophenanthrene when it undergoes enzymatic dehydrogenation. The compounds can be oxidised further to 1-hydroxy-2-naphthoic acid, 2-carboxybenzaldehyde, o-phthalic acid, protocatechuic acid as shown in Fig 1.2. (Ri-He *et al.,* 2008).

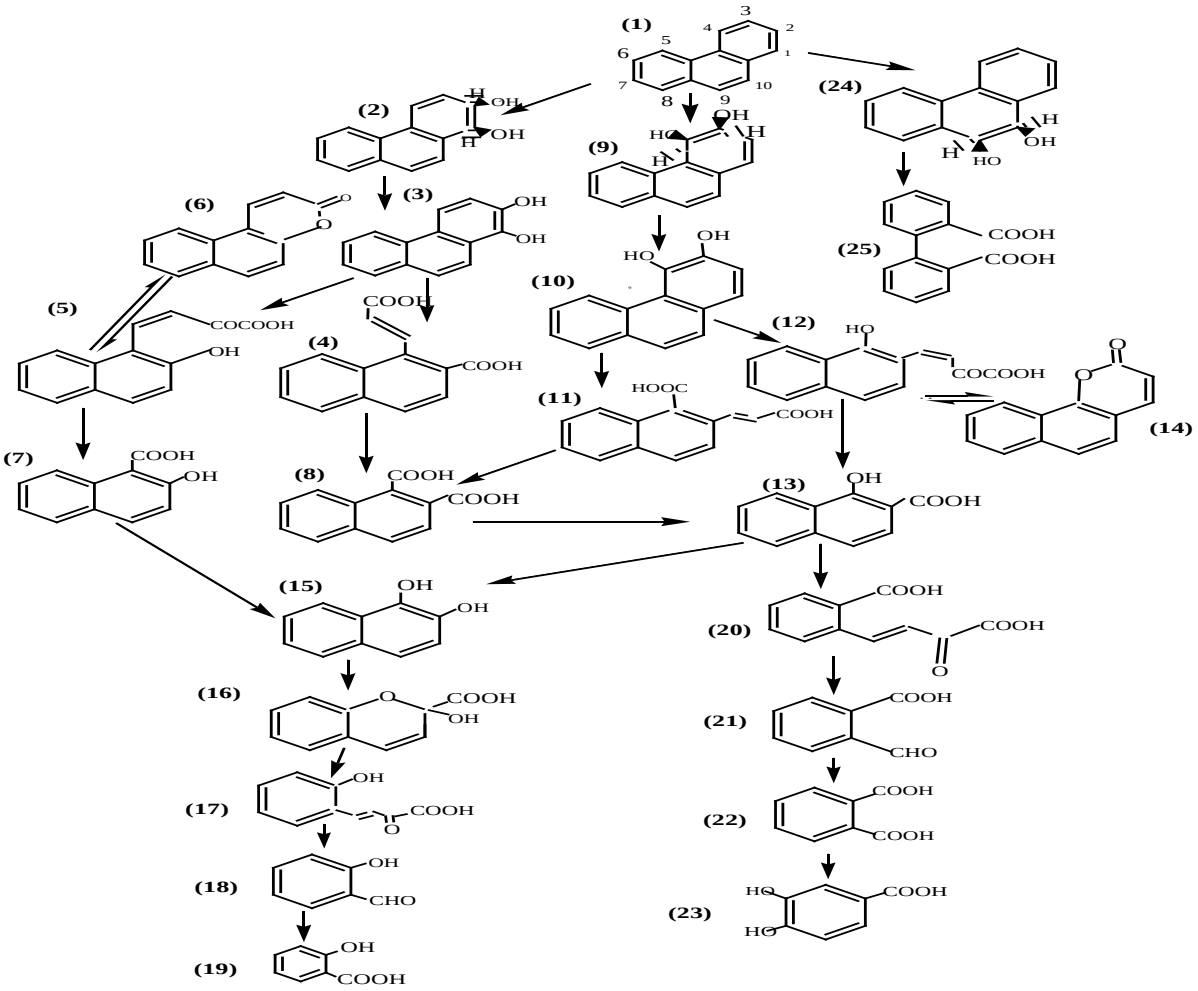


Fig.1.2. Proposed catabolic pathways of phenanthrene by aerobic bacteria. *The compounds are phenanthrene (1), cis -1,2-dihydroxy-1,2-dihydrophenanthrene (2), 1,2-dihydroxyphenanthrene (3), 2-[(E)-2-carboxyvinyl]-1-naphthoic acid (4), trans-4-(2-hydroxynaph-1-yl)-2-oxobut-3-enoic acid (5), 5,6-benzocoumarin (6), 2-hydroxy-1-naphthoic acid (7), naphthalene-1,2-dicarboxylic acid (8), cis-3,4-dihydroxy-3,4-dihydrophenanthrene (9), 3,4-dihydroxyphenanthrene (10), 1-[(E)-2-carboxyvinyl]-2-naphthoic acid (11), trans-4-(1-hydroxynaph-2-yl)-2-oxobut-3-enoic acid (12), 1-hydroxy-2-naphthoic acid (13), 7,8-benzocoumarin (14), 1,2-dihydroxynaphthalene (15), 2-hydroxy-2H-chromene-2-carboxylic acid (16), trans-o-hydroxybenzalpyruvic acid (17), salicylaldehyde (18), salyclic acid (19), trans-2-carboxybenzalpyruvic acid (20), 2-carboxybenzaldehyde (21), o-phthalic acid (22), protocatechuic acid (23), cis-9,10-dihydroxy-1,2-dihydrophenanthrene (24) and 2,2′-diphenic acid (25)* (Ri-He *et al.*, 2008).

1.1.3 Pyrene

Pyrene is a polycyclic aromatic hydrocarbon (PAH) consisting of four fused benzene rings. It is the smallest peri-fused PAH (the rings are fused through more than one face). Many microorganisms have shown the capability of utilising four ringed aromatic hydrocarbons such as pyrene (Heitkamp *et al.,* 1988; Meyer and Steinhart, 2001). Bacteria such as *Rhodococcus* sp. strain UW1 are capable of growing on pyrene as sole carbon source (Walter *et al.*, 1991). This organism was found to mineralise up to 72% of pyrene to CO_2 within two weeks (Walter *et al.,* 1991). Three percent of the labelled carbon was found in the organic phase and 25% was present as water-soluble metabolites in the aqueous phase. Pyrene-4, 5-dihydrodiol was identified as the initial ring oxidation product and 4-phenanthroic acid as the major metabolite of the degradation of pyrene by a *Mycobacterium sp* (Heitkamp *et al.* 1988). Also, a proposed catabolic pathway of pyrene by aerobic bacteria has been suggested as shown in Fig 1.3 (Vila *et al.*, 2001; Liang *et al.*, 2006, Ri-He *et al.,* 2008).

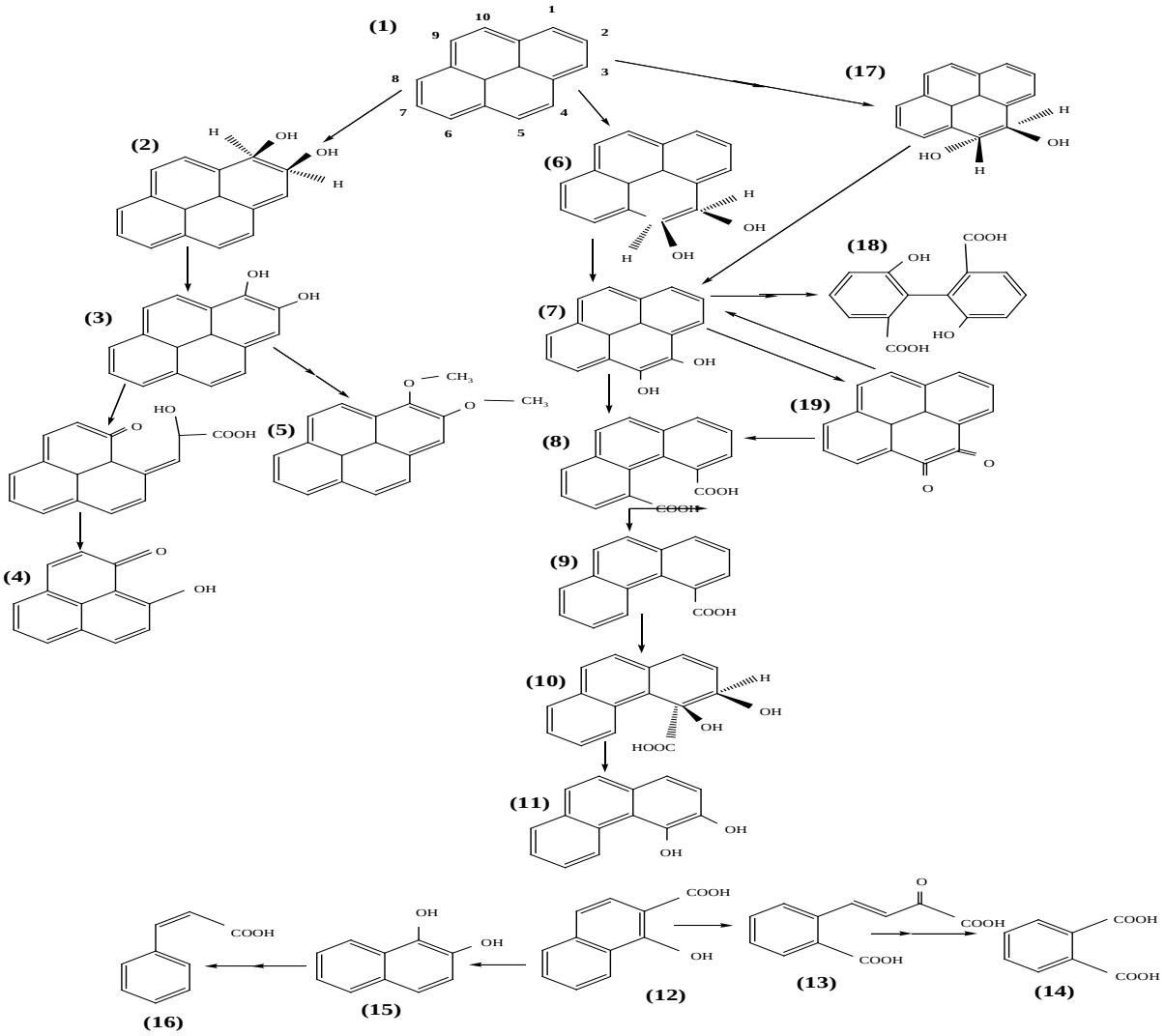


Fig.1.3. Proposed catabolic pathways of pyrene by aerobic bacteria. *The compounds are pyrene (1), cis-1,2-dihydroxy-1,2-dihydropyrene (2), 1,2- dihydroxypyrene (3), 4-hydroxyperinaphthenone (4), 1,2-dimethoxypyrene (5), cis-4,5-dihydroxy-4,5-dihydropyrene (6), P2,4,5-dihydroxypyrene (7), phenanthrene-4,5-dicarboxylate (8), phenanthrene-4-dicarboxylate (9), cis-3,4-dihydroxyphenanthrene-4-carboxylate (10), 3,4-dihydroxyphenanthrene (11), 1-hydroxy-2-naphthoate (12), trans-2′-carboxybenzalpyruvate (13), phthalate (14), 1,2-dihydroxynaphtharene (15), cinnamic acid (16) trans-4,5-dihydroxy-4,5-dihydropyrene (17), 6,6′-dihydroxy-2,2′-biphenyl dicarboxylic acid (18) and pyrene-4,5-dione (19)* (Ri-He *et al.*, 2008).

1.1.4 Fluorene

Fluorene is a polycyclic aromatic hydrocarbon. Fluorene has been found to be susceptible to microbial degradation to varying extents. (Gibson and Subramanian, 1984; Mueller *et al.*, 1991; Field *et al.*, 1992; Sutherland *et al.*, 1995). The initial attack on fluorene is catalysed by dioxygenase to yield 9-fluorenol and 1, 1a-dihydroxy-1-hydro-9-fluorenone. The catabolic pathway for fluorene degradation has been proposed as shown in Fig 1.4 (Kasuga *et al*., 2001; Wattiau *et al*., 2001; Habe *et al.*, 2004, Ri-He *et al.*, 2008).

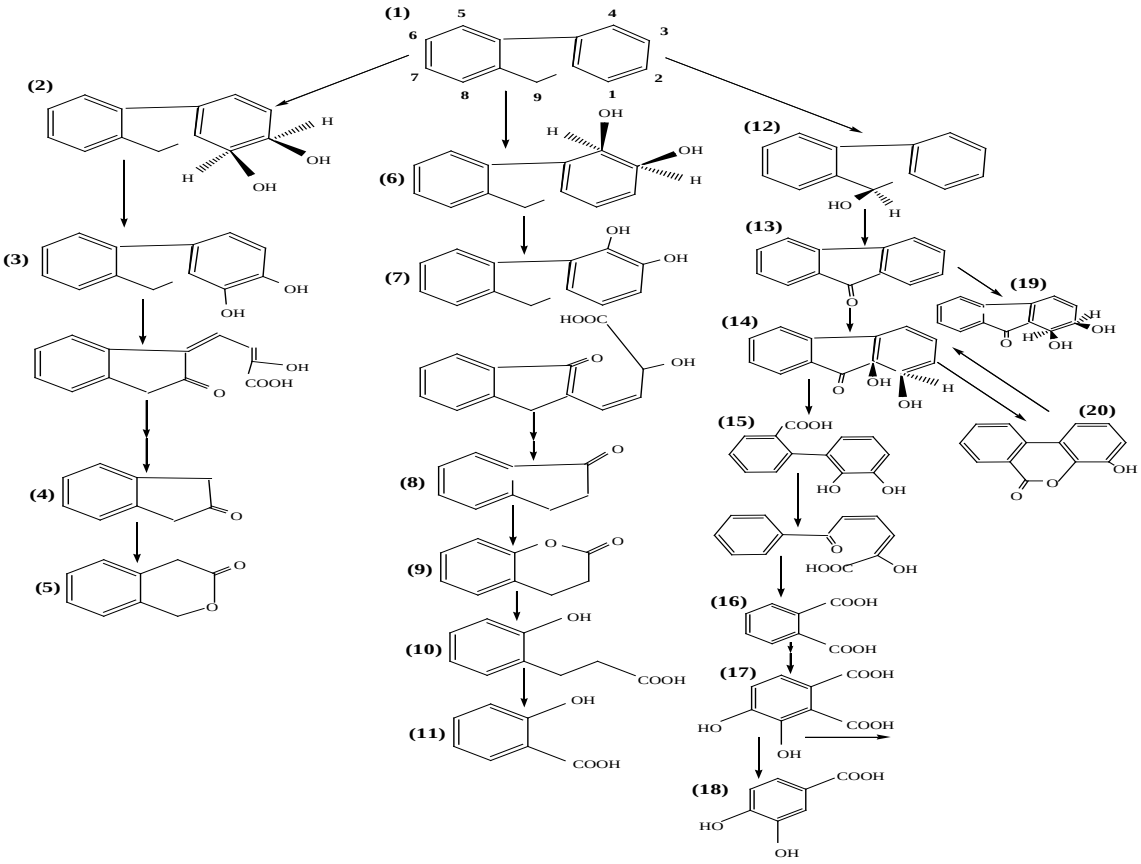


Fig.1.4. Proposed catabolic pathways of fluorene by aerobic bacteria. *The compounds are fluorine (1), cis-1,2-dihydroxy-1,2-dihydrofluorene (2), 1,2-dihydroxy fluorine (3), 2-indanone (4), 3-isochromanone (5), cis-3,4-dihydroxy-3,4-dihydrofluorene (6), 3,4-dihydroxyfluorene (7),*

1-indanone (8), 3,4dihydrocoumarin (9), 3-(2-hydroxyphenyl) propionic acid (10), salicylic acid (11), 9-fluorenol (12), 9-fluorenone (13), 1,1a-dihydroxy-1-hydro-9-fluorenone (14), 2/-carboxy-2,3-dihydroxybiphenyl (15), phthalic acid (16), 4,5-dihydroxyphthalate (17), protocatechuic acid (18), 1,2-dihydro-1,2dihydroxy-9-fluorenone (19) and 8-hydrixy-3,4-benzocoumarin (20) (Ri-He *et al.*, 2008).

1.1.5 Fluoranthene

This is a four fused benzene ring polycyclic aromatic hydrocarbon consisting of naphthalene. Many microorganisms showed the capability of utilising fluoranthene (Heitkamp *et al.,* 1988; Meyer and Steinhart, 2001). The catabolic pathway describing the biodegradation of fluoranthene by *M. Vanbaalenii* PYR-1 (Fig. 1.5), initiated by mono-and deoxygenated reactions was discovered recently (Ri-He *et al.*, 2008).

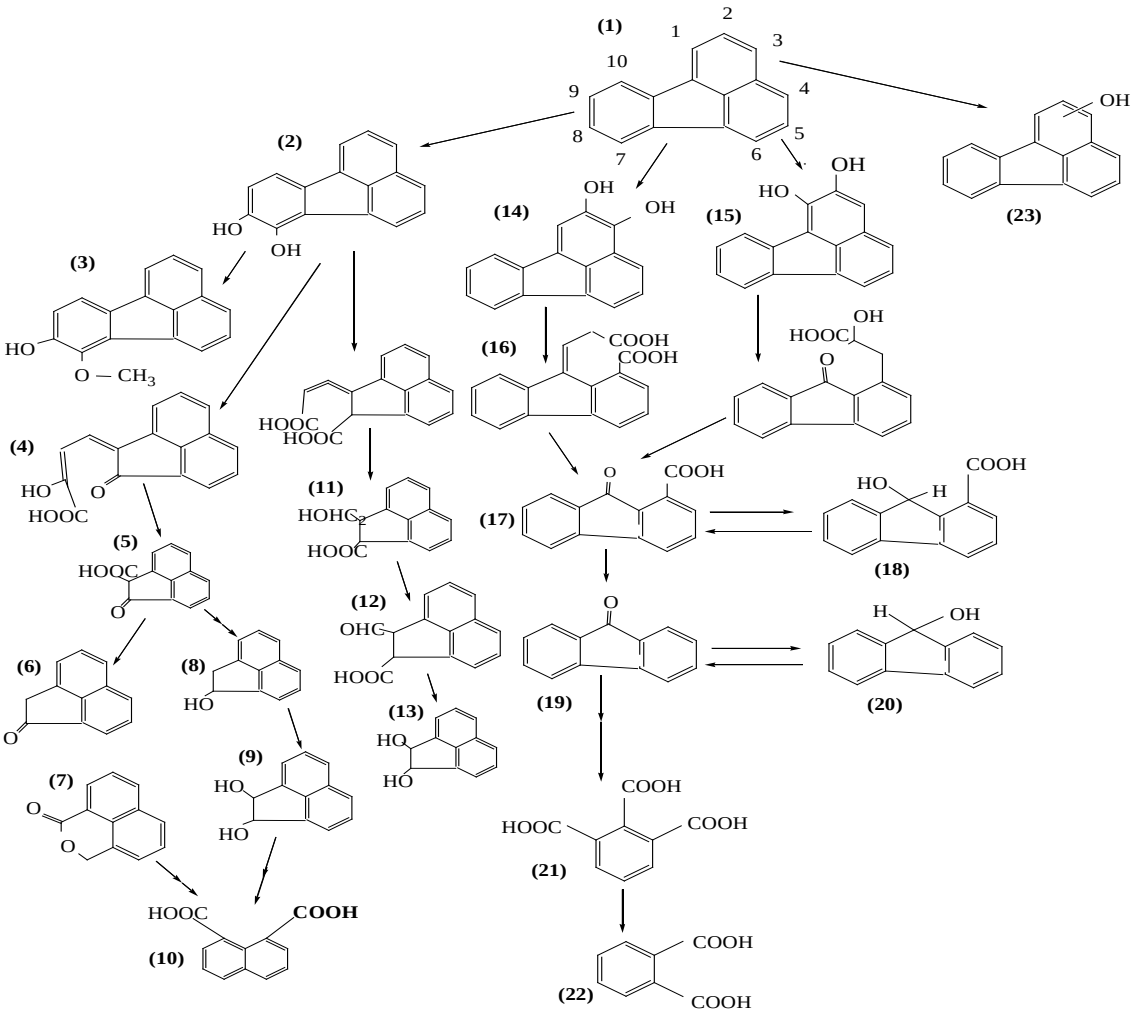


Fig.1.5. Proposed catabolic pathways of fluoranthene by aerobic bacteria. *The compounds are fluoranthene (1), 7,8-dihydroxy fluoranthene (2), 7-methoxy-8-hydroxy-fluoranthene (3), (2Z,4Z)-2-hydroxy-4-(2-oxoacenaphthylen-1(2H)-ylidene) but-2-enoic acid (4), 1-acenaphthenone-2-carboxylic acid (5), acenaphthylene-1(2H)- one (6), 1H,3H-benzo[de] isochromen-1-one (7), acenaphthylen-1-ol (8), acenaphthylen-1,2-diol (9), naphthalene-1,8-dicarboxylic acid (10), 2-(hydroxymethy)-acenaphthylene-1-carboxylic acid (11), 2-formylacenaphthylene-1-carboxylic acid (12), 1,2-dihydroacenaphthylene-1,2-diol (13), 2,3-dihydroxy fluoranthene (14), 1,2-dihydroxy fluoranthene (15), (9E)-9-(carboxymethylene)-9H-fluorene-1-carboxylic acid (16), 9-fluorenone-1-carboxylic acid (17), 9-hydroxy-9H-fluorene-1-carboxylic acid (18), 9-fluorenone (19), 9-hydroxyfluorene (20), 1,2,3-benzene-tricarboxylic acid (21), phthalic acid (23) and monohydroxyfluoranthene (22)* (Ri-He *et al.*, 2008).

1.1.6 Benzo[a]pyrene

This is a five ring polycyclic aromatic hydrocarbon ($C_{20}H_{12}$) whose metabolites are mutagenic and highly carcinogenic (Le Marchand *et al.,* 2002). Benzo[a]pyrene can be oxidised to various metabolites by different microorganisms, which include; *trans*-7, 8-dihydroxy-7, 8-dihydrobenzo[a]pyrene, 3-hydroxybenzo[a]pyrene and 9-hydroxybenzo[a]pyrene, *trans*-9,10-dihydroxy-9,10-dihydrobenzo[a]pyrene, benzo[a]pyrene-1,6-quinone and benzo[a]pyrene-3,6-quinone as shown in Fig 1.6 (Gibson and Subramanian, 1984; Cerniglia *et al.*, 1992, Ri-He *et al.*, 2008).

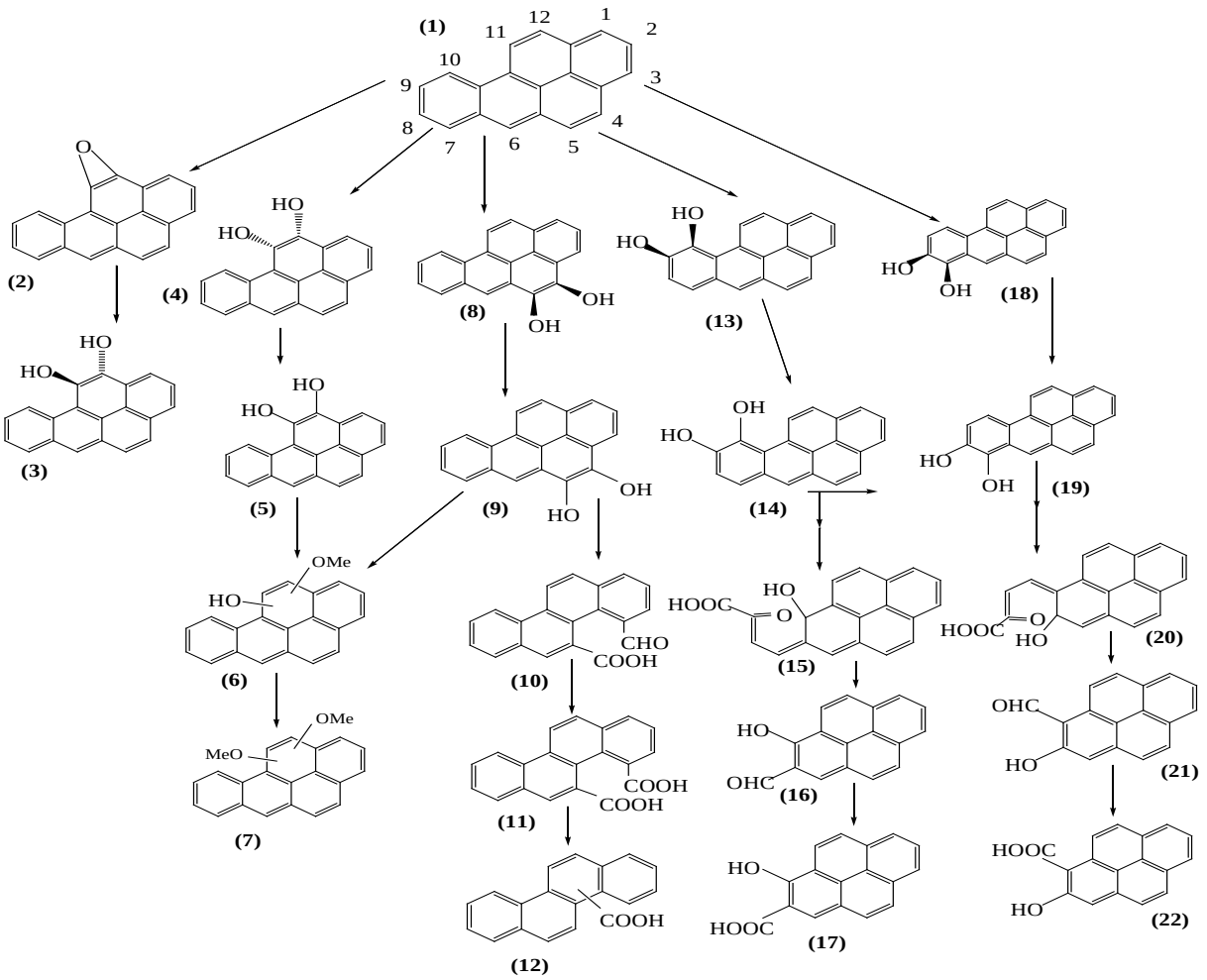


Fig.1.6. Proposed catabolic pathway of benzo[a]pyrene by aerobic bacteria**.** *The compounds are benzo[a]pyrene (1), benzo[a]pyrene-11,12-epoxide (2), trans-benzo[a]pyrene-11,12-dihydrodiol (3), cis-benzo[a]pyrene-11,12-dihydrodiol (4), 11,12-dihydroxy-benzo[a]pyrene (5), hydroxymethoxybenzo[a]pyrene (6), dimethoxybenzo[a]pyrene, (7), cis-benzo[a]pyrene-4,5-dihydrodiol (8), 4,.5-dihydroxy-benzo[a]pyrene (9), 4-formylchrysene-5-carboxylic acid (10), 4,5-chrysene-dicarboxylic acid (11), chrysene-4(5)-carboxylic acid (12), cis-benzo[a]pyrene-9,10-dihydrodiol (13), 9,10-dihydroxy-benzo[a]pyrene (14), cis-4-(8-hydroxypyrene-7-yl)-2oxobut-3-enoic acid (15), pyrene-8-hydroxy-7-aldehyde (16), pyrene-8-hydroxy-7-carboxylic acid (17), cis-benzo[a]pyrene-7,8-dihydrodiol (18), 7,8-dihydroxy-benzo[a]pyrene (19), cis-4-(7-hydroxypyrene-8-yl)-2-oxobut-3-enoic acid (20), pyrene-7-hydroxy-8-aldehyde (21) and pyrene-7-hydroxy-8-carboxylic acid (22)* (Ri-He *et al.*, 2008).

1.1.7 Anthracene

This is a solid polycyclic aromatic hydrocarbon consisting of three fused benzene rings. It is also component of coal tar (Iglesias *et al.*, 2010). The initial reactions in the bacterial degradation of anthracene involve the formation of *trans*-1, 2-dihydroxyanthracene prior to ring fission (Gibson and Subramanian, 1984). Other studies showed that *Pseudomonas putida* strain 199 and *Beijerinckia* sp. strain B-836 oxidised anthracene to (+)-*cis*-1, 2-dihydroxy-1, 2-dihydroxyanthracene. Bacteria grown in a medium of naphthalene are shown to oxidise anthracene, 1, 2-dihydroxyanthracene to 2-hydroxy-3-naphthaldehyde (Gibson and Subramanian, 1984; Sutherland *et al.*, 1995). Also, the reactions in the degradation of anthracene are catalyzed by multicomponent dioxygenases to produce cis-1, 2-dihydrodiols. The proposed catabolic pathway involves the ortho-cleavage of 1, 2-dihydroxyanthracene into 3-(2-carboxyvinyl) naphthalene-2-carboxylic acid for *Mycobacterium* sp. PYR-1 and *Rhodococcus sp.* Fig 1.7 (Dean-Ross *et al.*, 2001; Moody *et al.*, 2001).

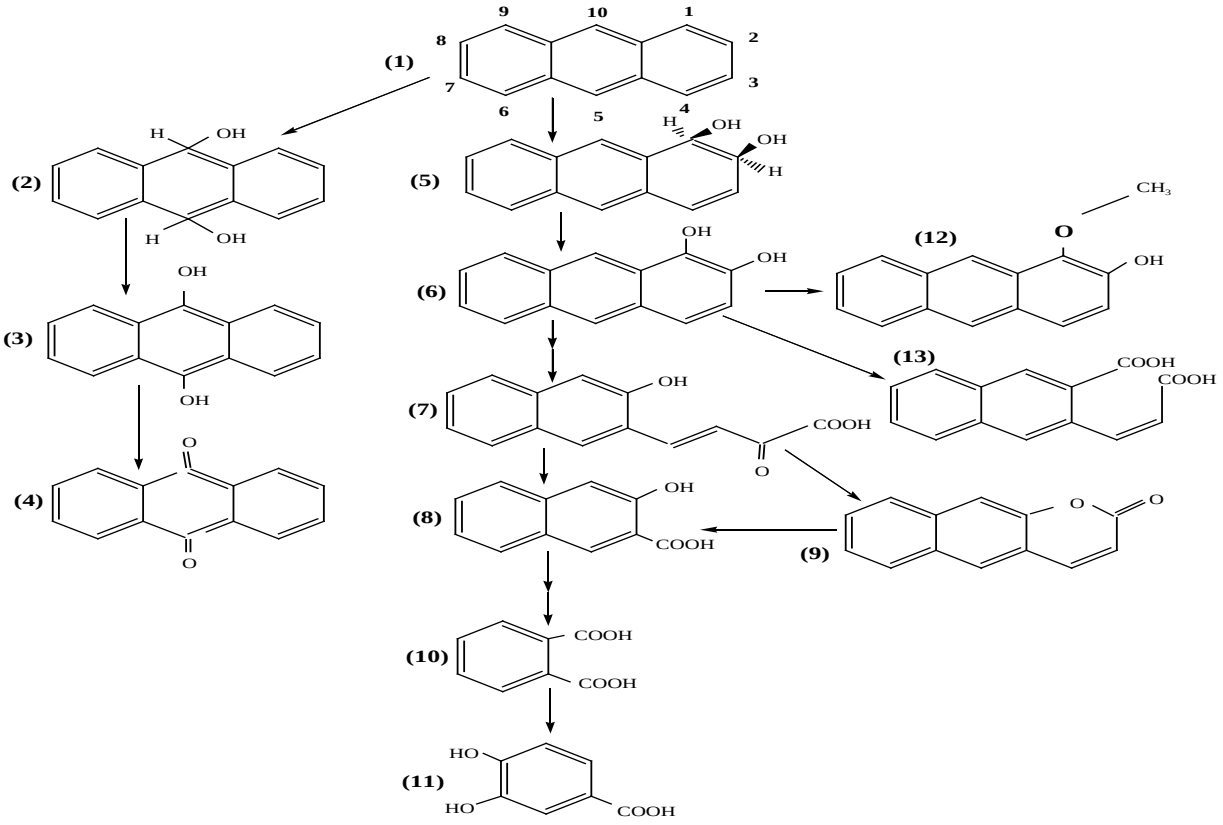


Fig.1.7. Proposed catabolic pathways of anthracene by aerobic bacteria. *the compounds are anthracene (1), anthracene-9,10-dihydrodiol (2), 9,10-dihydroxyanthracene (3), 9,10-anthraquinone (4), cis-1,2-dihydroxy-1,2-dihydroanthracene (5), 1,2-dihydroxyanthracene (6), cis-4-(2-hydroxynaphth-3-yl)-2-oxobut-3-enoic acid (7), 2-hydroxy-3-naphthoic acid (8), 6,7-benzocoumarin (9), o-phthalic acid (10), protocatechuic acid (11), 1-methoxy-2-hydroxyanthracene (12) and 3-(-2carboxyvinyl)-napthalene-2-carboxylic acid (13)* (Ri-He *et al.*, 2008).

1.1.8 Other PAHs

The other polycyclic aromatic hydrocarbons (PAHs) are classified as acute toxic hazardous organic compounds. They include compounds such as benzo[a]anthracene, benzo[k]fluoranthene with five fused benzene rings and indenol (1, 2, 3-cd) pyrene with six fused benzene rings. These 16 PAHs are present in substantial quantities in oil sludge and can also be susceptible to microbial degradation (Gibson and Subramanian, 1984; Mueller *et al.*, 1991; Field *et a*l., 1992; Sutherland *et al.*, 1995).

1.2 Oil Sludge in the Environment

It is generally recognised that land as a component of the environment deserves the same attention and protection as water and air (Okieimen and Okieimen, 2005). This recognition has perhaps risen because of increased incidents of land pollution, the scarcity of land, awareness and concern about long-term effects of land pollution on terrestrial and aquatic ecosystems. The adverse effects of oil sludge on soil ecology and fertility have been pivotal in the development of efficient technologies for the degradation of these contaminants in the environment (Okieimen and Okieimen, 2005). As oil sludge is dumped into the environment, lighter compounds volatilize and heavier ones remain. Most oil sludge components have high affinity for soil material and particulate matter. Overtime, they accumulate to the extent that they become difficult to eliminate because they reside in fine pores. Then, they become protected from attack by biota in the soil; hence they are not bio-available. Their fate and behaviour are controlled by factors such as soil type and their physico-chemical properties (Reid *et al.*, 2000). Such properties include their (concentration, structures of the components and their solubility), environmental conditions (temperatures, pH, moisture content and wind), and the available microorganisms (physiology and genetics). Their solubility is the key factor of their fate in the

environment. The solubility of oil sludge components is different, some are infinitely soluble polar compounds, and others are of low solubility (PAHs) (Mahmoud, 2004). As the time of contact between oil sludge constituents and the environments increase, there is a decrease in chemical and biological availability, a process termed “ageing”. This has attracted considerable attention in recent years.

Oil sludge can be biodegraded by microorganisms such as bacteria and fungi. A large number of bacteria species have the ability to degrade majority of natural hydrocarbon components from oil sludge especially low-molecular-weight contaminants (Ward *et al.*, 2003). Microbial biodegradation is an effective and inexpensive approach to the degradation of petroleum hydrocarbons from oil sludge. This is possible as long as a large population of degrading microorganisms is present and the conditions encouraged the microbial growth and activities (Philips *et al.*, 2000).

1.3 Microbial Biodegradation of Oil Sludge

Oil sludge indicates degradable and biodegradable properties in the environments (soil, water and sediments) (Leahy and Colwell, 1990; Research triangle institute, 1999; Laskova *et al.*, 2007, Paulauskiene *et al.*, 2009). To successfully exploit the microbial degradation of oil sludge, it is imperative to understand and master the mechanism needed in order to manipulate the microbial activities. For oil sludge containing large quantities of hydrocarbons, microorganisms must be able to use hydrocarbons as substrates (Tabuchi *et al.*, 1998). They must be able to synthesise enzymes that catalyse the reaction in which these contaminants are degraded to simpler, lower molecular chains and less toxic compounds (CO_2 and H_2O), through obtaining the nutrients and energy necessary for their survival in the process (Johnson and Scow, 1999). The initial step in this mechanism is the catabolism of oil sludge by bacteria and fungi. It involves the

oxidation of the substrate by oxygenases, in which molecular oxygen is required. Aerobic conditions are necessary for this route of microbial oxidation of hydrocarbons to take place (Marin Millan, 2004). Microbial bioremediation of oil waste sludge is very much dependent on the factors which include: characteristics of the oil sludge, choice of microbial consortium and factors affecting the biodegradation mechanisms (temperature, pH and moisture). However, the characteristics and fate of oil sludge depends on its molecular size and topology (Kanaly and Harayama, 2000). For low molecular weight petroleum hydrocarbons (4-ring or less), removal through evaporation is the first line of elimination. As the molecular sizes increases, biodegradation rates become slower. Oil sludge, albeit very slow, is susceptible to degradation by naturally occurring microflora, but this process reduces nutrient and oxygen level in soil which in turn impedes other environmental processes. In order to enhance the biodegradation processes and making it economically realistic and rapid, it is necessary that the bioavailability of petroleum hydrocarbons in soil be increased. This may be by biostimulation which is simply the addition of nutrients to stimulate the degradative capabilities of the indigenous microorganisms present (Piskonen and Itävaara, 2004). Many microbial strains are capable of degrading only specific components of oil sludge. However, oil sludge is a complex mixture of different petroleum hydrocarbon (Mac Naughton *et al.,* 1999). Single bacterial species has only limited capacities to degrade all the fractions of hydrocarbons presents (Loser *et al.,* 1998). Hence, a mixture of other bacterial species that can degrade a broad range of the hydrocarbon constituents of the oil sludge should be employed. Also, steps must be taken to ensure that the original indigenous bacterial communities be part of the regiment. Mishra *et al.,* (2001), suggested that indigenous microorganisms isolated from a contaminated site will assist in

overcoming this problem, as the microorganisms can degrade the components and have a higher tolerance to toxicity that may wipe off other introduced species.

1.4 Factors affecting the Biodegradation Mechanisms

There are many factors (physical, chemical and biological) that will ultimately determine the effectiveness of strategies of choice for microbial bioremediation of oil sludge (van Hamme *et al.,* 2003). These include: biosurfactants, effect of pH, nutrients, salinity, oxygen, temperature, enzymatic activities, pressure and water activities/ moisture contents.

1.4.1 Biosurfactants

Biosurfactants are important agents that enhance the effective uptake of oil sludge constituents by bacteria and fungi (Leahy and Colwell 1990; Cort and Bielefeldt, 2000; Shiohara *et al* 2001). Bacteria have been reported to be involved in the formations of emulsion in the presence of biosurfactants (Calvo *et al.*, 2004; Bayoumi, 2009; Liu *et al.,* 2011; Plaza *et al.,* 2011). In addition, the production of biosurfactant may be supplemented with additives and bulking agents, to enhance overall hydrocarbon catabolism (Ward *et al,* 2003). Bulking agents such as compost will enhance metabolism of organic contaminants because they provide extra nutrients, additional carbon source and assist in retaining moisture contents of the pile (Namkoog *et al*., 2002). Commercial chemical surfactants may be used to boost microbial degradation and desorption of oil sludge constituents (Sim and Ward, 1997). Different types of surfactants would have different effects during biodegradation processes (Cort and Bielefeldt, 2000; Shiohara *et al.,* 2001). Surfactants may inhibit the microbial degradation of oil sludge, depending on the concentration of the surfactants applied in the process (Cort and Bielefeldt, 2000). Surfactants

may enhance bioremediation processes depending on the faster biodegradation rate of the target compounds (Diehl and Borazjani, 1998; Shiohara *et al.,* 2001).

1.4.2 Effect of pH

The most important oil sludge degrading heterotrophic bacteria and fungi perform at their best when pH is neutral. However, fungi are known to be tolerant of acidic conditions (Al-Daher *et al.,* 1998). At pH 7 to 7.8, the mineralisation of oil sludge is also improved, thereby enhancing the overall biodegradation process (van Hamme *et al.,* 2003). The metabolic pathways for degradation differ in both fungi and bacteria (Cerniglia *et al*., 1979). According to report by Cerniglia *et al*. (1979), fungal decomposition of oil sludge components may produce mutagenic intermediates. In such instance, liming may be used to increase the pH from acidic to alkaline state so that bacterial growth may be favoured than fungi growth.

1.4.3 Nutrients

The growth of heterotrophic bacteria and fungi depends on a number of nutrient elements, an electron acceptor and organic compound that serves as the source of carbon and energy (Adriano *et al*., 1999; Boettcher *et al*., 2001). For aerobic microorganisms, the electron acceptor is oxygen. Some microorganisms can utilize some inorganic compounds such as nitrates, sulphates, carbon dioxide, ferric iron and some organic compounds, as electron acceptors for electrons released by the oxidation of the substrate carbon source. Some bacteria and fungi also require low concentrations of some amino acids, vitamins or other organic molecules as growth factors. The absence of any of these essential elements from the environment may prevent growth and metabolism of microorganisms (Atagana, 2003). Microorganisms that degrade oil sludge are dependent on fixed forms of nitrogen (NH_3, NO_3^-, NO_2^- and organic nitrogen) to meet their

nitrogen requirements. These forms of nitrogen are frequently limiting for microbial populations in soil, ground water and surface water (Atlas 1991). Microbial synthesis of adenosine triphosphate (ATP), nucleic acids and cell membranes require phosphorus. For nitrogen to be available to soil microorganisms it must be, in most cases, present in inorganic form such as ammonium or nitrate (Swindell *et al.,* 1988). Most microorganisms can utilize ammonia or nitrogen gas (Tate, 1995) while phosphorus is available in the form of orthophosphate (Alexander, 1999). According to report by van Hamme *et al.,* (2003), nitrogen and phosphorus contents have great effects on microbial degradation of oil sludge.

1.4.4 Salinity

Studies have shown that there are generally positive correlations between salinity and rates of mineralization of oil sludge components (PAHs) such as phenanthrene and naphthalene (Leahy and Colwell, 1990). However, it is noted that hypersalinity will result in the decrease in microbial metabolic rates.

1.4.5 Oxygen

Aerobic biodegradation is the most effective pathway for bioremediation. This means that, the presence and concentration of oxygen is the rate-limiting parameter in the biodegradation and catabolism of cyclic and aromatic hydrocarbons by bacteria and fungi (van Hamme *et al.*, 2003). Oil sludge components breakdown may possibly involve the utilisation of oxygenase, in which molecular oxygen is required. Great efficiency of natural microbial hydrocarbon degradation occurs mostly when oxygen is available (Ward *et al.,* 2003). Although anaerobic degradation of PAHs by microorganisms has been shown to occur, the rates are somewhat negligible and

limited to halogenated aromatics compounds such as the halobenzoates, chlorophenols and alkyl-substituted aromatic (Sulfita *et al.,* 1982; Boyd and Shelton, 1984; Angelidaki *et al.,* 2000).

1.4.6 Temperature

Temperature is another important variable that has effect on oil sludge biodegradation. Microorganisms can grow at temperatures below 0^{o}C to above 100^{o}C with good water supply (Atlas and Barther, 1987). Bacteria can tolerate a wide range of temperatures. Optimum temperature dictates the rate of oil sludge metabolism by microorganisms and also the pattern of the microbial community. Temperature has direct effect on the physical nature and chemical composition of oil sludge components (Atlas, 1981). An increase in temperature is proportional to the solubility of contaminants and induces higher metabolic activity of the compost (Gibb *et al.,* 2001). When temperatures are low, oil sludge tend to be more viscous and their water solubility is greatly reduced (Leahy and Colwell, 1990). Low temperature also affects microbial growth, propagation and subsequently results in decrease in the rate of degradation (Gibb *et al.,* 2001). Low temperature also results in a decrease in enzymatic activities. The optimum temperature is typically in the range of 30^{o}C to 40^{o}C. At temperatures above this range, enzymatic activities are inhibited as proteins denature at higher temperature (Leahy and Colwell, 1990).

1.4.7 Water activities/ Moisture contents

According to Vinas *et al.,* (2005), the rates at which oil sludge components are degraded are also determined by moisture level. The reason is that water is needed for microbial growth and enzymatic/biochemical activities (Leahy and Colwell, 1990). Elemental uptake by microorganisms is by absorption and transportation of solubilised molecules across the cell

membrane. The availability of target molecules to the microorganisms depends on the amount of water present in the treatment matrix. Optimal water content for aerobic bioremediation treatment matrix is usually between 10% and 20% by mass (Hinchee and Arthur, 1991). If the soil water holding capacity ranges from 30% to 90%, biodegradation rates are usually small because of water-logging (Hinchee and Arthur, 1991). However, water-logging may promote anoxic conditions, thereby reducing aerobic bioremediation efficiency.

1.5 Treatment Technologies and Disposal of Oil Sludge

Safe disposal and treatment of huge quantity of oil sludge generated during the processing of crude oil are some of the major challenges faced by oil refineries and petrochemical industries (Srinivasarao *et al.,* 2011). In recent years, most refineries treat oil sludge using conventional methods which includes; physical treatment (storage, combustion and incineration in a rotary kiln), chemical treatment (oxidative thermal treatment, treatment with fly-ash, lime stabilization, stabilization and solidification, pyrolysis treatment and solvent extraction) (Udotong *et al.,* 2011), and biological treatment (landfarming, landfill, bio-reactor treatment and composting) (Mahmoud, 2004, Srinivasarao *et al.,* 2011). Most of the physical and chemical methods require expensive equipments, machineries and high energy to treat oil sludge. Some of these methods convert (recycle) oil sludge into lighter products and reduce the quantity before disposal. Some of the methods may generate residual products that may need to be treated using other methods before disposal to a landfill, making them more expensive and partially effective. Examples of the conventional methods are discussed below;

1.5.1 Incineration

It is a technology used in large refineries. The most common are rotary kiln and fluidized bed incinerators. In rotary kiln incinerator, the combustion temperature is 980 – 1200^{o}C and the residence time is about 30 minutes. While in fluidized bed incinerators, the combustion temperature is 732 -760^{o}C, and the residence time may be in order of days for solids entrapped by the bed. The incineration process requires sophisticated equipments and experienced operation to achieve adequate combustion of oil sludge. Recent RCRA regulation requires that the destruction and removal efficiency of hazardous organics in oil sludge in an incineration facility should be greater or equal to 99.9%. The incineration of oil sludge was carried out using a fluidized bed technique. This process was successful and the products were ash scrubber sludge, and low content of heavy metals. These products were disposed of in a landfill (Liu *et al.,* 2010). Incineration is an expensive technique and oil sludge contains high concentration of hazardous compounds including those that are resistant to incineration such as ash. Incineration is not only expensive but generates toxic residues such as ash, scrubber water, scrubber sludges, sulphur dioxide, nitrogen monoxide, carbon monoxide, organic compounds etc (Srinivasarao *et al.,* 2011). Some of these residues such as ash containing metals need to be treated before being disposed of. The main parameters that should be controlled during the process are: waste feed rates, oxygen: air ratio, residence time, combustion temperature, and gas emission (Mahmoud, 2004).

1.5.2 Treatment with Fly-Ash

This is the treatments of oil sludge with aqueous slurry of fly-ash and a small amount of polymer. This process mixes light sludge in a small tank equipped with a mixer before thickening. Sludge with high oil and solids contents are de-watered in a centrifuge before being

treated with ash slurry in a screw mixer. The settled products from the thickener and mixer are transported in a closed truck containers directly to a landfill, which must be well drained to minimise leaching. During the dry season the deposit quickly become hard enough to be carried in trucks and it can be used for roadbeds. When disposed, after covering with a layer of soil the deposit area can be used to grow grass and trees (Atlas, 1984; Mahmoud, 2004). This method may be expensive, since equipments, energy and operating persons are needed for this process.

1.5.3 Lime Stabilization

Stabilization involves mixing a solid additive material to the oil sludge in order to produce a matrix within which the oil and metal are fixed and will not leach out. The use of lime for this purpose has being established in the literature, the addition of lime produces physical and chemical changes in the oil sludge which facilitates hydrocarbon adsorption and immobilisation of metals as insoluble salts (Wright and Noordhius, 1991; Mahmoud, 2004). The high pH provided by adding the lime is essential in this process, some additives can be added to produce hydrophobic matrix to prevent contaminants from becoming acidic due to rainfall percolation in the landfill (Mahmoud, 2004). This technique may also generate residual products that may need to be treated using other methods before disposal to a landfill which will make the techniques expensive.

1.5.4 Solvent Extraction

In this method the oil sludge is extracted with a solvent to remove oil and other organics, the solvent is recovered and recycled. Many refineries believe that recycling is the most desirable environmental option for handling oil sludge, due to the possibility of recovering valuable oil for reprocessing, reformulating and energy recovery (Bonnier *et al.,* 1980; Taiwo and Otolorin,

2009). Evaluation of the extent of sludge treatment before disposal can be done and can make significant impact on refinery and petrochemical industries. Condensed solvent and water are continuously separated in a trap. The condensed liquid containing water and hydrocarbon is transferred to a graduated cylinder. The extractable hydrocarbon oil contains about 73.24% of the sludge, and they are both volatile and non-volatile hydrocarbons. The solvent extraction technique has a tendency to greatly reduce sludge contaminants from 100% to 30 % water and solid wastes. The method may possibly reduce the pollution effects of oil sludge on the environment with the recovery of recyclable hydrocarbons. If the optimum conditions are carefully selected, solvent extraction approach can significantly mitigate the non-compliance to standard limit of industrial discharge into the environments and the permissible allowances for oil sludge. The advantage of solvent extraction techniques is that the recovery approach to oil sludge treatment explored can serve as a precursor to *in-situ* treatment and cleaning of oil storage facilities. It will also reduce economic losses and out of operation period, since there will be a reduction in time requirements for treatments, also the oil, water and mud can be effectively used and extraction solvents can be recycled. The limitation is the adaptation of selected solvent to the sludge treatment. Solvent extraction may not remove heavy metals such as arsenic, lead and selenium; these residues must be treated using other methods before disposal (Mahmoud, 2004, Taiwo and Otolorin, 2009).

1.5.5 Stabilization and Solidification Method

This technology is used to minimise potential environmental impact of oil sludge by enhancing the non-leachable properties of the treated oil sludge. The treatment uses advanced chemical oxidation (Fenton's reagents) followed by stabilization and solidification process with lime-clay and Portland cement-lime to yield oil sludge degradation and immobilization. In this process,

PAHs and BTEX compounds are reduced after stabilization and solidification process (Beech *et al.,* 2009). The reduction of these compounds may be due to the dilution which occurred by the addition of clay and lime, and by immobilization promoted by the lime and cement (Radetski *et al.,* 2006). The stabilization and solidification process is cheap compared to many other technologies for treating and disposing oil sludge. This technique reduces the mobility of hazardous substance and contaminants in the environments through physical and chemical means, and can be applied *ex-situ* and *in-situ* (Karamalidis and Voudrias, 2001). If the ecotoxicity potential of oil sludge is considered, the initial waste has high toxicity in PAHs and high concentration of phenolic compounds before treatment while after treatment the final products would be less toxic, and can be reused as concrete road bed blocks (Karamalidis and Voudrias, 2001). Despite the fact that the process enables the change of the initial dangerous waste to non-dangerous waste, the mass and volume ratio of residual product increases after the treatment (3kg of waste yield 20kg of commercial concrete block). In some cases, potential of oil sludge components leaching in long-term is possible. This calls for evaluation by a temporal series of leaching test to ensure environmental protection, in terms of public health and ecotoxicological perturbation of terrestrial and aquatic ecosystems (Karamalidis and Voudrias, 2001).

1.5.6 Oxidative Thermal Treatment

In this process, the oil sludge is not combusted but heated to remove organics and water from solids, the water is converted to steam to help strip off high boiling point semivolatile compounds, which can be condensed for recovery and disposal. The treatment is carried out using different concentration of oxygen at a constant heating temperature. This minimised waste and oil is recovered while producing a solid residue that meets environmental standards that are

directly disposed into landfill. The flaw with this process is its high energy consumption and complex operation (Shie *et al.,* 2004).

1.5.7 Pyrolysis Treatment

It is a technique for recovering oil and organic liquid gas by breaking down large molecules into smaller ones. The treated sediments that met the standard land disposal restriction level are directly discharged. In pyrolysis treatment of oil sludge, the initial step produces CO_2, hydrocarbons (volatile organics), water, CO, char and tar. The next stage, char and tar are combusted to release heat which is needed for the endothermic pyrolysis reaction. The hydrocarbons consist mainly of low molecular weights paraffins and olefins (C_1-C_2). The advantage of this process is that about 70 - 84% of the oil could be separated from the solids (Liu *et al.,* 2009). The disadvantage of pyrolysis is that a significant amount of vacuum residue is produced during the process. The energy required in pyrolysis of oil sludge is very high because it is close to energy required to distillate diesel from crude oil. Oxidative pyrolysis of oil sludge performed with insufficient oxygen produces alkyl and alkene compounds rather than being oxidised to produce CO_2, CO and H_2O. Therefore, oxygen is important in this technique to yield a better result (Liu *et al.*, 2010).

After considering the limitations of physical and chemical processes in treating and disposing oil sludge, the next step is to involve the biological process which is a cost effective and environmental friendly option for oil sludge treatment technique. It has numerous applications which include the clean-up of ground water, soil, surface water and effluent treatment of process waste streams. Most biological techniques are developed as a result of simple emulation of nature and how nature does bioremediation (Okieimen and Okieimen, 2005). Biological

methods have been proposed as the possible remedy for oil sludge treatments. However, most biological methods are economically unsound, prone to prolonged treatment time and they are not permanent solutions (Ward *et al.,* 2003). The observed lag time in biological treatment may be attributed to the stability of the compounds, the complex molecular structures and the ability of oil sludge components to adsorb onto sediments (Bach *et al.,* 2005). Despite the complications, the biological method is still being used (and studied) in the remediation of oil sludge (Leung, 2004).

1.6 Bioremediation

Bioremediation is defined as the use of living organisms to reduce or eliminate environmental hazards resulting from accumulations of toxic chemicals or other hazardous waste (Gibson and Sayler, 1992). Bacteria are generally used for bioremediation, but fungi, algae and plants could also be used. Bioremediation is not a new technology however, perspectives on the use of bioremedial technologies to treat contaminants vary. There are three classifications of bioremediation:

1. Biotransformation - the alteration of contaminant molecules into less or non-hazardous molecules
2. Biodegradation - the breakdown of organic substances in smaller organic or inorganic molecules
3. Mineralization - the complete biodegradation of organic materials into inorganic constituents such as CO_2 or H_2O (Leung, 2004).

These three classifications of bioremediation can occur either *in situ* (at the site of contamination) or *ex situ* (contaminant taken out of the site of contamination and treated

elsewhere) (Das and Mukherjee, 2007). There are advantages and disadvantages to both *in situ* and *ex situ* strategies. *Ex situ* strategies also known as 'pump and treat', removes the contaminants and places them in a contained environment. It involves excavation and transportation (relocation) from the natural or original contaminated site to elsewhere. This allows for easier monitoring and maintaining of conditions and progress, thus making the actual bioremediation process faster. However, the removal of the contaminant from the contaminated site is time consuming, costly and potentially dangerous. By bringing the contaminant to the surface, the workers and the general public have increased exposure to the toxic material. In contrast, the *in situ* strategy does not require removal of the contaminant from the contaminated site. *In-situ* bioremediation method involves the treatment of contaminants at the natural or original contaminated sites without relocation. The *in-situ* methods include biostimulation, and bioaugmentation, *in situ* remediation are cost effective because there is no need for excavation and transportation but it is less controllable and less effective. A major advantage of *ex situ* technique is that their products are reusable. Amidst all, Bioremediation of oil sludge are promising methods, where adapted microbial species are used for the degradation of the sludge. They are nature-compatible, reliable, cheaper and easy to adopt compared to physical and chemical methods (Machin-Ramirez *et al.,* 2008). The end products are usually harmless carbon dioxide, water and fatty acids. Bioremediation is often less expensive and disruption is minimal, it eliminates waste permanently, eliminates long term liability, and has greater public acceptance, with regulatory encouragement, it can also be coupled with other physical or chemical methods (Idris and Ahmed, 2003). Bioremediation has its limitations; some chemicals are not amenable to bioremediation, for instance, heavy metals, radionuclides and some chlorinated compounds. In some cases, microbial metabolism of contaminants may produce toxic metabolites.

Bioremediation therefore is a scientifically intensive procedure, which must be tailored to the site-specific conditions. There are different number of *ex-situ* and *in- situ* methods which include biostimulation, bioaugmentation, landfilling, landfarming, bioreactors, and composting.

1.6.1 Biostimulation

This involves the management of the natural environment to optimise the growth and activity of the natural microbial population (Crivelaro *et al.,* 2010). Biostimulation of indigenous degrading bacteria as a tool in bioremediation process should be encourage, because the process relies on the degrading bacteria that have already adapted to the site's conditions (Dzantor, 1999; Ausma *et al.,* 2002, Singh and Lin, 2010). The constraints in this technique are time and limited knowledge of microbial process, since if compared with other technologies, bioremediation is a slow process. Also, favourable conditions such as temperature, pH, nutrients and additives such as surfactants must be optimised to stimulate the microbial growth and activities during bioremediation (Atlas and Bartha, 1972; Kim *et al.,* 2004, Mahmoud, 2004).

1.6.2 Bioaugmentation

This technique refers to the introduction of specialized or genetically engineered microorganisms that target specific chemical compounds. These organisms have been developed to biodegrade most common organic contaminants ranging from polychlorinated biphenyls (PCBs), organic solvents and petroleum hydrocarbons (Mehrashi *et al.,* 2003; Atlas and Philip, 2005). The identification of the key microorganisms that play a major role in pollutant degradation processes is relevant to the development of optimal *in-situ* bioremediation strategies (Abed *et al.,* 2002; Watanabe, 2002). The use of such specialised formulations of microorganisms is often dictated whereby the indigenous bacteria cannot metabolise the contaminants concerned. It could be used

if the contaminants are toxic to the naturally occurring bacteria. Introduction of specialised bacteria also may be used to increase the biological activity (Van Veen *et al.,* 1997). The dynamic growth of a bacterial consortium on oil sludge has been studied. The results showed varied individual ability of the bacterial strains to grow on oil sludge. The growth was further improved by mixing the sludge with non-ionic surfactant and optimising favourable conditions such as temperature and nutrients (fertilizer). The reduction in petroleum hydrocarbon biodegradation rates varied from 16.75% to 95% (Lazar *et al.,* 1999, Mishra *et al.,* 2001, van Hamme *et al.,* 2003). This means that some bacterial strains have the ability to degrade oil sludge. The results have shown that the performance of the microbial cultures is dependent on several factors including the composition of the sludge that varies depending on the type of crude oil and the source of the sludge inside the refinery (Mahmoud, 2004). However, very little information is available about the use of microbial cultures to treat oil sludge on pilot scale and full scale studies. The limitation to successful bioaugmentation has always been attributed to poor survival of the introduced strains. The use of readily degradable substrate has been found as a limitation, due to low concentration and non biodegradability of targeted pollutants (Alexander, 1994). Again, if the soil (or the media that contains contamination) is heterogenous, there will be uneven flow of liquid or gas containing the nutrients or microbes, so different areas will undergo different rates of remediation. Del'Arco and de Franca, (2001) observed that various efforts have been attempted to improve the success of bioaugmentation process. Strategies that were employed to improve bioaugmentation process for the effective degradation of oil sludge include the use of adapted strains or the field application vector (Lajoie *et al.,* 1994). Bioaugmentation of oil sludge is a slow process if compared to landfarming and composting. Hence, more research

needs to be conducted to stimulate the growth of microorganisms on oil sludge and improve the performance of the bioaugmentation process.

1.6.3 Landfilling

Landfilling is a deliberate dumping of oil sludge into land (pit) with or without formal treatment. It has been the most common form of sludge disposal. This process has limitations as it requires a large land area and volatile organic compounds are emitted if the oil sludge is not treated before disposal. Most times, the locations of Landfill sites for oil sludge disposal have been selected according to availability of land and convenience rather than consideration of the hydro geological features of the sites. This calls for more strict legislative restrictions on landfilling (Bhattacharyya and Shekdar, 2003).

1.6.4 Landfarming

Landfarming involves the controlled application of the oil sludge on the land surface. This method requires tilling of the topsoil (for easy mixture with oil sludge), addition of water and addition of desired nutrient such as organic fertilizers and manures. Tilling in this process is important as it aids aeration, proper mixture of sludge and nutrient, thereby making the sludge bio-available for microbial degradation. Proper Landfarming practice has minimal impact on the environment (good site appearance, absence of odour, relatively low-cost compliance with sound industrial practices and government regulation, minimal residue disposal problems and compatibility of the method with the climate, location and type of sludge treated). Landfarming gained popularity over incineration and Landfilling following its advantages such as low energy consumption, low risk of pollution of the surface and groundwater due to the immobility of hydrocarbons and metals through the soil (Hejazi *et al.,* 2003; Besalatpour *et al.,* 2011).

Landfarming technique only lost its popularity when the USA Environmental Protection Agency (USEPA), issued the land disposal restriction conservation and recovery act (RCRA), establishing treatment standards under the land disposal restriction program (USEPA, 1997). The restriction prohibited the land disposal of untreated oil sludge. This led to treating the oil sludge to EPA treatment standards and making sure that there was no migration of hazardous constituents from the injection zone (Hejazi *et al.*, 2003). However, Landfarming was an acceptable disposal method as long as it is within EPA guideline that aims to minimise the possibility of wash out and groundwater contamination. Simplicity and cost-effectiveness are some of the major advantages of the technology (Hejazi *et al.*, 2003). It is simple in that, typical equipment used for landfarming is used widely in the farming community and is therefore readily available. Although, Landfilling is reported as the most cost effective oil sludge treatment method, Landfarming gained popularity among refineries following restrictions on Landfilling oil sludge (Mahmoud, 2004). The challenges of Landfarming include the release of hydrocarbon compounds (VOCs) during the application and degradation of oil sludge (greenhouse structure can help minimise emission), and its requirement of a large land area for treatment (just as in Landfilling). There is also risk of residues such as the branched n-alkanes not degrading. There are also health related concerns as the sludge poses serious carcinogenic risks to workers during the early sludge application period (Hejazi *et al.*, 2003).

1.6.5 Bioreactor

This uses petrozyme in a bioreactor process as a fermentation technology to degrade oil sludge into non-hazardous effluents with very low level of hydrocarbon (Singh *et al.*, 2001). This method uses a naturally selected and acclimated indigenous bacterial culture supplemented with a carefully designed blend of nutrients such as nitrogen, phosphate, essential minerals and a

surfactant for degradation. The design and process operating conditions of the technique promoted the growth of highly active microbial population, which rapidly converted the oil sludge components to carbon dioxide and water. It was further reported that the bacteria involved are known oil-degrading bacteria such as *Pseudomonas, Acinetobacter, Rhodococcus and Alcaligenes* (Singh *et al.*, 2001). In their (Singh *et al.*, 2001) study, more than 90% of the total petroleum hydrocarbons contained in the oil sludge were degraded. After a successful treatment, 50-80% of the processed materials were disposed of, leaving about 20-50% in the reactor. The 20-50% remaining in the reactor serves as inoculums for the next run if the reactor is reloaded with another batch of oil sludge. Otherwise all processed materials are disposed off. The analysis of the total petroleum hydrocarbons obtained from the petrozyme treatment process indicated that oil sludge was treatable to non-hazardous levels. The aqueous low TPHs can be sent to the wastewater system, solid residues can be disposed of in a landfarm, to a non-hazardous landfill, dewatered and reused in other industrial purposes (Singh *et al.*, 2001). This technique can be used in the process recovery of recyclable oil, biodegradation of oil sludge and disposal of treated oil sludge. It also eliminates the need to spray high concentration oil sludge on large areas of land. Bioreactor process has high rates and extent of degradation than Landfarming process due the minimization of mass-transfer limitation. This technique controls the environmental and nutritional factors such as pH, temperature, moisture, bioavailability of nutrients and oxygen promotes microbial growth and activity for the rapid degradation of oil sludge. The limitations faced by this technique are that it is an ex-situ process therefore; substantial cost can be incurred during excavation and transportation of oil sludge. The reactor mixer consumes energy and availability of well trained personnel is required for this method. It involves the risk of pollutant

exposure and the unravelling limiting factors during bioremediation (Piotrowski, 1991; Lees, 1996).

1.6.6 Composting

Despite decades of research, successful biological remediation of oil sludge in the environment remains a challenge. It is noticed that, there are physical, chemical and biological aspects of Landfilling, Landfarming, bioreactor treatments that can hamper the degradation processes of oil sludge, making them partially effective and prohibitively expensive. It is necessary to search for cheaper and environmental friendly options in enhancing bioremediation of oil sludge. Such options should be able to take care of the limitations of the previous methods while improving oil sludge bioremediation. Therefore, composting process which involves the careful control and addition of nutrients, watering, tilling, addition of suitable microbial flora and bulking agents (wood-chips or hay) were considered an alternative option to improve the bioremediation of oil sludge (De-qing *et al.*, 2007). The process leads to the production of carbon dioxide, water, minerals and stabilized organic matter (Pereira-Neta, 1987).

Composting is a controlled biological process of a mixture of substrates carried out by successive microbial population combining both mesophilic and thermophilic activities. It is applied to solid and semi-solid organic waste such as nightsoil, sludge, animal manures, agricultural residues and municipal refuse, whose solid content are usually higher than five percent. The process can be classified into mechanical and non-mechanical processes (aerobic and anaerobic composting system); using technology as the key (the classification is divided into static pile or windrow, and mechanical or "enclosed" composting) (http://www.lagoonsonline.com/composting.htm, accessed on 20/11/2011; Beffa, 2002; Norbu, 2002). Compost systems can be on three general

bases: oxygen usage, technological approach and temperature. Oxygen usage is divided into aerobic and anaerobic. Aerobic composting involves the activity of aerobic microbes, and hence the provision of oxygen during the composting process. Aerobic composting generally is characterized by high temperatures, the absence of foul odours and is more rapid than anaerobic composting. The addition of oxygen promotes bacterial and fungal growth within the compost pile. The organisms that grow in aerobic compost piles produce less methane and sulphur-based gases than in anaerobic composting, resulting in less odour. This method requires much higher maintenance, regular turning and mixing to incorporate air into the material than in anaerobic composting. Moisture loss is more likely in aerobic composting and frequent watering of the material is required. Anaerobic composting is characterized by low temperatures, the production of odorous intermediate products, and generally proceeds at a slower rate than aerobic composting (Eneji *et al.,* 2006). In anaerobic composting, the material stacks in layers to form an environment completely free of air within the layers. Bacteria, fungi and a higher form of bacteria, such as actinomycetes, that thrive in this environment begin to grow to breaking down the material. Anaerobic composting requires little maintenance, as there is no need to turn the material within the compost pile. The bacteria, however, produce more methane and sulphur-based gases as by-products, which can produce a strong odour. The odour indicates the composting process is progressing. Composting could be divided with respect to the modes of operations such as batch operation and continuous or semi-continuous operation. When temperature is the basis, composting can be divided into mesophilic composting (25 – 40^{o}C) and thermophilic composting (50 – 65^{o}C). The main advantage of composting is waste stabilization. The biological reactions occurring during composting will convert organic wastes into stable, mainly inorganic forms. These stable inorganic forms may cause little pollution effects if

discharged onto land or into water course (Eneji *et al.,* 2006). The degradation of organic matter in aerobic composting system depends on the presence of oxygen. Oxygen serves two functions in the metabolic reaction; the terminal electron acceptor in aerobic respiration and as a substrate required for the operation of the class of enzymes called oxygenase (Finstein *et al.,* 1980). Briefly, essential factors are those features of the physical, chemical, and biological background that are necessary to the establishment and proliferation of the microorganisms specific to the desired process. Five essential factors that have become key design features in recent compost technology are suitable microbial populations, aeration (oxygen availability), temperature, moisture content, and carbon availability (http://www.lagoonsonline.com/composting.htm, accessed on 20/11/2011).

Compost bioremediation relies on the mixing of primary ingredients of compost with the contaminants and oil sludge is compostable which is enhanced when bulking agents are added to the treatment process (Milne *et al*., 1998). As the compost matures, the pollutants are degraded by the active microflora within the mixture. It is called tailored compost (designed compost), in the sense that, it is specially made to treat specific contaminants at specific sites (US EPA, 1997). In most cases, temperature, pH and nutrients are the important factors. An increase in temperature in the compost pile increases solubility of contaminants and induces higher metabolic activity of the compost (Gibb *et al.,* 2001). Oil sludge degrading bacteria and fungi performance are affected by pH level; while on the other hand, nutrients like nitrogen and phosphorus have great effect on microbial degradation of oil sludge constituents (van Hamme *et al.,* 2003).

Jose *et al.*, (2006), attempted to ascertain the efficacy of composting technology in the reduction of hydrocarbon contents of oil sludge with a large total hydrocarbon content (250-300g kg^{-1}) in semiarid conditions. They designed three composting systems with open air piles, which were turned periodically over a period of 3 months. This system proved to be inexpensive and reliable. They (Jose *et al.*, 2006) also studied the effect of bulking agent (wood shavings) addition on the oil sludge biodegradation and inoculation of the composting pile with pig slurry (a liquid organic fertilizer which adds nutrients and microbial biomass to the pile). The most effective treatment was composting pile with the bulking agent. Initially, hydrocarbon content was reduced by 60% in 3 months. It seems that the bulking agent encourages the diffusion of oxygen inside the pile. It also facilitates microbial developments and raising the temperature quicker. The temperature increase in the composting process may be due to the differing capacity of microorganisms to degrade the hydrocarbons. Since oil sludge contains highly degradable materials, these microorganisms accept the hydrocarbons as substrates, which enhance their activities, leading to the higher increase in temperature (Bengtsson *et al.*, 1998, Jose *et al.*, 2006). The reduction of petroleum hydrocarbon achieved in the compost bioremediation was 85–90% over a period of 11 months. The composting pile without a bulking agent was reduced by 32% in 3 months. The introduction of the organic fertilizer did not significantly improve the hydrocarbon degradation because it only degraded 56% of the hydrocarbon content.

Oxygen content is known to be a key factor in composting. In a pile containing bulking agent, the oxygen content measured was always high after turning (10-14 %). However, in piles without a bulking agent, oxygen content remained at 2-9% (Zhou and Crawford, 1995). This result demonstrated the effectiveness of a bulking agent for fostering microbial activity during the composting process (Zhou and Crawford, 1995). The humidity of the pile maintained at 40-60%

encouraged microbial activities and the biodegradation of the hydrocarbons. Low moisture level and low oxygen content explains the low temperature reached in piles without bulking agent. As time progressed, the moisture level of the piles declined and water had to be added. The maintenance of humidity could be difficult in case of co-composting with oil sludge because oil sludge may not readily absorb water due to the high hydrophobic nature (Zhou and Crawford, 1995). This is one of the challenges which could arise in bioremediation process involving co-composting with oil sludge. Oil sludge does not readily absorb water and its hydrophobic nature makes it difficult to maintain humidity that may encourage microbial activities for degradation of hydrocarbons in compost systems (Mishra *et al.,* 2001; De-qing *et al*., 2007).

The initial degradation of the hydrocarbons in oil sludge may possibly be catalysed by mono and dioxygenase enzymes (Britton, 1984; Singer and Finnerty, 1984). The enzymes gradually oxidise the hydrocarbons to alcohol and aldehydes in the presences of oxygen, producing acids that finally follow a metabolic pathway to produce carbondioxide (CO_2) and water (H_2O) (Britton, 1984; Singer and Finnerty, 1984). This is synthesized by aerobic microorganisms, which is clearly the benefit from the addition of the bulking agent. Therefore, the addition of suitable bulking agent improves aeration and the performance of the composting process of the oil sludge. When the total hydrocarbons present in the composting undergo a great degree of degradation, the process results in detoxification of the mass and the loss of their toxic substances.

The limiting step of composting process is maintaining a suitable level of humidity in the pile. Furthermore, the challenges of compost bioremediation are the nature of the oil sludge, the composting conditions, microbial communities and time. Lack of sufficient readily

decomposable organic matter may give inadequate substrate to stimulate microorganisms in the decomposition of untreated disposed oil sludge (Cole *et al.,* 2003, Fountoulakis *et al.,* 2009). All these affect the mechanism of conversion in compost. Composting bioremediation tends to treat oil sludge in a cost-effective and environmental friendly way by utilizing effectively its biological, physical and chemical process. Many factors are considered in the design of an optimal oil sludge treatment process. These factors include time, nutrients, pH, moisture and microbial biodegraders; they are also considered as attributes of composting processes.

Amidst limitations that may hamper the composting processes, co-composting techniques for bioremediation of oil sludge have its advantages. It is economically sound, natural process that destroys organic contaminants and the residues obtained are no more harmful (UNIDOI, 2003; http://www.embiotech.org). The process eliminates the transfer of residue from one environmental medium to another. The biological reactions occurring during composting will convert organic wastes into stable, mainly inorganic forms. These stable inorganic forms may cause little pollution effects if discharged onto land or into a water course. As already stated, composting could be *ex situ* or *in situ* process depending on whether the oil sludge is taken out from its source or not. It is often less expensive and disruption is minimal (Jain *et al.,* 2011). It eliminates waste permanently, eliminates long term liability, and has greater public acceptance, with regulatory encouragement, it can also be coupled with other physical or chemical methods (Idris and Ahmed, 2003; Sharma and Mudhoo, 2010; Anyasi and Atagana, 2011). As far as the effectiveness of the by-products is concerned, the treated sludge is found enriched in organic matter along with sufficient amount of nutrients (nitrogen, phosphorus and potassium) (Fleming and Ford, 2002). This technique does not only reduce the PAHs concentrations, but tends to improve soil quality through the addition of organic matters. Also, if compared to Landfill or

Landfarming and destructive treatment methods, such as incineration, the use of composted material and co-composting as bioremediation technique may possibly promote soil sustainability and re-use.

The aim of this study was to measure the effect of co-composting oil sludge with pig, cow, horse and poultry manures on the reduction in Polycyclic Aromatic Hydrocarbons concentrations of oil sludge under laboratory conditions. Specific objectives are to isolate and characterise degrading bacteria present in the compost system, and to determine whether microbial growth and activities can be enhanced.

CHAPTER TWO

Bioremediation of Oil Sludge by Co-composting with Animal Manures

2.1. Introduction

All organic matter eventually decomposes under natural conditions. The decomposition process provides an ideal environment for bacteria and other decomposing microorganisms to breakdown organic matter. In composting, the final product is compost, a humus-like end product which looks like fertile garden soil. The end-product is dark, crumbly, earthly-smelling and provides vital nutrients to help both plants and microorganisms to grow. The decomposing organisms consist of bacteria, protozoa, actinomycetes, fungi and larger organisms such as worms, sow bugs and nematodes. The decomposition process relies on the action and interaction of these microorganisms which thrive within different temperature ranges to achieve the stabilization and minimization of waste (Diehl and Borazjani, 1998). Decomposing organisms need some key elements such as nitrogen, carbon, phosphorus, moisture and oxygen to thrive on.

Composting has been described in Chapter One section 1.6.6. In composting, best results are obtained from a mixture of materials high in nitrogen (such as clover, fresh grass clippings and livestock manure) and those high in carbon (such as dried leaves, wood chips and twigs) (De-qing *et al.,* 2007). In composting, moisture is provided by addition of water and oxygen is provided by mixing the composting pile. Composting systems are used to degrade and stabilize organics such as manure, municipal refuse, municipal sewage sludge, yard waste and food processing waste (Potter *et al.,* 1999). The process is generally accepted in the treatment of agricultural and municipal waste (Gray *et al.,* 2000). Compost systems range from relatively

simple compost pile (windrows) to highly engineered and controlled continuous-feed reactors (Atlas and Barther, 1987). Composting is an aerobic, mesophilic and thermophilic process where the maintenance of adequate oxygen level and appropriate temperature is of great importance when designing the system (Baker and Herson, 1994).

Compost bioremediation is an application of the principle of composting in remediation of contaminated environments (Barnes *et al.,* 2000). This technique is used to restore contaminated soils by degrading volatile organic compounds (VOC) (US EPA, 1997). Compost bioremediation is also the use of biological system of microorganisms in mature and cured compost to breakdown or reduce contaminants in water or soil. The microorganisms consume the contaminants by digesting, metabolizing and transforming them into humus and inert products such as carbon dioxide, water and salts (US EPA, 1997). However, compost bioremediation is not new. It has been used to treat contaminated soils (US EPA, 1997; Potter *et al.*, 1999). As stated in Chapter One, composting technology has been used in the reduction of hydrocarbon contents of oil sludge with large total hydrocarbon content (250-300g kg^{-1}) in semiarid conditions (Mahmoud, 2004). Three composting systems were designed with open air piles, which were turned periodically over a period of 3 months. This system proved to be inexpensive and reliable. Since oil sludge contains highly degradable materials, microorganisms in the composting piles accept the hydrocarbons as substrates, which enhance their activities, leading to the higher increase in temperature (Bengtsson *et al.,* 1998, Jose *et al.,* 2006). The reduction of petroleum hydrocarbon achieved in this compost bioremediation was 85–90% over a period of 11 months (Mahmoud, 2004, Jose *et* al., 2006). Compost can be specially made to treat specific contaminants at specific sites (US EPA, 1997). This technique, more than any other soil-clean-up

technique enriches the soil as the end-product provides some nutrients required for revegetation and leaving the soil without altering the soil mineral elements after treatment (Cole *et al.,* 1995).

Composting is characterized by different temperature ranges during degradation of organic compounds. At the initial stage, temperature can increase from ambient to approximately 40^{o}C. As degradation of organic matter proceeds, there is an increase in temperature, mesophilic to thermophilic range of 40^{o}C to 70^{o}C as a result of more heat evolving in the process. Optimal organic breakdown takes place in the thermophilic range of 55^{o}C to 60^{o}C. Increase in temperature may result in a decrease in the decomposition rate in the compost system due to loss of water from the system caused by high temperature rate and also decline in microbial population (US EPA, 1997). This could be resolved by more frequent watering of the system.

For effective composting, various operational procedures are necessary. For example, windrow composting may include amendments of the organic matter and bulking agent (such as wood chips), aeration of the compost pile, curing of the compost, product utilization and disposal. Compost bioremediation is carried out by co-composting the contaminants with suitable compost materials to effect biodegradation of the contaminant (Reid *et al.,* 2000).

Several studies have previously examined the degradation of organic pollutants in composts (Cole *et al.,* 1995; US EPA, 1997; Bengtsson *et al.,* 1998, Potter *et al.,* 1999; Barnes *et al.,* 2000; Reid *et al.,* 2000; Jose *et al.,* 2006). It has been shown that microorganisms present in windrow composts are capable of mineralizing organic pollutants (Valo and Salkinoja-Salonen, 1986). The growth of bacteria and fungi is dependent on the presence of a number of nutrient elements and electron acceptor, together with the organic compounds that serve as the source of carbon and energy (van Hamme *et al.,* 2003).

Composting has been used as a bioremediation technique, in the remediation of soils contaminated with a number of organic compounds including PAHs. Most of the studies on treatment of soils contaminated with PAHs by composting has been achieved but on lower concentration of the contaminating substances (low-molecular-weight PAHs), in spite of the fact that composts have been reported to have a good potential for remediation of heavily contaminated sites contaminated with high-molecular-weight PAHs (Whyte, 1997; Bastieans, 2000).

Oil sludge has been identified to constitute of both low and high- molecular weight PAHs of between two to more than six fused benzene rings that are of environmental concern (US EPA, 1997; van Hamme *et al.,* 2003). This high level of PAHs in oil sludge provided a good opportunity for this study, which aims to further understand the potentials and the efficacy of compost bioremediation in soils heavily contaminated with high-molecular weight PAHs. This will help to determine the requirements of each compost type and their practical application on field-scale treatment of contaminated soils or sites.

2.2. Materials and Methods

2.2.1. Soil samples

Garden soil (up to a depth of 20cm, as the highest concentration of organic matter and microorganisms are found in this layer) (Mann, 2008), was collected in a black plastic bag from a farm in Tembisa near Johannesburg, South Africa. The soil was transported to the laboratory at University of South Africa in Pretoria and stored at room temperature before use for the experiment. The soil was air-dried and homogenized by hand, to make the soil easy to mix with compost materials. The soil was then characterized for parameters such as soil type, organic

carbon content, total nitrogen content, total phosphorus content, soil pH and water holding capacity.

2.2.2. Crude Oil Refinery Sludge

The sludge was collected from an oil refinery in Durban, KwaZulu-Natal, South Africa. It was characterized for selected PAHs using automated soxhlet extractor coupled with gas chromatography/mass spectrometry (GC/MS) method. The concentrated acid digestion method (CADM) was used to determine metals present in the oil sludge.

2.2.3. Compost material

Cow, pig, horse, and poultry manures were collected from the University of Pretoria, Veterinary Campus Onderstepoort, Pretoria, South Africa. The manures were air dried and stored at room temperature before use. They were characterized for Carbon, Nitrogen and Phosphorus content.

2.2.4. Bulking agent

The bulking agent used was wood-chips. It was collected at the road construction site along Pretoria-Johannesburg free way R21. The wood-chips were pieces of wood from the bush cleared at the centre of the roads as construction was going on. The wood-chips were dried under the sun before collection from the site. It was not treated but was chopped into smaller sizes before use.

2.2.5. Carbon Tetrachloride reagent (CCl_4)

Carbon tetrachloride (99.55%), molar mass 153.8236g/mol, density 1.594g/ml) was purchased from Merck Johannesburg, South Africa. The reagent was used to dissolve the oil sludge before mixing with soil.

All reagents used, were of analytical grade and were used without further purifications. The composting treatments were designed based on the knowledge of the conditions prevalent in the laboratory used. The temperature and pressure of the laboratory was 22 ^{o}C at 100 kPa.

2.3. Soil Characterisation

2.3.1. Soil texture

Three soil separation tubes were placed on a rack and were labelled A, B and C. Homogenized soil sample was added to Tube A up to the 15 ml mark (the tube was gently tapped while adding the soil to pack the soil and eliminate air space). One ml of texture dispensing reagent was added to the soil in Tube A and was diluted to 45 ml mark with deionised water. The tube was capped and gently shaken for two minutes for thoroughly mixing. It was place on the rack and allowed to settle for 30 seconds undisturbed. The mixture in Tube A was carefully poured into Tube B and both tubes were placed on the rack. The content in Tube B was allowed to settle for 30 minutes undisturbed. Then, all the solution from Tube B was carefully poured into Tube C. One ml of soil flocculation reagent was added to Tube C and the tube was capped and shaked for one min. Tube C was placed in the rack and allowed to stand until all the clay in the suspension had settled. Due to the colloidal nature of clay in solution and its tendency to swell and form a gel, the portion of clay in Tube C was not used to determine the clay fraction in the soil. Rather, the addition of the reading on sand in Tube A and the reading of the silt from Tube B and subtracting the sum from initial volume of soil used for the separation gives the clay fraction. The soil texture analysis was conducted by Waterlab (Pty) Ltd, Persekor Techno Park, 41 De Havilland Crescent, Persequor, Pretoria, 0020. The procedures described above adopted from the standardized method were followed.

Reading the level of soil in Tubes A and B.

Percentage sand = reading on Tube A /15 X 100

Percentage silt = reading on Tube B / 15 X 100

2.3.3. Determination of Metals in Experimental Soil

The concentration of trace metals in the garden soil sample was determined by concentrated acid digestion method (CADM) using strong acids in digesting the soil samples. The aqua regia solution 20 ml (HCl and HNO_3 in a ratio of 3:1) was added to 10 g soil sample in a 100 ml volumetric flask. The soil was digested using open heat method on a hot plate with a low heat for 2 hours. After the digestion time, the flask was rinsed with deionised water to wash down the solution from the sides of the flask. The solution was filtered using Whatman filter paper (125 mm) and the filtrate was diluted to 100 ml mark with deionised water. The standard used was multi-element standard solution V for ICP (the reagent was purchased from Fluka Analytical, Johannesburg, South Africa, in accordance with ISO/IEC 17025 and ISO GUIDE 34). The standard solution was prepared in 8ppm, 4ppm, 2ppm, 1ppm concentration in mg/l (ppm = parts per million and mg/l = milligram per litre). The filtrate was measured for metals using the ICP. The results were calculated using the following formula:

$P_1 - P_0 \times F_x \times V / M$

Where P_1 is the original reading, P_o is the blank reading, F_x is the dilution factor, V is the dilution volume and M is the initial mass weighed.

2.3.4. Determination of pH level in Experimental Soil

The pH of the soil sample was measured by weighing 10 g of 2mm-sieved soil sample into a 60 ml Sterilin vial. 25 ml of deionised water was added to the vial and it was covered with the lid. The solution was shaken and allowed to stand for 30 minutes, and the procedure was repeated once again. After, the solution was shaken again and the pH of the supernatant was measured using Crison Micro pH 2000^{TM} meter.

2.3.5. Determination of Dry Matter Content of Experimental Soil

The Dry Matter content (DM) of the soil sample was determined by adding 10 g of 2mm sieved moist soil sample into pre-weighed crucibles. The crucibles with the soil were then placed in a metal tray and in a 105 ^{o}C oven for 24 hours. The crucibles were removed from the oven and cooled in desiccators containing blue silica gel. The weights were recorded and the percentage soil dry matter was calculated using the following:

Percentage soil dry matter content = dry weight of soil /fresh weight of soil x 100

Percentage soil moisture content = 100 – percentage soil dry matter content

2.3.6. Determination of Water Holding Capacity of Experimental Soil

The water holding capacity of the soil sample was determined by placing a measured glass wool (0.30 g each) firmly down into the 100 ml glass funnel in duplicates. 50 g of moist soil sample was weighed into the glass funnels. The blank in another glass funnel contain the glass wool only without the moist soil. A 100 ml measuring cylinder was placed beneath the glass funnel to collect the liquid dripping out from the short rubber tube attached to the mouth of the glass funnel stem and the tube was closed completely with a clip. The cylinder was carefully placed to

ensure that all the liquid dripped directly into the measuring cylinder. Then 100 g deionised water was added into the glass funnels. The glass funnels were covered with aluminium foil to prevent evaporation and allowed to stand on the clamp overnight. The clip was then opened to allow water to drip into the measuring cylinder beneath the glass funnels through the tube and the water was collected for 30 minutes. The volume of water collected was weighed with the collecting cylinder. The soil samples were weighed and transferred into a crucible. It was dried in an oven at 105 ^{o}C to constant mass. The samples were cooled in a dessiccator and re-weighed. The percentage –water-holding capacity (%WHC) was calculated as follows:

100 – (Volume water retained by glass wool + volume water collected) ml = A

(Note: the volume of water retained by the glass wool = 100 ml – the volume of water collected from the blanks).

To calculate the soil WHC (ml water held at 100% WHC per 100g oven dried soil)

2A + MC %(% soil moisture content) = WHC (ml 100g^{-1} fresh soil) = B ml

Then: (B ml/soil DM (soil dry matter) X 100 = mls of water held by 100 g oven dried soil at 100% WHC. This experiment was adopted from Forster, (1995).

2.3.7. Determination of Total Organic Nitrogen in Experimental Soil

The total organic nitrogen as N in soil was determined by a titration method. The digestion reagent was prepared by dissolving 134 g K_2SO_4 and 7.3 g $CuSO_4$ in 800 ml of distilled water. Concentrated H_2SO_4 (134 ml) was carefully added. The solution was allowed to cool to room temperature (22 ^{o}C) and was made up to 1 litre with distilled water, then mixed thoroughly. The solution was stored at 20 ^{o}C to prevent crystallization. 500 g NaOH and 25 g $NaS_2O.5H_2O$ was dissolved in distilled water and diluted to 1 litre in another bottle. Ten grams of the soil sample

was weighed into 800 ml kjeldahl flask, 300 ml distilled water was added and the solution was adjusted to pH 7. To remove ammonia, 25 ml borate buffer was added and 6M NaOH was added to the solution until pH 9.5 was reached. Five boiling chips such as Hengar granules #12 were added and 300 ml of the solution was boiled off to remove ammonia. The mixture was allowed to cool to 22 ^{o}C and 50 ml of the digestion reagent was added carefully to the flask. Five glass beads such as Hengar granules #12 were added and mixed.

The mixture was heated under a fume hood and the mixture was allowed to boil briskly for 2 hours until the volume was reduced to 100 ml. The mixture was allowed to cool. It was diluted to 300 ml with distilled water and mixed thoroughly. Then 50 ml of $Na_2S_2O_3.5H_2O$ reagent was added to form an alkaline layer at the bottom of the flask. The flask was connected to a steamed-out distillation apparatus and was swirl to ensure complete mixing. The solution exceeded pH 11.0. The solution was distilled and 50 ml indicating boric acid was used as absorbent solution. The condenser was extended well below level of the absorbent solution and temperature was allowed to rise above 29 ^{o}C and 200 ml distillate was collected using Erlenmeyer flask. The ammonia measurement was done by titration method. The distillate solution in the Erlenmeyer flask was titrated with 0.02M H_2SO_4 using 6 - 8 drops of mixed colour indicators. End point was reached when colour changed from blue to pink/grey. The blank was set-up following the same procedure but there was no soil used.

The ammonia nitrogen concentration was calculated as follows:

mg/L ammonia nitrogen as N = (titre value of sample – titre value of blank) x 280/ sample volume.

2.3.8. Determination of Total Phosphorus in Experimental Soil

2.3.8.1. Ammonium Molybdate Stock Solution

Ammonium molybdate tetrahydrate (40 g) [$(NH_4)_6Mo_7O_{24}.4H_2O$] was weighed into 1 litre bottle and diluted with 983 ml of distilled water. The solution was mixed with magnetic stirrer for 4 hours and stored in a refrigerator before use.

2.3.8.2. Antimony Potassium Tartrate Stock Solution

Antimony potassium tartrate (3g) (potassium antimony tartrate hemihydrates; $K(SbO)C_4H_4O_6.{}^1/_2H_2O$) was weighed into a dark 1 litre bottle and diluted with 995 ml of distilled water. The solution was mixed with a magnetic stirrer until all the powder had dissolved. The solution was stored in the dark bottle in a refrigerator before use.

2.3.8.3. The working Molybdate Reagent

Distilled water (680 ml) was measured into 1 litre bottle and 64.4g of concentrated sulphuric acid was added and then the container was swirled for thorough mixing. Then 213 g of stock ammonium molybdate solution from (section 2.3.8.1.) was added followed by 72 g of stock antimony potassium tartrate solution from (section 2.3.8.2.). The solution was shaken and degassed with helium.

2.3.8.4. Ascorbic Acid Solution

Granular ascorbic acid (60g) was weighed into 1 litre bottle, diluted with 975 ml distilled water. The solution was stirred until it dissolved. The reagent was degassed with helium, then 1g dodecyl sulphate, $CH_3(CH_2)_{11}OSO_3Na$ was added. The solution was stirred gently to mix.

2.3.8.5. The Stock Orthophosphate Standard

Primary standard grade anhydrous phosphate monobasic (25mg P/L: 0.1099g) (KH_2PO_4) was dissolved with 800 ml distilled water in 1 litre volumetric flask. The solution was diluted to mark with distilled water and inverted to mix.

2.3.8.6. The Standard Orthophosphate Solution

Orthophosphate standard was prepared using the stock standards in (section 2.3.8.5.) above and was diluted with distilled water. After the preparation of the reagents, 10 g of the sample was digested in a micro-kjeldahl flask with H_2SO_4 and HNO_3 in a ratio of 1:5 respectively. The solution was digested by heating gently on a hotplate to reduce the volume of the solution and until the solution became colourless to remove HNO_3. The solution was cooled and diluted with 20 ml of distilled water. A drop (0.05 ml) of phenolphthalein indicator and 1M NaOH solution was added in dropwise manner until a faint pink tinge colour was produced. The neutralised solution was transferred by filtering into a 100 ml volumetric flask. The filtrate was diluted to 100 ml mark with distilled water. 50 ml of the filtrate was transferred into 125 ml Erlenmeyer flask using a pipette and a drop (0.05 ml) of phenolphthalein indicator solution was added to the solution. 5M H_2SO_4 solutions were added in dropwise manner to discharge the red colour produced as a result of the addition of phenolphthalein indicator. Then 8 ml of the combined reagent solution (mixture of 50 ml 5M H_2SO_4, 5 ml potassium antimony tartrate solution, 15 ml ammonium molybdate solution and 30ml ascorbic acid solution) was mixed thoroughly. The solution was allowed to stand for 10 minutes. The absorbance of each sample was measured at 880nm. The blank was used as the reference solution. The blank was prepared following the same procedure and all reagents were added except ascorbic acid and potassium antimony tartrate. The blank absorbance was subtracted from the sample absorbance of each sample. The

calibration curve preparation was by preparing individual calibration curve from the standards within the phosphate range. A distilled water blank with the combined reagent was used to make photometric reading for the calibration curve. A straight line graph was plotted that passed through the origin (graph of absorbance vs. phosphate concentration) (US EPA, 1984).

Total phosphate calibration calculation = Absorbance X y ± a = answer X 2

Where y = 3.6828, a = 0.0217, X 2 = diluting titre volume to 100 ml.

2.3.9. Determination of Total Organic Carbon in Experimental Soil

The determination of total organic carbon was measured by weighing 10g of the sample into a digestive flask. 25ml of chromic acid mixture (10 ml 1M $K_2Cr_2O_7$ and 20 ml concentrated H_2SO_4) was added to the sample in the flask. The flask was heated on a digestion rack for 2 hours. The mixture was allowed to cool and was filtered through a whatman filter paper (125 mm). The filtrate was diluted to 100 ml with distilled water. Then 5ml of phenolphthalein indicator solution was added and a thick bluish colour was developed. The solution was titrated with 0.4M ferrous ammonium sulphate solution, until a greenish colour was produced. The volume of 0.4M ferrous ammonium sulphate solution gave the titre value. Total organic carbon was determined as follows:

%C (mg/L) = 27.5 – titre value X 0.12/ weight of sample digested.

The C: N: P analysis on all samples was conducted by Waterlab (Pty) Ltd. The procedures followed were standardised method for C: N: P analysis (US EPA, 1984).

2.4. Determination of concentration of Selected PAHs present in Oil Sludge

Oil sludge (5 g) was weighed and placed in an extraction thimble and was extracted with 50 ml of dichloromethane for 60 minutes in the boiling extraction solvent. The thimble with the sludge sample was then raised into the rinse position and extracted for another 60 minutes. The extract was concentrated to 1- 10 ml. The method for the blank was included following the extraction procedures. The solvent rinsing or extraction, prior to the use, was performed to eliminate or reduce interferences.

The extract was analysed using Gas Chromatography- Mass Spectrometry (GC/MS) following the US EPA method 8270. The semivolatile organics concentration was determined by GC/MS. The GC was an Agilent 7860/5975C inert mass-spectrometry-detector (MSD) (manufactured by Agilent Technologies Canada) with helium 30c/s constant flow as a carrier gas and fitted with an Agilent HP-5ms Ultra inert 30m capillary column with 0.25 mm internal diameter and 0.25µm film thickness and mass spectrometry detector. Two temperature programmes were run in order to obtain a good separation and quantification of the more volatile compounds. The table below shows the GC/MS conditions for the analysis of the semivolatile organics present in the oil sludge (US EPA method 8270, 2007). The Restek cat no. 8270-1 stock standard was used to prepare the calibration standard. The concentration of the stock standard was 1000ppm. Calibration standard of 10ppm, 30ppm and 50ppm were prepared and analysed using the GC/MS.

Table 2.0. Gas Chromatographic conditions for EPA Method 8270 calibration standard.

Instrument component ID	Description/condition of GC/MS
GC:	Agilent 7860/ 5975 MSD
Sampler:	Agilent 7683B, 5.0µL syringe (Agilent p/n 5181-1273) 1.0µL splitless injection
Carrier:	Helium gas 30 cm/s, constant flow
Inlet:	Splitless; 260^{o}C, purge flow 50mL/min at 0.5 min Gas saver 80mL/min at 3 min
Inlet liner:	Deactivated dual taper direct connect (Agilent p/n G1544-80700)
Column:	Agilent HP-5ms Ultra Inert 30m x 0.25mm x 0.25µm (Agilent p/n 19091S-433UI)
Oven:	40^{o}C (1min) to 100^{o}C (15^{o}C/min), 10^{o}C/min to 210^{o}C (1min), 5^{o}C/min to 310^{o}C, hold 8 min
Detection:	MSD source at 300^{o}C, quadrupole at 180^{o}C, transfer line at 290^{o}C, scan range 45 to 450 amu

2.4.1. Determination of Metals present in Oil Sludge

The metals present in the oil sludge were determined by concentrated acid digestion method (CADM) using strong acid (nitric acid) for digesting the oil sludge sample. The filtrate was measured for metals using the ICP as described in (section 2.3.3.) in soil characterization experiment.

2.5. Materials used for Composting

The cow, pig, horse, and poultry manures were characterized for carbon: nitrogen: phosphorus content following the procedures described in (sections 2.3.7, 2.3.8, and 2.3.9).

2.6. Preparation of Compost Mixture

Eight hundred grams (800g) of oil sludge was dissolved in 400 ml CCl_4. The mixture was mixed with 4kg (w/w) of homogenized soil. The mixture of soil + sludge + CCl_4 was allowed to air-dry at room temperature for about 2 hours to evaporate CCl_4. This also allows the hydrocarbons in oil sludge to penetrate the soil. Then, the mixture (4kg of soil + oil sludge) was mixed with the bulking agent (wood-chips) in a ratio of soil: bulking agent 1:2 (w/v), to improve aeration in the composting system. The mixture of soil + sludge + bulking agent was divided into 5 parts, 4 parts was used differently, each for different compost material (manure). 1 part was used for control. The control was a mixture of soil + sludge and bulking agent in the ratio of 1:2 without any manure. Then the soil + bulking agent was mixed with manures differently each in a ratio of soil/sludge + bulking agent: manure 2:1 (w/w). After mixing each with different manure separately, each was divided into 3 parts (triplicates) including the control, to get 15 bowls of compost and was set-up as static-compost pile.

Then, the 15 bowls of compost was incubated by covering the bowls with a black plastic, to maintain and control temperature, moisture and aeration at room temperature. Deionised water was added to the compost pile when necessary to maintain moisture level by not allowing the pile to get dried. The compost pile was turned once in every 3 days for 4 months and once in every 7 days for the remaining 6 months. This is for proper mixing and aeration during the composting period. The plastic was opened in small holes on top of about 10 places for aeration (carbon dioxide and oxygen flow). The experiment was incubated for a total of 10 months at room temperature in the laboratory at Chemistry Department, University of South Africa (UNISA). The static composting piles were monitored for temperature changes, moisture content

and CO_2 evolution to estimate the microbial activities at room temperature. Then, the isolation and identification of the degrading bacteria was done by biochemical tests (Holt, 1994; MacFaddin, 1980) and also by molecular techniques.

Fig. 2.0. The co-compost piles of oil sludge, soil, wood chips and animal manures incubated under laboratory conditions.

2.6.1 Determination of Temperature of Compost pile

The temperature was measured by dipping the thermometer in the middle of the compost heap to monitor the temperature changes daily for the first 7 days at the beginning of the composting. After which it was measured at interval of 3 days for 2 weeks, then it was measured at interval of 7 days for another 2 weeks. After then it was allowed for another 2 weeks. This procedure (1

week interval) was repeated for the period of incubation. During this period the compost was turned to maintain aeration and O_2 flow to make it an aerobic composting. All temperature measurements were taken at noon.

Fig. 2.1. Determination of compost pile temperature during incubation.

2.6.2 Determination of Moisture Content of compost pile

The moisture content of the compost was determined weekly as described in (section 2.3.5.) and when necessary water was added to the compost pile to maintain moisture level and not allowing it to get dried; it was measured as adopted from Forster, (1995).

2.6.3 Determination of Ash Content of compost pile

The ash content was measured at the beginning and at the end of the experiment. Ash content of each compost pile was determined by weighing 10 g of each compost mixture separately into a preweighed crucible and reweighed before heating in a furnace at 400^{o}C for 6 h. The crucible and ash were cooled to a constant in a desiccator before being weighed again.

2.6.4 Determination of pH changes in compost pile

The pH of the supernatant of the compost mixture was measured at weekly interval in triplicate using the pH meter as described in Section 2.3.4.

2.6.5 Determination of Carbon dioxide Evolution and Oxygen Consumption

This was conducted by soil respiration experiments using the closed jar method (Atagana, 2008). Moist soil sample (50g) from the static-composting- pile experiments were placed in a beaker and were lowered in a glass jar, all in duplicates. A plastic vial containing 40ml of 0.1M sodium hydroxide solution was suspended from a tripod stand in each jar. The jars were tightly closed and incubated for 3 days at room temperature. The vials contents were transferred into 250ml Erlenmeyer flask and 2ml barium chloride solution was added to each flask. The mixture was titrated with 2M HCl. The controls (blank) were set up as described above but without the compost-soil samples.

The results were calculated as follows:

$\mu g\ CO_2\text{- } C/g/day = V_{sample} - V_{blank}\ X\ 2.2\ x\ 0.27/\ dwt\ X\ day\ X\ 1000$

Where V_{sample} is the titre value of HCl for the samples, V_{blank} is the titre value of HCl for the blank for each experiment, 2.2 is the conversion factor (1ml 0.1M NaOH = 2.2mg CO_2), 0.27 is the mg CO_2-C and dwt is the dry mass of the samples.

2.6.6. Statistical Analysis

The results obtained from the temperature, moisture content, pH measurements, respiration experiments were subjected to one way analysis of variance (ANOVA) to determine if there was a significant difference (effect) on the parameters measured in all treatments. Also to determine if time (months) had any significant difference on the parameters. The statistical analysis was tested using excel spread sheet at p=0.05.

2.7. Results and Discussion

2.7.1 Soil Characteristics

Table 2.0 below describes the characteristics of the top garden soil used in this study. The total organic carbon content of the soil was higher than the total organic nitrogen and total phosphorus. The initial soil pH was slightly acidic (5.56). The soil sample was a fertile soil as shown by the properties of the soil. It is rich in nutrients necessary for plant growth and microbial activities, contains sufficient minerals, organic matter that improves soil structure as well as soil moisture retention. A range of microorganism could be found in this soil sample because in this type of soil most biological soil activities occurs (Mann, 2008).

Table 2.1 Characteristics of garden soil used for the experiment

Soil parameter	Characteristics [Conc]
Sand [% wt]	61.3
Silt [% wt]	21.3
Clay [% wt]	9.3
Texture	sandy loam
pH (H_2O)	5.56
Total organic carbon in %[mg/l]	1.02
Total organic N [mg/l]	20
Total P [mg/l]	4.4
Cr [mg kg^{-1}]	121.7
Pb [mg kg^{-1}]	31.91
Ni [mg kg^{-1}]	10.13
Cu [mg kg^{-1}]	38.08
Zn [mg kg^{-1}]	9.65
Mn [mg kg^{-1}]	92.38
Fe [mg kg^{-1}]	67.04
Co [mg kg^{-1}]	2.45
Mg [mg kg^{-1}]	22.37
Dry matter content [% DM]	90.48
Moisture content [% MC]	9.52

2.7.2 Characteristics of Oil Sludge

The properties of the oil sludge before the composting period is presented in Table 2.2, 16 PAHs were present in substantial quantity both low and high molecular weight hydrocarbons with few metals. The hazardous components present in the oil sludge included acenaphthene, acenaphtylene, pyrene chrysene, benzo[a] fluoranthene, benzo[a]pyrene, indenol (1,2,3-cd) pyrene among others (Table 2.2). They are a family of compounds that cause the cancer risk. These PAHs do not act directly as carcinogens but reacts in the body together to form PAH epoxides that are the active carcinogenic agents (IARC, 2007, CONCAWE report, 2001, 2005, Bayoumi, 2009). However, many components of oil sludge were not detected because they were below the detection limits (<0.01mg/kg) by GC/MS.

Table 2.2 Characteristics of oil sludge (mg/kg) used for the experiment

Compounds	Concentration (Soxhlet extraction with dichloromethane)
Naphthalene [mg kg-1]	95.32
1-Methyl Naphthalene [mg kg-1]	205.81
2- Methyl Naphthalene [mg kg-1]	195.70
Acenaphthene [mg kg-1]	7.94
Acenaphtylene [mg kg-1]	5.05
Fluorene [mg kg-1]	23.11
Anthracene [mg kg-1]	40.44
Phenathrene [mg kg-1]	1.44
Pyrene [mg kg-1]	10.83
Benzo [a] anthracene [mg kg-1]	1.44
Chrysene [mg kg-1]	44.77
Benzo [b] Fluoranthene [mg kg-1]	21.66
Benzo [k] Fluoranthene [mg kg-1]	2.17
Benzo [a]pyrene [mg kg-1]	7.22
Indenol (1,2,3-cd) pyrene [mg kg-1]	5.78
Zn [mg kg-1]	8.33
Fe [mg kg-1]	46.35
Mg [mg kg-1]	22.37

2.7.3 Characteristics of the Animal Manure

The animal manures were rich in nutrients necessary for basic microbial activities. Nitrogen, carbon and phosphorus were in substantial amounts which were necessary to stimulate microbial growth and activities in the compost pile as shown in Table 2.3 below.

Table 2.3 Characteristics of animal manures used for the experiment

Animal manures	Total organic C [%]	Total N [mg/L]	Total P [mg/L]
Poultry	49.2	277	254
Cow	54.9	109	46
Horse	52.7	81	50
Pig	50.6	904	252

2.7.4. Changes in Temperature during Composting

There was an increase in temperatures in all the composts except the control experiment which contained no manure. There was no observed increase in temperature in the cow manure experiment except until the first month. The initial decrease in temperature in cow manure compost may be due to the fact that the compost retained much water than other compost. The excess water in the cow compost pile must have led to an anoxic condition which is a factor that limits microbial activities. During this period, the temperature fluctuated in the composting piles, with the cow manure compost pile decreasing during the composting period (Fig. 2.2). The fluctuating temperature of the compost treatments indicates that the level of microbial succession and activities in all composting piles was enhanced. The temperature in the control composting pile remained low during the treatment period. It may be due to lack of compostable materials which are necessary for microbial proliferation and to produce increase in temperature. In co-composting piles with horse, pig, poultry and cow manure it was observed that microbial activities was responsible for the increase in temperature. This increase in temperature also was anticipated to affect positively the biodegradation of hydrocarbons within the compost contaminated soil mixtures.

However, initial increase in temperature recorded in the poultry, horse, and pig manure (Fig. 2.2), co-compost mixture can be attributed to the increase in microbial activities. When temperature increases, microorganisms may utilise the hydrocarbon because a low temperature affects PAHs and they tend to be more viscous and their water solubility is greatly reduced (Leahy and Colwell, 1990). Low temperature will also affect microbial growth and proliferation (Potter *et al.*, 1999). With low temperature, the biodegradation of the target contaminants

decrease under normal circumstances (Gibb *et al.*, 2001). This will also result to decrease in enzymatic activities.

The temperature was observed to be stable (22^{o}C) in all the composting pile except for the poultry composting pile for the last 2 months (Fig. 2.2). This may be due to low availability of substrates to the microorganisms. The observed decrease and stability in temperature may also be due to low hydrocarbon concentration in the compost pile during the treatment period.

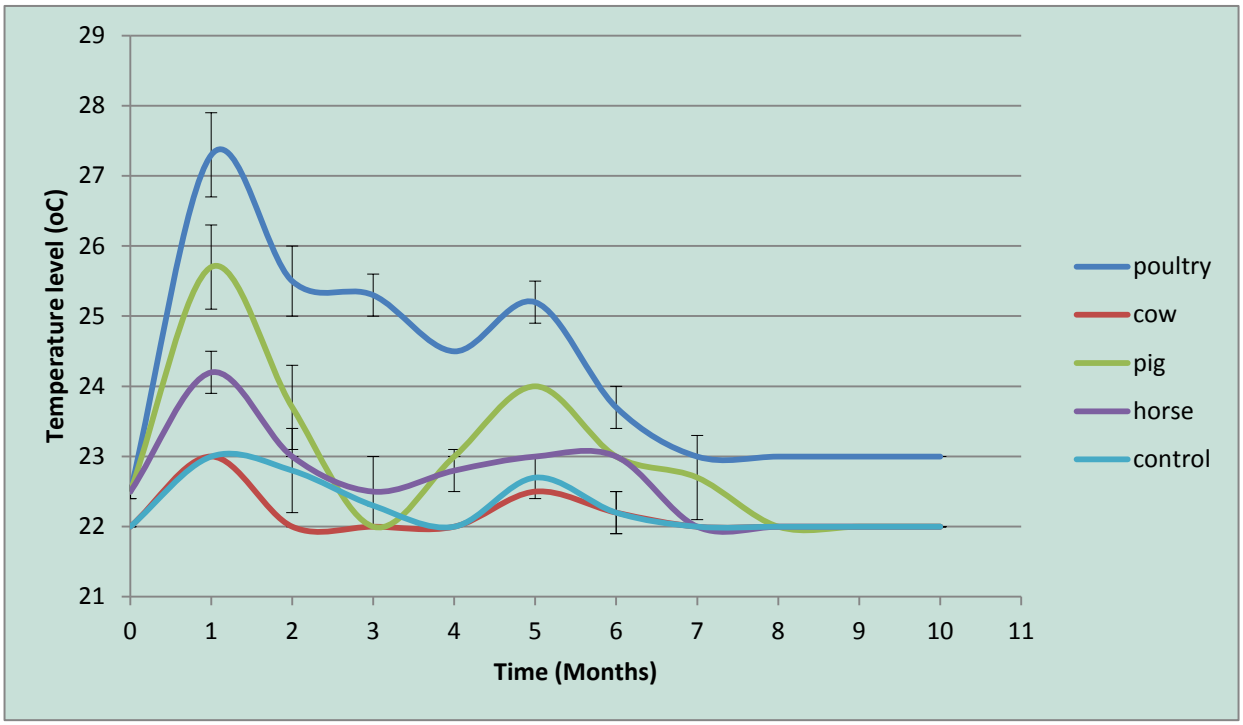


Fig. 2.2. The temperature of the composts during incubation of the co-composting of the contaminated soil. Values are mean of three replicates ± standard error for the compost piles**.**

2.7.5. Changes in Moisture content during Composting

The moisture and the dry matter content of soil used was 9.52% and 90.48% respectively. The water holding capacity of the soil was 32.62% (Table 2.0). The moisture levels in this experiment were observed to increase from the first to the eighth month and it was above 50% in all compost pile. This could be because the experiment was a closed pile system, moisture are trapped and returned back to the compost pile (Fig 2.0). The control set up did not exceed 44% within these months (Fig. 2.3). This agrees with the report by Alexander (1999), which states that water content between 50% and 80% enhanced the biodegradation of organic contaminants in soil. At this stage, microbial activities and growth are enhanced for the degradation of the target contaminant compounds.

Moisture enhanced microbial growth during the treatment period. Moisture has an advantage to the microorganism's activities in the soil. Adequate water supply within the soil-contaminated compost mixture enhanced biodegradation rate of the contaminants. This simply means that water is necessary not only to meet the physiological requirements of microorganism. It is also needed for the transportation of nutrients, metabolic by-products within and outside the microorganisms and for their activities. However, water is also needed to determine the oxygen status of the compost pile. Although, optimal moisture level for degradation of organic hydrocarbon compounds in compost pile vary from one soil type to the other (Baker and Herson, 1995).

In this study, the water content of the compost pile was measured weekly as described in (section 2.3.5.). Water was added to the compost pile when necessary and excess water was

avoided, as excess moisture can cause anoxic condition in the composting pile which in turn affects and limits the biodegradation of petroleum hydrocarbons.

In addition, aeration by turning the compost pile at interval of three days enhanced microbial growth and activities. The increase in microbial activities was reflected as an increase in respiration rate. This enhanced the decrease in concentration of the hydrocarbon contaminants. However, according to Gibson and Subramanian (1984), the oxidation of the hydrocarbon contaminants molecules requires molecular oxygen. Therefore, adequate amount of oxygen is necessary for bioremediation of petroleum hydrocarbon in the soil contaminated compost piles. In aerobic bioremediation, microorganisms responsible for the degradation of petroleum hydrocarbons in the soil compost pile require oxygen as their electron acceptor.

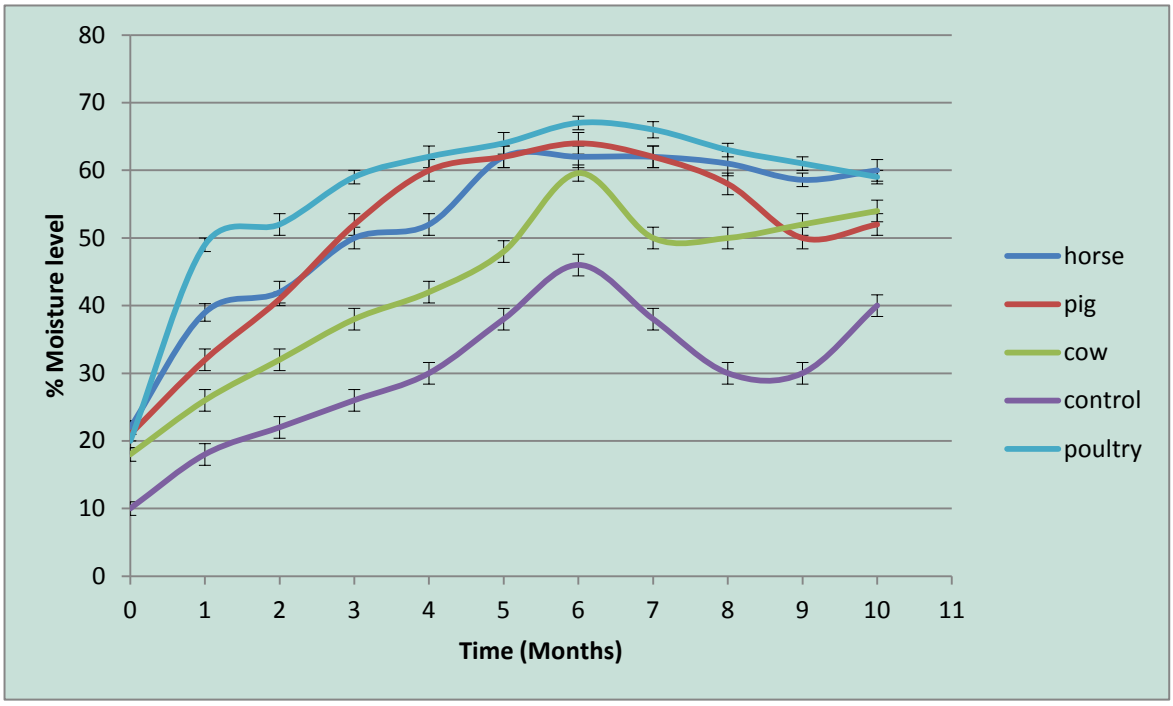


Fig. 2.3. The moisture level of the composts during the incubation of the co-composting of the contaminated soils. Values are mean of three replicates ± standard error for the compost piles.

2.7.6. Changes in pH during Composting

The soil pH before the treatment was 5.56 (Table 2.0). Naturally, most soils have an acid pH and it is necessary in bioremediation techniques to raise the pH to neutral or near neutral (Baker and Herson, 1995). The pH in all systems were observed to be increasing, including the control composting pile. The poultry composting pile pH increased from 5.9 to 7.9, the cow composting pile pH increased from 5.8 to 7.6, pig composting pile pH increased from 5.6 to 7.8, horse composting pile pH increase from 5.6 to 7.7 and in the control composting pile pH was 5.6 to 6.8. There was a sharp pH decrease after the fifth month in all treatment, then a slight increase was observed in the control, cow and horse compost pile. The poultry compost pile had a sharp increase to 7.7 while the pig decreased on the seventh month. It eventually became stable with little fluctuation during the remaining composting period (Fig. 2.4)

Biodegradation of organic contaminants in the soil has been reported to be faster at neutral or near neutral pH (Fu and Alexander, 1992). However, neutral pH or near neutral are more favourable to bacteria while fungi are known to be tolerant of acidic pH conditions (Al-Daher *et al.*, 1998).

The pH value were within the recommended pH range for composting organic compounds (van Hamme *et al.*, 2003). The increase in pH of the compost pile may be due to high content of ammonia from the manures. The decrease observed after the fifth month may be due to the degradation of the compost and the petroleum hydrocarbon contaminated in the soil. The decrease may be due to the release of intermediates and other products that have a low pH effect on the mixture according to Fava and Piccolo, (2002) and Lee *et al.*, (2007).

On the other hand, pH has effect on nutrients such as N and P in their solubility, bioavailability and chemical forms as well as the hydrogen and hydroxyl ions of the compost pile. The solubility of most metals are reduced at higher pH, therefore, their toxicity to the degrading microorganisms are also reduced (Winningham *et al.*, 1999). This reduced toxicity of metals allows microorganisms growth and activities that would have been inhibited.

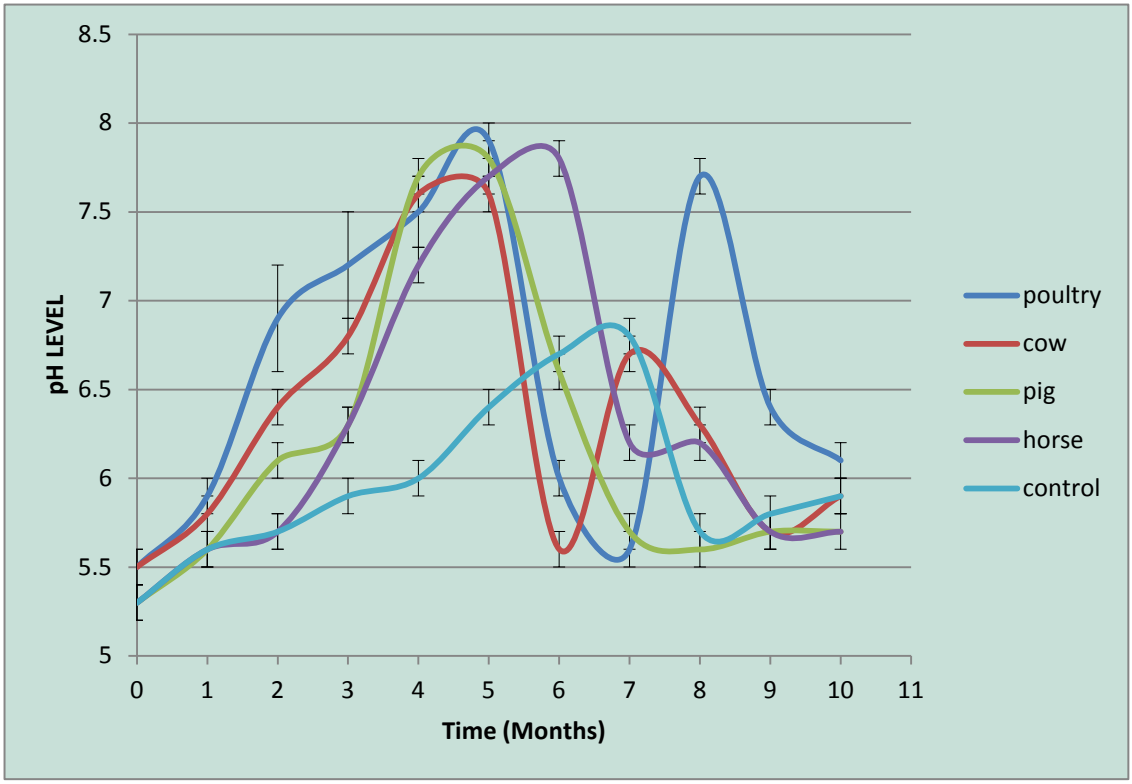


Fig. 2.4. The pH of the composts during the period of incubation of the co-composting of the contaminated soil. Values are mean of three replicates ± standard error for the compost piles.

2.7.7. Changes in Ash content of Compost pile

The ash content of the compost pile mixture showed that there were no significant difference from the initial soil-compost pile mixture and that of the end of the composting period (Table 2.5). Therefore, there were no significant changes in the mineral components of the soil at the end of the composting period. This further agrees that composting process do not alter the soil components after treatment (Atagana, 2008).

Table 2.4 Ash mass (g) initial stage and end-of-the composting period (10 months). Values are mean of three replicates ± standard error for the compost piles.

Soil-compost mixture	Initial	End
Poultry	4.03± 0.21	4.08± 0.16
Cow	4.01± 0.19	4.01± 0.20
Pig	3.77± 0.15	3.78± 0.14
Horse	3.34±0.04	3.36± 0.04
Control	4.04± 0.33	4.07±0.32

2.7.8. Respiration of Compost Organisms during Composting

The results showed that the respiration rate increased in the first six months in all the composting pile systems with the control set-up stable from the fourth month. The respiration rate of the soil microorganism decreased slightly afterwards with the horse and the control composting pile stable. This indicates the reduction in the microbial population by succession of mesophilic to thermophilic and availability of the target contaminants in the composting pile system. This also indicates that metabolic activities of the microorganisms contributed so much to enhance the reduction of the concentration of hydrocarbon contents in oil sludge in the compost pile mixture.

The control set up which had no manures showed increase in the respiration experiment. This may be due to fungi growth observed in the control compost pile system.

The results obtained from the respiration experiments showed that there was an increase in the microbial activities in the composting pile mixture (Fig 2.5). This may be due to the utilization of subatrates (nutrients and hydrocarbons) in the composting pile mixture by microorganisms. Carbon dioxide emission increased as the treatment proceeds and the composting piles were turned for aeration. This is the effect of oxygen consumption for the growth and activities of the degrading microorganisms. The decrease in the respiration rate observed towards the end of the treatment process may be due to the decrease in carbon from the oil-sludge components. This may have reduced the population of the degrading microorganisms present in the composting pile mixture. Respiration experiments have been used to study the aerobic biodegradation of contaminants in contaminated soils (Mahmoud, 2004). Naturally, biodegradation is a slow process in contaminated soil. Therefore, bioremediation is the process used to stimulate the microbial activities and growth in the contaminated soil to enhance contaminants degradation in no distant time. In this study, soil respiration experiments was helpful to quantify the effects of the nutrients and microorganisms from the animal manures as well as those from the soil in the bioremediation of the oil sludge.

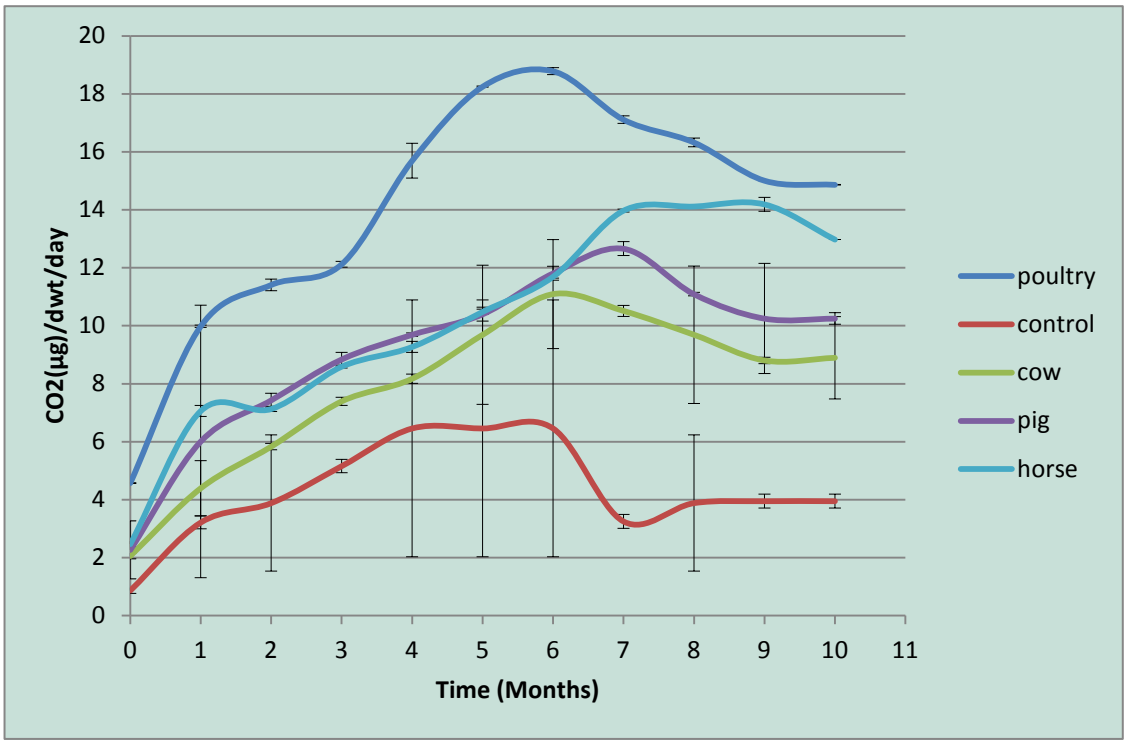


Fig. 2.5. The respiration rate of soil microorganisms in the composts during the incubation of the co-composting of the contaminated soils. Values are mean of three actual values ± standard error for the compost piles.

2.7.8. The Effect of Nutrients on Composting

The characterization of the soil sample showed that the total organic carbon content in the soil sample is 1.02%, total nitrogen 20% as well as total phosphorus 4.4 % (Table 2.0). The C: N: P ratio of the animal manures used in this study are shown in Table 2.2. The imbalance in nutrients ratio caused by the polluting carbon source affects the extent of hydrocarbon degradation in contaminated soil (Breedveld and Sparrevik, 2000). Nutrients encouraged the microbial growth and enhanced the utilization of the contaminant hydrocarbon in the soil composting pile (Atagana, 2003). Nitrogen and phosphorus are required for the growth of microorganisms. Nitrogen is needed for cellular protein and cell wall formation, while

phosphorus is needed for the nucleic acids, cell membrane and ATP formation (Swindell *et al.*, 1988). Therefore an adequate supply of these elements are needed for the active growth and metabolic activities of the hydrocarbon degrading microorganisms. However, high nitrogen concentration is required to facilitate effective compost bioremediation (van Hamme *et al.*, 2003). The results obtained from the characterization of the compost material used in this experiment showed a high nitrogen content in all the compost materials and in the soil sample used which may have helped to stimulate microbial activities (van Hamme *et al.*, 2003). The C : N ratios in the different compost pile treatment are expected to change with time as the incubation period proceeds. The C : N ratio in the treatment system may increase or decrease depending on the microbial activities and the release of ammonia. The moisture level, pH, temperature and carbondioxide evolution results obtained in this study showed that microbial activities in the compost pile were enhanced. Microbial activities could have enhanced the breakdown of the organic hydrocarbons substrates present in the composting pile system. This may have caused depletion of nutrients such as nitrogen in the compost system.

The results obtained from the characterized garden soil sample used in this treatment test showed that trace elements were present in the soil. The trace elements included copper, zinc, iron, chromium, lead, nickel, manganese, cobalt, and magnesium. Many of these metallic elements play an essential role in the function of microorganisms. However, excess of these essential trace metal elements and non-essential trace metal elements can be toxic to microorganisms. Environmental conditions such as pH also affects the presence of metals in soil. At low pH values, the presence of adsorbed extracellular polymer may enhance the adsorption of metals to surface (Roane *et al.*, 2001). The dissolved bacterial polymer may bind to trace metals in the aqueous phase and the adsorption of metals onto the soil may reduce at higher pH values (Todd

and Rania, 2003). This may occur when microorganisms use metals as electron acceptor (Roane *et al.,* 2001). Following the pH values obtained in the composting pile conducted in this study, metals present in the soil sample may not impose oxidative stress on the degrading microorganisms. Therefore, the inhibition of pollutant biodegradation through interaction with enzymes involved in the biodegradation may not occur.

The statistical analysis of the results obtained from the temperature, moisture content, pH measurements, respiration experiment of the compost pile using analysis of variance (ANOVA), showed that there was a significant difference ($p=0.05$) in the results obtained. This difference may be due to the microbial activities as the utilize nutrients (C: N: P), from the manures, oxygen consumption and reduction of the hydrocarbon content which affected these parameters in the composting piles. Time (months), also showed a significant difference ($p=0.05$) in these parameters. Time affected their changes. As time (months), increases these parameters decreases which may be due the low availability of nutrients and hydrocarbons to the microorganisms.

2.8. Conclusion

This study has shown that co-composting bioremediation under controlled conditions can enhance the growth and activities of microorgamism. Therefore, this techniques can be useful in remediating soils contaminated with oil sludge and soils contaminated with other organic compounds. This can be done using compost materials such as animal manures (poultry, pig, cow and horse). The treatment emulates nature and how nature does bioremediation with additional nutrient amendments. It was further explained in ash content results obatined in this treatment which showed that the soil mineral components did not change at the end of the experiment.

Changes in temperature and pH were within the accepted range in all the composting system with the compost materials used. There were no adjustments of pH using lime water to suite the experimental conditions. The adjustment in pH was by ammonia from the manure sources. Microbial activities were favourable in all the composting pile including the control compost pile system where fungi growth were active than other composting system. Fungi can degrade hydrocarbon even under acidic conditions. The bulking agent (wood-chip) played a vital role in the aeration of the composting system. This ensured maintenance of moisture level, pH, temperature, carbon dioxide evolution and oxygen consumption, making the static-pile an aerobic composting system. This enhanced the microbial activities in the system. The increase and decrease between the fourth and seventh months in all the parameters tested within the composting period showed that the reduction of hydrocarbon content may have been achieved within these period. The isolation and identification of the degrading bacteria using biochemical tests and molecular techniques are discussed in Chapters Three and Four.

CHAPTER THREE

Measurement of reduction in selected Polycyclic Aromatic Hydrocarbons (PAH) present in Oil Sludge during Co-composting with Animal Manures

3.1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) present in oil sludge are potent pollutants that consist of fused aromatic rings. They are environmental contaminants and are regulated as hazardous waste. These components of oil sludge (PAHs) can be broken down by the microorganisms found in the soil (Research triangle institute, 1999, Laskova *et al.,* 2007, Paulauskiene *et al.,* 2009).

Ouyang *et al.,* (2005), reported a study on oil sludge degradation using two bioremediation technologies which include comparison of bioaugmentation and conventional composting. Total hydrocarbon (THC) present in the oil sludge was 371.2g/kg and in oil polluted soil was 151.0g/kg. They were co-composted with straw, sawdust, top sand as well as pure soil in different proportions to oil sludge and soil. In composting, the sludge was mixed with crude manure and straw, the concentration of THC in oil sludge was 101.4g/kg. The experiments lasted for 56 days under the ambient temperature and THC in this study decreased by 31% - 53%. In the positive control which had only fertilizer the THC decreased by 13% – 23% while there was no degradation in the negative control which was just sludge and soil. The challenge in this study was that branched alkanes were resistant to microbial degradation than n-alkanes. This may be due to their molecular structure because large molecular weight hydrocarbons are less degradable than smaller ones (Ouyang *et al.*, 2005). In another study, *in-situ* bioremediation of petroleum waste sludge in Landfarming sites by Katsivela *et al.,* (2005). They (Katsivela *et al.,* 2005), reported the degradation of petroleum hydrocarbon by bacterial community within 14 months.

The analysis indicated a depletion of petroleum hydrocarbons approximately 75% - 100%. The challenge in this study was that the residual concentration of long-chain length n-alkanes was only slightly reduced. The reduction in PAHs between 40.3% and 81.1% was reported in a study of oil refinery sludge and green waste stimulated windrow composting (Fountoulakis *et al.*, 2009). Marin *et al.*, (2006), also reported 60% degradation of hydrocarbon content of oil refinery sludge mixed with wood shaving. In these studies, there was a weakness in the composting treatment. The residue quantity was highly recalcitrant to biodegradation due to possible strong biosorption to the solids (non-bioavailable fraction) (Leonardi *et al.,* 2007, Fountoulakis *et al.*, 2009). The biodegradation of total petroleum hydrocarbons in oil sludge was performed to assess the degradation potentials of the microbial species present in oil sludge through augmentation (Srinivasarao *et al.*, 2011). The optimum degradation of 89% TPH was observed at a concentration of oil sludge of 1g/L at pH 7, temperature 30^{o}C. In their study (Srinivasarao *et al.*, 2011), it was found that the microbial population was not adequate to degrade the oil sludge at a fast rate.

The analytical method commonly used for the residual concentration of petroleum hydrocarbons is solvent extraction and quantification techniques. This method can precisely be used to quantify hydrocarbon contamination in the environment. Gas chromatography (GC) coupled with a specific extraction techniques, can provide information on the product type relative to values from chromatogram with benchmarks. The use of GC techniques in the analysis of soil, water and sediments contaminants are growing and effective. The interesting aspect of this technique is that volatile and semivolatiles are determined separately. These volatile and semivolatile organic components of oil sludge are determined by the analysis of an extracts by GC/MS (US EPA method 8270, 2007). Analysis is expensive, time consuming and has limiting factors when

handling soil samples (Eriksson *et al.,* 2001*).* In recent years, a lot of research is aiming at faster extractions and analysis methods. These methods are such that can be used in screening techniques and is applicable for field analysis. In this case, the choice of suitable extractable solvents is important. The problem is that extraction solvents could overlap substances in a chromatogram and volatile substances may be lost during extraction (Eriksson *et al.,* 2001*).* Generally, solvents extraction processes are most attractive compared to other alternatives when the contaminant to be extracted is either volatile or semivolatile. This is because solvent extraction is suitable for volatile or semivolatile organic compounds with the most stable solvent (Eriksson *et al.,* 2001).

Automated soxhlet extraction method is one of the fast and effective extraction techniques. It is fast such that extraction is done within minutes and it is convenient such that it uses little quantity of solvents. Automated soxhlet extraction with GC/MS method is used to determine and quantify content of semivolatile compounds (PAHs) in oily sludge. This extraction technique is preferably based on the extraction efficiency, selectivity, its simplicity of operation, smallest amount of solvent used, extraction solvent, size of sample, rapidity, the ease of automation, cost and sample throughput (EPA method 3541). In most cases, this technique only requires a preconcentration step and not a cleanup step. Sample preparation and especially extraction is an important procedure in organic pollutant analysis. The preparation process needs to be adequately carried out with care because in many cases it is the origin of quantification errors (Doumenq *et al.,* 2004).

The aim of this study was to measure the reduction of selected polycyclic aromatic hydrocarbons contents of oil sludge spiked in soil which was co-composted with pig, horse, cow and poultry manures for 10 months.

3.2. Materials and Methods

3.2.1 Samples collected from the Compost piles

Samples were collected from the co-composted piles as described in Chapter Two (section 2.6). Analytes recovered from the samples were used to determine the reduction in selected PAHs present in the oil sludge.

3.2.2. Liquid Sample

3.2.3. Mineral Salt Medium (MSM)

Mineral salts medium (MSM) per litre of distilled water was prepared which contained; KH_2PO_4, 0.5 g, $MgSO_4$. $7H_2O$, 0.5 g, NaH_2PO_4. H_2O, 0.5 g, NH_4Cl, 0.5 g, NaCl, 4.0 g, the trace elements solutions contained in a (mg L^{-1} distilled water): $FeCl_2$ .H_2O, 1500 mg, NaCl, 9000 mg, $MnCl_2$. $4H_2O$, 197 mg, $CaCl_2$, 900 mg, $CoCl_2$. H_2O, 238 mg, $CuCl_2$. H_2O, 17 mg, $ZnSO_4$, 287 mg, $AlCl_3$, 50 mg, H_3BO_3, 62 mg, $NiCl_2.6H_2O$, 24 mg, Conc. HCl, 10 ml. The mineral salt medium was prepared in three stages; Stage 1; All the components of the medium except Na_2CO_3, $NaHCO_3$, and trace elements solution were dissolved in distilled water and diluted to 900 ml. This solution was dispensed into flasks and stoppered with cotton wool bungs wrapped in aluminium foil and autoclaved at 121^oC for 15 min. Stage 2:$NaHCO_3$ and Na_2CO_3 were dissolved in 97 ml of distilled water and autoclaved as in stage 1 above. One hundred ml of the solutions (MSM) were dispensed into 250 ml Erlenmeyer flasks under aseptic conditions using

sterile glassware on a laminar flow bench. Stage 3: Trace elements solution described above were filter sterilized through 0.2 µm Millipore filter membrane and 1 ml of each were added to the medium in the flask.

3.2.4. Enrichment Culture of Oil Sludge degrading Bacteria

In each of 250 ml Erlenmeyer flasks containing 100 ml sterile MSM, 15 g of compost sample was added. The flasks were spiked with 1ml oil sludge. The control flasks without oil sludge were also set up, the same as describe above and shown in Fig 3.1A. The flasks were stoppered with cotton wool bungs and aluminium foil and incubated in the dark at 30 ± 2^{o}C on a rotary shaker at 150 rpm for 21 days. All treatments were duplicated. Following incubation, 1 ml from each flasks were aseptically subculture into another set of 250 flasks each containing 100 ml sterile MSM, spiked with 1ml oil sludge as the only source of carbon for the bacteria growth, Fig 3.1B and incubated for a second 21 days at 30 ± 2^{o}C in a rotary shaker in the dark. The subculturing was repeated. At the end of each incubation period, samples were withdrawn from each flask for determination of concentration of the spiked oil sludge using gas chromatography/mass spectrometry.

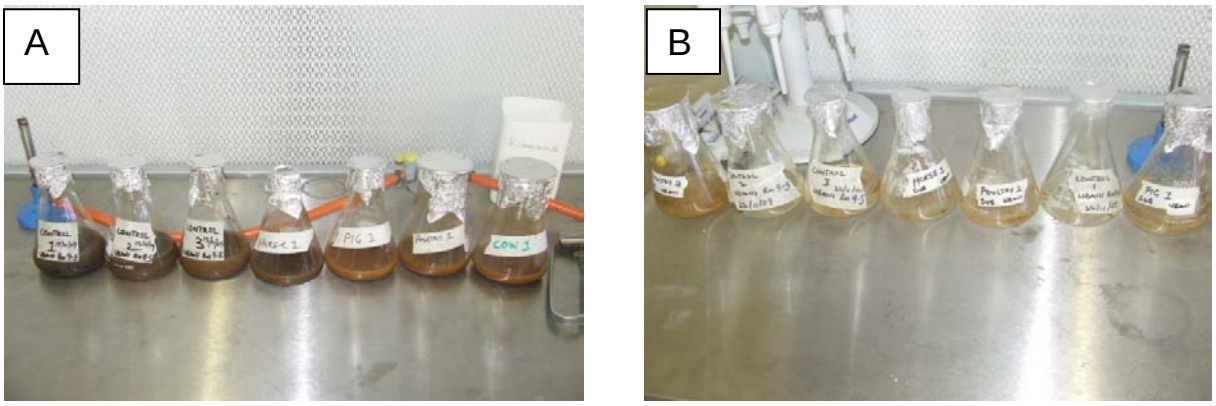


Fig. 3.1. A is the enrichment culture of oil refinery sludge degrading bacteria. B is the enrichment subculture of oil refinery sludge degrading bacteria.

3.3. Extraction of Polycyclic Aromatic Hydrocarbon from Compost Samples

The automated soxhlet extraction was performed using EPA method 3541 (Stewart, 1989; Lopez-Avila, 1991) for the extraction of organic analytes from composted soil sample and liquid samples. The method uses a commercially available, three unique stage extraction system to achieve analyte recovery in a much shorter time. Considering the time needed for extraction, to perform analysis and pretreatment of samples, automated soxhlet extraction EPA method 3541 was a good choice. The extraction time is short and has shown to be advantageous for handling large number of samples. The method was carried out in three stages which include extraction and rinsing, solvent evaporation and concentration of the extracts. The stages involve: (1.) the sample-loaded extraction thimble is immersed directly into the boiling solvent, this ensure very rapid intimate contact between the sample and solvent, which enhance rapid extraction of more than 70% of the organic analytes (Sun *et al.*, 2006). (2.) the thimble is elevated above the solvent and is rinse-extracted. (3.) the solvent is evaporated as the concentration step. Then the concentrated extract is used for the measurement of the organic analytes.

The automated soxhlet extractor was switched on and the cold water tap connected to the reflux condensers were opened. The flow of water was adjusted to 2L/min to prevent solvent loss through the condenser. 10g of the soil samples were weighed and were mixed with 10g anhydrous sodium sulphate in a beaker for easy precipitate (recovery) of the analyte. Then the samples were transferred into the cellulose extraction thimble (60mm x 26mm id). The thimbles were inserted into the condensers immediately and the knob was raised to the boiling position. The magnets were fasten to the thimbles and were placed in the rinsing position hanging just below the condenser valves. The extraction cups containing boiling chips were inserted each loaded with 50ml dichloromethane as the extraction solvent. (The choice for dichloromethane as the extraction solvent for semivolatile PAHs was because it is a common and stable solvent for extraction of semivolatile organic compounds (Jonker and Koelmans, 2002). Then, the locking handle was lowered using the holder, ensuring that the safety catch was engaged. At the moment, the cups were clamped into positions. The thimbles were immersed into the boiling solvent. The timer was set for 60 minutes and the condenser valves remained in the open position. The extraction was performed for the preset time. After 60 minutes, the extraction knobs were moved to the rinsing position making the thimbles to hang above the solvent surface. Then the timer was set for another 60 minutes and the condenser valves were left opened. Extraction was performed for the preset time. After the rinse time elapsed, the condenser valves were closed to allow solvents to evaporate, remaining about 5ml of solvent. The system was closed and the cups were removed. Then the content of the cups were transferred into 15ml graduated conical round bottom glass tubes. The cups were rinsed with dichloromethane; the rinsates were added to the conical round bottom glass tubes. The extracts were made up to 10ml using dichloromethane and

a portion was transferred to a GC vial. The vials were stored at $4^{o}C$ until analyses were performed by GC/MS. The extraction on liquid samples was carried out as described above.

The blank method was also performed following the same procedure as described above to ensure that all glass-wares and reagents were interference-free. This is as a safeguard against chronic laboratory contamination. The blank method was carried out through all stages of the sample preparation and measurements. This was important because of the possibility of interference being extracted from the extraction cup seal. All reagents used, were of analytical grade or the purest commercially available and was used without further purifications.

3.3.1. Determination of Polycyclic Aromatic Hydrocarbon from Compost Samples

The polycyclic aromatic hydrocarbons present in the extracts were quantified by GC/MS using US EPA 8270 (Bobak, 2010). The stock standard was restek cat No 8270-1 which contains semivolatile mix. It was purchased from Sigma Aldrich, South Africa. The concentration of the stock standard was 1000ppm and it was used to prepare the calibration standards of 10ppm, 30ppm and 50ppm. Working standard solution was prepared from the surrogate standard using dichloromethane. Calculation of the required concentrations was based on the chemical formula:

$C_1V_1 = C_2V_2$

Where C_1 = Concentration of stock solution, C_2 = Concentration to be made, V_1 = Volume to be determined, V_2 = Volume required.

These standards were first analysed using the GC/MS to register a known retention time to match with each compound.

The qualitative and quantitative analysis of the semivolatile compounds present in the sample extracts were carried out with the GC/MS Agilent 7860GC system and 5975C MSD, equipped

with a 7683B autosampler (Weavers *et al.*, 2006, Smith and Lynam, 2009). The sampler syringe was 5.0µl and splitless injection was 1.0µl. The carrier gas used was helium 30 cm/s and at a constant flow rate of 1ml/min. The inlet, splitless, 260^{o}C, purge flow was 50ml/min at 0.5 min and gas saver was at 80ml/min at 3min. Inlet liner was the deactivated dual taper direct connect. The column was Agilent HP-5ms ultra inert 30m x 0.25mm x 0.25µm film thickness. The oven program was started at 40^{o}C for 1minute to 100^{o}C (15^{o}C/min), 10^{o}C/min to 210^{o}C (1min), 5^{o}C/min to 310^{o}C and it was held for 8 minutes. The detection was MSD source at 300^{o}C, quadrupole at 180^{o}C, transfer line at 290^{o}C, scan range 45 to 450 amu. The vials were amber screw top glass vials and the vial cap was blue screw cap. The vial inserts were 100µl glass/polymer feet. The septum was advanced green. The ferrules were 0.4mm id short; 85/15 vespel/graphite. The magnifier was 20x magnifier loupe. This instrument works on principle that a small amount of liquid extract injected into the instrument id volatilized at the hot injection chamber. The volatilized molecules are swept by a stream of inert carrier gas through a heated column that holds a high boiling liquid as the stationary phase. As the mixture flows along the column, the components bombard each other at different rates between the gaseous phase, dissolved in the high boiling liquid and it is then separated into pure components. The compounds are passed through a detector which sends an electronic signal to the recorder which responds by peak formations. The peaks formed are quantified by mass selective detector using the retention time of the relative compounds registered from a known standard. PAHs are identified by retention times matching to standards concentration. The value of the chromatogram was quantified using peak area integration.

The extraction procedure and extract analysis on all samples was conducted by Waterlab (Pty) Ltd. The procedures described above and adopted from the standardized methods were followed (US EPA methods 3541 and 8270, revision no: 4, January 1998).

3.4. Results and Discussion

3.4.1. Reduction in Selected Polycyclic Aromatic Hydrocarbon from the Compost

The detectable PAHs recovery and quantification was achieved with automated soxhlet extractor coupled with GC/MS in a short time (LECO Corporation, EPA method 8270, Weavers *et al.,* 2006, Lynam, 2008, Lynam and Smith, 2008). These compounds recovered were both low and high molecular weight PAHs ranging from 2 to 6 fused benzene rings. They include naphthalene, 1-methyl naphthalene, 2-methyl naphthalene, acenaphtylene, acenaphthene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indenol(1,2,3-cd)pyrene.

In this study, most of the PAHs were not detected because their concentration were below the detection limits (<0.01mg/kg). The initial concentrations of the PAHs for the 2 to 6 rings detected were between 1.44 mg/kg to 205.81 mg/kg before the co-composting process of oil sludge. The results obtained showed reduction in selected PAHs (77% to 99.99%) in all co-composting piles over a period of ten months. This result is in agreement with the report from comparison of bioaugmentation and composting for remediation of oil sludge (Ouyang *et al.,* 2005). Percentage reduction obtained in this study are shown as follows; poultry (naphthalene 97.86%, 1-methyl-naphthalene 97.86%, 2-methyl-naphthalene 99.02%, fluorene 99.68%, anthracene 98.00%, pyrene 92.86%, chrysene 96.63%, benzo[b]fluoranthene 87.42%); horse (naphthalene 98.41%, 1-methyl-naphthalene 99.87%, 2-methyl-naphthalene 99.92%, fluorene

99.83%, anthracene 99.93%, pyrene 99.66%, chrysene 99.82%, benzo[b]fluoranthene 99.40%); cow (naphthalene 99.49%, 1-methyl-naphthalene 99.96%, 2-methyl-naphthalene 99.98%, fluorene 99.94%, anthracene 99.95%, pyrene 99.72%, chrysene 99.89%, benzo[b]fluoranthene 99.86%); pig (naphthalene 99.65%, 1-methyl-naphthalene 99.97%, 2-methyl-naphthalene 99.99%, fluorene 99.96%, anthracene 99.97%, pyrene 99.88%, chrysene 99.93%, benzo[b]fluoranthene 99.82%).

Table 3.1: Average final reduction of selected PAHs in Poultry, Horse, Cow, Pig and Control compost piles

Selected PAHs	Initial (mgkg^{-1}) for all	Poultry Ave. Final (mgkg^{-1})	% reduction on poultry	Horse Ave. Final (mgkg^{-1})	% reduction on horse	Cow Ave. Final (mgkg^{-1})	% reduction on cow	Pig. Ave. Final (mgkg^{-1})	% reduction on pig	Control Ave. Final (mgkg^{-1})	% reduction on control
Naphthalene	95.32	21.24	77.74	1.52	98.41	0.49	99.49	0.33	99.65	0.01	99.99
1-methyl naphthalene	205.81	4.41	97.86	0.26	99.87	0.90	99.96	0.06	99.97	0.13	99.94
2-methyl naphthalene	195.70	1.87	99.02	0.14	99.92	0.05	99.98	0.03	99.99	0.07	99.96
Acenaphtylene	5.05	1.09	78.42	0.08	98.42	0.03	99.47	0.01	99.74	0.04	99.14
Acenaphthene	7.94	0.47	94.12	0.04	99.54	0.01	99.83	0.01	99.87	0.02	99.79
Fluorene	23.11	0.68	99.68	0.02	99.83	0.01	99.94	0.01	99.96	0.02	99.91
Anthracene	40.44	0.81	98.00	0.03	99.93	0.02	99.95	0.01	99.97	0.02	99.95
Phenanthrene	1.44	0.09	93.98	0.02	98.61	0.01	99.08	0.01	99.08	0.01	99.08
Fluoranthene	1.44	1.05	26.85	0.05	96.30	0.02	97.92	0.02	98.61	0.03	97.92
Pyrene	10.83	0.78	92.86	0.04	99.66	0.03	99.72	0.01	99.88	0.04	99.63
Chrysene	44.77	1.50	96.63	0.08	99.82	0.04	99.89	0.03	99.93	0.10	99.78
Benzo[a] anthracene	1.44	1.08	24.77	0.06	95.60	0.03	97.45	0.02	98.61	0.03	97.69
Benzo[b] fluoranthene	21.66	2.72	87.42	0.13	99.40	0.03	99.86	0.04	99.82	0.03	99.86
Benzo[k] fluoranthene	2.17	0.07	96.62	0.13	94.01	0.03	98.47	0.04	98.31	0.03	98.47
Benzo[a]pyrene	7.22	0.46	93.58	0.08	98.94	0.04	99.40	0.08	98.85	0.11	98.43
Indenol (1,2,3-cd)pyrene	5.78	0.72	87.54	0.06	99.02	0.01	99.83	0.03	99.42	0.05	99.19

The reduction in PAH in control compost pile were: (naphthalene 99.99%, 1-methyl-naphthalene 99.94%, 2-methyl-naphthalene 99.96%, fluorene 99.91%, anthracene 99.95%, pyrene 99.63%, chrysene 99.78%, benzo[b]fluoranthene 99.86%), which was as a result of the fungi action because fungi invaded the control experiment. The result in Table 3.1 is the calculation of the remaining PAH concentration in this analysis using the mean value of three duplicates for each sample. The detailed results for each PAH extracted and analysed using GC/MS are summarised

in Table 3.1. In each case, the individual PAHs mentioned above were identified and quantified. In poultry compost pile, percentage reduction calculated for Fluoranthene (26.85%) and Benzo [a] anthracene (24.77%) were suggested to be human error because the percentage reduction of the same compound were above 95% in other compost piles as shown in Table 3.1.

The results obtained from the compost piles have shown that composting can be used to degrade PAHs present in oil sludge (Whyte, 1997, Bengtsson *et al.,* 1998, Bastieans, 2000; Jose *et al.,* 2006, Meintanis *et al.,* 2006). Parameters measured in all compost piles attributed to the degradation of PAHs. The increase in temperature of the compost treatments indicates that the level of microbial activities in all composting piles was encouraged. In the co-composting piles with horse, pig, poultry and cow manure it was observed that microbial activities were responsible for the increase in temperature. This increase in temperature also affects the biodegradation of hydrocarbons within the compost soil mixtures. At increased temperature, microorganisms break down the hydrocarbon because of increase in the solubility of the target contaminants (Leahy and Colwell, 1990, Gibb *et al.,* 2001). The pH value were within the recommended pH range for composting organic compounds (near neutral, Fig. 2.4) (van Hamme *et al.,* 2003). The increase in pH of the compost pile may be due to high content of ammonia from the manures. Increased pH was more favourable to microorganisms for their activities. The results obtained from the respiration experiments showed that there was an increase in the microbial activities in the composting pile mixture (Fig 2.5). This may be due to the utilization of substrates (nutrients and hydrocarbons) which had positive effect on the degradation of PAHs by microorganisms. The quantification results for the bioavailability of the PAHs to the microorganisms are shown in Table 3.2. The initial concentration obtained of the spiked enrichment culture before incubation with the results obtained from the samples withdrawn from

enrichment culture and subcultured media showed that microorganisms were able to degrade them, suggesting the existence of active bacterial community in this period (Katsivela *et al.,* 2003).

Table 3.2: Average final reduction of selected PAHs in Poultry, Horse, Cow, Pig and Control compost piles withdrawn from the enrichment culture media

Selected PAH	**Initial ($mgkg^{-1}$) for all**	**Initial Culture.Ave. Final($mgkg^{-}$)**	**% reduction**	**Subculture Ave.final ($mgkg^{-1}$)**	**% reduction**
Naphthalene	95.32	19.06	80.00	31.77	66.67
1-methyl naphthalene	205.81	41.16	80.00	51.38	75.04
2-methyl naphthalene	195.70	39.14	80.00	48.93	74.99
Acenaphtylene	5.05	1.01	80.00	1.68	66.73
Acenaphthene	7.94	1.59	79.98	2.65	66.63
Fluorene	23.11	4.62	80.01	5.78	74.99
Anthracene	40.44	9.09	77.52	13.48	66.67
Phenanthrene	1.44	0.29	79.86	0.48	66.67
Fluoranthene	1.44	0.36	75.00	0.38	73.61
Pyrene	10.83	2.71	74.98	3.61	66.67
Chrysene	44.77	8.95	80.00	11.19	75.01
Benzo[a] anthracene	1.44	0.26	81.94	0.55	61.81
Benzo[b] fluoranthene	21.66	4.33	80.00	7.20	66.67
Benzo[k] fluoranthene	2.17	0.43	80.18	0.72	66.82
Benzo[a]pyrene	7.22	1.44	80.06	1.81	74.93
Indenol (1,2,3-cd)pyrene	5.78	1.16	79.93	1.45	74.91

3.5. Conclusion

In this study, GC/MS was used to analyse the co-composted soil sample extracts. The detection rate, coupled with the powerful data manipulation and interpretation algorithms of the GC/MS, provided full mass range analysis of complex mixtures of organic compounds in a very short time. Therefore, using the GC/MS determination, quantification of PAHs in the co-composted soil was achieved. The automated soxhlet extractor coupled with GC/MS has demonstrated the use of an Agilent J/W HP-5 ms Ultra Inert capillary GC column for low and high molecular weight PAHs (<0.01 to 205.81mg/kg). The results obtained have shown that this column is an excellent choice for SVOCs analysis. The percentage reduction in selected PAHs was highest in pig co-compost pile, followed by cow and horse co-compost pile; the reduction in PAHs for poultry co-compost pile was the least (Table 3.1). The lower percentage reduction in poultry co-compost compared to the other treatment could possibly be due to the fact that increase in temperature was rapid in the poultry compost which affected microbial growth and activities (Fig 2.2). However, co-composting with animal manures could be efficiently used for bioremediation of oil sludge polluted soils.

CHAPTER FOUR

Identification and Characterization of Oil Sludge Degrading Bacteria Isolated from Compost

4.1. Introduction

Microorganisms are important agents in the natural biodegradation of contaminants in the environment. They play significant roles in the detoxification of environmental pollutants. Culture-dependent method has been employed to determine the composition of the metabolically active bacteria that play important role in the degradation of pollutants. These bacteria are characterized using biochemical–based techniques and molecular-based techniques. The molecular-based techniques are used for the retrieval of 16S rRNA (rDNA) sequences. This has become the most important tool in the characterization of bacterial from environmental samples (Nogales *et al.,* 1999).

Cultivation methods such as, viable plate count and most probable number (MPN) techniques have been used for quantification of active cells in environmental samples (Torsvik and Ovreas, 2002). However, the results are always biased because the medium used in these methods may select for certain organisms. Furthermore, it may be difficult to distinguish cocci from small rods. In addition, some bacterial cells may be viable but not be able to replicate under stress conditions. These problems have been realised by observation that direct microscopic counts of bacteria in aquatics and soil habitats exceeds viable plate counts by several orders of magnitude (Torsvik and Ovreas, 2002). In most cases, conventional cultivation methods can detect only a small fraction of the organisms. However, in order to study in details the microorganisms that can be detected, the organisms are first isolated (Torsvik and Ovreas, 2002).

Molecular identification describes the microbial structure based on deoxyribonucleic acid (DNA) sequences recovered from the microorganisms. The DNA sequencing of biological samples involves five main steps. These include; extraction of DNA from the cell, amplification of the number of DNA molecules, purification of the amplified products, tagging the ends of the fragments with fluorescent dyes and reading off the nucleotide sequence from the ends of the fragments. The DNA is extracted directly from microorganisms, so that the cultivation bias is eliminated. Certain gene fragments of the different organism are then cloned or amplified by polymerase chain reaction (PCR) from the extracted DNA in order to determine their sequences. Therefore, gene coding for small subunit of ribosomal RNA (16S rDNA for bacteria and archaea) is most common for this purpose (Amann *et al.,* 1995). The advantages of the use of this gene are; all organisms harbour this gene and from these genes their evolutionary relationship can be deduced (Woese, 1987), a large number of sequences of different organisms are stored in the databases (Maidak *et al.,* 1999). Universal PCR primers can be designed using sequence DNAs in several highly conserved regions and bacterial cells can be detected by *in-situ* hybridization targeting abundant ribosome in cells. Using the 16S rDNA sequences, bacteria are classified in the phylogenetic group proposed by Woese (1987). The identification of natural population also follows this phylogenetics classification. Since these molecular methods are capable of detecting microbial population that are hardly detected by conventional culture-dependent methods (Wagner *et al.,* 1994), researchers have started to apply them in environmental biotechnology processes.

Bacteria capable of degrading a number of the various components of petroleum hydrocarbons (low/high molecular weight PAHs) have been reported (Katsivela *et al.,* 2003). However, there are few reports about the characteristic and identification of bacteria that can catabolise oil

sludge components of more than five fused benzene rings PAHs (Katsivela *et al.*, 2003). One of the objectives of this experiment is to identify the bacteria involved in the degradation of oil sludge (PAHs) during the composting period. This will allow rational manipulation of the bacteria towards better performance. Identification of active bacteria in the degradation of petroleum hydrocarbon was done using molecular methods.

4.2. Materials and Methods

4.2.1. Sample collection and preparation of Culture media for Isolation of Oil Sludge Degrading Bacteria

The compost pile was monitored for oil sludge biodegradation by microorganisms for 10 months at room temperature. At the end of the 10^{th} month, samples were collected at random from the compost piles for analysis. Enrichment cultures, isolation and identification of organisms from the compost pile were conducted. The microbial population isolated from the liquid enrichment cultures comprises a wide range of microorganisms such as bacteria and fungi adapted to growing on the hydrocarbon compounds. The bacteria was selected by plating out on the mineral salt medium agar plates as interest was focused on bacteria species only. Only bacteria that can grow and utilize oil sludge are expected to grow on this mineral salt medium agar plates.

4.2.2. Mineral Salt Medium. (MSM)

The mineral salt medium was prepared as described in Chapter Three (section 3.2.2) and was used for the enrichment culture.

4.3. Enrichment Culture of Oil Sludge Degrading Bacteria

The enrichment culture was conducted as described in Chapter Three (section 3.2.3) and the final subculture was used for the isolation and molecular identification of the oil sludge degraders.

4.3.1. Mineral Salts Agar (MSA)

Into 900 ml of mineral salts medium, 20g of bacteriological agar was added and the mixture autoclaved at 121^{o}C for 15 min. The medium was cooled to about 50^{o}C and 1ml trace element solution previously filter through 0.2 µm membranes (Millipore) was added and mixed before dispensing into Petri dishes under aseptic conditions. To each of the (MSA) plates 50 µL of filter sterilized oil sludge was added by means of a syringe fitted with a 0.2 µm disposable filter membrane and spread with a sterile glass rod spreader as the only source of carbon for the bacteria growth. The plates were allowed to stand on the laminar flow for 24 hours to check for any possible contamination, then, they were stored at 4^{o}C before used for isolation of the bacteria.

The nutrient agar plates were prepared by mixing 31g of the nutrient agar powder with 1 litre of distilled water and the mixture was autoclaved at 121^{o}C for 15 min. It was allowed to cool to 50^{o}C before pouring into Petri dishes to solidify and was stored first under the laminar flow for about 24 hours to check for contamination before storing them in cold room at temperature of 4^{o}C. The preparation methods of MSM, enrichment culture, MSA and isolation was adopted from Atagana, (2003) and Mashreghi and Marialigeti, (2005) and was amended.

4.4. Isolation of Degrading Bacteria from Enrichment Cultures

Oil sludge degrading bacteria was isolated from the enrichment cultures by serially diluting the culture to 10^{8} to reduce the bacterial load, using test tubes. Eight autoclaved test tubes were used for each sample. The test tubes were filled with 10 ml autoclaved distilled water and 1ml of the culture was transferred into the first test tube, using the 5ml micropipette and was vortex to mix, from the first test tube, another 1ml was withdrawn and transferred into the second test tube,

vortex to mix until the 8 test tube. 0.1 ml of each of the serial dilutions from 10^{-6} to 10^{-8} was plated out on the mineral salts agar plates (MSA) and each plate was overlaid with 50 µL of the oil sludge as described in (section 4.3.1). All experiments were duplicated for all the samples. The plates were sealed in plastic bags and incubated for 21-28 days at 30 ± 2^{o}C and checked daily for bacteria growth to avoid overcrowding. Distinct colonies was picked from the mineral salt medium plates (MSA) using a sterilised wire loop and was used to produce pure colonies on nutrient agar plates by serially diluting the colony to 10^{3} using 3 sterile eppendorf tubes. The colonies picked from the MSA plates were serially diluted by transferring the colony into 1ml sterile distilled water in the eppendorf tube by sterile wire loop and was vortex to mix. 0.1ml of the serially diluted colony from the third dilution was plated out by spreading on the nutrient agar plate and was incubated for three days at 30 ± 2^{o}C. All experiments were duplicated for all the samples. The colonies that grew on the nutrient agar plates were further purified using streaking method to produce single colonies. Further identification was carried out using the gram-reaction test and molecular techniques

4.4.1. Gram-reaction test

The gram-reaction test was first used to ascertain purity of the colonies before proceeding to do molecular identification. The gram-reaction test was carried out for morphological characterisation of the bacterial samples. The Gram positive bacteria retained the primary stain (crystal violet) causing them to appear violet/purple under a microscope. The Gram negative bacteria, did not retain the primary stain, rather retained the secondary stain, causing them to appear red/pink when viewed under a microscope. With this approach, bacterial isolates that appeared the same under the microscope were successfully screened for further molecular identification (Rollins and Joseph, 2000; http://www.life.umd.edu/classroom/bsci424).

4.5. Genomic DNA Extraction of the Bacteria Isolates

Due to the key nature of DNA to organism, knowledge of DNA is useful in practical and biological research on organisms. DNA extraction was conducted using conventional method (CTAB).

4.5.1. Nutrient broth

The nutrient broth was prepared by weighing 16 g of the nutrient broth powder into 2 litre bottle and 1000 ml distilled water was added. The mixture was autoclaved at 121 ^{o}C for 15 minutes. It was allowed to cool at room temperature and 10 ml each was dispensed into autoclaved test tubes with cap and was stored at 4 ^{o}C before use.

4.5.2. Tris ethylene diamine tetraactic acid buffer (tris EDTA)

The TE buffer contained in a stock solution 10mM tris HCl, pH 8.0 and 1mM EDTA. 1 litre working solution of 1XTE buffer was prepared by mixing 10ml TE buffer with 990 ml of autoclaved distilled water.

4.5.3. Sodium Dodecyl Sulphate (SDS)

The 10% SDS was prepared by dissolving 10g of SDS in 100 ml distilled water; the solution was autoclaved at 121 ^{o}C for 15 minutes and the solution was allowed to cool to room temperature before use.

4.5.4. Proteinase K

The 10ml stock solution proteinase K was prepared by adding 10 ml of storage buffer (TE buffer) in a 15 ml screw-cap tube and 100mg of proteinase K was added. It was mixed by inverting the tube until the crystals were dissolved. 1ml of the aliquots was transferred into a 1.5 ml microfuge tube and was stored at -20^{o}C before use.

4.5.5. NaCl solution

The 5M NaCl solution was prepared by dissolved 29.22g of NaCl in 100ml distilled water, the solution was autoclaved at 121^{o}C for 15 minutes and the solution was allowed to cool to room temperature before use.

4.5.6. Cetyltrimethyl Ammonium Bromide (CTAB)

The 10% CTAB solution was prepared by dissolving 10g of cetyltrimethyl ammonium bromide in 100ml distilled water and the solution was autoclaved at 121^{o}C for 15 minutes, the solution was allowed to cool to room temperature before use.

4.5.7. Phenol, Chloroform and Isopropanol

These chemicals were purchased from Merck chemical company. They were analytical grade reagents or the purest commercially available and were used without further purifications.

4.5.8. Ethanol

Ethanol (70%) was prepared by adding 30 ml distilled water to 70 ml ethanol to make up to a 100 ml.

4.5.9. RNase ready to use

This was purchased from Sigma Aldrich, South Africa and it was ready to use. The RNase in solution contains 50% glycerol, 10Mm tris- HCl and pH 8.0.

The colonies from the streaked plates were inoculated into the liquid nutrient broth in the test tubes, by carefully picking the colonies with sterile wire loop. The test tubes were capped and incubated for three days at 30 ^{o}C ± 2 and was observed for bacteria growth. The cloudy colour change and sediment observed at the bottom of the test tubes showed bacteria growth in the nutrient broth.

Then, genomic DNA extraction was conducted using the above prepared reagents with some eppendorf tubes. Each liquid culture in test tubes was vortex and 1.5 ml of the culture was transferred into a sterile eppendorf tube using a micropipette. The tubes were centrifuged at maximum speed 14000 rpm for 5 minutes. The supernatant was discarded and the tubes were refilled to another 1.5 ml mark with the liquid culture. The tubes were centrifuged again at maximum speed 14000 rpm for 5 minutes, the supernatant was discarded. The pellets in each tube were resuspended in 567µl of tris ethylene diamine tetraacetic acid buffer (tris EDTA). 30 µl of 10% sodium dodecyl sulphate (SDS) was added to each tube. Three microlitre of proteinase K (20 mg/ml) was added to the tubes to give a final concentration of 100 µg/ml proteinase K and 0.5% SDS. The tubes were inverted to mix thoroughly and were incubate in the Accu block digital dry bath incubator at 65 ^{o}C for 1hour, Fig.4.3. Then, the tubes were removed from the incubator, 180 µl of 5M NaCl solution was added to each tube and mixed thoroughly. Eighty microlitre of 10% CTAB solution was added to the tubes. The solution was mixed thoroughly and incubated in the digital dry bath for 10 minutes at 65 ^{o}C. Then, equal volume of phenol and

chloroform was added to each tube (400 ml each); the solution was mixed thoroughly by inverting the tube. The tubes were centrifuged at 14000 rpm for 15 minutes. Three hundred microlitre of the supernatant was transferred into a new sterile eppendorf tube using the micropipette and DNA was precipitated by adding 0.6 volume of cold isopropanol to each tube. The solution was incubated in ice at -20 ^{o}C for 1 hour. The precipitates were collected by centrifuging the tubes at maximum speed for 15 minutes and the supernatants were discarded. Two hundred microlitre of 70% ethanol was added to each tube to wash DNA pellets and were centrifuged at maximum speed 14000 rpm for 10 minutes. Then the supernatants were carefully removed and the tubes were left open for several minutes on the bench to air dry the DNA pellets. When the DNA pellets were dried, 100 µl of TE buffer were added to the tubes and were incubated in the digital dry bath incubator at 37 ^{o}C for 1hour to dissolve the DNA pellets. One microlitre of RNAase was added to the tubes and the tubes were incubated with the same conditions as described above (37 ^{o}C for 1 h). The addition of the RNase is to produce clean and RNA free DNA extracts. The dissolved DNA was stored in the refrigerator at -20 ^{o}C before use. All the steps in this DNA extraction protocol were carried out under aseptic conditions and on ice to avoid loss of the DNA during the extraction. The concentration of DNA extracted were analysed spectrophotometrically and by agarose gel electrophoresis. The DNA extraction CTAB method using the organic solvents as described above, proved to be very efficient in providing large amounts of DNA templates.

Fig 4.1. The Accu block digital dry bath incubator for incubation of DNA.

4.5.10. Preparation of Agarose Gel Electrophoresis

Electrophoresis is a technique used to separate and sometimes purify macromolecules such as nucleic acids that differ in sizes, charges or conformation. In nucleic acids which have a consistent negative charge imparted by their phosphate backbone, they migrate towards the anode in the electrophoresis gel tank. The TAE buffer was the liquid used because it provides ions to support conductivity for the migration of DNA in the gel tank and maintain the pH at constant value. The TAE buffer provides the best resolution for large DNA, this means that it

requires a lower voltage and more time but a better product is achieved. If water is used instead of the TAE buffer, there will be no migration of DNA in the gel but concentrated buffer (10Xstock solution) will generate enough heat that will melt the gel.

The TAE buffer contained in a stock solution 40mM tris HCl, 20mM NaOAc, 1Mm EDTA and pH 8.5. 1 litre working solution of 1XTAE buffer was prepared by mixing 100 ml TAE buffer with 900ml of autoclaved distilled water. Agarose is a polysaccharide extracted from seaweed. It is best used at concentration of 0.5% to 2% and the higher the agarose concentration, the stiffer the gel. This is because higher concentration of agarose allows separation of small DNAs and low concentration of agarose allows the determination of large DNAs. Therefore, 1% agarose was used in this study and was prepared by mixing 1 g agarose gel powder in 100 ml tris acetate EDTA buffer (TAE) in an Erlenmeyer flask. The mixture was dissolved by heating the Erlenmeyer flask in a microwave oven. The solution was allowed to cool 50 ^{o}C and it was stained by adding 1 drop of ethidium bromide solution. The ethidium bromide is a fluorescent dye that interposes between bases of nucleic acids and allows very convenient detection of DNA fragments in the gel. It is a known mutagen and was handled with care by avoiding physical contact, precautions was taken by wearing gloves. The gel tray was sealed and the gel comb was placed on top of the gel tray. The gel was poured on the gel tray to produce wells with the comb. Bubbles were removed underneath the comb and on the surface in the gel before it set.

After the gel solidifies at room temperature, the comb was gently removed straight up to avoid tearing the wells. The tray with the gel was placed into the electrophoresis gel tank. The gel was covered with enough 1XTAE buffer to 2mm depth in the tank. Bubbles were removed from the gel wells and 1kb DNA ladder ready-to-use of concentration 0.1µg/µl was used as the DNA

marker. The 1kb DNA marker contains storage and loading buffer 10Mm tris-HCl (pH 7.6), 10mM EDTA, 0.025% orange G, 0.005% xylene cyanol FF and 10% glycerol. This is designed for wide range of double- stranded DNA fragments on agarose gel. This was to check the DNA base pair size. It also contains three reference bands for easy orientation (6000, 3000 and 1000 base pair). The DNA marker (ladder) was loaded first in the first well. The negative control that contains only the loading dye and autoclaved distilled water without the DNA template was loaded in the second well. This was to check for contamination in the DNA extract. Gel loading dye (2X1ml 6X orange DNA loading dye) was supplied with the DNA marker. The gel loading dye contains 10mM tris-HCl (pH 7.6), 0.15% orange G, 0.03% xylene cyanol FF, 60% glycerol and 60% Mm EDTA. It contained dense glycerol that allows the samples to fall into the sample wells. The loading dye has one or two tracking dyes (bromophenol blue or orange G and xylene cyanol dyes) which migrates in the gel and allows visual monitoring of the electrophoresis. 1µl of gel loading dye was dispensed with a micropipette on the laboratory parafilm which was later mixed with 5µl DNA template sample. Then the samples mixed with the loading dye were loaded in the appropriate wells and were recorded accordingly. The electrophoresis tank was closed with the lid and the gel was allowed to run at 80 volts for 1hour. After 1 hour, the power was switched off and the gel tray was removed with the gel from the tank. The gel was viewed in the dark room under ultraviolet (UV) light and the results were recorded. Then the DNA extract was amplified for 16S rDNA using PCR technique as described below.

4.6. Amplification of the genomic DNA Extract using Polymerease Chain Reaction (PCR)

This is a technique used to amplify a specific region of DNA such as 16S rDNA across several orders of magnitude. It generates millions of copies of the DNA sequence. This method uses a thermal cycler which involves the cycles of repeated heating and cooling of the reaction for

melting and enzymatic replication of the DNA. Primers (short DNA fragments) contain sequences complementary to the target region, as well as DNA polymerase that are key components which enable selective and repeated amplification. As the PCR proceeds, the DNA generated is itself used as a template for replication, setting in motion a chain reaction such that the DNA is amplified. The basic PCR set up requires several components and reagents which are used for PCR master mix.

4.6.1. Dream Taq

Dream taq ready to use, is the DNA polymerase for all standard PCR application at concentration of 5µ/µl. It is used for higher sensitivity, and to generate long PCR products as well as high yields. One unit of the enzyme catalyzes the incorporation of 10nmol of deoxyribonucleotides into a polynucleotide fraction in 30 minutes at 70^{o}C. The enzyme contains 20mM tris-HCl at pH 8.0, 1mM DTT, 0.1mM EDTA, 100mM KCl, 0.5%(v/v) Nonidet P40, 0.5% (v/v) Tween 20 and 50% (v/v) glycerol. The enzyme activity is assayed in the mixture of 67mM tris-HCl (pH 8.8 at 25^{o}C), 6.7mM $MgCl_2$, 1mM 2-mercaptoethanol, 50mM NaCl, 0.1 mg/ml BSA, 0.75mM activated calf thymus DNA, 0.2mM of each dNTP and 0.4 MBq/ml[^{3}H]-dTTP.

4.6.2. 10 X Dream Taq buffer

It contains KCl and $(NH_4)SO_4$ at a ratio optimized for strong performance of Dream Taq in PCR application.

4.6.3. $MgCl_2$

The optimized 10X Dream Taq buffer is provided with $MgCl_2$ at a concentration 0f 20mM.

4.6.4. Deoxyribonucleotide triphosphate (dNTPs) mix

This is the mixture of all four nucleotide (dATP, dCTP, dGTP and dTTP) in equal concentration, each dissolved in highly purified water to give a final concentration 10mM of each (0.2ml) at pH 8.3.

4.6.5. Sterile sabax water (nuclease free water)

This is used in mixing all the PCR reagents and reaction setup.

4.6.6. PCR Primers

The two sets of primers used in this study were (a) 16S-P1 PCR

($5^{/}$AGAGTTTGATCCTGGCTCAG$3^{/}$) with 20 base length. This primer is usually in pellet form that was vortexed and to prepare a stock solution of 100µm, 374.05µl of sterile sabax water was added to the pellet in the tube. The working solution 10µm16S-P1 PCR was prepared by mixing 10µl primer stock solution with 90µl of sterile sabax water. (b) 16S-P2 PCR

($5^{/}$AAGGAGGTGATCCAGCCGCA$3^{/}$) with 20 base length. It is also in a pellet form which was vortexed and the stock solution 100µm was prepared by adding 313.87µl of sterile sabax water. The working solution of 10µm 16S-P2 PCR was further prepared by mixing 10µl primer stock solution with 90µl of sterile sabax water.

4.6.7. DNA Template

This is taken from the dissolved DNA extract from DNA extraction (as described in Section 4.5.) stored in the refrigerator at -20 ^{o}C.

The PCR reaction was set up in parallel, however, to reduce the number of pipetting error and contamination, PCR master mix was prepared. The preparation of the master mix was by mixing

sterile sabax water, buffer, dNTPs, primers and Dream Taq DNA polymerase. All solutions were placed on ice during the experiment. The sufficient master mix for the number of sample plus one extra was prepared for 50µl per PCR tube and for each sample reaction. The master mix aliquot was dispensed into individual PCR tubes and then the different DNA template samples were added to each tube. Then the PCR tubes were labelled. The control set up (negative control) was used to check for contamination in the master mix. It was a portion of the master mix reaction but without DNA template. The PCR reagents in each tube amounted to 50µl per reaction as follows: buffer 5µl, $MgCl_2$ 1.5µl, primer1 2µl (forward), primer2 2µl (reverse), dNTP mix 1µl, Dream Taq 0.25µl, sterile sabax water 36.25µl and DNA template 2µl. The volume of water may be reduced if the volume of DNA template needed to be increased to correspond to 50µl. Then, the reactions were placed in a thermal cycler. The PCR was performed using the recommended thermal cycling conditions. The conditions were (a) initial denaturation 10 minutes at 95^{o}C for 1 cycle. (b) Denaturation at 95^{o}C for 30 seconds, this causes DNA template melting by disrupting the hydrogen bonds between complementary bases, yielding single strands of DNA. (c) Annealing at 94^{o}C for 30 seconds, in this step, the temperature is lowered to allow annealing of the primers to the single-stranded DNA template. Stable DNA-DNA hydrogen bonds are formed when the primer sequence closely matches the template sequence. The polymerase binds to the primer-template hybrid and DNA synthesis begins. (d) Elongation at 54^{o}C for 2 minutes, at this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template 5$^{/}$ to 3$^{/}$ direction. This was done by condensing the 5$^{/}$-phosphate group of the dNTPs with the 3$^{/}$-hydroxyl group at the end of the nascent (extending) DNA strand. All steps in denaturation, annealing and elongation were for 35 cycles and (e) final elongation 10 minutes at

72^{o}C for 1 cycle; this was to ensure that any remaining single-stranded DNA is fully extended. The reaction was held at 4^{o}C for 1 hour in the thermal cycler. The PCR product was checked by running the product on agarose gel. The agarose gel was prepared as previously described above in section 3.5.10. The DNA marker (ladder) was first loaded in the first well and the negative control that contains all PCR reaction reagents without the DNA template was loaded in the second well. The negative control was to check for contamination in the PCR product. The volume of the ladder loaded (4µl) was the same amount of gel loading dye 1µl and 3µl of PCR product to be loaded. The 3µl of PCR product was mixed with the 1µl of gel loading dye. Then the samples were loaded in the appropriate wells and were recorded accordingly. The gel electrophoresis was allowed to run as previously described in section 4.5.10 and the gel was viewed in the dark room under ultraviolet (UV) light and the results were recorded. The PCR products was cleaned and sequenced as described in Sections 4.7, 4.8 and 4.9 below.

4.7. Clean-up of PCR product

4.7.1 NaCl solution

The 5M NaCl solution was prepared as previously described above in section 4.5.5 for DNA extraction protocol.

4.7.2. Polyethylene Glycol (PEG)

PEG 8000 (13%) was prepared by dissolving 13g PEG 8000 in 100ml distilled water, autoclaved at 121^{o}C for 15 minutes and was allowed to cool at room temperature before use.

The PCR products were cleaned to efficiently remove primers and salts by a simple polyethylene glycol (PEG) precipitation. This is for DNA sequencing application. The following was mixed in a sterile eppendorf tubes for each PCR sample; 20µl of PCR product, 20µl of 5M NaCl solution

and 160µl 13% PEG 8000. The mixture in the tubes was incubated on ice for 30 minutes. After, the incubation time, the tubes were centrifuged at 13,500 rpm for 30 minutes at 4^oC and the supernatant was removed using pipette. Then 200µl of 70% ethanol was added to wash the pellet and it was centrifuged at 13,500 rpm at 4^oC for 5 minutes. The supernatant was carefully removed using a pipette and pellets formed in the tubes were air dried for 30 minutes and were resuspended in 12µl of sterile sabax water. The cleaned PCR products were verified by running gel electrophoresis as described above in section 4.5.10 and was viewed in the dark room under ultraviolet (UV) light. The results were recorded.

4.8. Sequencing of the PCR products

The DNA sequencing is the process of determining the nucleotide order of a given DNA fragment. The sequence of DNA encodes the necessary information for organisms. Therefore, determining the sequence is useful in their fundamental identification.

4.8.1. Sequencing primer

The sequencing primer 16S-PA SEQ (5′CTACGGGAGGCAGCAG3′) with 16 base length was used. It is also in a pellet form which was vortexed and the stock solution 100µm was prepared by adding 361.79µl of sterile sabax water. The working solution of 2µm 16S-PA SEQ was further prepared by mixing 2µl primer stock solution with 98µl of sterile sabax water.

4.8.2. Big Dye

The big dye is a ready to use reaction mix which contains ampli Taq polymerase, standard dNTPs, ddNTPs, FS, rTth pyrophosphate, $MgCl_2$ and buffer.

All solutions were placed on ice. Then the PCR tubes were labelled and were placed on ice during the reaction mix. The sequencing reaction was set up by preparing a master mix. The sequencing reagents in each tube amounted to 10µl per a reaction as follows: The master mix contains 2µl of sterile sabax water, 2µl of big dye, 1µl of 5Xsequencing buffer, 1µl of sequencing primer and 4µl of cleaned PCR products. The volume of water may be reduced if the volume of PCR products needed to be increased to correspond to 10µl. The sufficient master mix for the number of sample plus one extra was prepared for 10µl per sequencing tube and for each sample reaction. The master mix aliquot was dispensed into individual sequencing tubes and then the different cleaned PCR products were added to each tube. The control set up was prepared the same as the reaction mix but without PCR products. As a negative control it was used to check for contamination in the master mix reaction. Then, the reactions were placed in a thermal cycler. The sequencing was performed using the recommended thermal cycling conditions. The conditions were; initial denaturation 5 seconds at 96^{o}C for 1 cycle, denaturation at 96^{o}C for 10 seconds, annealing at 50^{o}C for 5 seconds, elongation at 60^{o}C for 4 minutes. The successive rounds of denaturation, annealing and elongation of the mix results in incorporation of one of the four dideoxynucleotide (ddNTPs), each tagged with a different fluorescent dye in each extension products. All steps in denaturation, annealing and elongation were for 35 cycles. The reaction was held at 4^{o}C for 1 hour in the thermal cycler.

4.9. Clean-up of sequencing products

The sequencing products were cleaned to efficiently remove primer, dye and salts by a simple ethanol precipitation. It is for DNA sequencing application. Clean up by ethanol precipitation requires very precise ethanol concentrations and centrifugation times. This is because if ethanol concentration is too high then the left over sequencing chemistry (big dye) is precipitated along

with the sequencing product. This may result to dye blobs at the start of the trace. On the other hand, if the ethanol concentration is too low then a failed reaction (no signal) results. Therefore, the right concentration of ethanol, salt, incubation time and centrifugation time was used. In addition, care was taken during ethanol wash step in order not to lose the DNA pellet. This was achieved by centrifuging the second time before drying the pellets. The second time was with a freshly prepared 70% ethanol which was discarded immediately at the end of each centrifugation.

4.9.1. Sodium Acetate solution

The 3M sodium acetate was prepared by dissolving 24.609g of sodium acetate in 100ml distilled water and adjusted the pH to 4.6 using glacial acetic acid.

The following was mixed in the eppendorf tubes containing each sequencing sample; 10µl of sterile sabax water, 2µl of 3M sodium acetate solution at pH 4.6, and 50µl of 95% ethanol. The tubes were tilted to mix and incubated on ice for 20 minutes. After incubation time, the tubes were centrifuged at 13,500 rpm for 30 minutes at 4^{o}C. The supernatants were removed using micropipette. 250µl freshly prepared 70% ethanol was added and was centrifuged at 13,500 rpm for 15 minutes. The ethanol was removed immediately, because the pellets will loosen if the ethanol is left longer after centrifuging, the product may be lost. The pellets were air dried for 30 minutes, and were stored in the refrigerator at -20^{o}C until used for sequencing analysis.

4.10. Sequencing Analysis

The sequencing analysis was conducted using the automated DNA sequencer (Perkin-Elmer) which was carried out according to the manufacturers' instruction. This was done at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. The DNA

sequencer carries out capillary electrophoresis for size separation, detection, recording of dye fluorescence and data output as fluorescent peak trace chromatograms. The machine consists of two parts (a) the gel electrophoresis apparatus, which performs a classical separation of the molecules according to weight. (b) the data acquisition and analysis system, which comprises a moving module combining an argon laser and a charge coupled device (CCD) camera, a data collection software, a data analysis software and a MacIntosh computer. The sequencing reaction product was loaded in a single lane and the fragments are size-separated by the process of capillary. An electric field was applied between the two ends of the capillary. The DNA migrates through the capillary taken different time. This is because DNAs are negatively charged and the fragments vary in lengths. Then the different extension products, each labelled with a specific fluorescent terminator dye separates according to weight all along the electrophoresis. They come at the bottom of the gel and they walk through the read region. There, the laser of the moving module excites the fluorescent dye. Then each dye emits its specific signal which is collected by the CCD camera. The signal were recorded and treated by the data collection software.

A number of commercial and non-commercial software packages can trim low-quality DNA traces automatically. These programs score the quality of each peak and remove low-quality base peaks which are seen at the end of the sequence. The raw data appears as electropherograms which shows the fluorescence peak emitted by each base. The corresponding names of the base called by the machine software are also shown. The raw data (electropherograms) were edited by controlling and reshaping using the edit-view or autoassembler softwares. This was done by opening the edit-view and the sequencer data. The base was controlled by checking all along the sequence, if the base calling was correctly translated by the appropriate letter. If there is any

error, it was corrected and ambiguities were resolved. Then the sequences were trimmed by removing the beginning and ending parts which are unsecured. The edited sequences were saved in an exportable file format for blasting.

4.11. Basic Local Alignment Search Tool (BLASTing) of DNA Sequences

This is used to compare the DNA sequences with the known nucleotide sequences on the gene bank database. It was done on blast program under National Centre for Biotechnology Information (NCBI) link available on the internet. The edited data or sequences were copied in a fasta format form, which was pasted on the link and the program was allowed to run. This was to check and compare the sequences with those on the database. Results from the database sequences show alignments with different colours. The lists of sequences produced significant alignments, along with the scores and E values. The scores are functions of length of the match with the pasted sequence query and the quality of the match. The E value indicates the probability of a match happening by chance. The first entries at the top of the list are most likely to be related to the pasted sequences. This means that the higher the scores, the greater the quality of the match and the number of bases that matched.

4.12. Results and discussion

The aim of this study was to identify the active bacterial community present in the compost during compost bioremediation of oil sludge after ten months of incubation. Bacteria were isolated from the compost piles using mineral salt medium and mineral salt agar as a control for growth as described in sections 4.3, 4.3.1 and 4.4 above. The bacterial community isolated using this mineral base medium were only those that can utilize the hydrocarbons in the oil sludge as sole source of carbon and energy Fig. 4.5A. They were further identified and characterized using

the DNA extraction and PCR amplification of 16S rDNA–based sequencing analysis for the metabolically active member fraction of the bacteria.

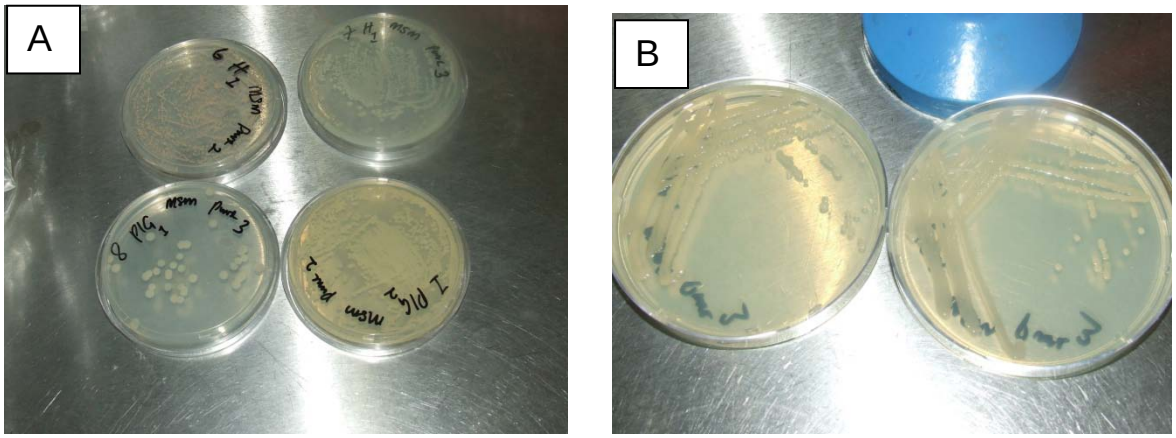


Fig. 4.2. A is the plates from the 10^3 serial dilutions bacteria growth that was plated out from the MSM agar plates. B is the plates of the further purification of colonies using streaking methods on nutrient agar plates.

The enrichment cultures were inoculated on 45 mineral salt medium agar plates following the duplication of the samples. All plates showed positive growth after incubation for 21 days at 28^{o}C, Fig. 4.5A. There was no growth observed until the 27th day on some of the mineral salt medium agar plates when inoculated with the serial dilution enrichment culture from the control sample set up.

The bacteria isolated includes both gram-positive and gram-negative bacteria. The results showed that the gram-positive bacteria were dominant at the tenth month in all the composting pile. The isolates were mostly short and long rods, a few cocci were also isolated (Table 4.1). This results shows that the soil samples contain more gram-positive than gram-negative bacteria that could be isolated (Wilson *et al.,* 1983; Smith *et al.*, 1985; Kampfer *et al.*, 1991). The

dominant gram positive bacteria isolated in this study could possibly be due to the manures and garden soil from a grazing field that was used.

Table. 4.1. The gram – reaction results as examined on the microscope under 100X objective lens. Bacteria morphology and arrangement from the pig manure sample.

Isolates from Pig samples	Morphology	Arrangement	Gram reaction
1	Short rod	cluster	+
2	Short rod	cluster	+
3	Short rod	cluster	+
4	Short rod	cluster	+
5	Long rod	diplo	_
6	cocci	single	_
7	Long rod	cluster	_
8	cocci	single	_
9	cocci	cluster	+
10	Long rod	cluster	_

Table. 4.2. The gram – reaction results as examined on the microscope under 100X objective lens. Bacteria morphology and arrangement from the cow manure sample.

Isolates from cow samples	Morphology	Arrangement	Gram reaction
1	Cocci	single	+
2	Long rod	Cluster	+
3	Long rod	cluster	+
4	Short rod	single	+
5	cocci	cluster	+
6	Cocci	single	+
7	cocci	single	+
8	Short rod	single	+
9	Short rod	single	+
10	Long rod	single	+
11	Short rod	cluster	+
12	cocci	single	_
13	cocci	single	+
14	Long rod	cluster	_
15	cocci	cluster	+
16	Long rod	single	+
17	Long rod	cluster	+
18	Long rod	single	_

Table 4.3. The gram – reaction results as examined on the microscope under 100X objective lens. Bacteria morphology and arrangements from the poultry manure sample.

Isolates from poultry samples	Morphology	Arrangement	Gram reaction
1	Long rod	single	+
2	Long rod	single	+
3	Long rod	single	+
4	Long rod	cluster	_
5	Long rod	cluster	+
6	Long rod	single	+
7	Short rod	cluster	+
8	cocci	chain	+
9	Short rod	cluster	+
10	Long rod	cluster	+
11	Long rod	cluster	+
12	Long rod	cluster	_
13	Long rod	cluster	+
14	Long rod	cluster	_
15	Long rod	cluster	+
16	Long rod	single	+
17	Long rod	cluster	+
18	cocci	cluster	+
19	Short rod	chain	+
20	Short rod	cluster	+

Table 4.4. The gram – reaction results as examined on the microscope under 100X objective lens. Bacteria morphology and arrangement from the horse manure sample.

Isolates from horse samples	Morphology	Arrangement	Gram reaction
1	Short rod	cluster	+
2	cocci	cluster	+
3	cocci	cluster	+
4	Long rod	single	+
5	Short rod	cluster	+
6	Long rod	cluster	+
7	Short rod	cluster	+
8	cocci	cluster	+
9	Long rod	diplo	_
10	cocci	chain	+
11	cocci	single	_
12	cocci	single	_
13	Short rod	cluster	+
14	cocci	single	_
15	Short rod	cluster	+
16	Short rod	cluster	+
17	cocci	single	_
18	Short rod	cluster	+
19	Long rod	single	+
20	Long rod	cluster	_
21	cocci	cluster	+
22	cocci	single	_
23	Long rod	single	+
24	Long rod	single	+
25	cocci	cluster	_
26	cocci	cluster	+
27	cocci	cluster	+
28	Long rod	cluster	+

Table 4.5. The gram – reaction results as examined on the microscope under 100X objective lens. Bacteria morphology and arrangement from the control samples without manure.

Isolates from control samples	Morphology	Arrangement	Gram reaction
1	Short rod	cluster	+
2	Long rod	single	+
3	Long rod	single	+
4	Short rod	cluster	+
5	Short rod	chain	+
6	Long rod	cluster	_
7	cocci	chain	+
8	Short rod	cluster	_
9	Long rod	cluster	+
10	Long rod	cluster	+
11	Long rod	cluster	+
12	Short rod	cluster	_
13	Short rod	cluster	_
14	Long rod	cluster	_
15	Long rod	cluster	+
16	Long rod	cluster	_
17	Long rod	single	+
18	Short rod	cluster	+
19	Short rod	cluster	+
20	Diplo	chain	+
21	Long rod	cluster	+
22	Short rod	cluster	+
23	Diplo	chain	+
24	Short rod	cluster	_
25	Short rod	cluster	_
26	Long rod	cluster	+
27	Long rod	cluster	+
28	cocci	cluster	+
29	Short rod	cluster	_
30	Long rod	cluster	+
31	Short rod	cluster	+
32	Short rod	cluster	+
33	Long rod	cluster	+
34	Short rod	cluster	+
35	Short rod	cluster	+
36	Long rod	cluster	+

A good amplification products were obtained during the PCR reaction Fig.4.6B. This may possibly be because growing (metabolically active) bacteria contain more ribosomes and rRNA than resting or starved cells (Nogales *et al.*, 1999). The primers amplified 1500 base pairs band of the 16S rDNA gene fragment using 3µl of the DNA template for the PCR reaction as described in section 3.6, (Fig.4.6B). Most of the PCR reaction did not work initially possibly because potential PCR inhibitors were coextracted with the DNA which inhibited the activity of Taq polymerase. It might also be due to non-amplifying DNA which may have disturbed the PCR detection of the gene. The PCR reaction was repeated for those that did not work initially.

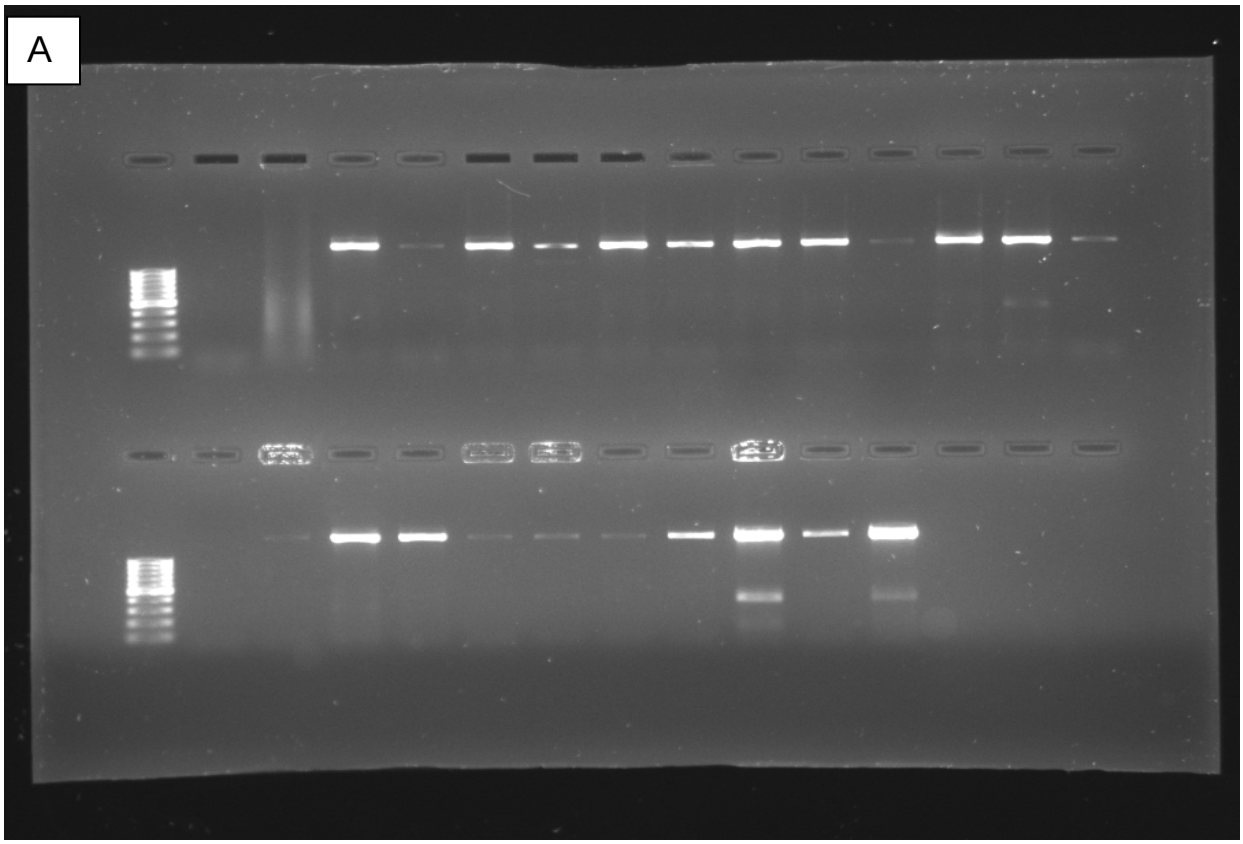


Fig 4.3. A, is the extracted DNA as viewed from the 1% agarose gel electrophoresis in the dark room under UV light, (Two combs were used in the gel to make more wells so as to run two sets of samples at the same time, bands with double strands were discarded due contamination).

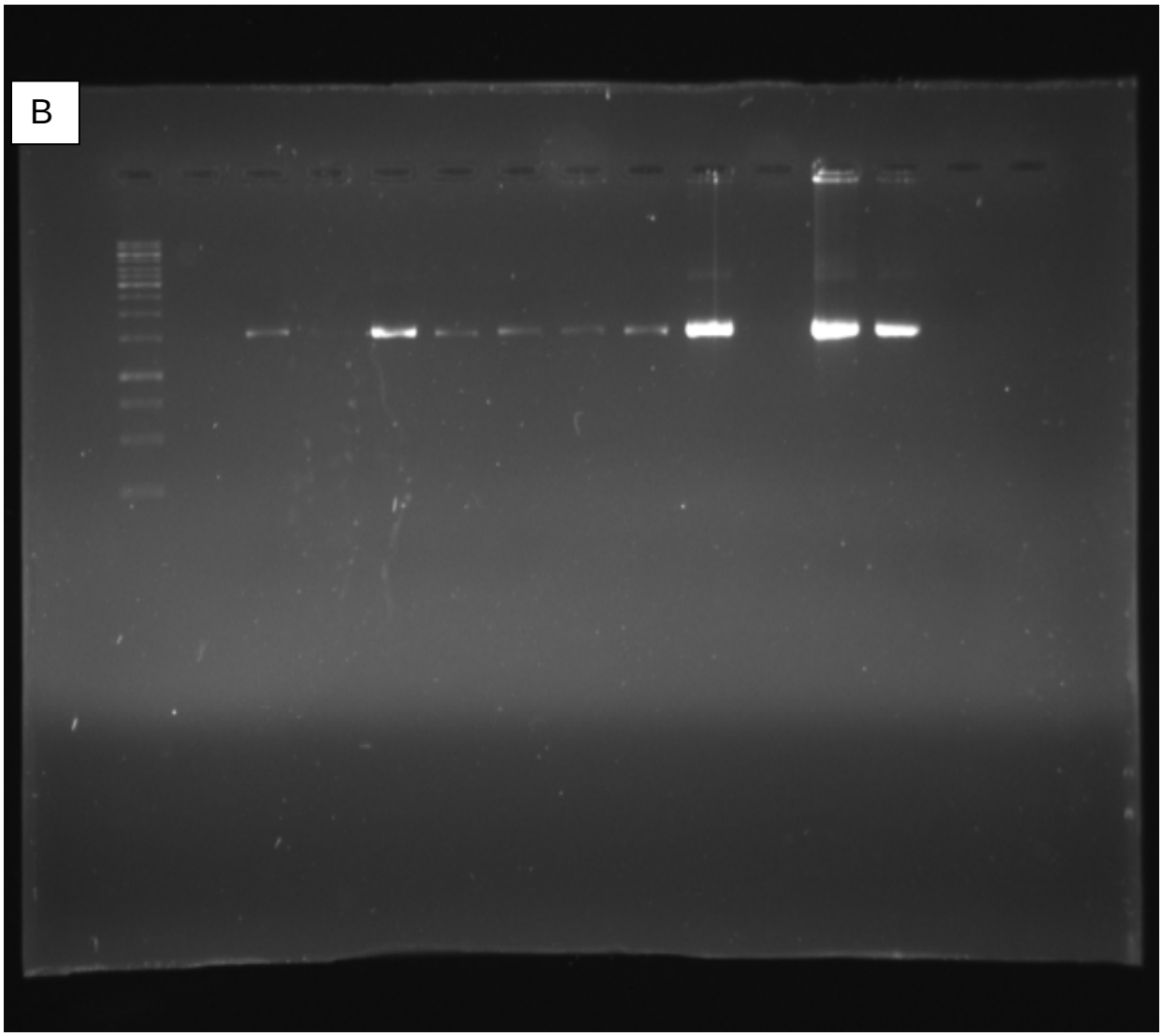


Fig 4.4 B, is the gel electrophoresis of the PCR product as viewed from the 1% agarose gel electrophoresis in the dark room under UV light.

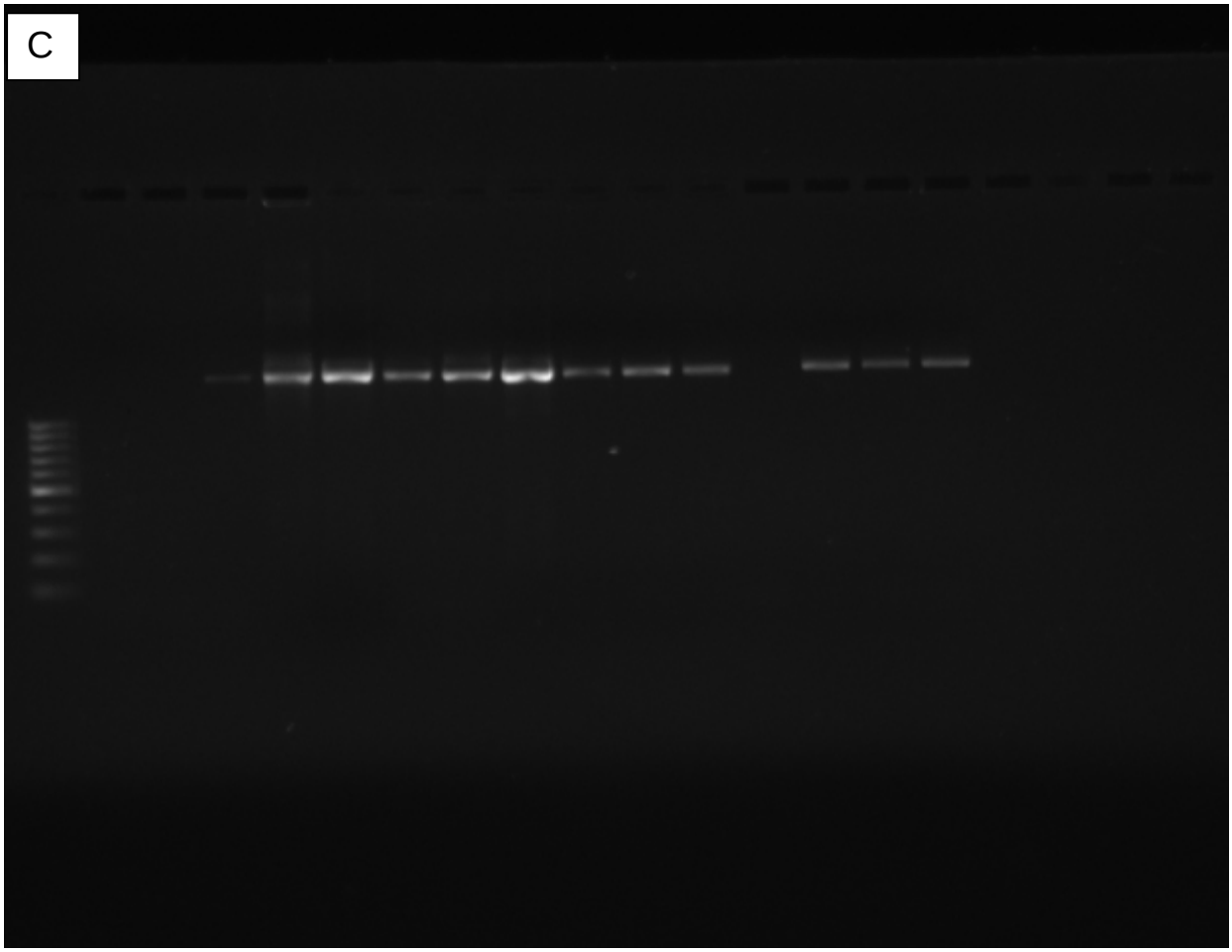


Fig 4.5. C, is the gel electrophoresis of the cleaned PCR product as viewed from the 1% agarose gel electrophoresis in the dark room under UV light.

The bacteria identified in the co-compost piles with pig manure include *Bacillus sp., Arthrobacter sp.* and *Brevibacterium frigoritolerans*. In the co-compost pile with cow manure bacteria identified include *Variovorax sp., Arthrobacter sp, Bacillus subtilis strain, Bacillus licheniforms strain, Staphylococcus succinus, Staphlococcus sp.,* and *Staphylococcus saprophyticus*. While in the co-compost pile with poultry manure the bacteria identified include *Paenibacillus sp. Bacillus sp., Bacillus licheniformis* and *Brevibacterium frigoritolerans.* In the co-compost pile with horse manure bacteria identified include *Bacillus circulan, Bacillus*

pumilus and *Arthrobacter globiformis.* In the control co-compost pile bacteria the bacteria include *Bacillus aryabhattai strain, Staphylococcus sp., Paenibacillus lautus strain, Ralstonia sp.* and *Geobacillus sp.*

The dominant bacteria species in all the treatment pile were the *Bacillus species*. The abundance of *Bacillus sp.* in all compost pile with manure and control set up without manure shows their indigenous origin from soil. *Bacillus* and other bacteria identified in this study, have been reported in different studies to be present in soils contaminated with petroleum hydrocarbon (Juhasz and Naidu, 2000; Kanaly and Harayama, 2000; Kanaly *et al.,* 2002; Bayoumi, 2009). They are also involved in the utilization of hydrocarbon in soil as their source of carbon and energy (Cooper and Goldenberg, 1987; Shimura *et al.,* 1999; Banat *et al.,* 2000; Kahng, 2002; Zhuang *et al.,* 2002; Prabhu and Phale, 2003; Toledo *et al.,* 2005; Das and Mukherjee, 2007; Bayoumi, 2009, Koukkou *et al.,* 2009).

Most of these bacterial strains identified in this study, are those that are efficient biosurfactant producers on petroleum hydrocarbon medium and in soil. The biosurfactants they produce can emulsify petroleum hydrocarbon in oil sludge so that they can be bioavailable to bacteria in the system. They do this by increasing the surface area of the substrates therefore, increased their solubility (Ahimuo *et al.,* 2000; Ron and Rosenberg, 2001; Maier, 2003; Mukherjee and Das, 2005). The production of biosurfactant is the advantage of continuous provision of natural, non-toxic and biodegradable surfactants by bacteria at low cost for solubilizing the hydrophobic oil sludge hydrocarbons during biodegradation (Calvo *et al.,* 2004; Bayoumi, 2009; Liu *et al.,* 2011; Plaza *et al.,* 2011). These biosurfactants secreted by bacteria are more effective than chemical surfactants in enhancing the solubility and biodegradation of petroleum hydrocarbons (Cybulski

et al., 2003; Wong *et al.*, 2004). The production of biosurfactant is proportional to the usage of hydrophobic PAHs substrates by the bacteria present in the system. Furthermore, there are several other factors that may be responsible for the uptake and high metabolism of the different PAHs in the compost system by these bacteria. These factors include growth condition of bacteria (pH, temperature), presence of a specific and higher amount of inducible enzymes secreted by the bacteria, substrate specificity of PAHs degrading enzyme and bacteria cell surface hydrophobicity (Gibson and Subramanian, 1984; Sharanagouda and Karegoudar, 2001; Mukherjee and Das, 2005) These factors helps the bacteria to utilize the PAHs because they are important survival tool for the bacteria. This is also related to higher breakdown and utilization of petroleum hydrocarbon by the bacteria strains (Ron and Rosenberg, 2001; Mukherjee and Das, 2005).

4.13 Conclusion

Studies on petroleum degrading bacteria are becoming more frequent due to their biotechnological importance to environmentalist and petrochemical industries. Hence, the studies on bacteria of petroleum contaminated sites is of high priority and the standard of an efficient protocol for the bacterial DNA extraction in such sites are necessary. This will be a good step for a detailed investigation and will help the improvement of bioremediation techniques of petroleum contaminants.

CHAPTER FIVE

General Discussion

Land and groundwater are important natural resources that should be protected and preserved in all parts of the world including South Africa. With the high rate of industrial activities and population growth, these two resources have been under increasing pressure of pollution over the past decades. Lack of legislation and standards defining acceptable disposal and treatment methods of pollutants has been an environmental challenge in past years. The South African National Environmental Management Act, no. 62 of 2008 have stressed on the minimization of pollution and protection of the environment by industries. Despite the regulation and guidelines on how to handle and manage environmental pollution, most industries pay little attention to safe disposal of hazardous waste (Quarterly Government Gazette No. 22, 2009).

In South Africa, refineries generate substantial quantity of oil sludge, a hazardous substance containing many known carcinogens (Bojes and Pope, 2007). In spite of the potential danger of oil sludge in the environment, pollution caused by poor handling has continued to increase because refineries hardly observe the regulatory legislation relating to oil sludge contamination in the environment. To the best of my knowledge, there was no record of any bioremediation project on oil sludge in South Africa when the present study started. One explanation for this may be that most of the work is performed by the refineries and they do not publish their results for proprietary reasons. It could also be that refineries cannot rely on bioremediation as a major treatment method because of the disparate results of bench-scale studies.

The main focus of this study was to develop a cost effective bioremediation technology for oil sludge. Although bioremediation technologies developed for the treatment of other contaminants

were adapted and optimized, the study further aimed at providing knowledge and information on the potentials of bioremediation for the treatment of oil sludge. Oil sludge used in this study was collected from one of the refineries in Durban, so as to provide useful information for the treatment of this oil sludge in South Africa.

5.1 Bacterial Degradation of Oil Sludge

Bioremediation techniques used in this study were compost bioremediation in which oil sludge was co-composted with various compostable materials in an *ex-situ* treatment system. A laboratory experiment was used to evaluate the potentials of composting and microbial bioremediation to treat the high molecular mass PAHs present in oil sludge. This technique is discussed in Chapters 1, 2 and 4. The biodegradation of PAHs entailed co-composting of oil sludge with animal manures (pig, cow, horse and poultry). It was observed that microbial activities in the compost mixture were responsible for the increase in temperature. When temperature increases, microorganisms degrade the hydrocarbon by utilizing the PAHs as sole source of carbon and energy. Moisture levels in this experiment were observed to increase above 50% in all compost pile except the control pile. At this stage, microbial activities and growth were encouraged. This simply means that water is necessary not only to meet the physiological requirements of microorganism. It is also necessary for the transportation of nutrients, metabolic by-products within and outside the microorganisms and for their activities. The observed pH value was near neutral which was more favourable to bacteria for composting organic compounds. Turning of the compost piles increased aeration in the system. Respiration experiments showed increased microbial activities. This may be due to bacteria utilization of nutrients from manures and hydrocarbons in the compost. The results from these parameters enhanced the degradation in concentration of the target hydrocarbon contaminants. Co-

composting of oil sludge with animal manures enhanced the reduction in PAHs, with pig manure compost pile performing better as compared to the other compost piles.

5.2 Recovery of selected PAHs from Co-composted Oil Sludge

Different treatments with animal manures enhanced the reduction in PAHs present in oil sludge. The initial concentration of the PAHs for the 2 to 6 rings detected was between 1.44 mg/kg to 205.81 mg/kg before the co-composting process of oily sludge. The results obtained showed the reduction in selected PAHs of all co-composting piles over a period of ten months. The reduction was between 77% and 99.99% in all the compost piles after the tenth month of incubation (Table 3.1). This experiment demonstrated that oil sludge can be degraded by co-composting with animal manures.

5.3 Isolation and Identification of Oil Sludge degrading Bacteria in the compost

It was observed that the oil sludge degrading bacteria isolates were both gram-positive and gram negative (shot/ long rods and cocci), Table 4.1, 4.2, 4.3, 4.4, 4.5, dominated by *Bacillus spp.* as described in Chapter Four section 4.12. This is evidence that *Bacillus spp.* among other bacteria identified are more tolerant to high level of PAHs and could be effective in treatment of oil sludge. The result obtained in this study showed that the microbial population exhibited good capability to degrade the selected PAHs. This was enhanced by the addition of animal manure which stimulated microbial activities in aerobic compost system. The ability of microorganisms to metabolise PAHs depends on the type, number of oil sludge-degrading microbes, nutrients, aeration and indigenous microbes in the system.

Another observation in this study is that applications of composting process to degrade PAHs present in oil sludge enabled the control of suitable operating parameter (temperature, moisture,

aeration and pH) to promote both microbial activities and contaminant biodegradation (Bayoumi, 2009). This application had two goals (1) to maximize degradation of PAHs; and (2) to produce residues that are not hazardous to the environment at the end of the process (CO_2 and H_2O).

The degradation of oil sludge in the compost system was expected since the animal manures used were rich in nutrients and has high microbial population. The microorganisms grew by utilising the nutrients present in the compost system. They readily metabolised the hydrocarbons in the compost system during the process. Animal manures were helpful in enhancing the degradation of oil sludge components (PAHs), and adequate ratio of contaminants to manure was essential for the biodegradation process. This is because higher mix ratio of organic amendments can inhibit the degradation rate while insufficient amount of manures may also retard the rate of biodegradation. Also, insufficient amount of manures may be less inoculum of microorganism and insufficient nutrient to stimulate microorganism growth and activities (Ling and Isa, 2006).

The temperature of the composting system was an important factor for biodegradation of oil sludge under laboratory conditions. At higher temperature, thermal desorption was expected. This must have helped to degrade contaminants in the compost system and making them bioavailable to microorganisms by transferring them to aqueous phase because it is easier for microorganisms to degrade organic contaminants in the aqueous phase. Therefore, it is expected that under normal full-scale operation, where the temperature would be higher, the increased bioavailability of organic contaminants would increase the amount as well as the rate of oil sludge degradation (Ling and Isa, 2006). This study has shown that there are much potential in using composting as a method for treating oil sludge or contaminated soil. The results showed that the soil used had a high amount of viable microorganisms (probably because of the site,

from which soils was collected). However, if the soil was not rich in microbes, effect of manure addition would be more pronounced. The encouraging results from this study supported the role of oil sludge degrading bacteria, as they could be successfully used in bioremediation and bioaugmentation procedures of soil treatments (Meintanis *et al.,* 2006). Particularly for the effective degradation of low and high molecular weight hydrocarbons (PAHs) present in oil sludge during composting processes. This is because these bacteria can exhibit high utilisation and cellular assimilation of the PAHs.

From the results obtained in this study, it can be concluded that a wide variety of bacteria identified are responsible for the degradation of the oil refinery sludge components in the compost piles. Since these bacteria can adapt, grow and survive in such compost systems, they may potentially degrade the oil sludge. The degradation of oil sludge is done through the production of enzymes, biosurfactants and using the hydrocarbons as source of carbon and energy to survive. Furthermore, the biosurfactant produced by these bacteria are capable of enhancing the solubility of PAHs in the media. As biosurfactants enhanced the solubility of PAHs, biodegradation rate of petroleum hydrocarbons (PAHs) increased in the media. Hence, there was 77 to 99% reduction of the PAHs as observed from the results obtained (Chapter Three, Table. 3.1.). Biosurfactants can also increase the cell surface hydrophobicity of the biosurfactant-producing strain that results in a high uptake of PAHs. This also means that as the cell surface hydrophobicity increased, there was bioavailability of PAHs in aqueous phase which made it easier for microorganisms to degrade organic contaminants (PAHs). This also helped to achieve 77 to 99% reduction of the PAHs as obtained in this study (Chapter Three, Table. 3.1.).

Therefore, it is concluded that co-composting may be suitable for practical field application for effective *in situ* and *ex-situ* bioremediation of oil sludge. It has been noted that co-composting process is an effective and controlled technology (with attributes such as nutrients, temperature, moisture, large population of microbes) for the degradation oil sludge. The results further agreed that composting process do not alter the soil components after treatment as shown in Section 2.7.7. At the end of the process, the residual products are not hazardous to the environment which is one of the advantages of composting process (UNIDOI, 2003).

5.4. Recommendations

It was noticed from previous studies that composting bioremediation tends to treat contaminants in a cost-effective and environmental friendly way, by utilising the removal efficiencies of its biological, physical and chemical process. This could possibly be through conversion of the oil sludge to CO_2 and H_2O. However, this aim may not be thoroughly achieved due to the limitations of the technique or the design applied. In many cases, an important fraction of the oil sludge and their metabolites remain untouched by the treatment process. The amount of contaminants residue remaining constitutes a major concern and source of debate in relation to risk assessment. Therefore, as composting techniques 'rely' on the biological process to remove or reduce the hydrocarbon content of oil sludge. There is a need to first gather and put into considerations all the information about the subsequent limiting factors during bioremediation (biological, chemical and physical limitation associated with composting), while looking for a way forward in the biotreatability studies. The limiting factors should help in the choice to design the process to optimise the treatment of oil sludge even after the removal of easily degradable constituents such as 2, 3 and 4 ringed PAHs. These limiting factors (time, nutrients, pH, moisture level, biodegraders, toxic metabolites), during composting processes should be investigated,

considered, adequately addressed and managed to optimise the biodegradation of both low and high molecular weight PAHs.

Following the conclusion drawn from this study, it is recommended that;

1. Composting techniques is cost effective and environmental friendly, which should be experimented on field-scales as a bioremediation process to treat sites contaminated with heavy deposit of oil sludge.
2. Composting should be combined with both bioaugmentation and biostimulation processes as a useful strategy for the degradation oil sludge. These could be used as a specific bioremediation process for practical field or full scale treatment.
3. Chemical surfactants may be added to composting process to support the biosurfactant produced by microorganisms to reduce the surface tension and increase the dissolution rates of total PAHs present in oil sludge. The application of biosurfactant and biosurfactant-producing bacteria in contaminated environments should be encouraged, as they are promising techniques in environmental biotechnologies. This is due to their potentials in biodegradability and low toxicity. Therefore, it is an important aspect regarding biological remediation technology to use biosurfactant in a large scale bioremediation process.

In all, research on (careful and controlled designed) composting process should be encouraged to achieve long term goals. Such goals includes reduced quantity of waste disposed of into Landfill, minimise greenhouse emission and cost of energy consumption during waste treatments in environments.

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APPENDIX 1

The sequencing blast results as compared on the NCBI gene bank (database)

Pig co-composting sequencing blast results

Sequencing code	Accession number	Description	Max score	Total score	Query coverage	E. value	Max identity
Pi1	JF508421.1	Bacillus Sp.Sd-20 16S rRNA gene	1367	1367	100%	0.0	96%
Pi2	DQ903961.1	Bacillus Sp.RM117 16S rRNA gene	1591	1591	98%	0.0	97%
Pi3	AY651317.1	Arthrobacter Sp.AGI 16S rRNA gene	1827	1827	100%	0.0	99%
Pi4	AM747813.1	Brevibacterium Frigoritolerans 16S rRNA gene, type strain DSM880	1062	1062	99%	0.0	99%
Pi5	DQ903961.1	Bacillus Sp.RM117 16S rRNA gene	1363	1363	100%	0.0	98%
Pi6	AM747813.1	Brevibacterium Frigoritolerans 16S rRNA gene, type strain DSM880	1044	1044	99%	0.0	99%
Pi7	DQ903961.1	Bacillus Sp.RM117 16S rRNA gene	1061	1061	100%	0.0	98%
Pi8	AM747813.1	Brevibacterium Frigoritolerans 16S rRNA gene, type strain DSM880	1061	1061	99%	0.0	99%
Pi9	HQ698840.1	Arthrobacter Sp.Mn5-7 16S rRNA gene	1062	1062	100%	0.0	99%
Pi10	Fm173523.1	Bacillus Sp. RM117 16S rRNA gene	1256	1256	99%	0.0	98%

Pi11	Fm173523.1	Paenibacillaceae bacterium CL2.1 partial seq 16S rRNA gene, isolate	1063	1063	99%	0.0	99%
Pi12	AY651317.1	Arthrobacter Sp.AGI 16S rRNA gene	1011	1011	100%	0.0	99%
Pi13	AM747813.1	Brevibacterium Frigoritolerans 16S rRNA gene, type strain DSM880	1066	1066	99%	0.0	99%
Pi14	GQ407190.1	Bacillus Sp.Sd-20 16S rRNA gene	1415	1415	99%	0.0	99%

Cow co-composting sequencing blast results

Sequencing code	**Accession number**	**Description**	**Max score**	**Total score**	**Query coverage**	**E. value**	**Max identity**
Co1	GQ478273.1	Variovorax Sp.B4M-V 16S RNA gene partial sequencing	1419	1419	100%	0.0	99%
Co2	HQ231927.1	Arthrobacter Sp. CNW2 16S rRNA gene	1247	1247	100%	0.0	96%
Co3	JF312740.1	Bacillus subtilis Strain ATF-4016S rRNA gene	1256	1256	100%	0.0	100%
Co4	EU257696.1	Bacillus licheniformis strain F1 16S rRNA gene	1247	1247	100%	0.0	98%
Co5	JF312740.1	Bacillus Subtilis Strain ATF-4016S rRNA gene	1496	1496	100%	0.0	99%

Co6	HQ018602.1	Staphylococcus Succinus subsp, succinus strain SSY00116S rRNA gene	1037	1037	100%	0.0	98%
Co7	HQ455044.1	Staphylococcus Saprophyticus strain CJ-5 16S rRNA gene	1142	1142	100%	0.0	99%
Co8	HQ327128.1	Staphylococcus Sp.TP-Snow-C19 gene 16S rRNA	1249	1249	100%	0.0	99%
Co9	HQ327128.1	Staphylococcus Sp.TP-Snow-C19 gene 16S rRNA	1288	1288	100%	0.0	99%
Co10	HQ018602.1	Staphylococcus Succinus subsp, succinus strain SSY00116S RNA gene	1018	1018	99%	0.0	98%
Co11	HM209761.1	Bacillus subtilis strain MJ01-PW1-OH-24 16S rRNA	1339	1339	100%	0.0	99%
Co12	HQ018602.1	Staphylococcus Succinus subsp, succinus strain SSY00116S RNA gene	1037	1037	99%	0.0	98%
Co13	GU982919.1	Baccillus Subtilis strainMUSc-1. W23, complete genome	1055	1055	100%	0.0	99%
Co14	JF312740.1	Bacillus subtilis strain	1496	1496	100%	0.0	99%

		ATF-4016S rRNA gene					
Co15	JF312740.1	Bacillus subtilis strain ATF-4016S rRNA gene	1256	1256	100%	0.0	100%
Co16	FJ655791.1	Bacillus licheniformis isolate D3A06 16S rRNA gene	1234	1234	100%	0.0	95%

Cow co-composting sequencing blast results continued

Poultry co-composting sequencing blast results

Sequencing code	Accession number	Description	Max score	Total score	Query coverage	E. value	Max identity
Po1	JF309262.1	Paenibacillus Sp.3504BRRJ 16S rRNA gene	1432	1432	99%	0.0	99%
Po2	JF768714.1	Bacillus Sp.PKS WIII 16S rRNA gene	1541	1541	100%	0.0	99%
Po3	EF173323.1	Bacillus licheniformis isolate MK19 16S rRNA gene	104	104	32%	2e-19	88%
Po4	FR749852.1	Bacillus simplex partial 16S rRNA gene,strain CNE22	1502	1502	100%	0.0	98%
Po5	AM747813.1	Brevibacterium frigoritolerans 16S rRNA gene, type strain	1068	1068	99%	0.0	99%

		DSM880					
Po6	JF508421.1	Bacillus Sp.Sd-2016S small subunit rRNA gene	1286	1286	100%	0.0	99%
Po7	JF309249.1	Bacillus Sp.3528BRRJ16S rRNA gene	1055	1055	99%	0.0	99%
Po8	HM755813.1	Bacillus Sp. MAN11 16S rRNA gene	1282	1282	100%	0.0	99%
Po9	FR749852.1	Bacillus simplex partial 16S rRNA gene,strain CNE22	1504	1504	100%	0.0	98%

Poultry co-composting sequencing blast results continued

Horse co-composting sequencing blast results

Sequencing code	**Accession number**	**Description**	**Max score**	**Total score**	**Query coverage**	**E. value**	**Max identity**
H1	FJ215785.2	Bacillus circulan strain 3399BRRJ 16S rRNA gene	1367	1367	100%	0.0	100%
H2	FJ215785.2	Bacillus circulan strain 3399BRRJ 16S rRNA gene	1369	1369	100%	0.0	99%
H2b	FJ215785.2	Bacillus circulan strain	1369	1369	100%	0.0	99%

		3399BRRJ 16S rRNA gene gene					
H3	CP000813.1	Bacillus pumilus SAFR-032,complete genome	1072	1.220et04	98%	0.0	99%
H4	CP000813.1	Bacillus pumilus SAFR-032,complete genome	1048	9187	98%	0.0	99%
H5	FN178364.1	Arthrobacter globiformis partial 16S rRNA gene OSB5	475	475	89%	1e-130	80%

Horse co-composting sequencing blast results continued

Control set up co-composting sequencing blast results

Sequencing code	**Accession number**	**Description**	**Max score**	**Total score**	**Query coverage**	**E. value**	**Max identity**
CT1	HQ284858.1	Bacillus aryabhattai strain 7L5 16S RNA gene	599	599	100%	4e-168	95%
CT2	HM566083.1	Staphylococcus Sp.08EPH15 16S rRNA gene	1238	1238	100%	0.0	99%
CT3	JF309264.1	Paenibacillus lautus strain 3566BRRJ16S rRNA gene	556	556	100%	3e-155	91%
CT4	HQ891976.1	Ralstonia Sp. SS33(2011) 16S rRNA	1048	1048	100%	0.0	97%

		gene					
CT5	CP001793.1	Geobacillus Sp. Y412 MC10 complete genome	601	7343	97%	2e-168	98%
CT6	JF309264.1	Paenibacillus lautus strain 3566BRRJ16S rRNA gene	551	551	100%	1e-155	91%
CT7	HQ284858.1	Bacillus aryabhattai strain 7L5 16S RNA gene	538	538	100%	8e-168	95%
CT7B	HQ891976.1	Ralstonia Sp. SS33(2011) 16S rRNA gene	1136	1136	99%	0.0	96%
CT8	HM566083.1	Staphylococcus Sp.08EPH15 16S rRNA gene	1472	1472	100%	0.0	98%
CT9	CP001793.1	Paenibacillus Sp. Y412 MC10 complete genome	601	7343	97%	2e-168	98%

Control co-composting sequencing blast results continued