# CHARACTERIZATION AND MOLECULAR DIVERSITY OF SOME SOIL PROKARYOTES WITH POTENTIALS FOR POLYCYCLIC AROMATIC HYDROCARBON DEGRADATION

**BY**

**HABIBA MUSTAPHA RAJI**

**DEPARTMENT OF MICROBIOLOGY**

**FACULTY OF SCIENCE**

**AHMADU BELLO UNIVERSITY, ZARIA**

**FEBRUARY, 2016**

# CHARACTERIZATION AND MOLECULAR DIVERSITY OF SOME SOIL PROKARYOTES WITH POTENTIALS FOR POLYCYCLIC AROMATIC HYDROCARBON DEGRADATION

**BY**

**HABIBA MUSTAPHA RAJI B.Sc. (A.B.U.) M.Sc. (A.B.U.)**

**Ph.D/Sci/02483/2009-2010**

**A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY IN PARTIAL FULFILMENT FOR THE AWARD OF PH.D MICROBIOLOGY**

**DEPARTMENT OF MICROBIOLOGY**

**FACULTY OF SCIENCE**

**AHMADU BELLO UNIVERSITY, ZARIA**

**FEBRUARY, 2016**

## DEDICATION

I humbly dedicate this thesis to my sweet mum, Hajia Hajara Iliyasu Atta, for her endless sacrifice in helping me achieve my goals.

## DECLARATION

I declare that the work in this thesis entitled, “Characterization and molecular diversity of some soil prokaryotes with potentials for polycyclic aromatic hydrocarbon degradation” had been performed by me in the Department of Microbiology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

| ____________________________ | ________________________ | _______________________ |
|---|---|---|
| Name of Student | Signature | Date |

## CERTIFICATION

This thesis entitled, "**CHARACTERIZATION AND MOLECULAR DIVERSITY OF SOME SOIL PROKARYOTES WITH POTENTIALS FOR POLYCYCLIC AROMATIC HYDROCARBON DEGRADATION**" by Habiba Mustapha RAJI meets the regulations governing the award of the degree of Ph.D. Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Prof. J.B. Ameh ______________________ Date ________________

Chairman, Supervisory Committee

Prof.S.A. Ado ______________________ Date_________________

Member, Supervisory Committee

Prof. S.E. Yakubu ______________________ Date_________________

Member, Supervisory Committee

Prof. I.O. Abdullahi ______________________ Date_________________

Head of Department

Prof. K. Bala ______________________ Date_________________

Dean, School of Postgraduate Studies

# ACKNOWLEDGEMENT

All praise to Allah for His countless bounties and blessings in my life, I pray Your light always leads the path in my life.

My special gratitude goes to my wonderful team of supervisors, Prof. J. B. Ameh, Prof. S. A. Ado and Prof. S. E. Yakubu, of the Department of Microbiology, Ahmadu Bello University, Zaria. Your collective effort in ensuring this research comes out in good quality is nothing short of amazing. Thank you so much. My profound appreciation goes to my supervisor in the United Kingdom, Prof. A.J. Weightman of the School of Biosciences, Cardiff University, Cardiff, Wales. Thank you for moulding my research to ensure it is of high standard.

I wish to acknowledge the management of Ahmadu Bello University, Zaria, for granting me fellowship to carry out the bench work of my research at Cardiff University. I also wish to appreciate the Petroleum Technology Development Fund, Nigeria for sponsoring my benchwork at Cardiff University, as part of the P.T.D.F Local Scholarship Award Scheme (L.S.S). I wish to thank the Science and Technology Post Basic scheme (STEP-B) in conjunction with the Federal Ministry of Education for the grant awarded me.

My appreciation goes to the following people for assisting me during the course of my research; Dr. Gordon Webster (School of Biosciences, Cardiff University), Dr. Michael Harbottle (School of Engineering, Cardiff University), and Prof. M.K. Usman (Head, Department of Plant Sciences, A.B.U., Zaria).

Special appreciation goes to my sweetheart, best friend and husband, Muhammad Mustapha Raji, your encouragement and support mean the world to me, you are a treasure. To the loves of my life, my children, Farida, Umar-Faruq and Feerozah, thank you for being my backbone and for always giving me a reason to smile even on very

difficult days. My darling mum, Hajia Hajara Atta, words could never express how fortunate I am to be your daughter. Your never-ending support and prayers got me where I am today, I pray I measure up to be even half the woman you are. Thank you to my late dad, Alhaji Iliyasu I. Atta, for instilling in me and so many others the value of good education, your legacy will forever live in our hearts. May Allah grant you “al jannatul firdaus”, Ameen. A very special thank you to my loving sister, Amina Atta for her valuable assistance all through my study. You are a true gem, May Allah reward you. And also to my brother, AbdulWahab Atta, I appreciate your timely support in helping me complete my study, God bless you. A million thanks to the rest of my wonderful siblings, Ibrahim, Mustapha, Suleiman (R.I.P), Aisha, Shehu (R.I.P), Nana-Hauwa and Salamatu; for their prayers and love through my study. I am very grateful to Barr. Talib S. Raji and Hajia R. Raji for their unwavering support and prayers, as well as the entire Raji family. I am very lucky to be a part of your amazing family.

I would like to say a big thank you to the following people who made a very significant impact on my research; Dr. (Mrs) M.A. Mukhtar of the Dept. of Microbiology, A.B.U., Zaria; Mr. Z.A. Kolawole of Total Ltd., Abuja; Mr. Abdullateef of U.B.A. Plc and Mr. Y. Adoke. May Allah reward you all for your priceless assistance through my research.

A thousand thanks to my colleagues at the Department of Microbiology especially, Dr. (Mrs) U.A. Asuke, Dr. (Mrs) S.L. Afegbua, Prof. (Mrs) H.I. Inabo, Prof. I.O. Abdullahi, Dr. A.B. Suleiman, Dr. D.A. Maccido, and Mr. Bishir Musa.

To my amazing friends, Mrs Sakeenah Nasir, Mrs Jamilah Makarfi, Arch. Zainab Sani, Arch. Hassana Gambo, Mrs Fatima Dasin, Dr. Fatima Ibrahim, Mrs Arwah Wali, Mrs Grace Oshagbemi, Mrs Maimuna Achimugu, and Mrs Zainab Achimugu. Thank you for always being there for me. God bless you all. And finally, a hearty thank you to Umar Musa for his assistance during my sample collection.

## TABLE OF CONTENTS

TITLE PAGE ---------------------------------------------------------------------- i

APPROVAL PAGE ---------------------------------------------------------------- iii

DEDICATION ------------------------------------------------------------------- iv

DECLARATION ------------------------------------------------------------------ v

CERTIFICATION --------------------------------------------------------------- vi

ACKNOWLEDGEMENT ------------------------------------------------------ vii

TABLE OF CONTENTS ------------------------------------------------------ ix

LIST OF TABLES -------------------------------------------------------------- xv

LIST OF FIGURES ---------------------------------------------------------- xvii

LIST OF PLATES------------------------------------------------------------- xix

LIST OF APPENDICES ---------------------------------------------------- xx

ABBREVIATIONS AND SYMBOLS ------------------------------------- xxii

ABSTRACT------------------------------------------------------------------------ xxiii

**1.0 INTRODUCTION ------------------------------------------------------- 1**

**1.1 Background ---------------------------------------------------------------- 1**

**1.2 Statement of Research Problem ---------------------------------------- 3**

**1.3 Justification --------------------------------------------------------------- 4**

**1.4 Aim ------------------------------------------------------------------------- 5**

**1.5 Objectives ------------------------------------------------------------------ 5**

**2.0 LITERATURE REVIEW ------------------------------------------------ 7**

**2.1 Polycyclic Aromatic Hydrocarbons ------------------------------------ 7**

**2.2 Polycyclic Aromatic Hydrocarbons: Source, Distribution and Characteristics ----------------------------------------- 10**

**2.3 Microbial degradation of PAHs in the environment ------------------ 11**

**2.4 Genes encoding PAH degradation in Bacterial species --------------- 16**

**2.5 PCR Primers used in the detection of Functional genes important in Bacterial degradation of High Molecular Weight-Polycyclic Aromatic Hydrocarbons -------------------------------------------------------------- 20**

**2.6 Metabolic Pathway for PAH Degradation in Bacteria ---------------- 24**

2.6.1 Pathway for the bacterial degradation of phenanthrene -------------- 24

2.6.2 Pathway for the bacterial degradation of chrysene ------------------- 24

**2.6.3** Pathway for the bacterial degradation of benzo[a]pyrene ----------- 25

**2.7 Identification of PAH-degrading Bacteria based on Non-culture Methods ---------------------------------------------------- 28**

2.7.1 Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) Analysis ------------------------------- 30

2.7.2 Single strand conformation polymorphism (SSCP) ------------------ 31

2.7.3 Terminal restriction fragment length polymorphism (T-RFLP) ----- 32

2.7.4 Amplified ribosomal DNA restriction analysis (ARDRA) and Amplified functional DNA restriction analysis (AFDRA) Profile --- 33

2.7.5 Microarray technologies ------------------------------------------------ 34

**2.8 Studies On Bacterial Degradation Of Polycyclic Aromatic Hydrocarbons In Nigeria ------------------------------------------------- 35**

**3.0 MATERIALS AND METHODS ---------------------------------------- 39**

**3.1 Sampling Sites and Soil Samples ---------------------------------------- 39**

**3.2 Physico-chemical Analysis of Soil Samples ---------------------------- 40**

3.2.1 Textural classification of soil samples --------------------------------- 40

3.2.2 Determination of pH --------------------------------------------------- 42

3.2.3 Determination of organic carbon --------------------------------------- 42

3.2.4 Determination of nitrogen in soil -------------------------------------- 43

3.2.5 Determination of phosphorus ------------------------------------------ 44

3.2.6 Determination of sodium, potassium, calcium, magnesium and lead - 44

3.2.7 Determination of some microelements --------------------------------------- 45

3.2.8 Determination of Cation Exchange Capacity ------------------------- 45

**3.3 Polycyclic Aromatic Hydrocarbon Analyses in Soil ------------------ 46**

**3.4 PCR-DGGE Analysis Of Bacterial 16S ribosomal RNA**

**Gene in Soil Samples ------------------------------------------------------- 47**

3.4.1 Extraction of DNA from soil ------------------------------------------- 47

3.4.2 PCR amplification and Denaturing gradient gel electrophoresis

analysis (DGGE) of partial 16S rRNA gene --------------------------- 48

3.4.3 Sequencing of DNA from excised DGGE bands and

phylogenetic analysis ----------------------------------------------------- 49

**3.5 Quantitative PCR (Real-time PCR) Analysis of**

**16S rRNA genes in Soil Samples ----------------------------------------- 50**

**3.6 Spiking Of Petroleum-Contaminated Soils with Polycyclic**

**Aromatic Hydrocarbons and DNA Extraction ------------------------ 52**

**4.0 RESULTS ------------------------------------------------------------------ 56**

**4.1 Textural classification and physico-chemical properties of**

petroleum-contaminated soils obtained in first sampling ----------------- 56

4.2 Macronutrients in petroleum-contaminated soils in first sampling------ 56

4.3 Microelements (mg/kg) of petroleum-contaminated

soils in first sampling -----------------------------------------------------------57

4.4 Polycyclic aromatic hydrocarbon content in

petroleum-contaminated soils in first sampling ---------------------------- 57

4.5 PCR Amplification of 16S ribosomal RNA in the

Petroleum-contaminated Soils -------------------------------------------------- 57

4.6 DGGE Analysis of 16S Sequences from Petroleum-

contaminated Soils in First Sampling ---------------------------------------- 65

4.7 Sequencing of DNA from Excised DGGE Bands and

Phylogenetic Analysis of 16S rRNA sequences from

Petroleum-contaminated Soils ------------------------------------------------- 65

4.8 Quantitative Polymerase Chain Reaction (Q-PCR) Analysis

of Bacterial and Archaeal 16S Gene in Petroleum-contaminated

Soils ------------------------------------------------------------------------------- 70

4.9 Physico-chemical and textural classification of

petroleum-contaminated soils in Second Sampling -------------------------- 71

4.10 Macronutrients of petroleum-contaminated soils in

Second Sampling -------------------------------------------------------------------- 71

4.11 Microelements of petroleum-contaminated soils in second sampling-- 80

4.12 Polycyclic aromatic hydrocarbon content in petroleum-contaminated

soils in second sampling ---------------------------------------------------------- 80

4.13 PCR amplification of bacterial 16S gene in

petroleum-contaminated soils spiked with phenanthrene,

**chrysene and benzo[a]pyrene ---------------------------------------------------------- 80**

**4.14 Analysis of the 16S rRNA amplicon from the**

**petroleum-contaminated soils using Denaturing Gradient**

**Gel Electrophoresis (DGGE) ---------------------------------------------------------- 85**

**4.15 Diversity of bacteria in petroleum-contaminated soils**

**and Phylogenetic analyses ---------------------------------------------------------- 91**

**4.16 Quantitative PCR (qPCR) analysis of 16S rRNA genes in soil**

**treated with PAHs ---------------------------------------------------------------- 92**

**5.0 DISCUSSION ---------------------------------------------------------------------- 109**

**5.1 Physico-chemical Properties and Heavy Metal Content**

**of the Petroleum-contaminated Soils in First Sampling ----------------- 109**

**5.2 PCR-DGGE Analysis of partial 16S rRNA of bacteria in**

**petroleum-contaminated soils in First Sampling -------------------- 110**

**5.3 Sequencing and phylogenetic analysis of 16S rRNA sequences**

**from petroleum-contaminated soils ------------------------------------ 111**

**5.4 Quantitative PCR (Real-time PCR) Analysis of Bacterial and**

**Archaeal 16S Gene in Petroleum-contaminated Soil---------------- 114**

**5.5 Physicochemical characteristics of petroleum-contaminated soils**

**spiked with polycyclic aromatic hydrocarbons ----------------------- 116**

**5.6 PCR-DGGE Analysis of Bacterial 16S gene in**

**Petroleum-contaminated Soils Spiked with**

**Phenanthrene, Chrysene and Benzo[a]pyrene ----------------------- 118**

**5.7 Diversity and Phylogenetic Analysis of Bacterial Population in the**

**Petroleum-contaminated Sites -------------------------------------------- 120**

**5.8 Quantitative PCR (qPCR) analysis of 16S rRNA genes**

**in spiked soil samples --------------------------------------------------------- 128**

**6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS ------ 132**

**6.1 Summary ---------------------------------------------------------------------- 132**

**6.2 Conclusion ------------------------------------------------------------------ 133**

**6.3 Recommendations ---------------------------------------------------------- 135**

**REFERENCES ------------------------------------------------------------------- 137**

**APPENDIX --------------------------------------------------------------------- 159**

## LIST OF TABLES


| Table | Title | Page |
|---|---|---|
| 2.1 | Some Common Reference Strains of PAH-degrading Bacteria | 14 |
| 2.2 | Some genes coding for enzymes for the ring hydroxylation of polycyclic aromatic hydrocarbons | 18 |
| 2.3 | Some genes coding for enzymes for the ring cleavage of polycyclic aromatic hydrocarbons | 19 |
| 2.4 | Some functional PAH degradative genes in Gram negative bacteria | 21 |
| 2.5 | Some functional PAH degradative genes in Gram positive bacteria | 22 |
| 3.1 | Determination of DNA concentration using fluorometer | 54 |
| 3.2 | PCR Primers used in this research | 55 |
| 4.1 | Textural classification and physico-chemical properties of petroleum-contaminated soils obtained in first sampling | 58 |
| 4.2 | Macronutrients in petroleum-contaminated soils in first sampling | 59 |
| 4.3 | Microelements (mg/kg) of petroleum-contaminated soils in first sampling | 60 |
| 4.4 | Polycyclic aromatic hydrocarbon content in petroleum-contaminated soils in first sampling | 61 |
| 4.5 | Phylogenetic affiliations of 16S rRNA sequences obtained from Mechanic workshop Soil using $BLAST_N$ Analysis | 72 |
| 4.6 | Phylogenetic affiliations of 16S rRNA sequences obtained from Trailer park Soil using $BLAST_N$ Analysis | 73 |
| 4.7 | Phylogenetic affiliations of 16S rRNA sequences obtained from Refinery effluent Soil using $BLAST_N$ Analysis | 74 |
| 4.8 | Physico-chemical properties and textural classification of petroleum-contaminated soils used in second sampling | 78 |

| | | |
|---|---|---|
| 4.9 | Macronutrients of petroleum-contaminated soils in second sampling | 79 |
| 4.10 | Microelements of petroleum-contaminated soils in second sampling | 81 |
| 4.11 | Polycyclic aromatic hydrocarbon content in petroleum-contaminated soils in second sampling | 82 |
| 4.12 | Shannon Index of Diversity of Bacteria in Spiked Petroleum-contaminated Soils | 93 |
| 4.13 | Phylogenetic affiliations of 16S rRNA sequences obtained from Mechanic workshop Soil treated with Polycyclic Aromatic Hydrocarbons | 94 |
| 4.14 | Phylogenetic affiliations of 16S rRNA sequences obtained from Trailer park Soil treated with Polycyclic Aromatic Hydrocarbons | 97 |
| 4.15 | Phylogenetic affiliations of 16S rRNA sequences obtained from Refinery effluent Soil treated with Polycyclic Aromatic Hydrocarbons | 100 |

## LIST OF FIGURES


| Figure | Title | Page |
|---|---|---|
| 2.1 | Chemical structure of some polycyclic aromatic hydrocarbons | 8 |
| 2.2 | Pathways involved in the degradation of PAH by microorganisms | 15 |
| 2.3 | Proposed catabolic pathway of phenanthrene by bacteria | 25 |
| 2.4 | Proposed catabolic pathway of chrysene in *Pseudoxanthomonas* sp. PNK-04 | 27 |
| 2.5 | Proposed Pathway for metabolic degradation of benzo[a]pyrene by bacteria | 29 |
| 3.1 | Sampling sites for collection of soils for molecular identification of bacteria in petroleum-contaminated soils | 41 |
| 4.1 | Neighbour-Joining Tree showing Phylogenetic Affiliation of 16S rRNA sequences obtained from petroleum-contaminated soils | 75 |
| 4.2 | Analysis of bacterial 16S gene in soils obtained from petroleum-contaminated sites in Kaduna state | 76 |
| 4.3 | Analysis of Archaeal 16S gene in soils obtained from petroleum-contaminated sites in Kaduna state | 77 |
| 4.4 | Summary of bands excised from DGGE gel for phylogenetic analysis of 16S rRNA gene amplified from petroleum-contaminated soils | 90 |
| 4.5 | A Maximum Parsimony Tree showing Phylogenetic Affiliation of 16S rRNA sequences obtained from a Mechanic workshop soil in Kaduna state. | 96 |
| 4.6 | A Maximum Parsimony Tree showing Phylogenetic Affiliation of 16S rRNA sequences obtained from a | 99 |

| | Trailer park soil in Kaduna state. | |
|---|---|---|
| 4.7 | A Maximum Parsimony Tree showing Phylogenetic Affiliation of 16S rRNA sequences obtained from a Refinery effluent soil in Kaduna state | 101 |
| 4.8 | Abundance of bacterial species in the PAH-spiked soils from Petroleum-contaminated sites based on depth of sampling | 102 |
| 4.9 | Abundance of bacterial species in the PAH-spiked soils from the petroleum-contaminated sites based on the PAH treatment | 103 |
| 4.10 | Distribution of bacterial taxonomic groups in PAH-spiked soils from petroleum-contaminated sites | 104 |
| 4.11 | The abundance of bacterial 16S gene in a Mechanic workshop soil treated with polycyclic aromatic hydrocarbons | 106 |
| 4.12 | The abundance of bacterial 16S gene in a Trailer park soil treated with polycyclic aromatic hydrocarbons | 107 |
| 4.13 | The abundance of bacterial 16S gene in a Refinery effluent soil treated with polycyclic aromatic hydrocarbons | 108 |

## LIST OF PLATES


| Plate | Title | Page |
|---|---|---|
| I | PCR amplification of 16S rRNA gene in Mechanic workshop soil | 62 |
| II | PCR amplification of 16S rRNA gene in Trailer park soil | 63 |
| III | PCR amplification of 16S rRNA gene in Refinery effluent soil | 64 |
| IV | PCR amplification of 16S rRNA gene in petroleum-contaminated soils | 66 |
| V | DGGE profile of amplicons of 16S rRNA gene from petroleum-contaminated soils | 67 |
| VI | DGGE profile of amplicons of 16S rRNA gene from petroleum-contaminated soils showing bands excised for sequencing | 68 |
| VII | DGGE profile of amplicons of 16S rRNA gene from triplicates of petroleum-contaminated soils showing bands excised for sequencing | 69 |
| VIII | Amplification of 16S rRNA gene in a Mechanic workshop soil treated with polycyclic aromatic hydrocarbons | 83 |
| IX | Amplification of 16S rRNA gene in a Trailer park soil treated with polycyclic aromatic hydrocarbons | 84 |
| X | Amplification of 16S rRNA gene in a Refinery effluent soil treated with polycyclic aromatic hydrocarbons | 86 |
| XI | DGGE profile of amplified 16S rRNA from Mechanic workshop soil treated with PAHs | 87 |
| XII | DGGE profile of amplified 16S rRNA from Trailer park soil treated with PAHs | 88 |
| XIII | DGGE profile of amplified 16S rRNA from Refinery effluent soil treated with PAHs | 89 |

## LIST OF APPENDICES


| Appendix | Title | Page |
|---|---|---|
| I | Mechanic workshop site and the soils sampled | 159 |
| II | Trailer park site and the soils sampled | 160 |
| III | Refinery effluent site and the soils sampled | 161 |
| IV | A Sampling point from the Mechanic workshop site showing the core from which soil was obtained | 162 |
| V | Electrophoregram of genomic DNA extracted from petroleum-contaminated soils in first sampling | 163 |
| VI | Electrophoregram of genomic DNA extracted from petroleum-contaminated soils spiked with PAHs. | 164 |
| VII | Reamplification of excised DGGE bands using sequencing primers | 165 |
| VIII | Molecular marker used during research | 166 |
| IX | DGGE Gel in a tank containing TAE Buffer undergoing Electrophoresis | 167 |
| X | Standard curve of bacterial 16S gene copies in petroleum-contaminated soils from Real-Time amplification assays | 168 |
| XI | Standard curve of archaeal 16S gene copies in petroleum-contaminated soils from Real-Time amplification assays | 169 |
| XII | Standard curve for Real-time PCR of DNA extracted from Mechanic workshop soils treated with PAHs | 170 |
| XIII | Quantification of bacterial 16S rRNA in soil collected from a Mechanic workshop site. | 171 |
| XIV | Standard curve for Real-time PCR of DNA extracted | 172 |

| | | |
|---|---|---|
| | from Trailer park soils treated with PAHs | |
| XV | Quantification of bacterial 16S rRNA in soil collected from a Trailer park site. | 173 |
| XVI | Standard curve for Real-time PCR of DNA extracted from Refinery effluent soils treated with PAHs | 174 |
| XVII | Quantification of bacterial 16S rRNA in soil collected from a Refinery effluent site. | 175 |
| XVIII | Consolidated report of Q-PCR run during quantification of bacterial 16S gene in Mechanic workshop soil | 176 |
| XIX | Consolidated report of Q-PCR run during quantification of bacterial 16S gene in Trailer park soil | 177 |
| XX | Consolidated report of Q-PCR run during quantification of bacterial 16S gene in Refinery effluent soil | 178 |
| XXI | Publications from Conference proceedings of Nigerian Society for Microbiology (NSM), 2014 | 179 |
| XXII | Publication from Conference proceedings of Biotechnology Society of Nigeria (BSN), 2014 | 180 |
| XXIII | Details of Poster presented during the Conference of American Society for Microbiology (ASM), 2015 at New Orleans, Louisiana, United States. | 181 |

## ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| PAH | Polycyclic Aromatic Hydrocarbon |
| L.M.W | Low molecular weight |
| H.M.W | High molecular weight |
| Bap | benzo[a]pyrene |
| USEPA | United States Environmental Protection Agency |
| GC-MS | Gas Chromatography Mass Spectrometry |
| DGGE | Denaturing Gradient Gel Electrophoresis |
| PCR | Polymerase Chain Reaction |
| Q-PCR | Quantitative Polymerase Chain Reaction |
| rRNA | Ribosomal ribonucleic acid |
| bp | base pair |

# ABSTRACT

This research was focused on using molecular tools to identify and determine the diversity of soil bacteria in petroleum-contaminated sites in some parts of Kaduna State, Nigeria. Soil samples were obtained from three sites with a history of petroleum hydrocarbon contamination in Kaduna state – a mechanic workshop in Zaria that has been in operation for at least thirty years, a trailer park in Zaria where trailers are serviced and repaired, and a site close to a stream receiving effluent from the petroleum refinery in Kaduna. The soils were collected from two different depths (17 – 20 cm, and 37 – 40 cm); and subjected to physico-chemical analyses and DNA extraction. Amplification of the 16S rRNA gene was carried out using the universal primers, 357F and 518R. Amplicons of the 16S ribosomal RNA gene from the soil samples were further analysed to obtain the different nucleotide sequences using denaturing gradient gel electrophoresis (DGGE), a forty base pair (40 bp) GC clamp was attached to the forward primer to aid separation during DGGE analysis. The prominent bands on the DGGE gel were excised and sequenced. Based on the similarity search conducted on the NCBI website, the identities of the bacteria in the soils were closely affiliated to the following phylogenetic groups, Gammaproteobacteria, Betaproteobacteria, Alphaproteobacteria, Firmicutes, Actinobacteria, Acidobacteria. Quantification of the bacterial and archaeal 16S gene in the soils by Real-time PCR using SYBR-Green fluorescence revealed that the bacterial 16S gene was more abundant than the archaeal 16S gene. The second phase of the experiment involved spiking the soil samples with PAHs having three, four and five benzene rings respectively. Genomic DNA was extracted from the spiked soil samples and used for phylogenetic studies and Real-time PCR ampification. The effect of the PAHs on the indigenous bacteria in the soils was

determined using the Shannon diversity index, phylogenetic analysis and quantification of the bacterial 16S rRNA gene. Soils from the Mechanic workshop and Trailer park sites had similar diversity indices however; the former had a slightly higher value. Thus, making it the site with the highest diversity of bacterial species. The closest relatives of the 16S sequences from the Refinery effluent site were all species belonging to the genus, *Bacillus*. On the other hand, the sequences from the Mechanic workshop and Trailer park were closely affiliated to a wide variety of bacterial groups such as, Gammaproteobacteria, Firmicutes, and to a less extent, Actinobacteria and Chloroflexi. The Trailer park soil had two sequences that had high similarity to the sequences of two novel strains in the Genbank. A good number of the bacteria identified in the Refinery effluent and Trailer park sites were obtained from the soils spiked with polycyclic aromatic hydrocarbons. This was not the case with the Mechanic workshop site, as most of the bacteria identified were from the untreated soil samples. Quantification of the 16S ribosomal RNA gene in the soils spiked with PAHs revealed higher gene copies in the treated soils than the soils that were not spiked with polycylic aromatic hydrocarbons. The Mechanic workshop site had the highest number of 16S gene copies and it was observed in the soil spiked with chrysene. Thus, chrysene likely favoured the proliferation of bacteria in the Mechanic workshop soil. Culture methods should not be solely relied on for the identification of PAH-utilizing bacteria in soils since most of these species do not grow on laboratory media. Metagenomics is a powerful tool in studying the ecology of the bacterial community, as well as in the determination of the microbial functional and phylogenetic diversity. Bioremediation studies in petroleum-polluted regions in Nigeria should focus on both culture and culture-independent methods in order to fully harness the potentials of hydrocarbon-degrading bacteria.

# CHAPTER ONE

## 1.0 INTRODUCTION

### 1.1 Background

Polycyclic aromatic hydrocarbons (PAHs) are produced during fossil fuel combustion, waste incineration or as by-products of industrial processes, such as coal gasification, production of aluminum, iron, steel and petroleum refining, component of crude oil, wood preservatives, smoke houses, wood stoves and also found in emissions from power generators and motor vehicles (Christensen and Bzdusek, 2005; Wilcke, 2007). The soil environment is home to a diverse group of bacteria; and they adapt to the presence of pollutants by utilizing these compounds for their metabolic needs.

Polycyclic aromatic hydrocarbons are compounds formed from the incomplete combustion of organic substances such as petroleum, wood, and coal. Bacteria indigenous in soils polluted with these hydrocarbons are important in Ecology studies. Generally, PAHs could be from any of three sources, petrogenic, biogenic or pyrogenic (Seo *et al*., 2009). Petrogenic PAHs are from petroleum and petroleum-derived products, those of biogenic origin are from aromatic amino acids, lignin compounds and their derivatives (Seo *et al*., 2009); while the PAHs of pyrogenic origin are produced from combustion processes. Polycyclic aromatic hydrocarbons as well as compounds such as hexachlorobenzene, dichlorodiphenyl trichloroethane (DDT), organometallic compounds, polychlorinated biphenyls (PCBs) are classified as persistent organic pollutants (POPs), and they are also referred to as persistent bioaccumulative and toxic chemicals (PBTs) (Seo *et al*., 2009).

Low molecular weight (L.M.W) polycyclic aromatic hydrocarbons consist of two to three fused aromatic rings while, the high molecular weight (H.M.W) polycyclic aromatic hydrocarbons have four or more fused aromatic rings. The HMW PAHs are of principal concern due to their recalcitrance, persistence, bioaccumulation, carcinogenicity, genotoxicity and mutagenicity (Xue and Warshawsky, 2005; Castorena-Torres *et al*., 2008). Microbial degradation of PAHs offers a more cost effective and eco-friendly way of removing these pollutants from the environment as opposed to physico-chemical methods which might introduce other dangerous substances as well as being expensive (Habe and Omori, 2003; Reddy *et al*., 2011). Several studies have shown that micro-organisms can be used to degrade PAHs in soils and sediments as well as in the aquatic environment (Kim *et al*., 2006; Cui *et al*., 2008; Kumar and Khanna, 2009; Niepceron *et al*., 2009; Huijie *et al*., 2011; Tikilili and Nkhalambayausi, 2011; Narancic *et al*., 2012). However, most of these microorganisms are able to degrade these hydrocarbons as a community through a process known as co-metabolism, whereby some bacteria degrade these compounds partially and the metabolites from the initial reaction are broken down further by other bacteria (Demaneche *et al*., 2004; Guo *et al*., 2010).

The ideal way of studying the structural and functional characteristics of PAH-degrading bacteria in the environment is through the use of culture-independent methods such as denaturing gradient gel electrophoresis (DGGE), phylogenetic analysis of the 16SrRNA gene and PCR amplification of PAH functional genes, the use of gene probes in restriction fragment length polymorphism and stable isotope probing (Desai *et al*., 2010; Jones *et al*., 2011; Zhang *et al*., 2011). However, these methods mentioned are not exhaustive, there are other molecular and "omic" technologies for the analysis of microbial community profile with relation to pollutant degradation. Culture-dependent

methods of determining the bacterial community in the environment are often inadequate because a large percentage of these bacteria are not culturable thus are poorly represented, both in abundance and function. In recent studies, the genes coding for enzymes involved in the degradation of PAHs are often targeted as a means of providing information on the total microbial community in PAH-contaminated environments (Junca and Pieper, 2003; Witzig *et al*., 2006; Gomes *et al*., 2007; Brennerova *et al*., 2009; Ding *et al*., 2010).

Several primers specific to PAH degradation have been designed over the last two decades and they have successfully amplified functional genes important in the degradation of PAHs (Wikstrom *et al*., 1996; Lloyd Jones *et al*., 1999; Johnsen *et al*., 2007; El Azhari *et al*., 2010; Vazquez *et al*., 2013). Most of these studies have focused on Gram negative bacteria, *Pseudomonas* and *Sphingomonas*, however, it has been discovered that members of the Actinobacteria subclass, such as *Mycobacterium*, *Rhodococcus* show remarkable PAH degradative ability especially to the high molecular weight (HMW) PAHs (Wilcke *et al*., 2007).

## 1.2 Statement of Research Problem

The incomplete combustion of fossil fuels leads to the production of polycyclic aromatic hydrocarbons. Petroleum forms a major part of the economy of Nigeria thus, activities leading to petroleum exploration and production exposes the environment to contamination with PAHs (Adoki and Odokuma, 2007).  The toxic, mutagenic and carcinogenic capabilities of PAHs make them important pollutant in the ecosystem. A large percentage of soil bacteria capable of degrading these compounds do not grow on culture media as such their metabolic capabilities are not adequately studied using culture-dependent methods. Most studies on microbial PAH degradation in Nigeria have

been centred on isolation of these hydrocarbon-degrading bacteria from the soil (Ayotamuno *et al*., 2006; Igwo-Ezikpe *et al*., 2006). The metabolic capabilities of these species are not fully explored on the molecular level, thus creating paucity in information on key aspects of their identification. The molecular approach seeks to address these problems. PAHs adsorb to solid particles in the soil, air and water due to their hydrophobic nature and consequently, they are able to persist in the environment and result in contamination. The toxic nature of these compounds could lead to adverse effects in plant, animal and even human life (Johnsen *et al*., 2006; Nkpaa *et al*., 2013).

## 1.3 Justification

Metagenomics offers a comprehensive approach to studying bacterial communities capable of degrading polycyclic aromatic hydrocarbons. The focus of these methods is centred on the highly conserved gene, 16S rRNA, since analysing this gene provides a lot of information on the metabolic, phylogenetic and functional characteristics of the bacterial population. The use of Polymerase Chain Reaction (PCR) and Q-PCR (real time PCR) in the amplification of the 16S gene offers valuable information on the bacteria based on their phylogenetic affiliation and quantity, respectively (Cebron *et al*., 2008; Yagi and Madsen, 2009; Yergeau *et al*., 2009). Molecular methods are also vital in the detection of the presence of functional genes thus highlighting the enzymatic capabilities of bacteria present in environmental samples (Ding *et al*., 2010; Iwai *et al*., 2011; Korotkevych *et al*., 2011). However, it is of utmost importance that taxonomic and functional gene analyses are performed simultaneously in order to have comprehensive data on the diversity of the soil bacterial population.

Polycyclic aromatic hydrocarbons (PAHs) persist in the environment and can be toxic to living organisms. Biodegradation of these compounds in the soil by bacteria plays a

major role in eliminating the potential threat they pose. Molecular approach in studying the bacteria with these degradative capabilities is not only crucial in understanding the metabolic pathway(s) in which these pollutants are being utilised but it also provides knowledge about the bacterial community possessing such catabolic genes (Takizawa *et al*., 1994; Vinas *et al*., 2005; Sipila *et al*., 2006). Analysis of the highly conserved gene, 16S rRNA can be achieved by PCR amplification and Q-PCR (real time PCR) using universal primers.

### 1.4 Aim

The aim of this research was to use molecular tools to characterize soil bacteria with polycyclic aromatic hydrocarbon (PAH) degradation ability.

### 1.5 Objectives

1. To determine the physico-chemical properties and textural classification of soils obtained from petroleum-contaminated sites.
2. To characterize the bacteria in soils obtained from petroleum-contaminated sites using PCR-DGGE analysis of the partial 16S rRNA sequence, and phylogenetics.
3. To quantify the bacterial and archaeal 16S rRNA gene in the petroleum-contaminated soils using Real-time PCR amplification.
4. To analyze the 16S rRNA gene in petroleum-contaminated soils spiked with phenanthrene, chrysene and benzo[a]pyrene, using PCR-DGGE.
5. To determine the diversity of the bacterial community in the petroleum-contaminated soils before and after spiking with polycyclic aromatic hydrocarbons using phylogenetic analyses and Shannon diversity indices.

6. To quantify the bacterial 16S ribosomal RNA gene in the soils spiked with polycyclic aromatic hydrocarbons using Real.time PCR amplification.

# CHAPTER TWO

## 2.0 LITERATURE REVIEW

### 2.1 Polycyclic Aromatic Hydrocarbons (PAH)

Polycyclic aromatic hydrocarbons are made up of three or more fused benzene rings, and they are often found in the environment as a result of anthropogenic activities. They are found in petroleum and as by-products from fossil fuel processes such as wood processing facilities and petroleum refineries. Polycyclic aromatic hydrocarbons are known for their toxic, mutagenic, teratogenic and carcinogenic nature (Mrozik *et al*., 2003; Nkpaa *et al*., 2013) as such there are acceptable limits for their presence in drinking water (USEPA, 2000). The low molecular weight PAHs are not as toxic as the high molecular weight PAHs due to their higher solubility. The low molecular weight (LMW) PAHs are composed of two or three rings (naphthalene, phenanthrene) and are less recalcitrant than the higher molecular weight (HMW) PAHs which are composed of four or more rings (benzo[a]pyrene, pyrene). The HMW PAHs are more stable and thus more difficult to degrade than the LWW PAHs. The LMW-PAHs (phenanthrene, naphthalene) show more acute toxicity to aquatic life while, the HMW-PAHs are associated with a higher degree of carcinogenicity (Kanaly and Harayama, 2000).

Sixteen PAHs have been identified as priority pollutants by the United States Environmental Protection Agency (USEPA) based on their abundance and toxic nature (Habe and Omori, 2003). The chemical structures of some polycyclic aromatic hydrocarbons are depicted in Figure 2.1. The number and configuration of the benzene rings in each PAH greatly affects their physical and chemical properties such as their metabolic reactions, persistence and attack by microbial enzymes.

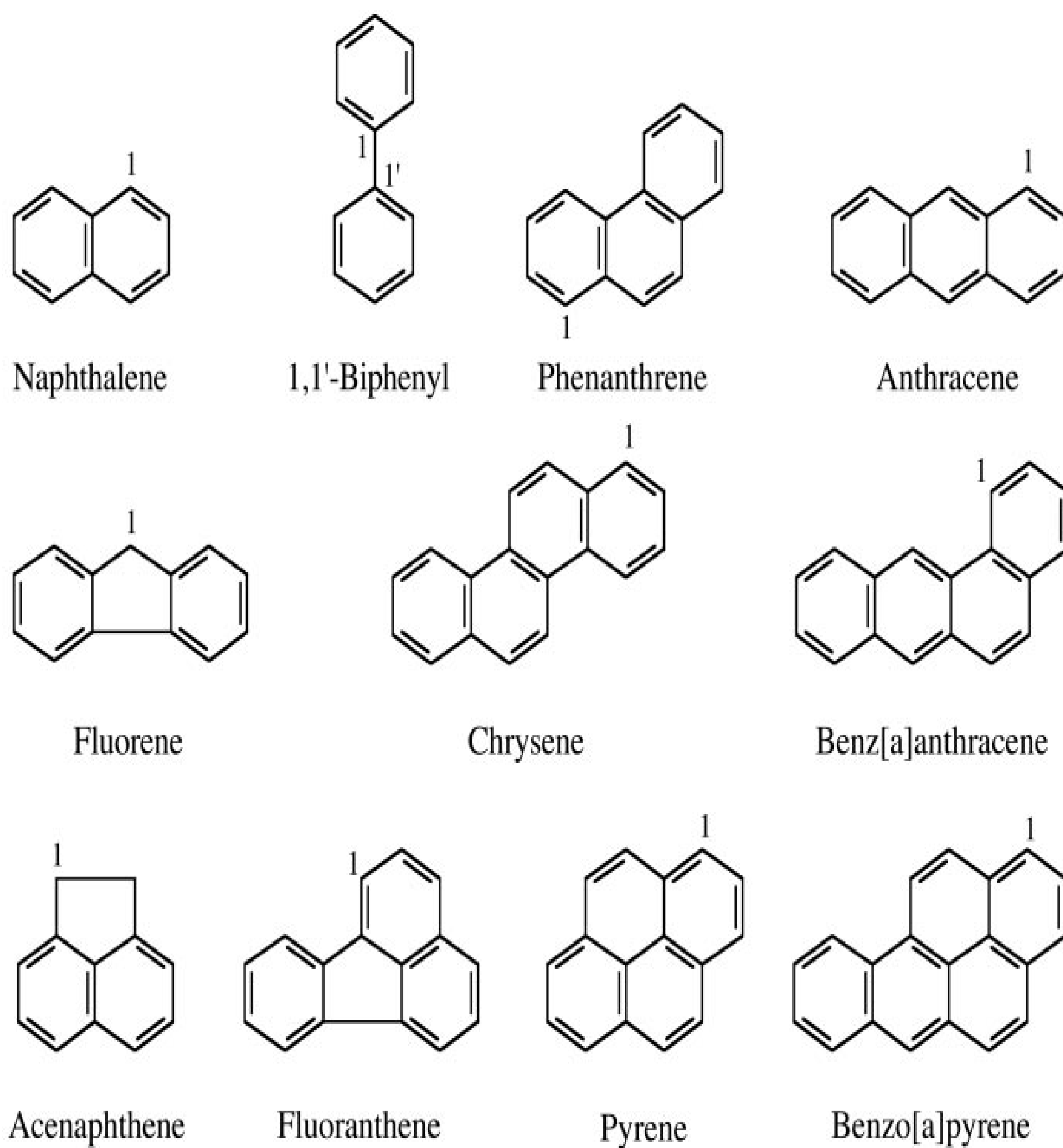



**Figure 2.1 Chemical structure of some polycyclic aromatic hydrocarbons** (Reference: Demaneche *et al*., 2004)

Among the ways in which PAHs are degraded is photolysis, but microbial degradation is thought to have a much greater effect in the breakdown of these hydrocarbons. Microorganisms capable of utilizing PAHs include bacteria, fungi and algae. However, phytoremediation and rhizoremediation are excellent ways of enhancing microbial degradation of PAHs because these approaches are cost-effective and cause negligible disturbance at the polluted site. The rhizosphere is rich in certain compounds (such as tryptophan, glutamate, glucose, xylose, oligosaccharides, benzoate, acetate, citrate) which aid the growth of bacteria and fungi. This is achieved by the overall activation of the metabolic capacity of these microbes in degrading pollutants (Louvel *et al*., 2011); the rhizosphere also induces the genes responsible for the breakdown of hydrocarbons (Khan *et al*., 2013). Several studies have highlighted the role of the rhizosphere of certain plants in increasing the rate of PAH utilization by microorganisms (Daane *et al*., 2001; Rentz *et al*., 2005; Sipila *et al*., 2008; Louvel *et al*., 2011; Khan *et al*., 2013), endophytic bacteria as well as fungi possess enzymes such as peroxidases, laccases, dioxygenases, cytochrome P450 monoxygenases important in degrading contaminants in the soil (Gerhardt *et al*., 2009). Plants such as legumes, cereals, willow and poplar trees have been shown to exhibit enhanced rates of biodegradation of PAHs in laboratory scale studies as well as in the field (Gerhardt *et al*., 2009; Afzal *et al*., 2011; Korotkevych *et al*., 2011).

Earthworms have also contributed to the degradation of these hydrocarbons (Dendooven *et al*., 2011) by virtue of their ability to increase aeration in the soil and by making the hydrocarbons more available for attack by the microorganisms. Other practices used to enhance biodegradation of PAHs by bacteria and fungi include the addition of compost, and organic materials such as straw, and manure.

## 2.2 Polycyclic Aromatic Hydrocarbons: Source, Distribution and Characteristics

Polycyclic aromatic hydrocarbons can occur naturally in the environment as a result of volcanic eruptions and forest fires. Combustion of fossil fuels such as wood, coal, and industrial processes in the metal and petroleum industries are all anthropogenic sources of PAHs. These manmade and natural processes contribute to the ubiquitous nature of PAHs, thus causing their wide spread distribution worldwide. The major man-made sources of PAHs are from power plants and domestic heating while other significant contributors include aluminium and steel processing industries as well as incinerating plants and outdoor combustion (Skupinska *et al*., 2004). Polycyclic aromatic hydrocarbons get adsorbed to particulate matter and persist in the environment particularly due to their hydrophobic nature. Polycyclic aromatic hydrocarbons in the air in urban areas occur as a result of emissions from mobile vehicles, airplanes, ships, *et cetera* (Lee and Vu, 2010); their presence in the air is most often linked to aerosols from anthropogenic and natural sources (forest fires, volcanoes, meteoritic dust, some vegetation) (Al-Turki, 2009). They tend to be more abundant in indoor air of residences having cigarette smoking than those without cigarette smoking (Lee and Vu, 2010). Another important source of PAHs in the air in domestic setting is cooking (Lee and Vu, 2010), although movement of air from outdoors into houses also contributes to the presence of these hydrocarbons in indoor air. Polycyclic aromatic hydrocarbons in the air, soil or water get sorbed into particulate matter; this property increases their persistence and recalcitrance in the environment. The presence of polycyclic aromatic hydrocarbons in water has received relatively less attention as their concentrations in air. Groundwater has understandably less quantity of these compounds than freshwater bodies such as lakes and rivers, presumably due to the different proximities to point sources. The comparatively low solubility of PAHs in water implies that a large

quantity of them is adsorbed onto suspended solids (Al-Turki, 2009). PAHs accumulate in soils, and their sources in soil include, sludge used as fertilizer, dust from the air, compost, runoff water from asphalt roads and then accidental spillage from petroleum products (Skupinska *et al*., 2004). The soil is regarded as "one of the major sinks of PAHs" (Wang *et al*., 2009).

The size as well as the arrangement of the Carbon atoms in a polycyclic aromatic hydrocarbon greatly determines its physical and chemical properties (Canadian Council of Ministers of the Environment, 1999). Generally, PAHs are classified based on their properties which include; boiling and melting points, solubility and vapour pressure (Lee and Vu, 2010). The high molecular weight PAHs have less solubility than the LMW PAHs, the higher the number of benzene rings, the more insoluble the PAH. Polycyclic aromatic hydrocarbons are hydrophobic thus, they have a low solubility in water (Skupinska *et al*., 2004), but are soluble in organic solvents such as acetone, n-hexane, Dichloromethane (DCM).

PAHs are highly sensitive to light, and can be cracked at high temperatures (Skupinska *et al*., 2004) but are mostly chemically inactive. However, in the environment they could be found substituted with the following functional groups, hydroxyl, methyl, nitrite and others (Skupinska *et al*., 2004).

### 2.3 Microbial degradation of PAHs in the environment

Bacteria and fungi are important role players in the degradation of these compounds and microbial degradation is thought to be the major contributor in the elimination of PAHs from the environment. These microorganisms carry out the degradation aerobically however, in environments where the hydrocarbons are found in very deep parts of the

aquatic or terrestrial ecosystems where there is little or no oxygen, the anaerobic pathway is utilized.

The presence of fossil fuels in these terrestrial and aquatic environments greatly influences the utilization of PAHs by indigenous microorganisms. The native microorganisms typically become adapted to these hydrocarbons and the expression of enzymes specific to the degradation of the compounds is initiated.

Microbial degradation of PAHs has been studied extensively over the decades using culture-dependent methods however, molecular methods of identifying these hydrocarbon-degrading microorganisms has proven to be more useful in providing vital information on their metabolic and taxonomic characteristics (Watanube and Hamamura, 2003; Ding *et al*., 2010; Kweon *et al*., 2011). Culture techniques generally involve isolation of fungi or bacteria on solid laboratory media usually following enrichment on the PAH(s) of interest. Isolates obtained are subsequently subjected to further testing to confirm degradation of the hydrocarbon by growing them on liquid media having the PAH(s) as the sole source of Carbon. Other parameters analysed during biodegradation of these compounds by the isolates are focused on metabolites such as catechol (Guo *et al*., 2010). Culture-independent methods in the identification and characterization of PAH-degrading bacteria are mostly centered on the PCR amplification of 16S rRNA gene and catabolic genes. The reference strains of bacteria important in the degradation of PAHs play a vital role in the PCR amplification of the genes encoding PAH degradation and/or utilization as they are often used as a positive control during these procedures. These reference strains possess genes coding for enzymes that are important in the metabolism of PAHs, some of these strains are as shown in Table 2.1.

The diversity of PAH-degrading bacteria in soil has been investigated in several studies based on the phylogenetic affiliation of the catabolic genes, in this case the dioxygenases (Bordenave *et al*., 2008; Bengtsson *et al*., 2013). Primers targeting the dioxygenases have been used in end time PCR as well as real-time PCR in order to determine their presence and number respectively (Cebron *et al*., 2008). The PAH dioxygenase belongs to a large family of enzymes important in the degradation of aromatic compounds (Johnsen *et al*., 2005).

Some fungi have been identified to mineralise PAHs more efficiently than bacteria; these include fungi belonging to the following groups, Basidiomycetes, white rot fungi, deuteromycetes and filamentous fungi (Peng *et al*., 2008). Fungi responsible for the breakdown of PAHs release extracellular enzymes that aid the process, those capable of degrading HMW-PAHs are grouped into lignolytic and non-lignolytic fungi (Wang *et al*., 2012). The former is capable of mineralisation of PAHs by cleaving the aromatic rings using lignolytic enzymes while, the non-lignolytic fungi only oxidise the PAH to produce a detoxified or hydrophilic product (Wang *et al*., 2012). Non-lignolytic fungi capable of PAH utilization include; *Cunninghamella elegans*, *Penicillium janthinellum*, *Syncephelastrum* sp., they utilize PAHs catalysed by cytochrome P450 monooxygenase (CYPs) to form trans-dihydrodiols (Cerniglia, 2003). The extracellular enzymes produced by lignolytic fungi can attack several substances due to their low substrate specificity (Haritash and Kaushik, 2009); they metabolize PAHs in nonspecific radical oxidation to yield PAH quinones (Cerniglia, 2003). Some of such lignolytic fungi include *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Trametes versicolor*; some other lignolytic fungi can mineralize the PAH quinones further to $CO_2$ (Cerniglia, 2003).

**Table 2.1: Some Common Reference Strains of PAH-degrading Bacteria**

| Strain | PAH catabolic gene | Reference |
|---|---|---|
| *Pseudomonas putida* NCIB 9816-4 | nahAc | Ni Chadhain *et al*., 2006 |
| *Pseudomonas putida* G7 | nahAc | Lloyd-Jones *et al*., 1999 |
| *Pseudomonas putida* OUS82 | pahAc | Takizawa *et al*., 1994 |
| *Pseudomonas aeruginosa* PaK1 | pahAc | Laurie and Lloyd-Jones, 1999 |
| *Burkholderia* sp. strain RP007 | phnAc | Lloyd-Jones *et al*., 1999 |
| *Sphingomonas yanoikuyae* B1 | bphA1f | Cho *et al*., 2005, Cebron *et al*., 2008 |
| *Novosphingobium aromaticovorans* | bphA1f | Cebron *et al*., 2008 |
| *Ralstonia* sp. U2 | nagAc | Cebron *et al*., 2008 |
| *Mycobacterium vanbaalenii* PYR1 | nidA | Sho *et al*., 2004 |
| *Mycobacterium vanbaalenii* PYR1 | pdoA | Sho *et al*., 2004 |
| *Mycobacterium* sp. S65 | pdoA1 | Cebron *et al*., 2008 |
| *Nocardioides* sp. Kp7 | phdA | Cebron *et al*., 2008 |
| *Rhodococcus opacus* | narA | Cebron *et al*., 2008 |

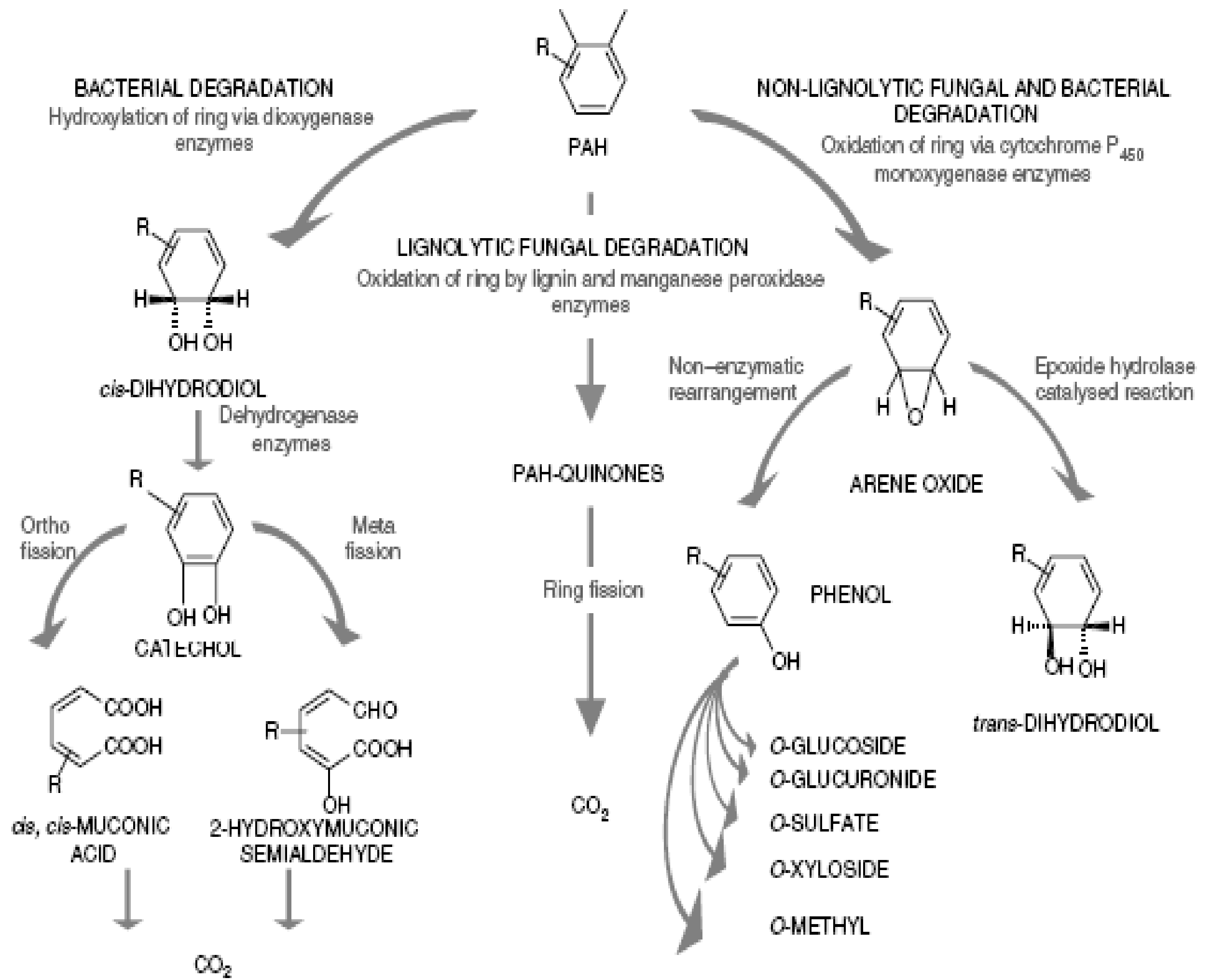



**Figure 2.2: Pathways involved in the degradation of PAH by microorganisms** (Reference: Maigari and Maigari, 2015).

The laccases which are a group of lignolytic enzymes are very efficient in the degradation of PAHs as well as other phenolic compounds, but cannot be harnessed in non-fungal systems (Chauhan *et al*., 2008); conversely, the CYPs are found in many organisms.

The pathway for the metabolism of PAHs by bacteria and fungi are shown in Figure 2.2. The non-lignolytic fungi and some bacterial species share the pathway involving the use of cytochrome P450 monooxygenase enzyme in the catabolism of these hydrocarbons; while other PAH-degrading bacteria utilize the dioxygenase enzyme system. The pathway used by lignolytic fungi appears relatively simpler than the other two mechanisms.

### 2.4 Genes encoding PAH degradation in Bacterial species

The functional genes coding for the enzymes which enable the utilisation of PAHs form a diverse group. Identification of bacteria in PAH-contaminated sites show that they possess similar dioxygenase genes, this plays a role in their abundance but not necessarily their phylogenetic affiliation. The most studied polycyclic aromatic hydrocarbon is naphthalene, a two-ringed aromatic hydrocarbon that possesses a ring hydroxylating enzyme system called the naphthalene dioxygenase. It has been the focus of many studies centred on the degradative pathways used by bacteria in the utilization of PAHs; and it consists of many components which include the oxygenase, a ferrodoxin, and a ferrodoxin reductase (Habe and Omori, 2003; Peng *et al*., 2008). The catalytic portion of the naphthalene dioxygenase system (NDO) consists of the large (α) and small (β) subunits, which are found on the active site, the oxygenase. The large subunit (α) contains an Iron-Sulphur centre: the Rieske centre [2Fe-2S], it also determines the substrate specificity of the enzyme, thus it is often the target in studies

involving PAH catabolic genes (Wilson *et al*., 1999; Baldwin *et al*., 2003; Witzig *et al*., 2006; Cebron *et al*., 2008; Ding *et al*., 2010; Vazquez *et al*., 2013).

Dioxygenases catalyze the introduction of two hydroxyl groups to polycyclic aromatic hydrocarbons during aerobic degradation (ring hydroxylation). The second step involves the ring cleavage/fission of the hydroxylated substrates by either the *ortho* or *meta* pathway; the resulting intermediates such as catechol are then channelled into the tricarboxylic cycle (Kanaly and Harayama, 2000; Habe and Omori, 2003). The details of both ring hydroxylation and ring fission stages are presented in Tables 2.2 and 2.3 respectively.

Genes coding for enzymes that enable bacteria to degrade polycyclic aromatic hydrocarbons are located on the chromosome or plasmids (Gomes *et al*., 2005). Most of the PAHs that have been degraded by pure cultures of bacteria are of low molecular weight. The H.M.W PAHs are more difficult to degrade due to the number of benzene rings they possess but there has been evidence of their breakdown as well (Igwo-Ezikpe *et al*., 2010). More attention had been focused on the Gram negative bacteria in the past few decades but Gram positive bacteria have shown just as much capability in the breakdown of these hydrocarbons, and in some instances, even better ability in degrading H.M.W PAHs. Both types of bacteria have different functional genes for PAH degradation. Tables 2.4 and 2.5 highlight these genes and the bacteria harbouring them.

**Table 2.2 Some genes coding for enzymes for the ring hydroxylation of polycyclic aromatic hydrocarbons**

| S/No. | Target gene | Bacteria | Target PAH | Reference |
|---|---|---|---|---|
| 1 | nahAc | GN | Naphthalene | Gomes *et al*., 2005; Lu *et al*., 2012; Vazquez *et al*., 2013 |
| 2 | pahAc | GN | Naphthalene Phenanthrene | Lloyd Jones *et al*., 1999, Johnsen *et al*., 2007 |
| 3 | phnAc | GN | Phenanthrene | Lloyd Jones *et al*., 1999; Edlund and Jansson, 2006; Isaac *et al*., 2013 |
| 4 | nidA | GP | L.M.W-PAHs H.M.W-PAHs | Brezna *et al*., 2003; Guo *et al*., 2010, Klankeo *et al*., 2009 |
| 5 | narA | GP | L.M.W PAHs | Larkin *et al*., 1999; Cebron *et al*., 2008 |
| 6 | nidB | GP | L.M.W-PAHs H.M.W-PAHs | Brezna *et al*., 2003; Klankeo *et al*., 2009 |
| 7 | pdoA | GP | L.M.W-PAHs H.M.W-PAHs | Johnsen *et al*., 2007; Cebron *et al*., 2008 |
| 8 | pdoB | GP | L.M.W-PAHs H.M.W-PAHs | Sho *et al*., 2004; Guo *et al*., 2010 |

GP: Gram negative bacteria; GN: Gram negative bacteria; L.M.W-PAHs: Low molecular weight polycyclic aromatic hydrocarbons; H.M.W-PAHs: High molecular weight polycyclic aromatic hydrocarbons

**Table 2.3 Some genes coding for enzymes for the ring cleavage of polycyclic aromatic hydrocarbons**

| S/No. | Target gene | Bacteria | Target PAH | Reference |
|---|---|---|---|---|
| 1 | catA | GN and GP | L.M.W PAHs | El Azhari *et al*., 2010; Cebron *et al*., 2013 |
| 2 | nahH | GN | Naphthalene | Wikstrom *et al*., 1996; Vazquez *et al*., 2013 |
| 3 | xylE | GP, GN | L.M.W PAHs | Marquez-Rocha *et al*., 2005; Edlund and Jansson, 2006 |
| 4 | Catechol 2,3-dioxygenase | GP, GN | L.M.W PAHs | Sei *et al*., 1999; Nayak *et al*., 2011; Philips *et al*., 2012 |
| 5 | Catechol 1,2-dioxygenase | GP, GN | L.M.W PAHs | Sei *et al*., 1999 |
| 6 | Lower pathway extradiol catechol dioxygenases (subfamilies 1,2A, 1.2B, 1.2C, 1.2D, and 1.2E) | GN, GP | L.M.W PAHs | Sipila *et al*., 2008 |
| 7 | Upper pathway extradiol catechol dioxygenases (subfamilies 1.3A, 1.3B, 1.3C, 1.3D, and 1.3E) | GN, GP | L.M.W PAHs | Sipila *et al*., 2008 |

GP: Gram negative bacteria; GN: Gram negative bacteria; L.M.W: Low molecular weight; H.M.W: High molecular weight

## 2.5 PCR Primers used in the detection of Functional genes important in Bacterial degradation of High Molecular Weight-Polycyclic Aromatic Hydrocarbons

The choice of primer set for the amplification of dioxygenase genes is critical to the detection of these genes in environmental samples. Three important characteristics of primer for the amplification of dioxygenase genes include its specificity, coverage and product length (Iwai *et al.*, 2011). The use of bacterial species isolated on laboratory media for the design of primers specific for genes important in the degradation of PAHs restricts the discovery of novel strains with unique metabolic pathway. Such studies involving primer design produce better results using DNA extracted from environmental samples. Several of these primers were designed *in silico* using bioinformatics software. This scheme avoids limitations arising from set-backs with degenerate bases at the 5' end.

Different primers are often seen to amplify the same functional gene. The alpha sub-unit of the naphthalene dioxygenase gene (nahAc) has been amplified in bacteria in many studies using different primer sets (Wilson *et al.*, 1999; Gomes *et al.*, 2005; Lu *et al.*, 2012; Philips *et al.*, 2012; Vazquez *et al.*, 2013). Same goes for other catabolic genes which include, phnAc (Lloyd-Jones *et al.*, 1999; Wilson *et al.*, 1999; Johnsen *et al.*, 2006; Wang *et al.*, 2007; Edlund and Jansson, 2006); nahH (Wikstom *et al.*, 1996; Sei *et al.*, 1999; Vazquez *et al.*, 2013); C23O (Sei *et al.*, 1999; Brennerova *et al.*, 2009); nidA (Brezna *et al.*, 2003); nar (Larkin *et al.*, 1999; Cebron *et al.*, 2008); xylE (Whyte *et al.*, 1996; Marquez-Rocha *et al.*, 2005, Edlund and Jansson, 2006). This list of genes mentioned is certainly not exhaustive however, it is imperative to point that the design of primers for the detection of PAH catabolic genes is at the discretion of the researcher.

**Table 2.4 Some functional PAH degradative genes in Gram negative bacteria**

| S/No. | Gene | PAH | Bacterial genera | Reference |
|---|---|---|---|---|
| 1 | *nah* | naphthalene | *Pseudomonas* spp. | Gomes *et al*., 2005; Wang *et al*., 2007; Philips *et al*., 2012; Isaac *et al*., 2013 |
| 2 | *nag* | naphthalene | *Comamonas* spp., *Ralstonia* spp. | Dionisi *et al*., 2004; Klankeo *et al*., 2009 |
| 3 | *phn* | phenanthrene | *Burkholderia* spp. | Lloyds-Jones *et al*., 1999; Guo *et al*., 2010; Isaac *et al*., 2013 |
| 4 | *bph* | Naphthalene, phenanthrene | *Novosphingobium* *Sphingobium* *Burkholderia* | Klankeo *et al*., 2009 Iwai *et al*., 2011, Korotkevych *et al*., 2011 |
| 5 | *arh* | Acenaphthylene acenanphthene | *Sphingomonas* | Klankeo *et al*., 2009 |

**Table 2.5 Some functional PAH degradative genes in Gram positive bacteria**

| S/No. | Gene | PAH | Bacteria | Reference |
|---|---|---|---|---|
| 1 | *nar* | L.M.W | *Rhodococcus* spp. | Larkin *et al*., 1999; Cebron *et al*., 2008 |
| 2 | *phd* | L.M.W, H.M.W | *Nocardioides* spp. | Krivobok *et al*., 2003; Cebron *et al*., 2008 |
| 3 | *pdo* | L.M.W, H.M.W | *Mycobacterium* spp. | Sho *et al*., 2004; Cebron *et al*., 2008 |
| 4 | *nid* | L.M.W, H.M.W | *Mycobacterium* spp. *Terrabacter* spp. | Guo *et al*., 2010; Zhou *et al*., 2008 |

L.M.W: Low molecular weight; H.M.W: High molecular weight

It appears that the initial ring-hydroxylating enzymes (*nahAc*, *phnAc*, *nidA*, *et cetera*) have been covered more extensively in studies than either the *meta* or *ortho* cleavage enzymes (C12O, C23O, *catA*, *nah*, *et cetera*), the reason for this may be the fact that the large subunit (alpha subunit) of the dioxygenase enzyme determines the substrate specificity of the enzymes. Many factors come into play during the design and choice of a primer set, and ultimately, the specificity and coverage are the two main factors affecting the outcome. The number of reference sequences used *in silico* in the design of the primers might play a big role in the successful amplification of the target gene. For example, the primer set, BPHD-f3/BPHD-r1, for the amplification of the biphenyl dioxygenase gene by Iwai *et al*. (2011) was done by using 31 reference sequences. This paid off because they were able to guarantee specificity of the primer set, and it also provided useful information pointing to the need for new schemes in detecting similar genes in the environment (Iwai *et al*., 2011).

In some studies, the ring hydroxylating enzymes are cloned into a complex (PAH-RHD)α, and a single primer pair used in amplifying these genes (Cebron *et al*., 2008; Ding *et al*., 2010; Jurelevicius *et al*., 2012); the primers used in these studies were found to be very specific. However, since the PAH degradative genes of Gram negative and Gram positive bacteria belong to different groups, primers targeting each of these two groups of bacteria were used. The primer set designed by Ding *et al*. (2010) was based on the various PAH-RHDα sequences depositied in the Genbank as at December 2007, while the primer set designed by Cebron *et al*. (2008) was based on a good number of previously described PAH-RHDα genes. They were able to use the same primers in Real-time PCR assays as well and achieved successful amplification of these genes.

## 2.6 Metabolic Pathway for PAH Degradation in Bacteria

### 2.6.1 Pathway for the bacterial degradation of phenanthrene

Phenanthrene is composed of three benzene rings, and it is the smallest PAH that possesses a bay and K region (Fig. 2.3). The bay region is defined as an open inner corner of a phenanthrene moiety while the K region is defined as the external corner of a phenanthrene moiety (Loganathan and Lam, 2011). These regions are responsible for the formation of epoxide which is thought to be a potential carcinogen. Its structure as well as that of anthracene is found in compounds such as, benzo[a]pyrene and benz[a]anthracene (Mrozik *et al*., 2003). Thus, phenanthrene is used as a model in studies relating to carcinogenic PAHs such as benzo[a]pyrene and chrysene. Several studies have documented the catabolic pathway of phenanthrene by bacteria belonging to the following genera, *Sphingomonas*, *Pseudomonas*, *Mycobacterium*, *Nocardia* (Mrozik *et al*., 2003). The strain, *Stenotrophomonas maltophila* is capable of mineralizing phenanthrene The metabolism of phenanthrene by soil bacteria occurs basically by any of two ways; either the compound, 1-hydroxy,2-naphthoic acid is oxidized to 1,2-dihydroxynaphthalene which is then broken down to salicylate before additional metabolism (naphthalene pathway), or 1-hydroxy,2- naphthoic acid is cleaved before further metabolism (phthalate pathway) (Ouyang, 2013). It however, shares a similar catabolic pathway to naphthalene (Fig. 2.3).

### 2.6.2 Pathway for the bacterial degradation of chrysene

Chrysene is a H.MW polycyclic aromatic hydrocarbon, made up of four fused benzene rings. It is listed as a priority pollutant (USEPA, 1996), and there are less studies on its bacterial degradation (Demaneche *et al*., 2004) compared to other four-ringed PAHs such as pyrene and fluoranthene (Johnsen *et al*., 2007; Zhou *et al*., 2008; Klankeo *et al*.,

2009; Guo *et al*., 2010). Its carcinogenic isoforms include, 5-methylchrysene, 6-nitrochrysene, and dibenzo[c,p]chrysene (Patel and Ali, 2014). Chrysene is very resistant to degradation and only a few species have been shown to mineralize the compound, *Rhodococcus* sp. strain UW1 (Walter *et al*., 1991), *Stenotrophomonas maltophila* strain (Boonchan *et al*., 1998).

The strain, *Sphingomonas* sp. strain CHY-1 capable of growing on chrysene as a sole source of carbon and energy was used in a study analysing the various proteins important in the degradation of PAHs (Demaneche *et al*., 2004), and it exhibited highest mineralization rates when chrysene was the only source of carbon compared to when a combination of phenanthrene and/or other organic substrates were used, thus showing very good potential in the degradation of chrysene. There has not been comprehensive reports about the complete catabolic pathway of chrysene by bacteria thus, the pathway shown in Fig. 2.4 is a tentative degradative pathway proposed by Nayak *et al*. (2011) in the likely event of complete mineralization of the compound.

### 2.6.3 Pathway for the bacterial degradation of benzo[a]pyrene

Benzo[a]pyrene (Bap) is a five ringed H.M.W polycyclic aromatic hydrocarbon. It is highly recalcitrant and resistant to degradation due to its low water solubility. It is often used as a standard for the measurement of carcinogenic PAH in environmental samples due to its low availability in the environment (Seo *et al*., 2009) and its high carcinogenic potential. There has been evidence of mineralization of Bap by bacterial species (Luo *et al*., 2009) however, its metabolic or cometabolic degradation by bacteria has been extensively documented (Kanaly and Bartha, 1999; Kanaly and Harayama, 2000).

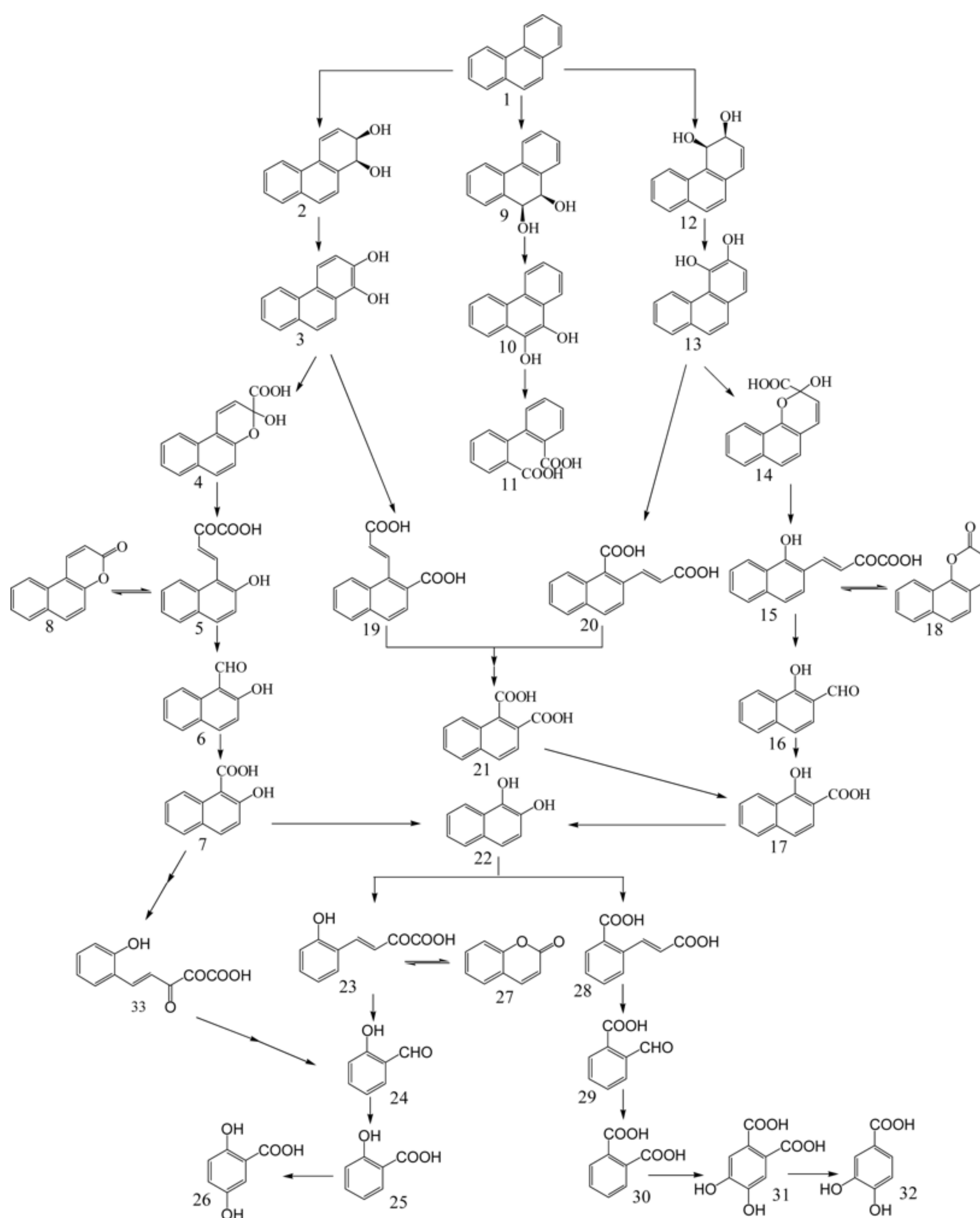


**Figure 2.3 Proposed catabolic pathway of phenanthrene by bacteria (Reference: Seo *et al*., 2009)**

Designations: 1, phenanthrene; 2, *cis*-1,2-dihydroxy-1,2-dihydrophenanthrene; 3, 1,2-dihydroxyphenanthrene; 4, 3-hydroxy-3*H*-benzo[*f*]chromene-3-carboxylic acid; 5, 4-(2-hydroxy-naphthalen-1-yl)-2-oxo-but-3-enoic acid; 6, 2-hydroxy-naphthalene-1-carbaldehyde; 7, 2-hydroxy-1-naphthoic acid; 8, 5,6-benzocoumarin; 9, *cis*-9,10-dihydroxy-9,10-dihydrophenanthrene; 10, 9,10-dihydroxyphenanthrene; 11, 2,2'-diphenic acid; 12, *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene; 13, 3,4-dihydroxyphenanthrene; 14, 2-hydroxy-2*H*-benzo[*h*]chromene-2-carboxylic acid; 15, 4-(1-hydroxynaphthalen-2-yl)-2-oxo-but-3-enoic acid; 16, 1-hydroxy-naphthalene-2-carbaldehyde; 17, 1-hydroxy-2-naphthoic acid; 18, 7,8-benzocoumarin; 19, 1-(2-carboxy-vinyl)-naphthalene-2-carboxylic acid; 20, 2-(2-carboxy-vinyl)-naphthalene-1-carboxylic acid; 21, naphthalene-1,2-dicarboxylic acid; 22, naphthalene-1,2-diol; 23, 2-hydroxybenzalpyruvic acid; 24, salicylic aldehyde; 25, salicylic acid; 26, gentisic acid; 27, coumarin; 28, 2-carboxycinnamic acid; 29, 2-formylbenzoic acid; 30, phthalic acid; 31, 3,4-dihydroxyphthalic acid; 32, protocatechuic acid; 33, *trans*-2,3-dioxo-5-(2'-hydroxyphenyl)-pent-4-enoic acid.

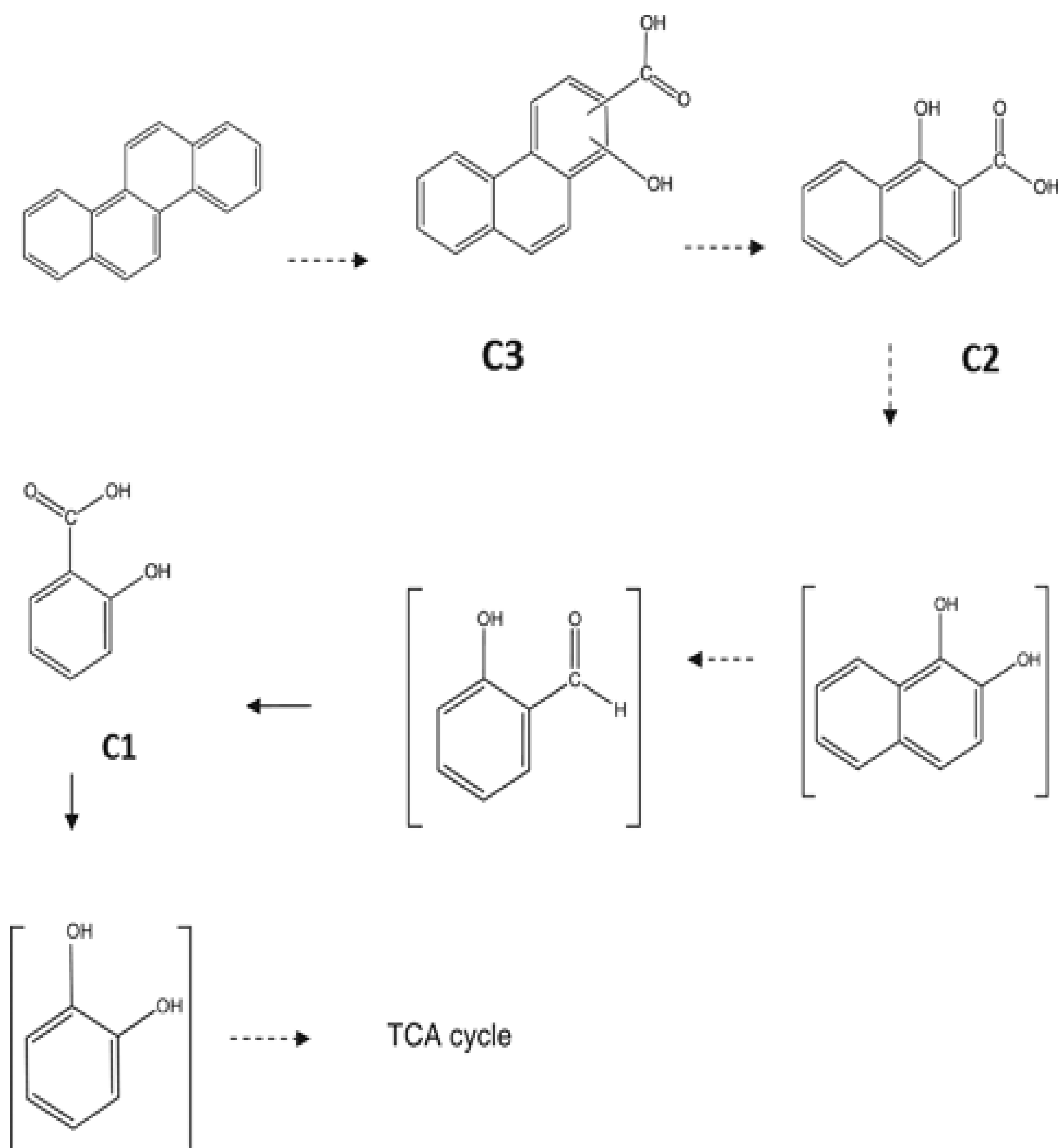



**Figure 2.4 Proposed catabolic pathway of chrysene in *Pseudoxanthomonas* sp. PNK-04.** (Reference: Nayak et al., 2011).

Designations: C1: salicylic acid; C2: 1-hydroxy,2-naphthoic acid; C3: hydroxyl phenanthroic acid.

Bacteria belonging to the genera, *Mycobacterium*, *Sphingomonas*, *Rhodococcus*, *Pseudomonas*, *Bacillus*, *Burkholderia* have shown the ability to degrade Bap to various levels (Ye *et al*., 1996; Aitken *et al*., 1998). The bioavailability of Bap during its mineralization in soil seems to occur at a faster rate in the presence of petroleum products such as diesel and crude oil (Kanaly and Bartha, 1999). The metabolic pathway for the degradation of Bap by bacteria is as shown in Figure 2.5.

## 2.7 Identification of PAH-degrading Bacteria based on Non-culture Methods:

Culture-dependent methods in the identification of hydrocarbon-degrading bacteria in the soil often results in the selective enrichment of only a few species, thus leading to the creation of a wide dearth in information concerning bacteria important in pollutant degradation. The isolation of these bacteria on culture media typically entails the introduction of one or more PAH either in solution with an appropriate organic solvent (Sho *et al*., 2004; Kim *et al*., 2006; Ding *et al*., 2010; Sun *et al*., 2010).

Even though many studies have combined both culture and molecular methods in the identification of bacteria capable of degrading PAHs (Luo *et al*., 2009; Lors *et al*., 2010; Guitierrez *et al*., 2015), some of these studies have confirmed that the culture dependent methods do not identify nearly as much species as the non-culture dependent techniques.

Another advantage of the molecular methods in the study of the structural and functional diversity of such PAH-degrading bacteria is the ability to detect active enzyme function in the polluted environments (Yagi and Madsen, 2009; Korotkevych *et al*., 2011). Needless to say, the methods based on culture inevitably yield pure isolates that can be used as a model for further studies including, the elucidation of metabolic pathway and as the basis for the *in silico* design of primers.

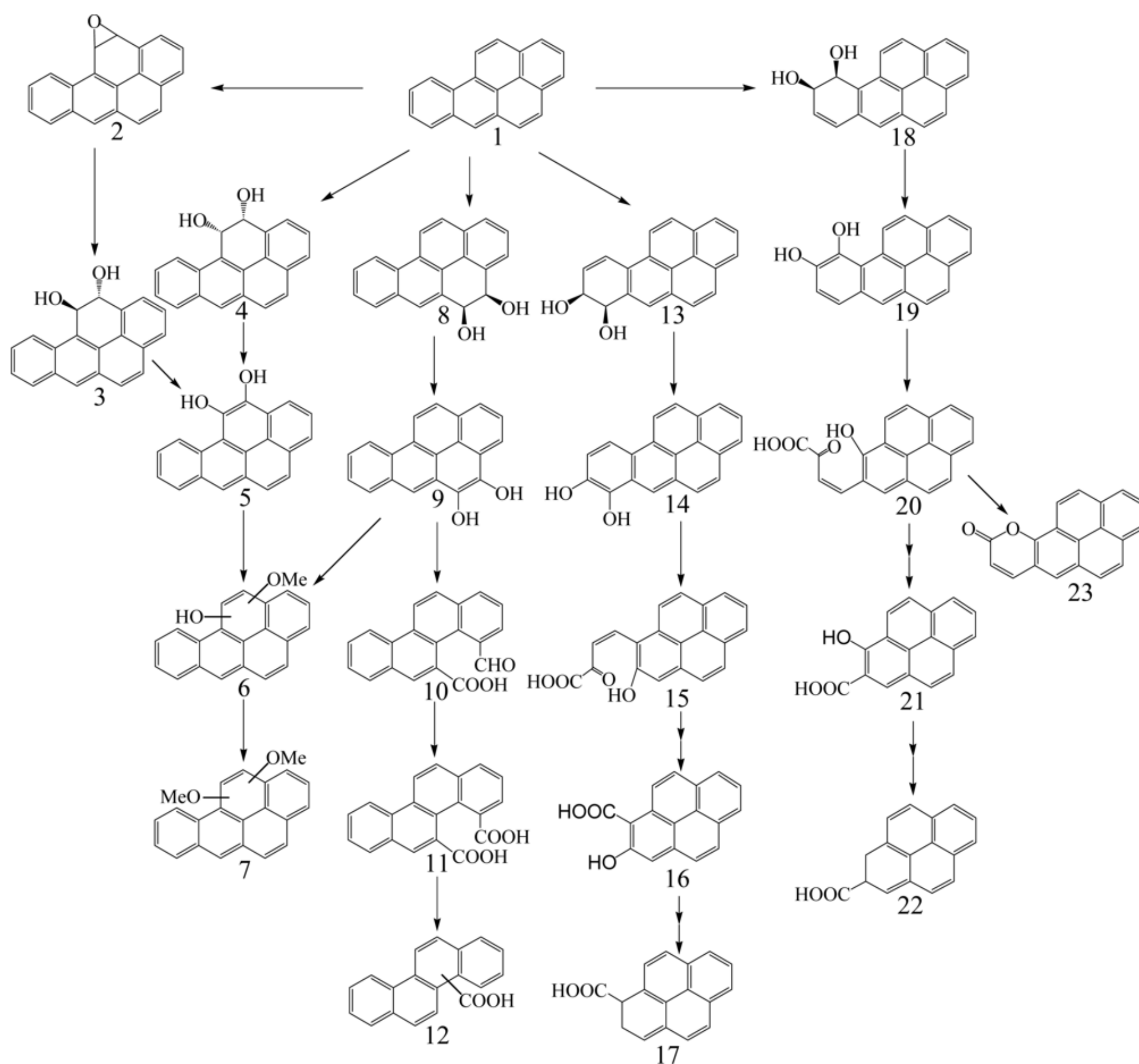



**Figure 2.5 Proposed Pathway for metabolic degradation of benzo[a]pyrene by bacteria** (Reference: Peng *et al*., 2008).

*Compound designations*: 1, benzo[*a*]pyrene; 2, benzo[*a*]pyrene-11,12-epoxide; 3, benzo[*a*]pyrene trans-11,12-dihydrodiol; 4, benzo[*a*]pyrene cis-11,12-dihydrodiol; 5, 11,12-dihydroxybenzo[*a*]pyrene; 6, hydroxymethoxybenzo[*a*]pyrene; 7, dimethoxybenzo[*a*]pyrene; 8, benzo[*a*]pyrene cis-4,5-dihydrodiol; 9, 4,5-dihydroxybenzo[*a*]pyrene; 10, 4-formylchrysene-5-carboxylic acid; 11, 4,5-chrysene-dicarboxylic acid; 12, chrysene-4 or 5-carboxylic acid; 13, benzo[*a*]pyrene *cis*-7,8-dihydrodiol; 14, 7,8-dihydroxybenzo[*a*]pyrene; 15, *cis*-4-(7-hydroxypyrene-8-yl)-2-oxobut-3-enoic acid; 16, pyrene-7-hydroxy-8-carboxylic acid; 17, 7,8-dihydro-pyrene-8-carboxylic acid; 18, benzo[*a*]pyrene *cis*-9,10-dihydrodiol; 19, 9,10-dihydroxybenzo[*a*]pyrene; 20, *cis*-4-(8-hydroxypyrene-7-yl)-2-oxobut-3-enoic acid; 21, pyrene-8-hydroxy-7-carboxylic acid; 22, 7,8-dihydro-pyrene-7-carboxylic acid; 23, 10-oxabenzo[*def*]chrysene-9-one.

### 2.7.1 Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis:

DGGE is a method of DNA fingerprinting that separates nucleotide sequences based on effect of two concentrations of denaturant. It has been used successfully in many studies to study the population dynamics of active bacterial communities during pollutant degradation (Gomes *et al*., 2005; Vinas *et al*., 2005; Korotkevych *et al*., 2011; Festa *et al*., 2013). Although the procedure involved in DGGE is laborious, often involving two days, it is very useful in analysing the different bacterial groups in an environmental sample. The bands generated from a DGGE profile obtained from the amplicons of 16S rRNA gene are excised and the nucleotide sequence determined, thus revealing the likely taxonomic groups present. The PCR amplification of the gene of interest is sometimes carried out using a primer having a GC clamp attached to aid the separation of the bands during DGGE. The use of a GC clamp in primer pairs during PCR preceding DGGE has been highlighted in details in a study by O'Sullivan *et al*. (2008), whereby the effect of various primer pairs with GC clamps were analysed in the complete sequencing of DNA obtained from bacteria and archaea. The diversity of bacterial populations as exhibited by the DGGE band patterns can be determined using tools such as dimensioning methods which include Principal Component Analysis (PCA) and multidimensioning scaling (MDS); cluster analysis as well as Shannon and Simpson diversity indices. These methods mentioned all analyse the band patterns resulting from DGGE in their respective ways however, the outcome leads to an elucidation of the bacterial population dynamics. The PCA and MDS as means of statistically analyzing data from DGGE-generated profiles inevitably lead to the creation of dendograms which are based on calculated distances. PCA and MDS have been used individually or together in determining changes or shifts in bacterial

population based on the band intensities in the DGGE profile (Vinas *et al*., 2005; Alonso-Gutierrez *et al*., 2009; Kostka *et al*., 2011). In a study by Korotkevych *et al*. (2011), the effect of pollutant concentration on the 16S rRNA fingerprint in bulk and control soils was assessed using the software, GELCOMPAR. Subsequent analysis of sequences in the study revealed that the two types of mesocosms had different predominant phylotypes. The DGGE analysis of the 16S rRNA fragments from sediments of a lake in the Antarctica showed that the diversity of the prokaryotic population in the sediments was likely a function of climatic events (Tang *et al*., 2013), this was arrived at from the dominant phyla identified in the different depths and texture of the sediments. This fact highlights the importance of DGGE in illuminating key aspects of microbial ecology and the impact of varying environmental conditions on prokaryotic population composition.

The diversity of PAH-degrading bacteria in soils and aquatic environment based on functional genes has also been studied extensively by DGGE (Gomes *et al*., 2007; Korotkevych *et al*., 2011; Adetutu *et al*., 2013). Polymerase Chain Reaction-Thermal Gradient Gel Electrophoresis (PCR-TGGE) works the same way as DGGE except for the fact that the temperature is used to create a gradient for the separation of same-sized nucleotide sequences into their respective sequences. It has been used in a few studies concerning the community profile of PAH-degrading bacteria from environmental samples (Cebron *et al*., 2009; Cebron *et al*., 2013) however; it is less commonly used compared to PCR-DGGE.

### 2.7.2 Single strand conformation polymorphism (SSCP):

This culture independent method involves the separation of equal-sized DNA based on the different nucleotide sequences; the principle is based on the folding of single

stranded DNA into secondary structures which ultimately display the corresponding position during electrophoresis, based on the different sequences. Witzig *et al*. (2006) used this method to study the profile of a complex bacterial community structure as well as the presence of the iron sulphur moiety which is important in the degradation of aromatic compounds (such as BTEX). The relatedness between the dioxygenase enzymes in the contaminated soil samples was thus determined from the resulting SSCP fingerprints. In another study by Junca and Pieper (2004) involving the diversity of C23O gene in environmental samples, PCR-SSCP was successfully employed in showing the effect of various sampling sites on the diversity of the catabolic gene (C23O), which is important in meta cleavage of the catechol ring during the degradation of aromatic compounds. The diversity of the gene was observed to be "site specific" (Junca and Pieper, 2004).It is important to note that SSCP uses a non-denaturing polyacrylamide gel unlike the DGGE method. SSCP remains a powerful tool in analysing sequence diversity in functional gene of pure bacterial strains and in environmental samples.

### 2.7.3 Terminal restriction fragment length polymorphism (T-RFLP):

Terminal restriction fragment length polymorphism is a technique used in analysing microbial community structure and is analogous to ARDRA however; it is a much better modification (Chikere, 2013). The primers used in this process are fluorescent-labelled at the 5' end, and unlike ARDRA, only the terminal restriction fragments for each organism detected are identified (Chikere, 2013). T-RFLP is an excellent tool in studying changes in bacterial composition and function; it has been used by Zhang *et al*. (2011) in determining dynamics in soils irrigated with reclaimed wastewater. The effect of PAH amendments in the soil samples in this study was also analysed using T-RFLP fingerprints of the bacterial community. In another study by Yrjala *et al*. (2010), the

impact of PAH amendments as well as plant rhizosphere effects was determined using T-RFLP and RFLP; the advantage of T-RFLP over RFLP is the fact that the former only elucidates the end fragments of PCR unlike RFLP in which all the fragments of the PCR are visualized (Yrjala *et al*., 2010). Thus, making it more labour-intensive. Even though in the study by Yrjala *et al*. (2010), the two methods were used simultaneously, they were both serving different purpose; T-RFLP was used in determining the community structure of the bacteria in the soils sampled by analysing the 16S rRNA sequences while RFLP was employed in detecting the diversity of the gene responsible for ring cleavage in PAHs. Bordenave *et al*. (2008) were able to detect population dynamics in the diversity of bacteria based on T-RFLP analysis of ring-hydroxylating dioxygenase gene (RHD); they employed the use of mRNA transcripts of the dioxygenase gene (RHD). The disadvantage of T-RFLP as a DNA fingerprint method is that, unlike DGGE/TGGE, the T-RF obtained are usually too short to obtain adequate information for sequencing, as such will not be ideal for phylogenetic analysis (Chikere, 2013).

2.7.4 Amplified ribosomal DNA restriction analysis (ARDRA) and Amplified functional DNA restriction analysis (AFDRA) Profile:

Amplified ribosomal DNA restriction analysis (ARDRA) is a procedure whereby restriction enzymes are used to cut PCR amplicons at specific sites and then the products separated by gel electrophoresis. The resulting fingerprint gives the profile of bacterial community of the sample being analyzed. This method is specific for 16S rRNA genes, while a similar technique known as, amplified functional DNA restriction analysis (AFDRA), applies the same principle but with functional genes. In a study by Junca and Pieper (2003), bacteria sharing the ability to degrade aromatic hydrocarbons were analysed for metacleavage activity by applying AFDRA to screen for the type of C23O gene harboured by the bacteria in soil samples. Both ARDRA and AFDRA have

been successfully combined with other fingerprinting methods in determining the community structure and function of pollutant-degrading communities (Junca and Pieper, 2003).

### 2.7.5 Microarray technologies

DNA microarray technology is a powerful tool in the study of bacteria important in pollutant degradation in relation to both taxonomic and functional genes. It is utilized in the analysis of gene expression in the fields of microbial ecology (Johnsen *et al*., 2007) and medicine. The technology has been used in pure culture studies however; it has proven challenging in environmental studies due to specificity, quantification and sensitivity in analysing the genes in question (Chikere, 2013). The sensitivity of glass-based microarray methods is lower than membrane-based hybridization methods probably because the probe-binding ability of on porous membranes is higher than that of glass surfaces (Schadt and Zhou, 2006). The challenges related to sensitivity and specificity of the microarray technologies can be overcome by employing a cohesive methodology (Yergeau *et al*., 2009), by focusing on specific environments or functional genes as opposed to wider groups. Regardless, analysis of 16S rRNA gene sequences in environmental samples has been successfully conducted using an appropriate set-up of this method (Desai *et al*., 2010). The possibility of biases occurring during PCR amplification of taxonomic or functional genes makes microarray technology a very reliable means of quantifying genes being studied. The other advantages of this method include becoming increasingly affordable and easy to use (Schadt and Zhou, 2006).

Based on the type of probes used in environmental studies, DNA microarray methods are grouped into three categories; Phylogenetic oligonucleotide arrays (POAs) based on "sequence probes derived from ribosomal RNA genes", thus are important in

phylogenetic studies; Functional gene arrays (FGAs) based on "sequence probes for genes encoding proteins important in certain functions in the environment"; Community genome arrays (CGAs) based on "whole genome or large fragment of DNA derived from pure culture of microorganisms" (Schadt and Zhou, 2006). Schadt *et al*. (2005) have proposed that the use of FGA in studying the diversity of bacteria capable of degrading PAHs such as naphthalene, the alpha (α) subunit is the ideal component of the multi-enzyme complex (NDO) that should be analysed since it confers substrate specificity. FGA was utilized in analysing several functional genes important in heavy metal transformation, alkane degradation, aromatic hydrocarbon degradation, nitrogen cycle and other processes in a study by Yergeau *et al*. (2009); in the same study 16S rRNA gene probe was also used to determine the community structure of the bacteria in cold environments. The soil samples in the study by Yergeau *et al*. (2009) were obtained from two sites in the Arctic region that had a large amount of diesel spillage a few years before the study (2004). An improvement on FGA analysis of microbial communities is the development of FGAs containing oligos (synthetic oligonucleotides) which are simpler to use compared to DNA-based FGAs; due to the fact that they can be "synthesized based on sequence information from public databases" (Schadt *et al*., 2005).

## 2.8 Studies on Bacterial Degradation Of Polycyclic Aromatic Hydrocarbons In Nigeria

Nigeria being one of the major oil exporting countries in the world is likely to have issues relating petroleum spillage in the environment. As a result of this there has been numerous studies focused on the use of microorganisms in the bioremediation and biodegradation of these pollutants (Ayotamuno *et al*., 2006; Igwo-Ezikpe *et al*., 2006; Adoki and Odokuma, 2007; Onifade and Abubakar, 2007; Chikere *et al*., 2012). Even

though a good number of these studies are based on culture dependent methods, they still cover a lot of depth in terms of the type of hydrocarbon degraded as well as methods of identification of the hydrocarbon utilizing microorganisms. Biostimulation is a technique that has been extensively highlighted in studies concentrated on petroleum degradation in Nigeria (Ijah *et al*., 2008; Raji *et al*., 2012).

The polycyclic aromatic hydrocarbons as constituents of petroleum and its products have received a fair amount of attention in studies relating to microbial ecology with respect to pollutant degradation (Chikere and Ekwuabu, 2014). Their toxic, carcinogenic and mutagenic properties cause them to be the subject of numerous researches in order to determine eco-friendly means of removing them from the environment (Eziuzor and Okpokwasili, 2013). Such studies often involve isolating bacteria and/or fungi from contaminated environments and optimizing these isolates in subsequent biodegradation studies (Ayotamuno *et al*., 2006; Akpe *et al*., 2015). However, studies based on only culture methods do not provide adequate information due to biases arising from the very small proportion of the bacterial community analyzed since most bacteria in soil do not grow on culture media. Culture methods cannot be used to obtain information on the effects of varying conditions such as pollutant concentration on the community structure. In light of this, culture independent methods (molecular techniques) based on the use of DNA or mRNA are more comprehensive in deducing the dynamics involved in microbial community structure and function in relation to pollutant degradation. However, attention is being given to soil metagenomics and its role in PAH and petroleum hydrocarbon degradation (Ichor *et al*., 2015).

Igwo-Ezikpe *et al*. (2010) evaluated the role of plasmids in the degradation of PAHs by isolating bacteria from engine-oil polluted soils in Lagos State, Nigeria. Their findings

revealed that some of the isolates lost the ability to degrade PAHs after curing them of plasmids however; the other isolates retained their PAH-degrading ability despite plasmid curing. This indicates that the genes encoding PAH degradation were located either on plasmids or chromosome, or both. The study is an improvement on just relying on the ability to isolate the hydrocarbon-degrading bacteria on culture media.

Some studies on microbial degradation of petroleum hydrocarbons in Nigeria inevitably lead to focus on polycyclic aromatic hydrocarbons. An example of this is a study by Nwinyi *et al*. (2014) in which different species of bacteria was isolated from soils contaminated by diesel; polycyclic aromatic hydrocarbons such as fluorene and pyrene were detected in the contaminated soils.

Interesting review articles written by Nigerian scholars on the biodegradation of PAHs as well as other key aspects of these compounds have shed a lot of light on the subject. Chikere (2013) wrote a review about the molecular techniques employed in identifying PAH-degrading microbial populations. These techniques are very recent technologies which are being widely used in developed and in some developing countries; they also offer a lot of information on the functional capabilities of these hydrocarbon-degrading bacteria. The methods covered by Chikere (2013) are centred on metagenomics and transcriptomics. Ukiwe *et al*. (2013) reviewed the various means of degrading PAHs including physical, chemical and biological methods; as well as a combination of two or more of these methods.

Not all the studies on microbial degradation of PAHs in Nigeria are based on petrogenic sources of PAHs. A study by Igwo-Ezikpe *et al*. (2010) involved the isolation and enrichment of HMW-degrading bacteria from soils obtained from not only petroleum-contaminated soils but also from coal gasification and wood processing sites. The sites

studied were not located in only one region of the country but from three different geographic zones, South-South or Niger delta region (Port-Harcourt City), South-East (Enugu City) and South-West (Lagos State). The isolates belonging to the genera, *Sphingomonas* and *Pseudomonas* were shown to be able to utilise the HMW PAHs as sole source of Carbon and energy (Igwo-Ezikpe *et al*., 2010).

These studies on the biodegradation of PAHs only reflect a few of the numerous studies conducted in Nigeria. There is a lot of attention on the use of microorganisms and plants in the bioremediation of PAH-contaminated environments in Nigeria due to the burden of environmental pollution prevalent in the country. The exploration of petroleum in the Niger delta region of the country has caused widespread pollution in the terrestrial and aquatic habitats thus grossly affecting the socio-economic life of the indigenes. The ability of these hydrocarbons to have potential carcinogenic and mutagenic effects only makes matters worse. However, a more comprehensive approach to these bioremediation efforts in Nigeria will be to include metagenomics and other molecular techniques in studying these PAH-degrading microbial populations.

# CHAPTER THREE

## 3.0 MATERIALS AND METHODS

### 3.1 Sampling Sites and Soil Samples:

The petroleum-contaminated sites where soil samples were obtained are located in Zaria and Kaduna, in Kaduna State. Kaduna state is located in the Northwest region of Nigeria. The sampling sites were selected on the basis of their long history of contamination (at least two decades) with petroleum products. The sites used in this study are as shown in the map in Figure 3.1. Sampling in this study was done in two phases, the first sampling was carried out in April, 2013, to cover the first three objectives while, the second phase was done in December, 2014 for the remaining three objectives. The soil samples were collected from the following sites:

- ✓ Mechanic workshop in Tudun Wada, Zaria (Long. 7°43’22’’ Lat. 11°05’19’’)
- ✓ The bank of Romi stream receiving effluent from Kaduna refinery (Long. 7°25’55’’ Lat. 11°26’39’’)
- ✓ Trailer park in Dan Magaji*, Zaria (Long. 7°40’14’’ Lat. 11°04’10’’)
- ✓ Trailer park in Gwargwaje**, Zaria (Long. 7°40’55’’ Lat. 11°01’50’’)

(*first phase of sampling; **second phase of sampling)

Samples were collected from the same mechanic workshop as well as refinery effluent site during both phases of sampling however; two different trailer parks were used for sampling for the first and second phase, respectively.

Core samples were collected using a clean auger from random points at the sites and pooled together. The soil samples were obtained at two different depths of 17 – 20 cm and 37 – 40 cm respectively. The samples were kept in sterile brown glass bottles and

stored in the laboratory at a temperature of 4°C for physico-chemical analyses, and at -20°C for DNA extraction.

Photographs of the sampling sites as well as the shallow and deep soil samples are as shown in Appendices I – III.

**3.2 Physico-chemical Analysis of Soil Samples:**

3.2.1 Textural classification of soil samples:

Fifty gram of sieved soil was weighed into a 250mL plastic beaker and 100 mL of sodium hexametaphosphate was added to the beaker and stirred with a glass rod. About 100 mL of distilled water was added and stirred in; the beaker was left to stand for about thirty minutes with occasional stirring. The sample and all the contents were washed into a mixing cup and stirred for five minutes; the mixture was transferred into a measuring cylinder (one litre), and filled to the mark with water. The suspension was mixed vigorously and hydrometer readings taken at 40 seconds and 2 hours respectively. The hydrometer reading of a blank solution (100mL of 50% sodium hexametaphosphate made up to one litre) was also taken at the same time intervals. The temperature of the suspension as well as the blank was measured using a thermometer. The suspension was then poured into a 0.2 mm sieve; the sand grains retained on the sieve were washed with water, transferred into a beaker and dried in an oven at 105°C. Finally, the sand was weighed and the weight recorded. This weight gave the coarse sand fraction. The calculation of percentages of the other particles is given below:

$$\%Clay = \frac{\text{Corrected 2hrs reading} - \text{Blank}*100}{\text{Weight of soil taken}}$$

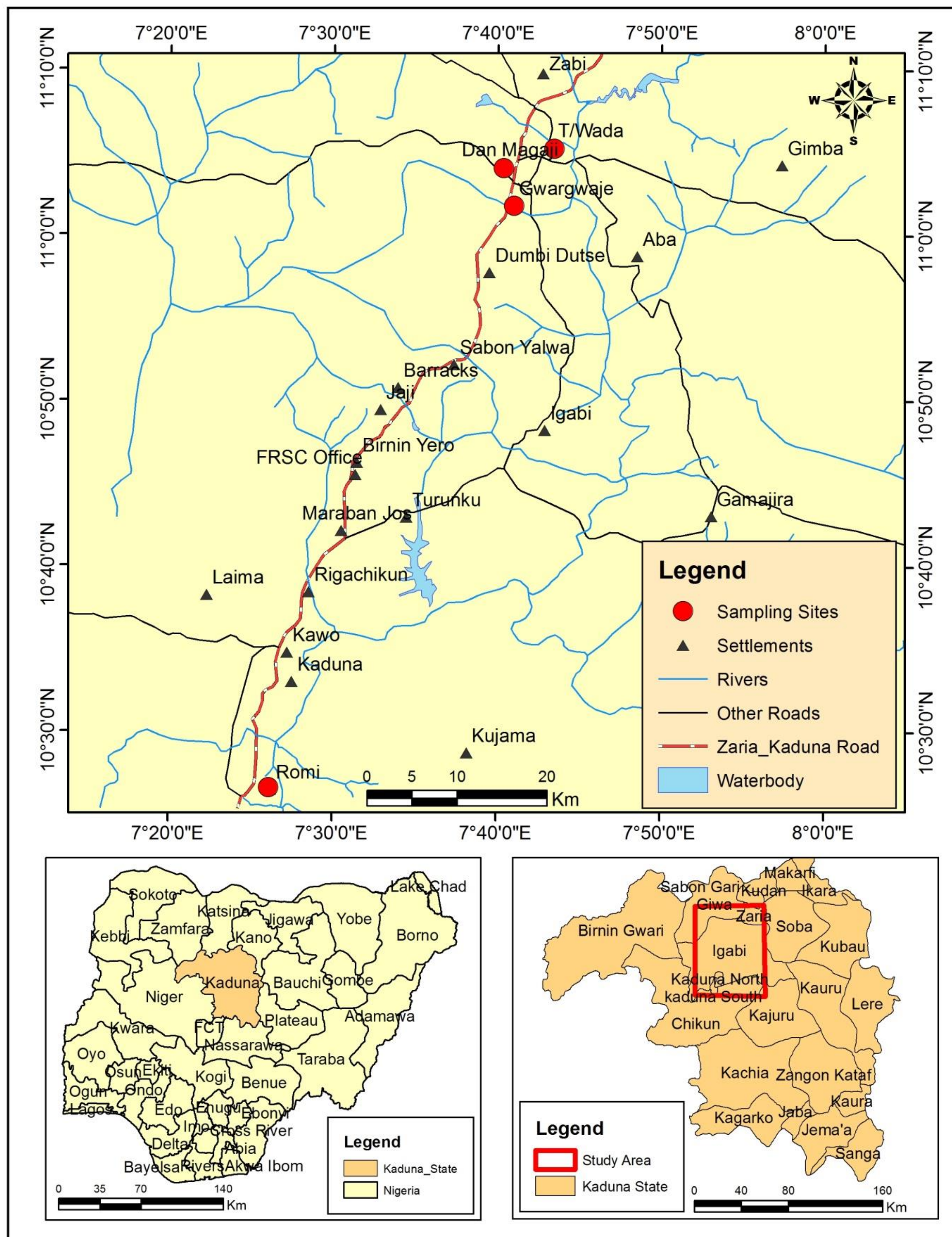



**Figure 3.1 Sampling sites for collection of soils for molecular identification of bacteria in petroleum-contaminated soils**

$$\%Silt = \frac{\text{Corrected 40 sec. reading} - \text{Blank}*100 - \%\text{Clay}}{\text{Weight of soil taken}}$$

%Sand = 100 – (%Clay + %Silt)

(Uyovbisere *et al*., 2013)

3.2.2 Determination of pH:

To ten gram of air-dried soil in a fifty millilitre plastic beaker, twenty five millilitre of distilled water was added and the suspension stirred several times for 30 minutes, after which it was left to stand for thirty minutes. The pH meter was calibrated using pH buffer 4, 7, and 9. The electrode was immersed into the suspension without touching the bottom of the beaker, and the pH read after thirty seconds (Uyovbisere *et al*., 2013).

3.2.3 Determination of organic carbon (Walkley-Black method):

One gram of air-dried soil was weighed into an Erhlenmeyer flask (250 mL), five millilitre of 1N $K_2Cr_2O_7$ was added and the flask swirled to ensure dispersion of the soil. Concentrated sulphuric acid (ten millilitre) was rapidly added and the suspension swirled again for one minute. The flask was allowed to stand on asbestos sheets for thirty minutes, and 100 mL of distilled water added and the flask was allowed to cool. Five drops of the indicator (O-phenanthroline ferrous sulphate) was added. The suspension was titrated with ammonium ferrous sulphate solution against a white background. A blank (without soil) determination was determined in the same manner.

$$\%\text{Organic Carbon} = \frac{(\text{blank titre} - \text{actual titre})*0.3*m*f}{\text{Weight of air-dried soil}}$$

Where, m = concentration of ferrous ammonium sulphate

f = correction factor (1.33)

(%Organic matter = %Organic Carbon*1.724)

3.2.4 Determination of nitrogen in soil:

Fine mesh soil (one gram) was weighed into a 500 mL Kjeldahl flask and few drops of water was added and allowed to stand for thirty minutes. Five gram of Kjedahl catalyst mixture and twenty millilitres of concentrated sulphuric acid were added to the flask, and heated on a sand block until frothing ceases. The temperature was then increased until the digest clears and then for another two hours. The flask was cooled and little quantity of water added with care before washing the contents into a 100 mL volumetric flask, allowed to cool and then made up to the mark. Ten millilitres of the digest was pipetted into a distillation flask, twenty millilitres of 2% boric acid was measured into a 100mL conical flask and introduced into the bottom of the condenser. Three drops of the mixed indicator (0.2 gram of methylene blue in 100 mL ethanol and 0.4 gram of methyl red in 1000 mL ethanol) was added. Ten millilitres of 10N NaOH was added to ten millilitres of the digest in the distilling flask, the distilling was immediately introduced and the digest distilled off, thus collecting fifty millilitres of the distillate. The distillate was titrated with 0.01N $H_2SO_4$ from green to purple at end point.

Calculation:

$$\%Nitrogen = \frac{0.014 * (T - B) * NA * \text{Volume of digest} \times 100}{\text{Weight of soil} * \text{Aliquot distilled}}$$

(Uyovbisere *et al*., 2013)

3.2.5 Determination of phosphorus:

One gram of soil was weighed into a tube fitted with a stopper, seven millilitres of the extraction solution (0.031N $NH_4F$ + 0.05N HCl) was added and the container shook for 60 sec. The suspension was filtered into a dry beaker and two millilitres transferred to a dry twenty five millilitres beaker, to which five millilitres of ammonium molybdate reagent and one millilitre stannous chloride dilute solution were added. The suspension was mixed and after 10 minutes, the colour development was measured at 890 nm on a Spectronic 20 machine. The absorption was read, the standards were measured in the same manner.

3.2.6 Determination of sodium, potassium, calcium, magnesium and lead (Total Digestion method):

A quantity of finely ground soil (0.1 gram) was placed in a thirty millilitre platinum crucible and the soil was moistened with a few drops of water. Hydrofluoric acid (5 mL), nitric acid (three millilitre) and perchloric acid (one millilitre) were added to the crucible. The soil-acid mixture was heated on a hot plate until fumes of perchloric acid disappeared. The crucible was left to cool and 5 mL of hydrofluoric acid was added; it was covered with a platinum lid and placed on a sand bath. The crucible was heated to 200°C - 250°C, and evaporated to dryness, after the crucible had been cooled, 2 mL of water and a few drops of perchloric acid were added. The

crucible was replaced in the sand bath and evaporated to dryness; 6N HCl (5 mL) and water (5 mL) were added and then heated over a burner till the solution boiled gently. After the sample had dissolved, it was fitered into 100 mL capacity volumetric flasks and made up to the mark with distilled water. The solution was then stored for analysis using photometer for sodium and potassium, and atomic absorption spectroscopy for calcium and magnesium.

3.2.7 Determination of some microelements (copper, manganese, zinc and iron):

Five grams of two millimetre-sieved soil was weighed into a 100 mL plastic bottle, and fifty millilitres of 0.1M HCl was added, the suspension was placed on a shaker for fifty minutes. It was then filtered through Whatman No. 42 filter paper. The quantities of the metals were determined using an atomic absorption spectrophotometer (AA500, PG Instruments, U.K) with the method described in the user manual.

3.2.8 Determination of Cation Exchange Capacity (C.E.C):

Ten grams of two millimetre sieved soil was weighed into a 100 mL plastic beaker, and about forty millilitres of 1N ammonium acetate (pH 7.0) was added, stirred with a glass rod and left overnight. The soil was filtered with light suction using a 55 mm Buchner funnel. The soil in the funnel was leached with 1N ammonium acetate to a volume of 250mL; the leachate was preserved for the determination of calcium, magnesium, potassium, and sodium. The leachate was tested with ammonium oxalate to ensure that it was calcium free. The soil was leached with 1N $NH_4Cl$ four times with twenty five millilitres portion at a time, and once with twenty five millilitres of 0.25N $NH_4Cl$, the solution was discarded. The electrolyte was washed with 150 – 200 mL of isopropyl alcohol, the soil was washed gradually with

acidified sodium chloride to a volume of 250 mL. Fifty millilitres of 2% boric acid was measured into 250mL conical flask and a few drops of mixed indicator added. The acidified sodium chloride leachate was poured into a 500mL Kjedahl flask and connected to a still. Some quantity of antibump and 1N sodium hydroxide were added to the flask and distilled over the boric acid in the conical flask.

The distillate (150mL) was collected; the ammonium borate titrated with a standard acid (0.1N HCl). The CEC was calculated in meq per 100 gram of soil.

Calculation:

$$\frac{(\text{Titre - B}) * \text{NA} * 100}{\text{Weight of soil}}$$

**3.3 Polycyclic Aromatic Hydrocarbon Analyses in Soil:**

The extraction of the PAHs was first carried out by weighing two gram of each soil into an amber-coloured reagent bottle, and twenty millilitres of dichloromethane (DCM) was added to the soil and vigorously shaken using a vortex mixer for five minutes and the solvent was decanted. The soil was washed again with DCM (twenty millilitres) and the solvent decanted. The extracts were collected and the solvent allowed to air-dry to two millilitres. It was then reconstituted with 4mL hexane, mixed thoroughly by shaking and then the solvent was allowed to evaporate to leave a residue of two millilitres. The column was stuffed with glass wool and the activated amorphous, twenty millilitres of hexane was used to condition the column. A volume of the sample (two millilitres) was loaded into the column and twenty millilitres of DCM was used to elute the sample, the solvent was allowed to

evaporate and then it was reconstituted with four millilitres of hexane, and subsequently allowed to evaporate to a final volume of two millilitres. A volume of twenty millilitres was transferred into a vial and used for Gas Chromatograph-Flame Ionizing Detector (GC-FID) analysis. The following conditions were used in the gas chromatograph column (OPTIMA-5MS, Macherey-Nagel, Germany); injection temperature of 250ºC; injection split of 10:1; oven temperature corresponding to 45 ºC/min to 130ºC, 10 ºC to 180 ºC, 6 ºC/min to 240 ºC, and finally, 13 ºC/min to 310 ºC for ten minutes (modified method of Massachusetts Department of Environmental Protection, 2004).

## 3.4 PCR-DGGE Analysis of Bacterial 16S ribosomal RNA Gene in Soil Samples:

### 3.4.1 Extraction of DNA from soil:

Isolation of DNA from the soil samples was carried out using the Fast DNA SPIN Kit for Soil (MP Biomedicals, U.K), the procedure was carried out as follows:

Soil sample (0.5 gram) was weighed aseptically and added to a Lysing Matrix E tube. To the sample in Lysing Matrix E tube was added 120 µL MT Buffer and 800µL Sodium Phosphate Buffer respectively. The tube was then homogenized in the Fastprep Instrument for thirty seconds at a speed setting of 5.5m/s (twice, allowing a five minute rest in-between); and centrifuged at 18,500g (14,000 rpm) for eight minutes to pellet debris. The supernatant was transferred to a clean 1.5mL microcentrifuge tube, and 250 µL PPS was added and mixed by shaking the tube by hand 10 times. The content of the tube was centrifuged at 18,500g for five minutes to pellet precipitate. The supernatant was transferred to a clean fifteen millilitres

tube (or universal bottle). The Binding Matrix suspension was resuspended by shaking the bottle and one millilitre was added to the supernatant in the universal bottle. The universal bottle was inverted by hand for two minutes to allow binding of DNA and then left to stand on the bench for thirty minutes to allow settling of silica matrix. Binding matrix was resuspended in the remaining amount of supernatant; approximately 600μL of the mixture was transferred to a SPIN Filter and centrifuged at 14,000g for one minute. The catch tube was emptied and the remaining mixture added to the SPIN Filter and centrifuged as before. The catch tube was emptied again. Prepared SEWS-M (ethanol added to concentrated SEWS-M) was added at a volume of 500 μL and the pellets gently resuspended using the force of the liquid from the pipette tip. It was centrifuged at 14,000g for one minute; the catch tube was emptied and replaced. Without any addition of liquid, it was centrifuged a second time at 14,000g for one minute to "dry" the matrix of residual wash solution. The catch tube was discarded and replaced with a new, clean catch tube. The Binding Matrix was gently resuspended (above the SPIN Filter) in 100μL of DES (DNase/Pyrogen-free Water) and left on the bench for 30 min. Finally, it was centrifuged at 14,000g for two minutes to bring eluted DNA into the clean catch tube. The SPIN Filter was discarded. The eluted DNA was stored at -20ºC for subsequent use.

3.4.2 PCR amplification and Denaturing gradient gel electrophoresis analysis (DGGE) of partial 16S rRNA genes:

The forward primer 357F*GC and the 518R amplify a product of about 201bp. For the DGGE procedure, a GC clamp with a length of 40bp was attached to the 5' end of the forward primer. The amplification was done in 50μL reaction mixtures containing: 1μL of template DNA, 20pmol/μL of each primer (MWG, U.K), 10μL 5

X PCR buffer (Promega, U.S), 10µg bovine serum albumin (Promega, U.S) and 1.25U DNA Polymerase (BioTaq, U.K). The amplification was carried out using a DYAD DNA Engine (MJ Research, U.S.A), the reaction mixture was incubated at 95ºC for five minutes and then decreased by 1ºC for thirty seconds. The annealing temperature was 55ºC for 30 seconds, and 72ºC for one minute, nine additional cycles was carried out at 94ºC then 25 cycles of 92ºC for thirty seconds, 52ºC for thirty seconds, 72ºC for one minute, and a final extension at 72ºC for ten minutes. The PCR products as well as DGGE marker were separated on 1.2% agarose gels, visulaized by ethidium bromide staining and quantified using a Gel-Doc molecular imaging software. A BioRad Detection Code was used in the DGGE procedure in separating the amplicons in sample volumes of 15µL using an 8% w/v polyacrylamide gel in 50 X TAE. The PCR amplicons obtained using the primers 357F-GC and 518R were separated with a parallel 30% and 60% urea-formamide denaturing gradient. The gels were electrophoresed for fifteen minutes at 80Volts; and then for a further 285 minutes at 200 volts, and stained with SYBR Gold (1 : 10 000; Promega) for twenty minutes. The gel was viewed using a Geldoc u.v illuminator, prominent bands were excised using sterile scapel amd stored at -4ºC until required for sequencing.

### 3.4.3 Sequencing of I6S rRNA amplicons and phylogenetic analysis

The excised DGGE bands were prepared for another round of amplification using the same primers and subsequently, the reamplified amplicons subjected to sequencing. The frozen excised DGGE bands in tubes were washed with fifty microlitres of sterile molecular grade water (Severn Biotech, U.K) while ensuring that the bands settled at the bottom of the tube. The water from the tubes was taken out using pipette and left open to enhance drying of the bands, pipette tip was used

to crush the bands (changing tips for each sample). Some quantity of sterile molecular grade water (fifteen microlitres for bright bands and ten microlitres for weak bands) was added to the crushed bands, (DNA contained in the water is used as template for reamplification procedure) and then stored in the freezer at 4ºC until ready for PCR (as a freeze-thaw process is necessary). Reamplification of the DGGE bands was carried out in 50µL reactions containing, one microliter (1µL) of the elute (DNA template), 200Nm each primer (357F-GC and 518R-M13), 10µg bovine serum albumin (Promega), 1.25 U DNA Polymerase (BioTaq, U.K) in 5 X PCR buffer (Promega). The following program was used in the PCR reaction: Incubation at 95ºC for five minutes., and nine cycles of 94ºC for thirty seconds., 55ºC for thirty seconds, and 72ºC for one minute; and then nineteen cycles of 92ºC for thirty seconds, 52ºC for 30 secs., 72ºC for one minute, and finally at 72ºC for ten minutes. Aliquots of fifteen microlitres of the reamplified amplicon were sequenced (Eurofins MWG, U.K), the resulting sequences were cleaned by removing GC clamp sequences using Finch tv program. The BLASTN program from the National Centre for Biotechnology Information (NCBI) website was used to conduct DNA similarity searches. The alignment and phylogenetic analysis of the resulting 16S rRNA sequences was conducted using MEGA4 program (Tamura *et al*., 2007).

### 3.5 Quantitative PCR (Real-time PCR) analysis of 16S rRNA genes in soil samples

A quantitative PCR method was used in determining the exact quantity of bacterial and archaeal 16S rRNA genes in the petroleum-contaminated soil samples. The bacterial and archaeal strains used as standards were *Anaerolineae thermpophila* DSM 14523 and

*Methanococcoides methylutens* DSM 2657 respectively. The primers for the 16S rRNA bacterial genes were 518F (Muyzer, 1993) and 907R (Muyzer and Smalla, 1998) while the Archaeal primers were ARCH – 0025 – AS17F and ARCH – 00344 AS20R (Vetriani, 1999).

The protocol used was adapted from Wilms (2007). Ten-fold serial dilutions of the standards was carried out ($10^{-1}$ – $10^{-8}$) and pipetted into the wells in triplicates. Twenty microlitres reaction mixtures containing 1µL of soil DNA, 20pmol/µL of each primer, 10µL 5 X SYBR Green qPCR SuperMix (Invitrogen, U.S.A.) and BSA 0.5µL were used for the amplification. Also included in the run was a no-template control (NTC) which is a mixture of all the PCR agents without any DNA. The qPCR runs were carried out: annealing temperature of 95ºC for seven minutes, 55ºC for thirty seconds, 72ºC for one minute, 60ºC for thirty seconds, 55ºC for thirty seconds, and 95ºC for thirty seconds. The resulting data after the qPCR run was analysed using MxPro, QPCR Software, version 4.10d (Stratagene, Agilent Technologies Division, Germany), the final number of target genes, was an average of triplicate measurements from three independent DNA extractions made from each soil sample.

The concentration of DNA in the standards (Archaeal and Bacterial) was measured using the Qubit Fluorometer (Quant-It Assay Kit) and Molecular Probes (Invitrogen, U.S.A.). All assay reagents were kept on the bench to attain room temperature. The Quant-It Working solution was made by diluting the Quant-iT reagent 1:200 in Quant-iT buffer. The assay tubes were prepared using the reagents' formulation depicted in Table 3.1. The tubes were vortexed and incubated at room temperature for 2 mins. They were read in Qubit flourometer and observations recorded. The concentration of the Archaeal and Bacterial DNA contained in the standards were input in the Q-PCR software for calculation of the gene copy number (Table 3.1).

### 3.6 Spiking Of Petroleum-Contaminated Soils with Polycyclic Aromatic Hydrocarbons and DNA Extraction

Another set of soils were collected from the same petroleum-contaminated sites described earlier and were treated with three polycyclic aromatic hydrocarbons (Sima-Aldrich), phenanthrene, chrysene and benzo[a]pyrene (Bap) respectively. The treatment was carried out as follows; five grams of each seeding soil was contaminated with phenanthrene, chrysene and Bap, respectively, that was dissolved in acetone (200 mg/mL) to reach a final concentration of two milligram of the PAH per gram of soil (Ding *et al*., 2010). The soil was mixed carefully, and kept at room temperature overnight to ensure evaporation of acetone. Control soils were mixed with only acetone. The moisture content was adjusted to 60% of the water holding capacity by adding the appropriate volume of sterile molecular grade water; this was repeated on a weekly interval through the period of incubation. Each soil treatment was kept in glass bottles covered in foil to avoid loss of the PAHs by photolysis, and incubated at room temperature for a period of sixty days. At the end of the incubation period, 0.5 gram of each soil was weighed and genomic DNA was extracted using the Fast DNA Spin kit for soil samples according to the manufacturer's instructions. The DNA elute obtained for the soil samples were stored at -20°C until required for further experiments.

The soils spiked with PAHs were subjected to DNA extraction, PCR-DGGE analysis, phylogenetics, and Real-time PCR analysis, as earlier described.

The DGGE profile of 16S amplicons from each of the contaminated site was subjected to Shannon-Weaver index to determine the species diversity (H), species richness (S) and evenness of specie distribution (E). The formulae below were used in the calculations:

$\mathbf{H = -sum\ (P_i ln[P_i])}$ (natural log)

**S = sum of the different species present**

$\mathbf{E = H/ln(S)}$ (natural log)

(Pi: proportion of each specie based on the total number of species; In[Pi]: natural log of Pi)

The nucleotide sequences obtained in this research were submitted in the Genbank database.

**Table 3.1: Determination of DNA concentration using fluorometer**

| Description | Standard Qubit assay tubes | User sample assay tubes (0.5µL) |
|---|---|---|
| Volume of working solution | 190µL | 198µL |
| Volume of standard from kit (Low and High, respectively) | 10µL | - |
| Volume of user sample to add | - | 2µL |
| Total volume in each assay tube | 200µL | 200µL |

µL: microliters; -: no volume added

**Table 3.2: PCR Primers used in this research**

| Primer | Sequence | Amplicon size | Reference |
|---|---|---|---|
| 518F (Bacteria) | CCAGCAGCYGCGGTAAN | 389 bp | Muyzer, 1993 |
| 907R (Bacteria) | CCGTCAATTCCTTTRAGTTT | | Muyzer and Smalla, 1998 |
| Arch – 0025 – AS17F (Archaea) | CTGGTTGATCCTGC CAG | 319 bp | Vetriani, 1999 |
| Arch – 00344– AS20R (Archaea) | ACGGGGCGCAGCAGGCGCGA | | Vetriani, 1999 |
| 357F*GC | CGCCCGCCGCGCGCGGCGGG CGGGGCGGGGGCACGGGGGG CCTACGGGAGGCAGCAG | 201 bp | Muyzer *et al.*, 1993 |
| 518R | ATTACCGCGGCTGCTGG | | |

*GC: GC clamp of forty (40) base pairs to aid separation of sequences during DGGE

# CHAPTER FOUR

## 4.0 RESULTS

### 4.1 Textural classification and physico-chemical properties of petroleum-contaminated soils obtained in first sampling

The textural classification and physico-chemical characteristics of the soil samples obtained is shown in Table 4.1. The soil texture was observed to be mostly loamy; however some of the samples had more quantity of sand than others. There was not much variation in the soil type in the two depths except for the Mechanic Workshop soils. It was noticeable that regardless of the site, the soils having a sandy loam texture had very similar electrical conductivity (0.30; 0.35) while, the soils having a loamy texture had relatively lower values of electrical conductivity (0.06 – 0.12). All the soils had acidic pH (5.0 – 5.9), oil and grease content ranged from 140 to 1590 mg/L with the shallow samples having higher values than their respective deeper samples. The moisture content was highest in the shallow soil from the Trailer park site, only the Mechanic workshop soil was observed to have higher moisture content in the deep soil. The cation exchange capacity of the Refinery effluent soil was very similar at both depths; however, the soils from the Mechanic workshop and Trailer park sites had strikingly different values at both depths.

### 4.2 Macronutrients in petroleum-contaminated soils in first sampling

The quantities of macronutrients in the soils from petroleum-contaminated sites are as shown in Table 4.2. The Organic Carbon content in soils from the sites was higher in the shallow soils with the exception of the Trailer park site. The same trend was observed with respect to the Nitrogen content. However, Phosphorus was present in higher quantities in the shallow soils than the deep soils for all three sites. The other

nutrients including Calcium, Magnesium, Sodium and Potassium didn't exhibit a particular pattern with respect to their quantity at both depths.

## 4.3 Microelements (mg/kg) of petroleum-contaminated soils in first sampling

The quantities of micronutrients in the soils sampled are shown in Table 4.3. Zinc was found to be in quantities higher than the recommended limit set by the Department of Petroleum Resources (2002) in all the soils sampled. Copper also exceeded the DPR standards in all the deep samples of the three sites as well as the shallow soil from the Refinery effluent site. Lead only exceeded its recommended limit in the Refinery effluent soil (shallow). Generally, the soils obtained from the Refinery effluent site had samples (5) with heavy metals exceeding the recommended limit, compared to the other sites (Mechanic workshop: 3; Trailer park: 3). Microelements such as, Manganese, Nickel, Cadmium, Chromium and Cobalt were present in the soils in quantities within the recommended limits (DPR, 2002).

## 4.4 Polycyclic aromatic hydrocarbon content in petroleum-contaminated soils in first sampling

None of the 16 priority PAHs was detected on analysis of the soil samples using Gas chromatography-Mass spectrometry (GC-MS) as depicted in Table 4.4.

## 4.5 PCR Amplification of 16S ribosomal RNA in the Petroleum-contaminated Soils

The 16S rRNA gene was amplified in the soils from all the sites; the primer pair, 357F-GC and 518R produced amplicons of 201 bp. The band intensity of the amplicons from the Mechanic workshop samples (Plate I) were not as bright as the amplicons from the Trailer park (Plate II) and Refinery effluent (Plate III) samples respectively.

**Table 4.1: Textural classification and physico-chemical properties of petroleum-contaminated soils obtained in first sampling**

| Sample | Textural Class | pH (1:2.5 $H_2O$) | O and G Content (mg/L) | Moisture Content (%) | Electrical Conductivity (dsm) | C.E.C |
|---|---|---|---|---|---|---|
| T.D | Sandy loam | 5.30 | 140 | 0.60 | 0.35 | 8.10 |
| T.S | Sandy loam | 5.50 | 1 590 | 3.10 | 0.30 | 5.90 |
| M.D | Loamy | 5.80 | 1 220 | 1.40 | 0.10 | 3.80 |
| M.S | Sandy loam | 5.90 | 1 340 | 0.80 | 0.35 | 5.70 |
| R.D | Loamy | 5.40 | 770 | 0.75 | 0.06 | 4.70 |
| R.S | Loamy | 5.00 | 940 | 1.10 | 0.12 | 4.80 |

T.D: trailer park deep soil; T.S: trailer park shallow soil; M.D: mechanic's workshop deep soil; M.S: mechanic's workshop shallow soil; R.D: refinery effluent site deep soil; R.S: refinery effluent site shallow soil

O and G: oil and grease; C.E.C: cation exchange capacity

**Table 4.2 Macronutrients in petroleum-contaminated soils in first sampling**

| Sample | Organic Carbon mg/kg | Nitrogen mg/kg | Available Phosphorus mg/kg | Ca mg/kg | Mg mg/kg | K mg/kg | Na mg/kg |
|---|---|---|---|---|---|---|---|
| T.D | 43.09 | 2.80 | 12.95 | 4.00 | 1.30 | 0.45 | 0.25 |
| T.S | 8.78 | 0.98 | 47.60 | 3.20 | 1.10 | 0.44 | 0.27 |
| M.D | 4.79 | 0.06 | 6.48 | 1.00 | 0.30 | 0.42 | 0.21 |
| M.S | 16.36 | 1.33 | 20.30 | 3.60 | 1.20 | 0.41 | 0.19 |
| R.D | 9.58 | 1.05 | 5.43 | 2.00 | 0.90 | 0.31 | 0.18 |
| R.S | 10.77 | 1.19 | 6.30 | 2.20 | 0.80 | 0.34 | 0.21 |

T.D: trailer park deep soil; T.S: trailer park shallow soil; M.D: mechanic's workshop deep soil; M.S: mechanic's workshop shallow soil; R.D: refinery effluent site deep soil; R.S: refinery effluent site shallow soil

**Table 4.3: Microelements (mg/kg) of petroleum-contaminated soils in first sampling**

| | T.D | T.S | M.D | M.S | R.D | R.S |
|---|---|---|---|---|---|---|
| Copper | [a]10.2 | 7.8 | [a]11.1 | 5.03 | [a]24.11 | [a]13.61 |
| Manganese | 23.1 | 40.2 | 53.7 | 29.6 | 79.22 | 112.46 |
| Zinc | [a]210 | [a]114.3 | [a]133.2 | [a]304.44 | [a]2725.53 | [a]147.87 |
| *Iron | 143 | 126.1 | 280.7 | 108.3 | 330.33 | 20442.9 |
| Lead | 5.2 | 10.3 | 11.2 | 6.09 | 23.07 | [a]351.42 |
| Nickel | 11.4 | 5.5 | 14.1 | 8.3 | 10.2 | 26.3 |
| Cadmium | 0.4 | 0.6 | 0.4 | 2.2 | 0.2 | 1.8 |
| Chromium | 0.12 | 0.1 | 0.3 | 0.1 | 0.12 | 0.7 |
| Cobalt | 0 | 0.02 | 0 | 0.02 | 0.02 | 0.05 |

T.D: trailer park deep soil; T.S: trailer park shallow soil; M.D: mechanic's workshop deep soil; M.S: mechanic's workshop shallow soil; R.D: refinery effluent site deep soil; R.S: refinery effluent site shallow soil

Superscript [a]: Quantity exceeding the recommended intervention values set by Department of Petroleum Resources (2002)

*Not a heavy metal

**Table 4.4: Polycyclic aromatic hydrocarbon content in petroleum-contaminated soils in first sampling**

| Sample | *PAH |
|---|---|
| Mechanic workshop shallow soil (M.S) | Not detected |
| Mechanic workshop deep soil (M.D) | Not detected |
| Trailer park shallow soil (T.S) | Not detected |
| Trailer park deep soil (T.D) | Not detected |
| Refinery effluent shallow soil (R.S) | Not detected |
| Refinery effluent deep soil (R.D) | Not detected |

*phenanthrene, chrysene and benzo[a]pyrene

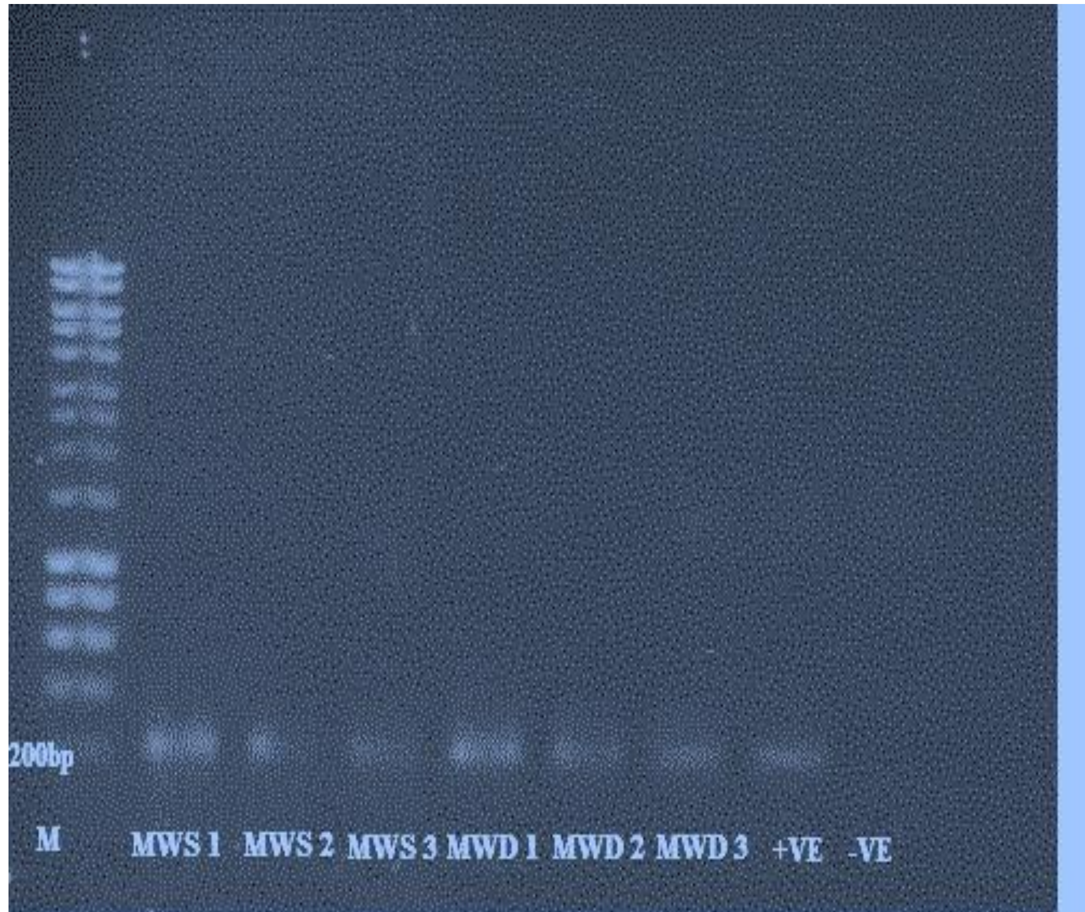



**Plate I: PCR amplification of 16S rRNA gene (201bp) in Mechanic workshop soil**

**KEY:**

M: Molecular marker

MWS 1 – 3: Mechanic workshop shallow soil sample (replicates 1 – 3)

MWD 1 – 3: Mechanic workshop deep soil sample (replicates 1 – 3)

+VE: Positive control

-VE: Negative control

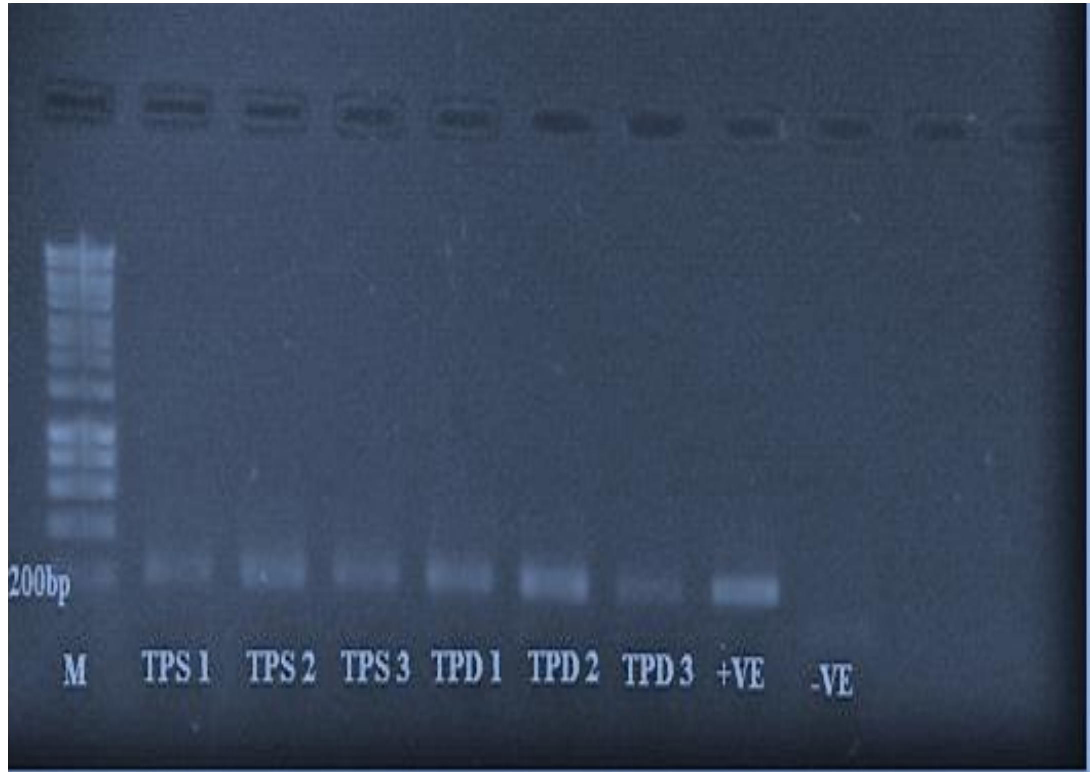



**Plate II: PCR amplification of 16S rRNA gene (201bp) in Trailer park soil**

**KEY:**

M: Molecular marker

TPS 1 – 3: Trailer park shallow soil sample (replicates 1 – 3)

TPD 1 – 3: Trailer park deep soil sample (replicates 1 – 3)

+VE: Positive control

-VE: Negative control

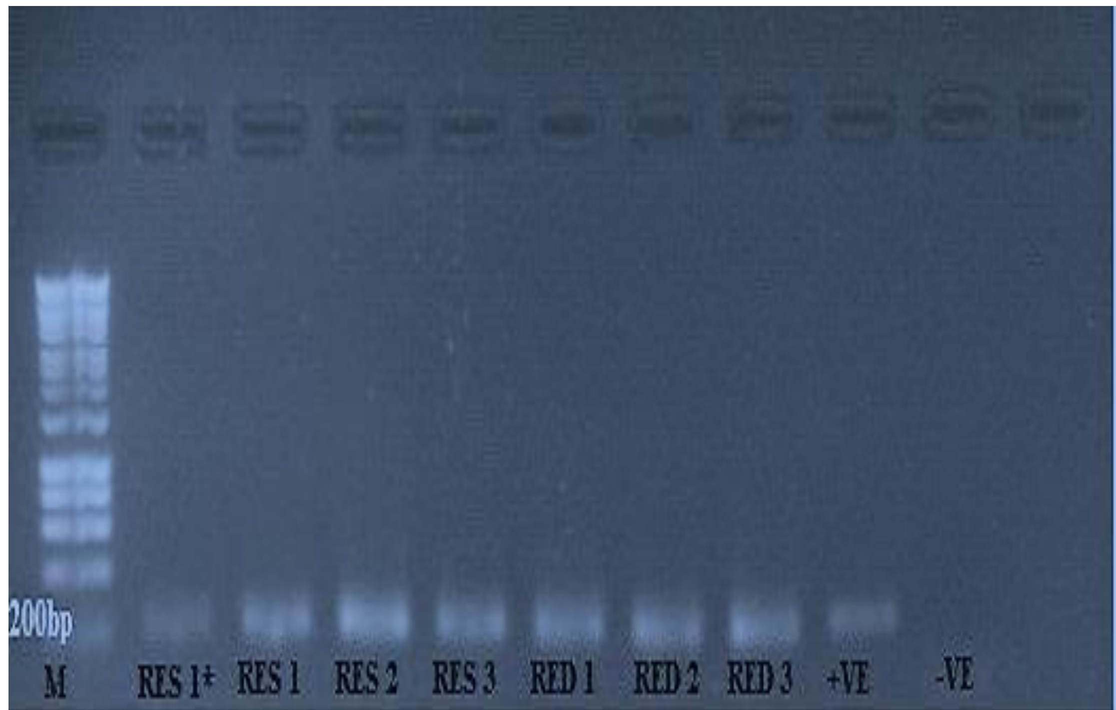



**Plate III: PCR amplification of 16S rRNA gene (201bp) in Refinery effluent soil**

**KEY:**

M: Molecular marker

RES 1*: Refinery effluent shallow soil sample replicate 1 (pipetting error)

RES 1 – 3: Refinery effluent shallow soil sample (replicates 1 – 3)

RED 1 – 3: Refinery effluent deep soil sample (replicates 1 – 3)

+VE: Positive control

-VE: Negative control

Plate IV shows the amplification of the bacterial 16S rRNA gene in the shallow and deep soils from the petroleum-contaminated sites.

### 4.6 DGGE Analysis of 16S Sequences from Petroleum-contaminated Soils in First Sampling

The resulting DGGE fingerprints of the bacterial community in the soils sampled was analyzed using the 16S gene amplicons. Each of the six samples were analysed in triplicates (Plate V) and the bands depicted in each lane in the gel signify varying sequences of same size. The bands excised for sequencing were indicated by numeric tags (Plate VI: 1 – 20; Plate VII: 21 – 32). The Mechanic Workshop soil showed relatively more fingerprints than the Refinery effluent and Trailer Park samples respectively. It was also observed that the Mechanic workshop deep sample had one of its replicates (MWD 2) having a band at a position distinct from the other two replicates. However, the replicates of the Refinery effluent and Trailer park samples all showed identical profiles.

### 4.7 Sequencing of DNA from Excised DGGE Bands and Phylogenetic Analysis of 16S rRNA sequences from Petroleum-contaminated Soils

The total number of bands sequenced was thirty, of which the Mechanic workshop samples consisted of twelve, the Trailer park and Refinery effluent samples were nine respectively. Only twenty five of the thirty were successfully used for phylogenetic analysis based on the quality of the sequences following analysis with the Finch tv software for chromatograms. The similarities between the 16S rRNA sequences from the petroleum-contaminated soils and the 16S sequences from the Genbank database were observed to be close with most of the values ranging between 92% – 100%.

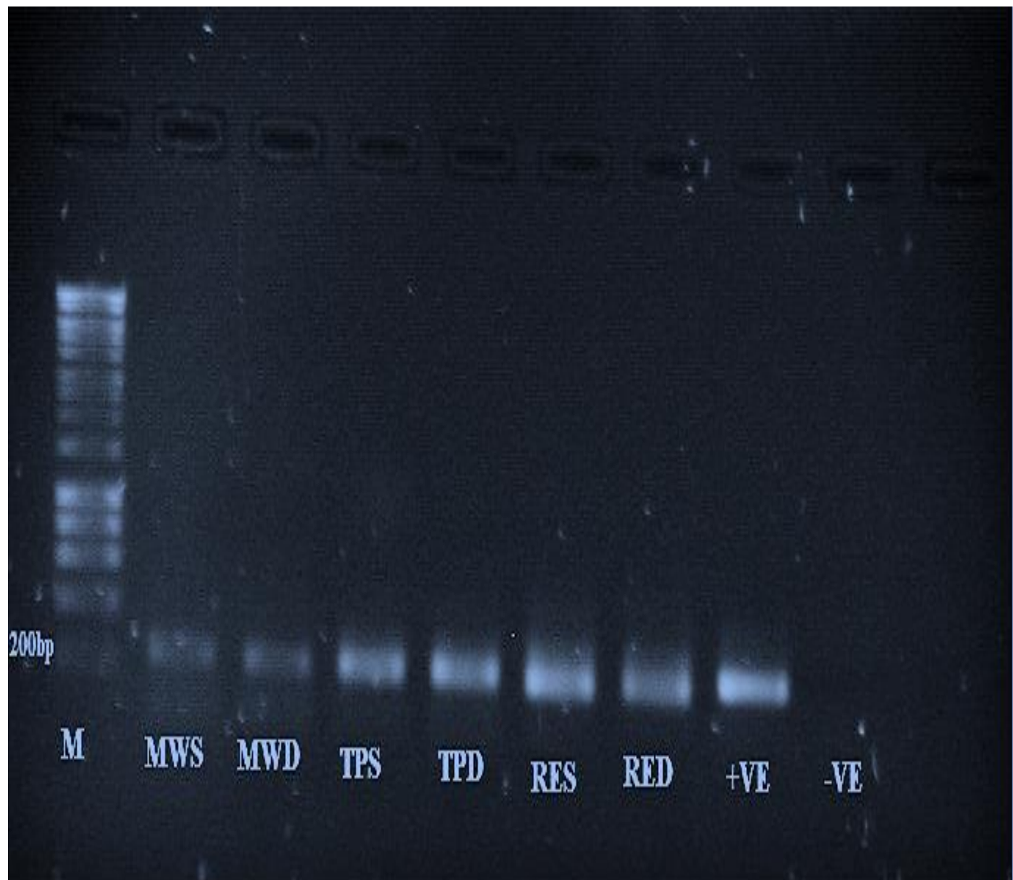



**Plate IV: PCR amplification of 16S rRNA gene (201bp) in petroleum-contaminated soils**

**KEY:**

M: Molecular marker
MWS: Mechanic workshop shallow soil sample
MWD: Mechanic workshop deep soil sample
TPS: Trailer park shallow soil sample
TPD: Trailer park deep soil sample
RES: Refinery effluent shallow soil sample
RED: Refinery effluent deep soil sample
+VE: Positive control
-VE: Negative control

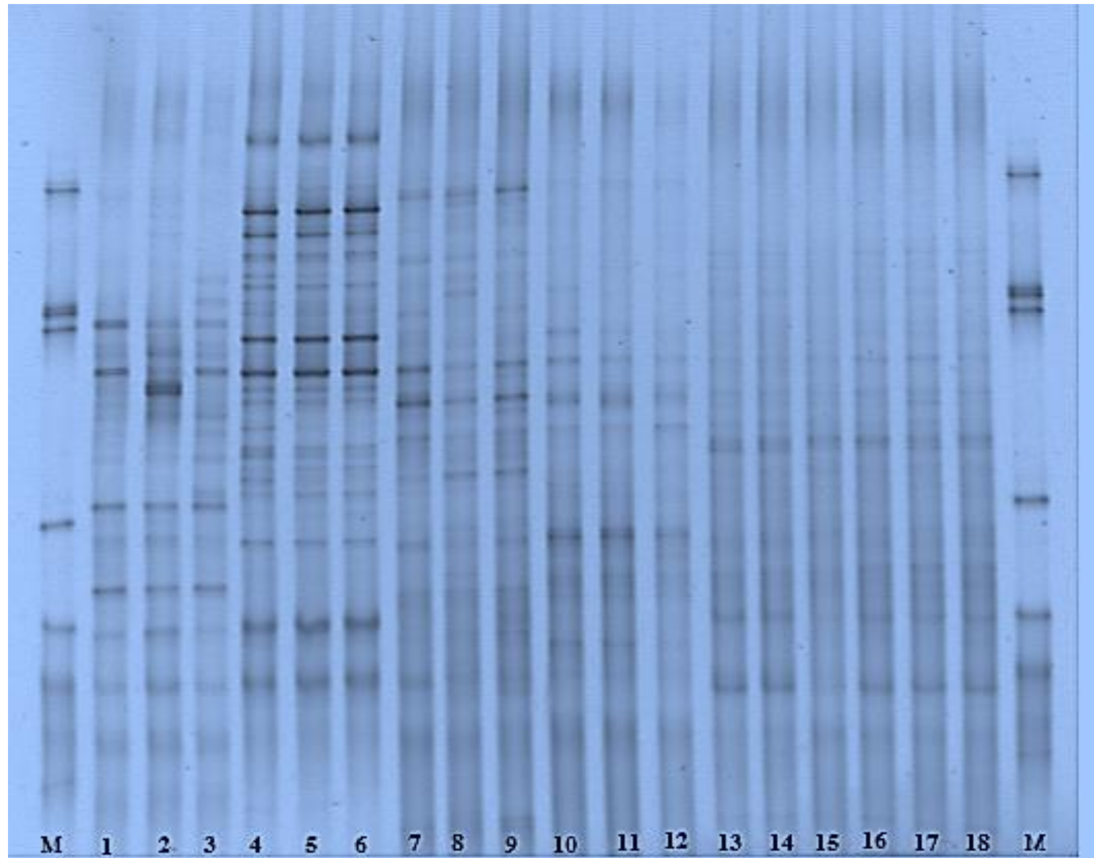



**Plate V: DGGE profile of amplicons of 16S rRNA gene from petroleum-contaminated soils**

**KEY:**

M: Molecular marker

Lanes 1 – 3: MWD (Mechanic workshop deep)

Lanes 4 – 6: MWS (Mechanic workshop shallow)

Lanes 7 – 9: TPS (Trailer Park shallow)

Lanes 10 – 12: TPD (Trailer park deep)

Lanes 13 – 15: RES (Refinery effluent shallow)

Lanes 16 – 18: RED (Refinery effluent deep)

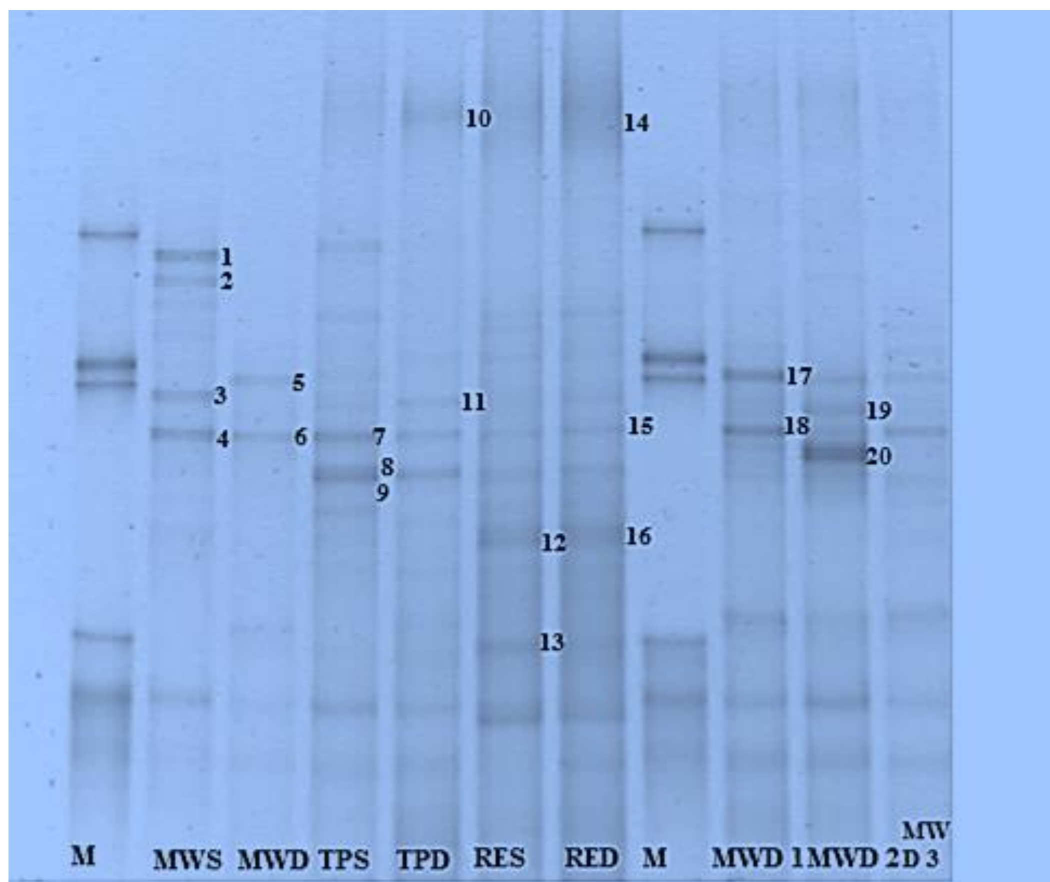



**Plate VI: DGGE profile of amplicons of 16S rRNA gene from petroleum-contaminated soils showing bands excised for sequencing**

**KEY:**

M: Molecular marker
MWS: Mechanic workshop shallow soil sample
MWD: Mechanic workshop deep soil sample
MWD 1- 3: Mechanic workshop deep soil sample (replicates 1 – 3)
TPS: Trailer park shallow soil sample
TPD: Trailer park deep soil sample
RES: Refinery effluent shallow soil sample
RED: Refinery effluent deep soil sample

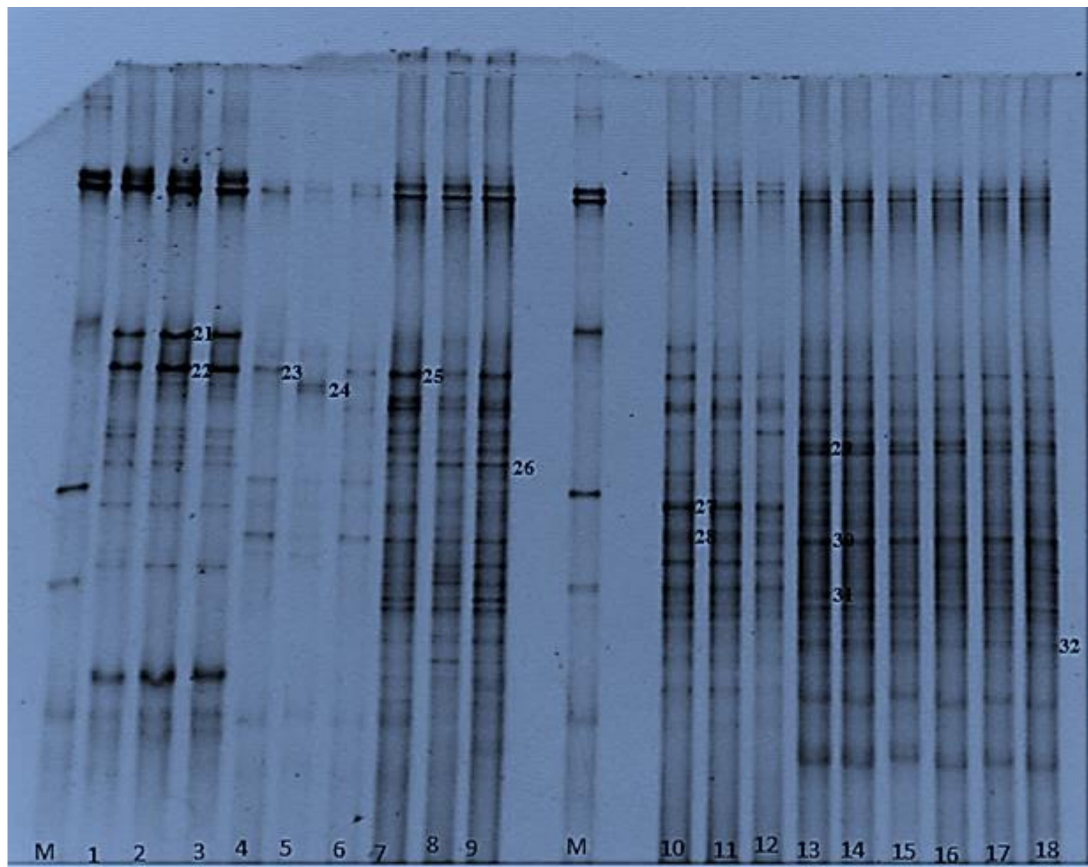



**Plate VII: DGGE profile of amplicons of 16S rRNA gene from triplicates of petroleum-contaminated soils showing bands excised for sequencing**

**KEY:**

M: Molecular marker
Lanes 1 – 3: MWS (Mechanic workshop shallow)
Lanes 4 – 6: MWD (Mechanic workshop deep)
Lanes 7 – 9: TPS (Trailer Park shallow)
Lanes 10 – 12: TPD (Trailer park deep)
Lanes 13 – 15: RES (Refinery effluent shallow)
Lanes 16 – 18: RED (Refinery effluent deep)

The 16S rRNA sequences from the Mechanic workshop soil had similarities identities in the range 99 – 100%, with the exception of the sample MS2, which had a value of 86% (Table 4.5). The sequences in the Mechanic workshop (Table 4.5) were all closely related to bacteria in the phylum, Proteobacteria.

The sequences from the Trailer park soil (Table 4.6) were all closely affiliated to Gram positive low G+C bacteria (Firmicutes) and Gram positive high G+C bacteria (Actinobacteria), while the sequnces from the Refinery effluent soil (Table 4.7) had strong affiliations to bacteria in the following phyla, Proteobacteria, Actinobacteria and Firmicutes. The alignment and phylogeny of the 16S rRNA sequences from soils in the three sampling sites were carried out using *MEGA* version 4. An unrooted Neighbour-joining tree was used in determining the evolutionary relationship between the sequences (Fig. 4.1). The sequences obtained from the Mechanic workshop soil had separate clades in the shallow and deep samples respectively. However, the Trailer park and Refinery effluent soils had sequences from both shallow and deep soils appearing in the same clade. The bootstrap values at the nodes of the clades indicate the reliability of relationships of the sequences within the clades.

### 4.8 Quantitative Polymerase Chain Reaction (Q-PCR) Analysis of Bacterial and Archaeal 16S Gene in Petroleum-contaminated Soils

The average gene copies of 16S rRNA for bacterial and archaeal populations in the samples are as shown in Figure 4.2 and Figure 4.3, respectively. The final number of target genes, was expressed as an average of triplicate measurements from three independent DNA extractions made from each soil sample. Generally in all three sites, the bacterial 16S gene was more abundant than the archaeal 16S gene. The Trailer park shallow (TS) sample had the most abundant bacterial 16S gene copies while the

Refinery effluent shallow (RS) sample had the most abundant archaeal 16S gene copies. In both the bacterial and archaeal 16S gene copies, the Refinery effluent soil exhibited relatively similar 16S gene copies. Whereas in the Mechanic workshop and Trailer park soils, the shallow and deep samples had markedly different gene copy numbers. The replicates of each soil sample were observed to have clear dissimilarities in the number of gene copies.

### 4.9 Physico-chemical and textural classification of petroleum-contaminated soils in Second Sampling

The texture of the soils was mostly loamy but with varying quantities of sand and clay. Clay was present in higher quantities in the deep soils for all the sites however; the Trailer park soil had the highest clay content. Generally, the pH of the soils from the sites (Table 4.8) was observed to be neutral with one of the samples being slighty acidic (MD). Moisture content was highest in the Refinery effluent soils. The Mechanic workshop shallow soil had the highest content of oil and grease while the deep soil from the same site had the lowest oil and grease content. The soils from the sites had higher values of oil and grease in the shallow soils with the exception of the Refinery effluent soil.

### 4.10 Macronutrients of petroleum-contaminated soils in Second Sampling

The Mechanic workshop shallow soil had the highest ratio of Carbon to Nitrogen (Table 4.9). Phosphorus and Potassium were observed in higher quantities in the shallow soils for all the sites, Sodium showed a similar pattern with the exception of the Refinery effluent soil which had equal quantities of the element at both sampling depths.

**Table 4.5: Phylogenetic affiliations of 16S rRNA sequences obtained from Mechanic workshop Soil using $BLAST_N$ Analysis**

| S/No. | DNA from soil sample | DGGE band No. | Closest match in Genbank database | Sequence Identity (%) | Accesion No. |
|---|---|---|---|---|---|
| 1 | MS1 | 1 | *Acinetobacter radioresistens* | 100 | LM994723.1 |
| 2 | MS2 | 2 | *Pseudomonas aeruginosa* strain B | 86 | HE862283.1 |
| 3 | MS3 | 3 | *Acinetobacter baumannii* strain-X2A | 100 | KJ806334.1 |
| 4 | MS4 | 4 | *Acinetobacter* sp. MU02 | 99 | KF261600.1 |
| 5 | MS5 | 21 | *Acinetobacter baumannii* strain C-X2A | 100 | KJ806334.1 |
| 6 | MS6 | 22 | *Acinetobacter* sp. MU02 | 100 | KF261600.1 |
| 7 | MD7 | 17 | *Burkholderia* sp. 6hN46 | 99 | KJ879969.1 |
| 8 | MD8 | 18 | *Burkholderia* sp. RPE64 (DNA, chromosome 2, complete genome) | 99 | AP013059.1 |
| 9 | MD9 | 19 | Uncultured *Burkholderia* sp. Clone AG10B | 99 | JF522220.1 |
| 10 | MD10 | 20 | *Burkholderia cenocepacia* strain DWS 37E-2 (chromosome 1, complete sequence) | 99 | CP007781.1 |
| 11 | MD11 | 23 | *Burkholderia* sp. RPE64 (DNA, chromosome 2, complete genome) | 99 | AP013059.1 |
| 12 | MD12 | 24 | *Burkholderia cenocepacia* strain DWS 37E-2 (chromosome 1, complete sequence) | 99 | CP007781.1 |

**KEY:**
MS: Mechanic workshop shallow; MD: Mechanic workshop deep

**Table 4.6: Phylogenetic affiliations of 16S rRNA sequences obtained from Trailer park Soil using $BLAST_N$ Analysis**

| S/No. | DNA from soil sample | DGGE Band No. | Closest match in Genbank database | Sequence Identity (%) | Accession No. |
|---|---|---|---|---|---|
| 1 | TS13 | 7 | Uncultured bacteria, clone McL 103 | 100 | FN567942 |
| 2 | TS14 | 8 | Uncultured *Bacillus* sp., clone SBK22 | 100 | KM108632.1 |
| 3 | TS16 | 25 | *Arthrobacter ramosus* strain F34 | 98 | KM019839.1 |
| 4 | *TD18 | 28 | Uncultured Actinobacterium clone | 94 | JF989586.1 |
| 5 | TD20 | 11 | *Bacillus* sp. THG-S3 | 93 | KJ810591.1 |
| 6 | *TD21 | 27 | Uncultured Actinobacterium clone | 98 | KJ662631.1 |

**KEY:**

TS: Trailer park shallow

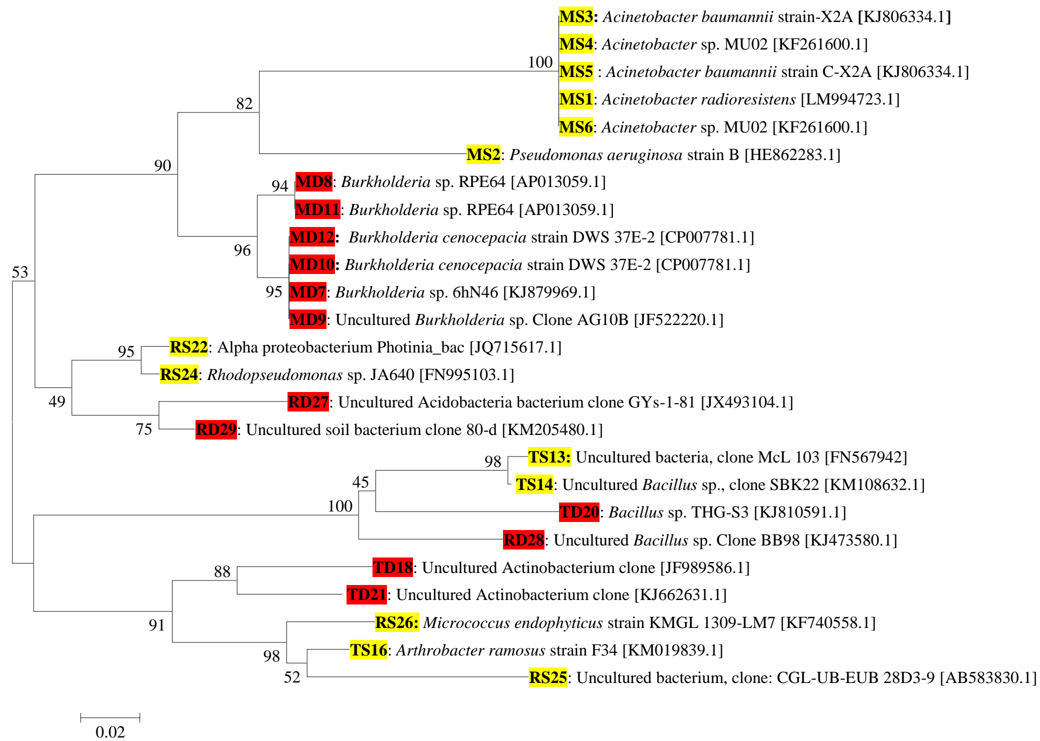
TD: Trailer park deep

*All closest hits on Genbank database are uncultured species

**Table 4.7: Phylogenetic affiliations of 16S rRNA sequences obtained from Refinery effluent Soil using $BLAST_N$ Analysis**

| S/No. | DNA from soil sample | DGGE Band No. | Closest match in Genbank database | Sequence Identity (%) | Accession No. |
|---|---|---|---|---|---|
| 1 | RS22 | 12 | Alpha proteobacterium Photinia_bac | 93 | JQ715617.1 |
| 2 | RS24 | 29 | *Rhodopseudomonas* sp. JA640 | 93 | FN995103.1 |
| 3 | RS25 | 30 | Uncultured bacterium, clone: CGL-UB-EUB 28D3-9 | 93 | AB583830.1 |
| 4 | RS26 | 31 | *Micrococcus endophyticus* strain KMGL 1309-LM7 | 95 | KF740558.1 |
| 5 | RD27 | 14 | Uncultured Acidobacteria bacterium clone GYs-1-81 | 92 | JX493104.1 |
| 6 | RD28 | 15 | Uncultured *Bacillus* sp. Clone BB98 | 92 | KJ473580.1 |
| 7 | RD29 | 16 | Uncultured soil bacterium clone 80-d | 92 | KM205480.1 |

**KEY:**
RS: Refinery effluent shallow
RD: Refinery effluent deep




Bootstrap values carried out for 500 replicates; the numbers at the nodes indicate the bootstrap value in percent. The bar represents 2% nucleotide divergence

**Figure 4.1: Neighbour-Joining Tree showing Phylogenetic Affiliation of 16S rRNA sequences obtained from petroleum-contaminated soils**

KEY:
MS: Mechanic workshop shallow; MD: Mechanic workshop deep
TS: Trailer park shallow; TD: Trailer park deep
RS: Refinery effluent shallow; RD: Refinery effluent deep
Yellow highlight: Shallow sampling sites (17 – 20 cm)
Red highlight: Deep sampling sites (37 – 40 cm)

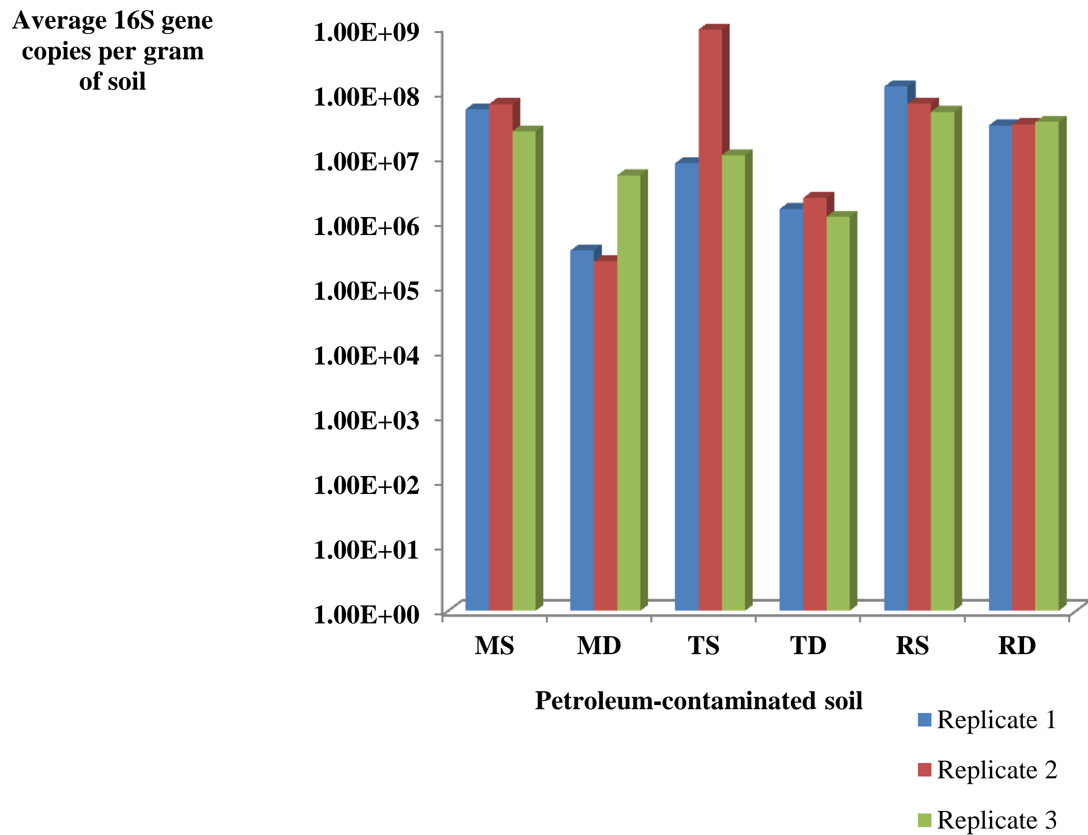



**Figure 4.2: Analysis of bacterial 16S gene in soils obtained from petroleum-contaminated sites in Kaduna state**

**KEY:**

MS: Mechanic workshop shallow; MD: Mechanic workshop deep

TS: Trailer park shallow; TD: Trailer park deep

RS: Refinery effluent shallow; RD: Refinery effluent deep

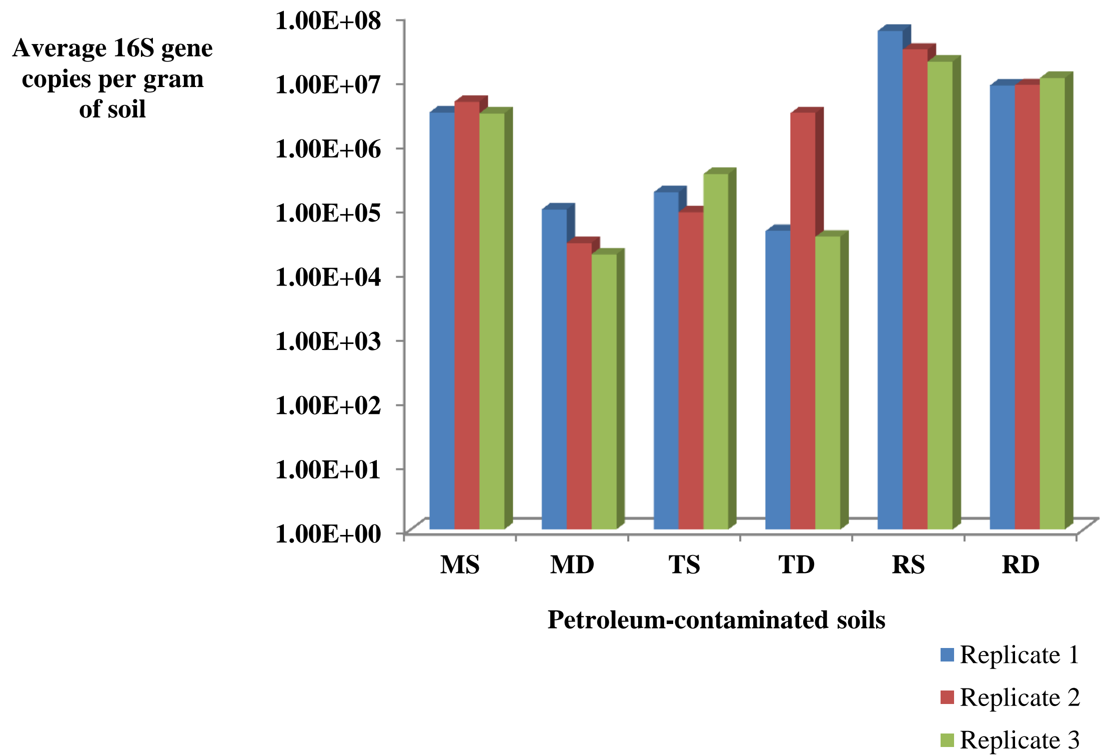



**Figure 4.3: Analysis of Archaeal 16S gene in soils obtained from petroleum-contaminated sites in Kaduna state**

**KEY:**

| | |
|---|---|
| MS: Mechanic workshop shallow; | MD: Mechanic workshop deep |
| TS: Trailer park shallow; | TD: Trailer park deep |
| RS: Refinery effluent shallow; | RD: Refinery effluent deep |

**Table 4.8: Physico-chemical characteristics of petroleum-contaminated soils used in second sampling**

| Soil sample | MS | MD | TS | TD | RS | RD |
|---|---|---|---|---|---|---|
| **Sand g/kg** | 690 | 450 | 410 | 350 | 430 | 810 |
| **Silt g/kg** | 300 | 440 | 500 | 460 | 500 | 140 |
| **Clay g/kg** | 10 | 110 | 90 | 190 | 70 | 150 |
| **Textural class (USDA)** | Sandy loam | Clayey loam | Silty loam | Clayey loam | Silty loam | Loamy silt |
| **pH (1:2.5 $H_2O$)** | 7.3 | 5.8 | 7 | 6.9 | 6.9 | 7.1 |
| **Moisture content (%)** | 0.05 | 0.11 | 0.06 | 0.12 | 0.13 | 0.18 |
| **Oil and Grease content (mg/L)** | 1760 | 290 | 830 | 390 | 1080 | 1350 |

**KEY:**
MS: Mechanic workshop shallow; MD: Mechanic workshop deep
TS: Trailer park shallow; TD: Trailer park deep
RS: Refinery effluent shallow; RD: Refinery effluent deep

**Table 4.9 Macronutrients of petroleum-contaminated soils in second sampling**

| Soil sample | MS | MD | TS | TD | RS | RD |
|---|---|---|---|---|---|---|
| **Organic Carbon g/kg** | 29.3 | 4.3 | 5.3 | 3.2 | 6.2 | 3.2 |
| **Nitrogen g/kg** | 0.84 | 0.42 | 0.56 | 0.42 | 0.56 | 0.28 |
| **Available Phosphorus mg/kg** | 10.15 | 4.73 | 11.73 | 5.43 | 5.25 | 4.38 |
| **Calcium mg/kg** | 0.169 | 0.134 | 0.146 | 0.179 | 0.074 | 0.086 |
| **Magnesium mg/kg** | 0.016 | 0.017 | 0.016 | 0.017 | 0.014 | 0.017 |
| **Potassium mg/kg** | 0.38 | 0.15 | 0.19 | 0.17 | 0.17 | 0.16 |
| **Sodium mg/kg** | 0.78 | 0.17 | 0.78 | 0.43 | 0.43 | 0.43 |

**KEY:**
MS: Mechanic workshop shallow; MD: Mechanic workshop deep
TS: Trailer park shallow; TD: Trailer park deep
RS: Refinery effluent shallow; RD: Refinery effluent deep

However, Magnesium was found in higher amounts in the deeper soils than the shallow soils. Calcium was observed to be more abundant in the deeper soils than the shallow soils in the Trailer park and Refinery effluent sites while the Mechanic workshop soil exhibited higher values of the element in the shallow soils. The Refinery effluent soil had the least quantity of nutrients.

### 4.11 Microelements of petroleum-contaminated soils in Second Sampling

Soils sampled from the Mechanic workshop site had the highest contamination of heavy metals and Iron (Table 4.10); it was also the only site having Lead in quantities higher than the recommended limit. The Refinery effluent site had the highest quantity of chromium. In all the soils sampled, Copper was observed to exceed the DPR recommended limit however; the Mechanic workshop site had the highest quantity of the heavy metal. The shallow soils from all the sites had higher values of Copper than the deep soils; the same trend was observed for Iron and Zinc with the exception of the Refinery effluent soil that had slightly higher values in the deep soils compared to the shallow soils. Cadmium was only present in the Mechanic workshop shallow soil.

### 4.12 Polycyclic aromatic hydrocarbon content in petroleum-contaminated soils in second sampling

None of the sixteen priority polycyclic aromatic hydrocarbons was detected in the soil samples (Table 4.11) using Gas-Chromatography Mass Spectrometry (GC-MS).

### 4.13 PCR amplification of bacterial 16S gene in petroleum-contaminated soils spiked with phenanthrene, chrysene and benzo[a]pyrene

The DNA extracted from petroleum-contaminated soils collected from three sites were analysed by amplifying the 16S gene, the size of the amplicon is 201 bp. In the Mechanic workshop site (Plate VIII) and Trailer park site (Plate IX), the amplicons

**Table 4.10: Microelements (mg/kg) in the petroleum-contaminated soils treated with PAHs**

| | MS | MD | TS | TD | RS | RD | DPR Standard |
|---|---|---|---|---|---|---|---|
| Copper | 8.15 | 3.79 | 4.19 | 2.34 | 3.79 | 3.66 | 10 |
| **Iron | 349.91 | 337.41 | 187.84 | 40.43 | 304.66 | 305.52 | |
| Zinc | 52.39[a] | 8.79[a] | 13.38[a] | 7.22[a] | 6.38[a] | 6.98[a] | 0 |
| Lead | 310.8[a] | 22.3 | 1.61 | 0 | 1.61 | 0 | 210 |
| Cadmium | 0.15 | 0 | 0 | 0 | 0 | 0 | 380 |
| Chromium | 0 | 0 | 40 | 0 | 0 | 90 | 240 |

**Not a heavy metal; Superscript[a]: Values exceeding limit set by DPR (2002)

**KEY:**
MS: Mechanic workshop shallow; MD: Mechanic workshop deep
TS: Trailer park shallow; TD: Trailer park deep
RS: Refinery effluent shallow; RD: Refinery effluent deep

**Table 4.11: Detection of Polycyclic aromatic hydrocarbons in spiked petroleum-contaminated soils using Gas-chromatography Mass-spectrometry (GC-MS)**

| Sample | *PAH |
|---|---|
| Mechanic workshop shallow soil (M.S) | Not detected |
| Mechanic workshop deep soil (M.D) | Not detected |
| Trailer park shallow soil (T.S) | Not detected |
| Trailer park deep soil (T.D) | Not detected |
| Refinery effluent shallow soil (R.S) | Not detected |
| Refinery effluent deep soil (R.D) | Not detected |

*phenanthrene, chrysene, benzo[a]pyrene

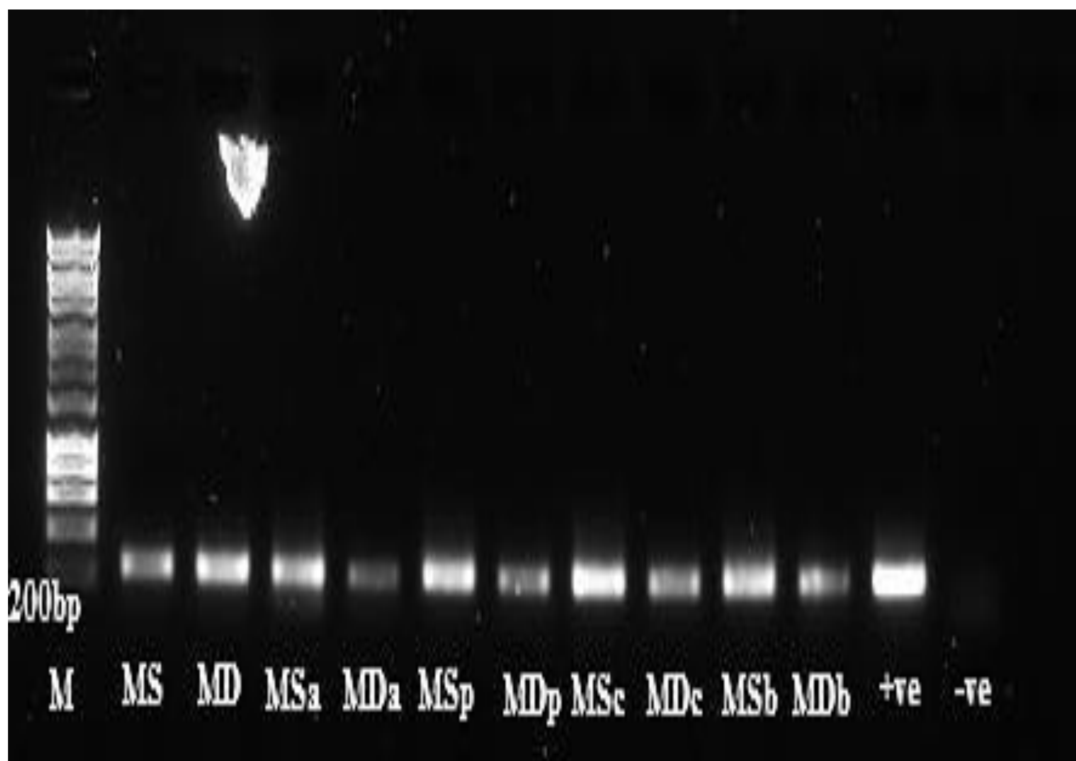



**Plate VIII: Amplification of 16S rRNA gene (201 bp) in a Mechanic workshop soil treated with polycyclic aromatic hydrocarbons**

**KEY:**

M: Molecular marker

MS: Untreated Mechanic workshop shallow

MD: Untreated Mechanic workshop deep

MSa: Acetone-treated Mechanic workshop shallow

MDa: Acetone-treated Mechanic workshop deep

MSp: Phenanthrene-spiked Mechanic workshop shallow

MDp: Phenanthrene-spiked Mechanic workshop deep

MSc: Chrysene-spiked Mechanic workshop shallow

MDc: Chrysene-spiked Mechanic workshop deep

MSb: B[a]P-spiked Mechanic workshop shallow

MDb: B[a]P-spiked Mechanic workshop deep

+ve: Positive control

-ve: Negative control

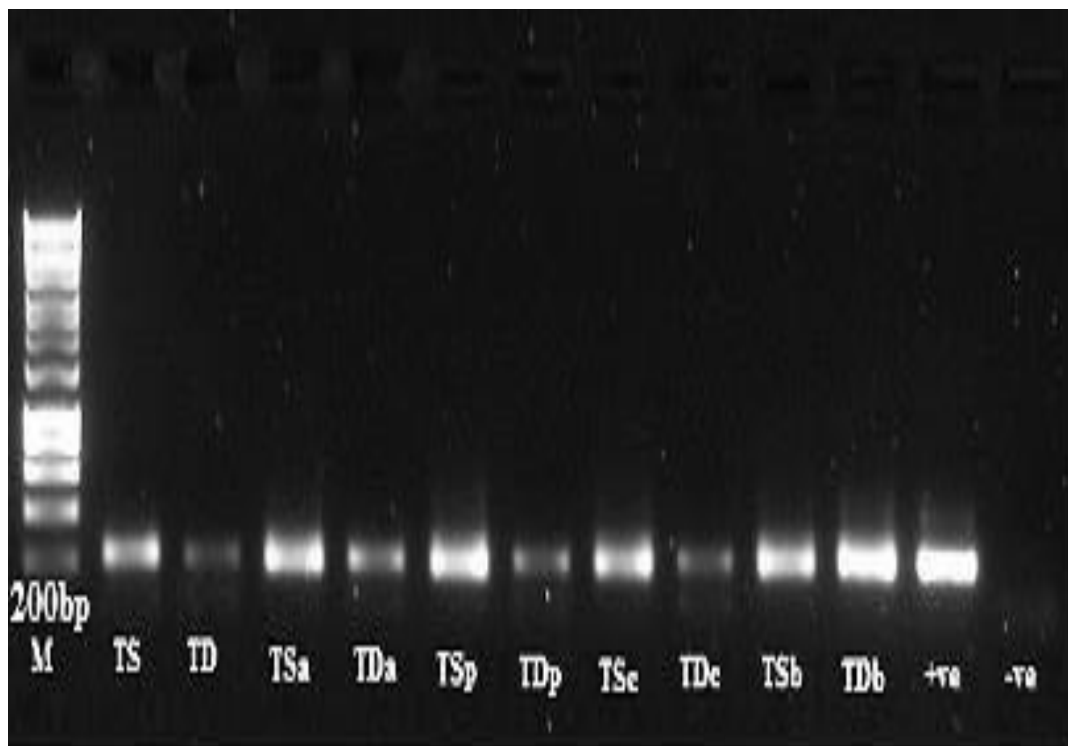



**Plate IX: Amplification of 16S rRNA gene (201 bp) in a Trailer park soil treated with polycyclic aromatic hydrocarbons**

**KEY:**

TS: Untreated Trailer park shallow
TD: Untreated Trailer park deep
TSa: Acetone-treated Trailer park shallow
TDa: Acetone-treated Trailer park deep
TSp: Phenanthrene-spiked Trailer park shallow
TDp: Phenanthrene-spiked Trailer park deep
TSc: Chrysene-spiked Trailer park shallow
TDc: Chrysene-spiked Trailer park deep
TSb: B[a]P-spiked Trailer park shallow
TDb: B[a]P-spiked Trailer park deep
+ve: Positive control
-ve: Negative control

from the shallow sites appeared brighter on the gel compared to bands from the deeper soils.

However, the Refinery effluent site (Plate X) had similar intensity in the brightness of the bands at both sampling depths. The 16S amplicons from soils spiked with PAHs had brighter bands compared to the amplicons from unspiked soils.

### 4.14 Analysis of the 16S rRNA amplicon from the petroleum-contaminated soils using Denaturing Gradient Gel Electrophoresis

Plates XI – XIII depict the DGGE profile of the bacterial population in petroleum-contaminated sites, as well as the bands excised for sequencing and phylogenetic studies. Generally, it was observed that in all the three sites, the control (soil samples not treated with acetone) and the soils treated with neither PAHs nor acetone, had less prominent bands than those treated with PAHs (phenanthrene, chrysene and benzo[a]pyrene). In the Mechanic workshop and Trailer park sites, the shallow soils had brighter bands than the deep soils (Plate XI, Plate XII); while the Refinery effluent site had very similar intensities in the brightestness of the bands at both sampling depths (Plate XIII). The DGGE fingerprint in all the sites was similar in the soils treated with PAHs as well as the control soils however there were a few exceptions, band 78 in Traiker park soil (Plate XII); bands 131 and 148 in Mechanic workshop soil (Plate XI). A total of one hundred and forty four bands were excised from all the gels (Fig. 4.4). The Mechanic workshop soil had the highest number of excised bands (39%) followed by the Trailer park soil with 37% while, the Refinery effluent soil had the least number of excised bands (24%).

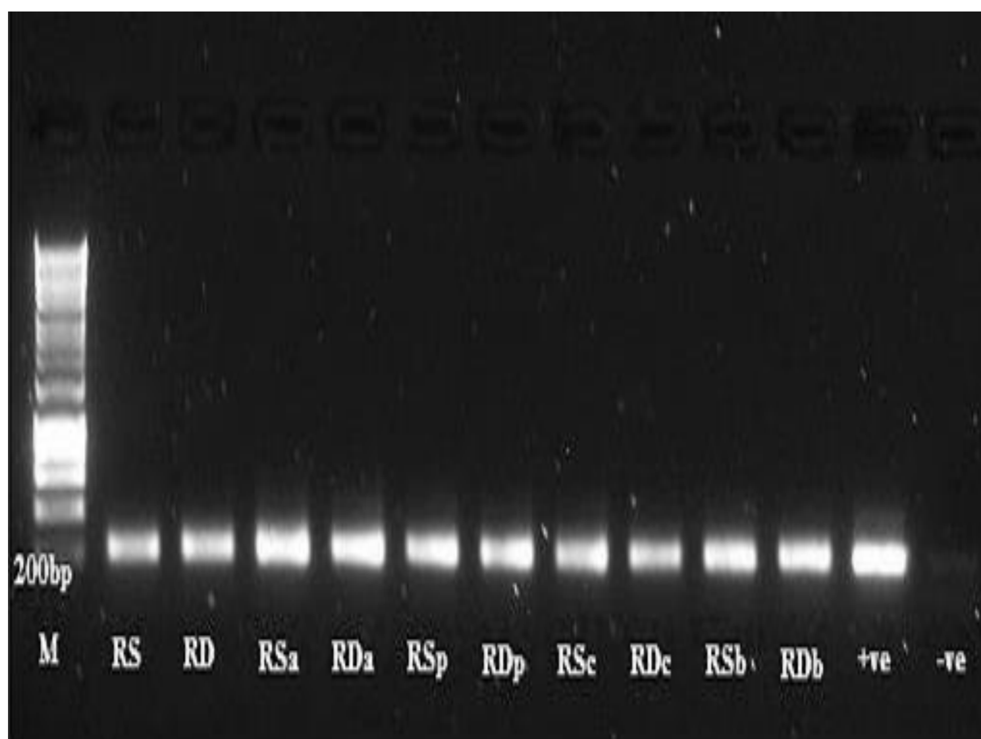



**Plate X: Amplification of 16S rRNA gene (201 bp) in a Refinery effluent soil treated with polycyclic aromatic hydrocarbons**

**KEY:**
M: Molecular maker
RS: Untreated Refinery effluent shallow
RD: Untreated Refinery effluent deep
RSa: Acetone-treated Refinery effluent shallow
RDa: Acetone-treated Refinery effluent deep
RSp: Phenanthrene-spiked Refinery effluent shallow
RDp:Phenanthrene-spiked Refinery effluent deep
RSc: Chrysene-spiked Refinery effluent shallow
RDc: Chrysene-spiked Refinery effluent deep
RSb: B[a]P-spiked Refinery effluent shallow
RDb: B[a]P-spiked Refinery effluent deep
+ve: Positive control
-ve: Negative control

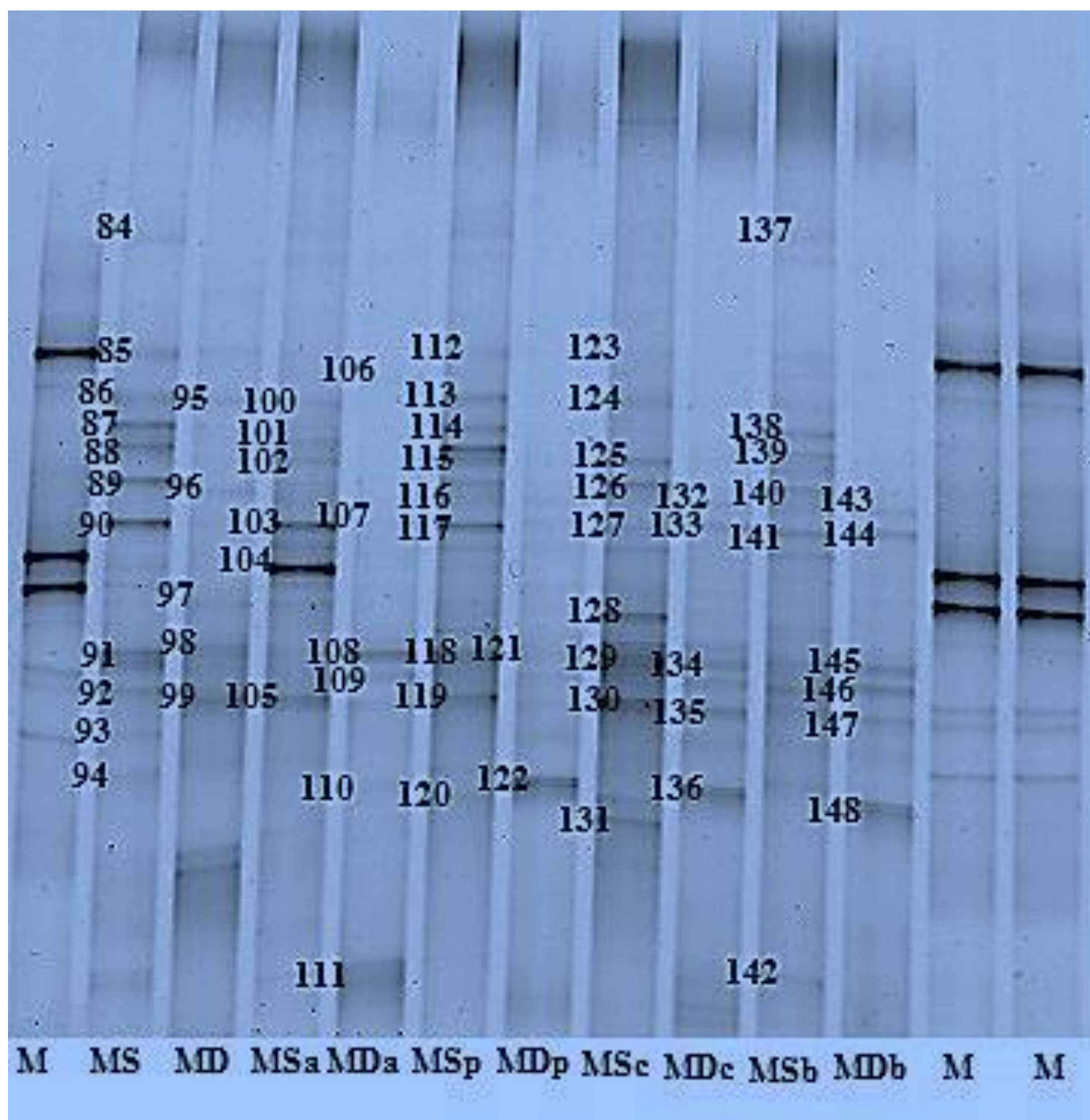



**Plate XI: DGGE profile of amplified 16S rRNA from Mechanic workshop soil treated with PAHs (bands excised for sequencing are as indicated); Shannon Index of diversity = 2.8292**

**KEY:**

M: Molecular marker

MS: Untreated Mechanic workshop shallow

MD: Untreated Mechanic workshop deep

MSa: Acetone-treated Mechanic workshop shallow

MDa: Acetone-treated Mechanic workshop deep

MSp: Phenanthrene-spiked Mechanic workshop shallow

MDp: Phenanthrene-spiked Mechanic workshop deep

MSc: Chrysene-spiked Mechanic workshop shallow

MDc: Chrysene-spiked Mechanic workshop deep

MSb: B[a]P-spiked Mechanic workshop shallow

MDb: B[a]P-spiked Mechanic workshop deep

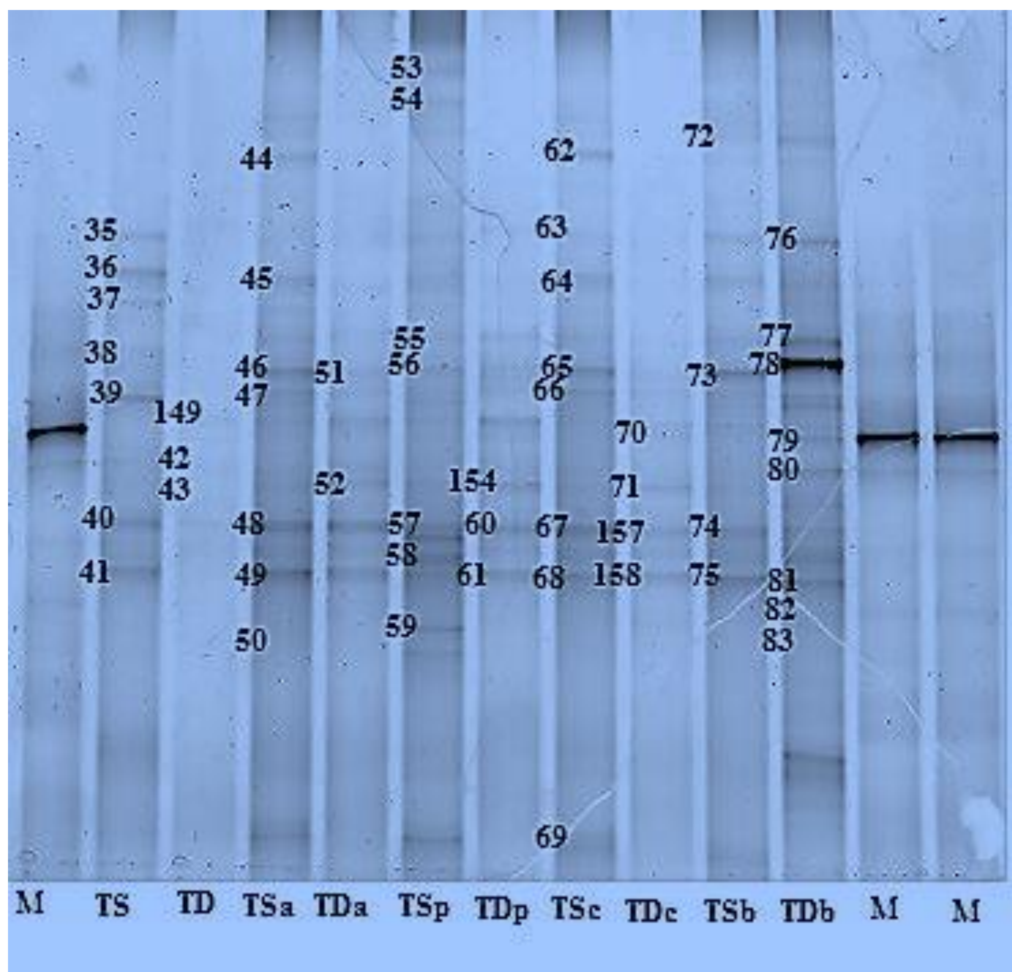



**Plate XII: DGGE profile of amplified 16S rRNA from Trailer park soil treated with PAHs (bands excised for sequencing are as indicated); Shannon Index of diversity = 2.8319**

**KEY**:
M: Molecular marker
TS: Untreated Trailer park shallow
TD: Untreated Trailer park deep
TSa: Acetone-treated Trailer park shallow
TDa: Acetone-treated Trailer park deep
TSp: Phenanthrene-spiked Trailer park shallow
TDp: Phenanthrene-spiked Trailer park deep
TSc: Chrysene-spiked Trailer park shallow
TDc: Chrysene-spiked Trailer park deep
TSb: B[a]P-spiked Trailer park shallow
TDb: B[a]P-spiked Trailer park deep

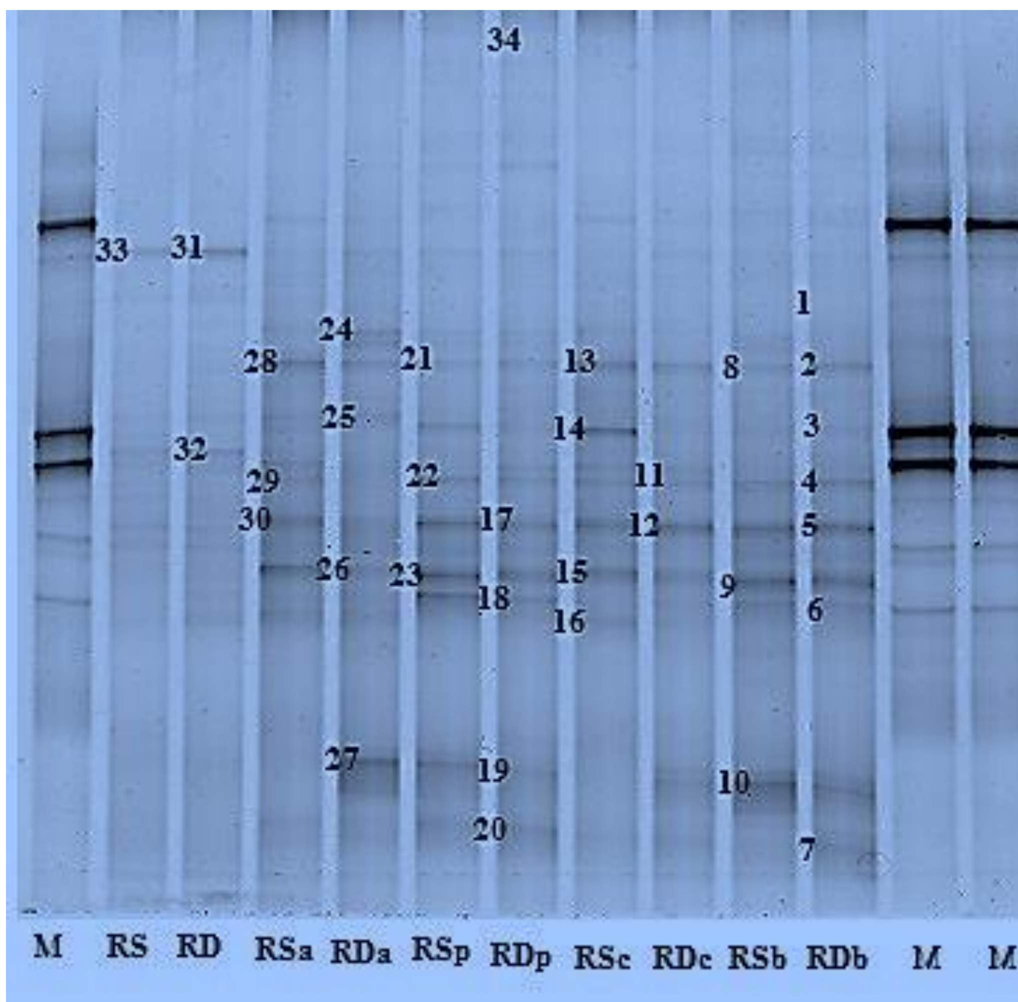



**Plate XIII: DGGE profile of amplified 16S rRNA from Refinery effluent soil treated with PAHs (bands excised for sequencing are as indicated). Shannon Index = 2.4424**

**KEY:**

M: Molecular marker

RS: Untreated Refinery effluent shallow

RD: Untreated Refinery effluent deep

RSa: Acetone-treated Refinery effluent shallow

RDa: Acetone-treated Refinery effluent deep

RSp: Phenanthrene-spiked Refinery effluent shallow

RDp:Phenanthrene-spiked Refinery effluent deep

RSc: Chrysene-spiked Refinery effluent shallow

RDc: Chrysene-spiked Refinery effluent deep

RSb: B[a]P-spiked Refinery effluent shallow

RDb: B[a]P-spiked Refinery effluent deep

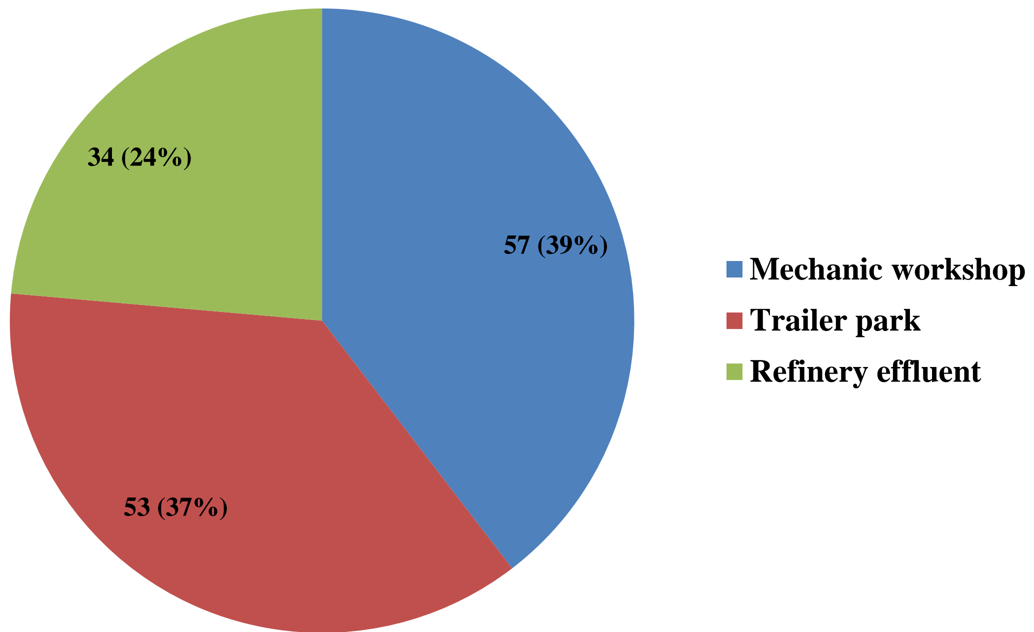



**Figure 4.4: Summary of bands excised from DGGE gel for phylogenetic analysis of 16S rRNA gene amplified from petroleum-contaminated soils**

## 4.15 Diversity of bacteria in petroleum-contaminated soils and Phylogenetic analyses

The diversity of the bacterial populations from each of the sites was analysed (Table 4.12) using the Shannon Weiner Index of diversity (H). The Trailer park soil showed the highest diversity, while the Refinery effluent soil had the least. The diversity of the Trailer park was only slightly higher than the Mechanic workshop.

The closest relatives of the sequences obtained in this study were determined using the BLAST tool to compare the 16S sequences with the sequences in the Genbank database (Tables 4.13 – 4.15). Members of Gamma-proteobacteria, Firmicutes, as well as a specie belonging to the phylum, *Chloroflexi*, were obtained in the Mechanic workshop (Fig. 4.5). The Trailer park site had most species belonging to the phylum, Firmicutes; while the phyla Gammaproteobacteria and Actinobacteria had a representative each (Fig. 4.6). The Refinery effluent site only had bacterial species belonging to the Firmicutes group specifically, the genus, *Bacillus* (Fig. 4.7). The Maximum parsimony method was used in constructing the phylogenetic trees and bootstrapping was carried out to determine the evolutionary history of the taxa analysed. Generally, the depth of sampling did not seem to affect clustering of the taxa.

Fig. 4.8 depicts the abundance of the bacterial species based on depth of sampling; the shallow soils had more identified bacteria than the deep soil in the sites except for the Refinery effluent site where the reverse was the case. The impact of spiking the soils with three different PAHs on the abundance of bacteria in the sites was analysed in Fig. 4.9; a high number of bacterial species was detected in the Refinery effluent soil treated with chrysene, the Mechanic workshop soil had more bacteria in the untreated soil than those treated with PAHs. For the phenanthrene-treated soils, the Trailer park site had the

highest number of bacterial species while, the Mechanic workshop site had the least number of species. The most abundant bacteria identified in Bap-treated soils was observed in the Refinery effluent site while no bacteria was identified in the Mechanic workshop soils having the same treatment (Fig. 4.9). The different phyla of bacteria detected with respect to the site are as depicted in Fig. 4.10, the Firmicutes group featured prominently in all three sites.

Phyla such as Chloroflexi and Actinobacteria were only identified in a single site while, only the phylum, Firmicutes specifically the genus, *Bacillus* was identified in the Refinery effluent site. The Trailer park soil had the highest number of uncultured bacteria, followed by the Mechanic workshop while, the Refinery effluent had the least number (Fig. 4.10).

The accession numbers of some of the sequences (32) obtained in this research submitted to the Genbank database are**: KU362728 – KU362759**.

### 4.16 Quantitative PCR (qPCR) analysis of 16S rRNA genes in soil treated with PAHs:

The number of bacterial 16S gene was analysed in the petroleum-contaminated soils using Real-time PCR, and they are as shown in Figures 4.11 - 4.13. The mean obtained from three runs of each sample is represented in the figures showing the average 16S gene copies in Mechanic workshop soil (Fig. 4.11), Trailer park soil (Fig. 4.12), and Refinery effluent soil (Fig. 4.13). In all three sites, the shallow samples had higher gene copies per gram of soil compared to the deep samples. The soils treated with either acetone or polycyclic aromatic hydrocarbons (phenanthrene, chrysene and benzo[a]pyrene) had higher gene copies than the soils without any treatment.

**Table 4.12 Shannon Index of Diversity of Bacteria in Spiked Petroleum-contaminated Soils**

| | Mechanic workshop | Trailer park | Refinery effluent |
|---|---|---|---|
| **Shannon Weiner index (H)** | **2.8292** | **2.8319** | **2.4424** |
| **Species richness (S)** | **140** | **124** | **108** |
| **Evenness (E)** | **0.5725** | **0.5875** | **0.5216** |

**Table 4.13a: Phylogenetic affiliations of 16S rRNA sequences obtained from Mechanic workshop Soil treated with Polycyclic Aromatic Hydrocarbons (using $BLAST_N$ analysis)**

| S/No. | DNA from soil sample | DGGE Band No. | Closest match in Genbank database (Accession No.) | Sequence Identity (%) | E-value |
|---|---|---|---|---|---|
| 1 | MS | 85 & 91 | *Pantoea* sp. Iso10-19 gene* (AB795554.1) | 99 | 5E-94 |
| 2 | MS | 88 | Uncultured *Acinetobacter* sp. Clone yantianma (LN833524.1) | 99 | 5E-94 |
| 3 | MS | 89 | Bacterium JNKLA9 (KR0270002.1) | 100 | 4E-95 |
| 4 | MS | 90 | *Acinetobacter variabilis* strain NIPH 2171 (KP278590.1) | 100 | 3E-96 |
| 5 | MS | 92 & 93 | *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* strain 33676, complete genome* (CP012681.1) | 100 | 1E-95 |
| 6 | MS | 94 | *Cronobacter sakazakii* strain NCTC 8155, complete genome (CP012253.1) | 100 | 1E-95 |
| 7 | MD | 96 | Uncultured bacterium clone SF-183 (KM207371.1) | 99 | 5E-94 |
| 8 | MD | 97 & 98 | *Bacillus* sp. THG-HS227* (KF815074.1) | 100 | 1E-95 |
| 9 | MD | 99 | *Bacillus* sp. KHA2 (KT368972.1) | 99 | 2E-92 |
| 10 | MSa | 101 | Uncultured *Acinetobacter* sp. clone W1109-275 (JQ754329.1) | 100 | 9E-52 |
| 11 | MSa | 102 | *Acinetobacter lwoffii* strain Na6MB-1 (KT159363.1) | 100 | 2E-49 |
| 12 | MSa | 103 | *Acinetobacter variabilis* strain NIPH 2171 16S ribosomal RNA gene, complete sequence (KP278590.1) | 96 | 5E-84 |

KEY:
MS: Untreated Mechanic workshop shallow
MSa: Acetone-treated Mechanic workshop shallow
MSp: Phenanthrene-spiked Mechanic workshop shallow
MSc: Chrysene-spiked Mechanic workshop shallow
MD: Untreated Mechanic workshop deep
MDa: Acetone-treated Mechanic workshop deep
MDp: Phenanthrene-spiked Mechanic workshop deep
MDc: Chrysene-spiked Mechanic workshop deep
E-value: Expect value
*The sequence with the higher significant E-value and percentage is shown

**Table 4.13b: Phylogenetic affiliations of 16S rRNA sequences obtained from Mechanic workshop Soil treated with Polycyclic Aromatic Hydrocarbons (using $BLAST_N$ analysis)**

| S/No. | DNA from soil sample | DGGE Band No. | Closest match in Genbank database (Accession No.) | Sequence Identity (%) | E-value |
|---|---|---|---|---|---|
| 13 | MSa | 104 | *Acinetobacter* sp. IHBB 9150 (KR085771.1) | 100 | 3E-96 |
| 14 | MSa | 105 | Uncultured bacterium clone S11-129 (EU669615.1) | 99 | 2E-93 |
| 15 | MDa | 108 | Uncultured *Chloroflexi* bacterium clone De1172 (HQ183887.1) | 99 | 2E-77 |
| 16 | MDa | 110 | Uncultured bacterium gene for clone: SK27B-25 (AB300066.1) | 97 | 2E-73 |
| 17 | MSp | 119 | *Bacillus* sp. CanL-51 (KT580578.1) | 98 | 2E-71 |
| 18 | MDp | 122 | Uncultured bacterium gene for clone: SK27B-25 (AB300066.1) | 97 | 6E-57 |
| 19 | MSc | 124 & 126 | **Bacillus subterraneus* strain MD-A13 (KP307809.1) | 100 | 5E-49 |
| 20 | MSc | 130 | Uncultured bacterium gene for clone: TSNIR003_L13 (AB487421.1) | 99 | 3E-91 |
| 21 | MSc | 131 | *Bacillus* sp. THG-HS227 (KF815074.1) | 96 | 4E-80 |
| 22 | MDc | 136 | Uncultured bacterium clone BiphS1_35 (EU651885.1) | 96 | 6E-68 |

KEY:
MS: Untreated Mechanic workshop shallow
MSa: Acetone-treated Mechanic workshop shallow
MSp: Phenanthrene-spiked Mechanic workshop shallow
MSc: Chrysene-spiked Mechanic workshop shallow
**Present in >1of the sites studied**
MD: Untreated Mechanic workshop deep
MDa: Acetone-treated Mechanic workshop deep
MDp: Phenanthrene-spiked Mechanic workshop deep
MDc: Chrysene-spiked Mechanic workshop deep
E-value: Expect value
*The sequence with the higher significant E-value and percentage is shown

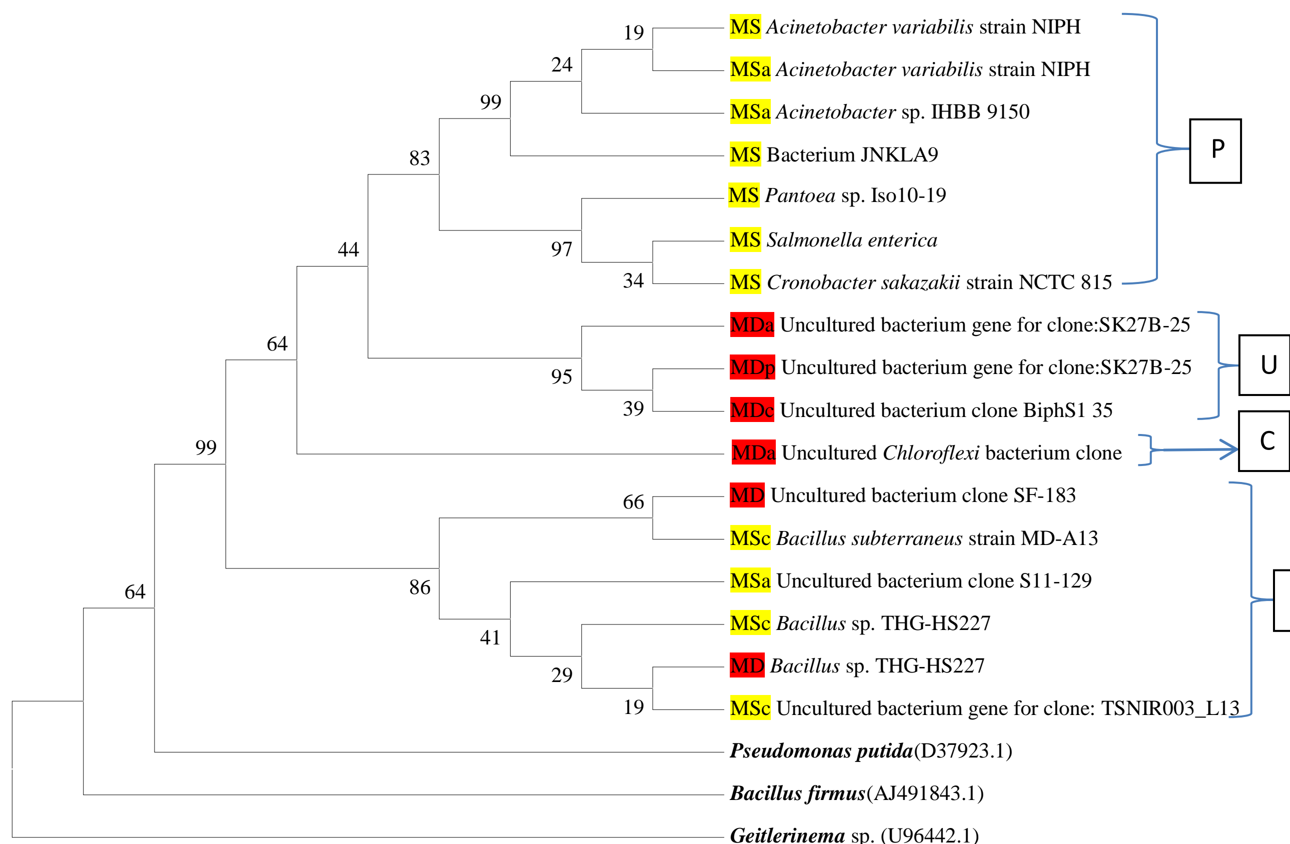



**Figure 4.5: A Maximum Parsimony Tree showing Phylogenetic Affiliation of 16S rRNA sequences obtained from a Mechanic workshop soil in Kaduna State**

**The tree was rooted with a genus from the Cyanobacteria group (*Geitlerinema* sp.); reference strains included in the tree are indicated in bold type. Bootstrap values carried out for 500 replicates; the numbers at the nodes indicate the bootstrap value in percent.**

**KEY:**

MS: Untreated Mechanic workshop shallow

MSa: Acetone-treated Mechanic workshop shallow

MSp: Phenanthrene-spiked Mechanic workshop shallow

MSc: Chrysene-spiked Mechanic workshop shallow

Yellow highlight: Shallow sampling sites (17 – 20 cm)

MD: Untreated Mechanic workshop deep

MDa: Acetone-treated Mechanic workshop deep

MDp:Phenanthrene-spiked Mechanic workshop deep

MDc:Chrysene-spiked Mechanic workshop deep

Red highlight: Deep sampling sites (37 – 40 cm)

P: Proteobacteria; C: Chloroflexi; U: Uncultured bacteria; F: Firmicutes

**Table 4.14a: Phylogenetic affiliations of 16S rRNA sequences obtained from Trailer park Soil treated with Polycyclic Aromatic Hydrocarbons (using $BLAST_N$ analysis)**

| S/No. | DNA frpm soil sample | DGGE Band No. | Closest match in Genbank database (Accession No.) | Sequence Identity (%) | E-value |
|---|---|---|---|---|---|
| 1 | TS | 40 | *Bacillus drentensis* strain SBB22 (KP790035.1) | 99 | 5E-94 |
| 2 | TS | 41 | Uncultured bacterium, clone 1-89-8-6-8 (AB929759.1) | 100 | 1E-95 |
| 3 | TD | 42 | *Bacillus panaciterrae* strain 351 BIHB (FJ859695.1) | 97 | 1E-85 |
| 4 | TSa | 46 | *Bacillus megaterium* strain WN603 (DQ275182.1) | 99 | 7E-93 |
| 5 | TSa | 47 | *Bacillus azotoformans* strain (KJ575025.1) | 99 | 2E-92 |
| 6 | TSa | 48 | Uncultured bacterium gene, clone 2-3-12-4-6 (AB930008.1) | 99 | 2E-92 |
| 7 | TSa | 49 | Uncultured bacterium gene, clone 1-25-12-4-6 (AB929976.1) | 100 | 1E-95 |
| 8 | TDa | 51 | Uncultured bacterium clone 60A (KJ600894.1) | 93 | 5E-74 |
| 9 | TDa | 52 | *Bacillus* sp. kyS1 (KP126757.1) | 98 | 2E-54 |
| 10 | TSp | 53 | *Cellulosimicrobium* sp. (KT368939.1) | 99 | 8E-82 |

**KEY:**
M: Molecular marker
TS: Untreated Trailer park shallow
TSa: Acetone-treated Trailer park shallow
TSp: Phenanthrene-spiked Trailer park shallow
TSc: Chrysene-spiked Trailer park shallow
TSb: B[a]P-spiked Trailer park shallow
**Present in >1of the sites studied**
TD: Untreated Trailer park deep
TDa: Acetone-treated Trailer park deep
TDp: Phenanthrene-spiked Trailer park deep
TDc: Chrysene-spiked Trailer park deep
TDb: B[a]P-spiked Trailer park deep
E-value: Expect value

**Table 4.14b: Phylogenetic affiliations of 16S rRNA sequences obtained from Trailer park Soil treated with Polycyclic Aromatic Hydrocarbons (using $BLAST_N$ analysis)**

| S/No. | DNA from soil sample | DGGE Band No. | Closest match in Genbank database(Accession No.) | Sequence Identity (%) | E-value |
|---|---|---|---|---|---|
| 11 | TSp | 57 | *Mahella australiensis* complete genome (CP002360.1) | 98 | 2E-78 |
| 12 | TSp | 59 | *Caloribacterium cisternae* strain (NR118109.1) | 99 | 1E-79 |
| 13 | TDp | 60 | Uncultured bacterium clone (KJ600894.1) | 95 | 1E-80 |
| 14 | TDp | 154 | *Bacillus* sp. kyS1 (KP126757.1) | 100 | 7E-77 |
| 15 | TSc | 67 | Uncultured bacterium clone 60A (KJ600894.1) | 96 | 5E-84 |
| 16 | TSb | 74 | Uncultured bacterial clone SBL 128F13 (FN356690.1) | 96 | 2E-82 |
| 17 | TSb | 75 | *Bacillus* sp. CanL-51 (KT580578.1) | 98 | 5E-89 |
| 18 | TDb | 78 | *Acinetobacter* sp. zf-IRht15 (DQ223660.1) | 98 | 1E-89 |
| 19 | TDb | 81 | Uncultured bacterium gene, clone: 1-47-12-4-6 (AB929987.1) | 95 | 5E-79 |

**KEY:**
M: Molecular marker
TS: Untreated Trailer park shallow
TSa: Acetone-treated Trailer park shallow
TSp: Phenanthrene-spiked Trailer park shallow
TSc: Chrysene-spiked Trailer park shallow
TSb: B[a]P-spiked Trailer park shallow
**Present in >1of the sites studied**
E-value: Expect value
TD: Untreated Trailer park deep
TDa: Acetone-treated Trailer park deep
TDp: Phenanthrene-spiked Trailer park deep
TDc: Chrysene-spiked Trailer park deep
TDb: B[a]P-spiked Trailer park deep
**Novel strain**

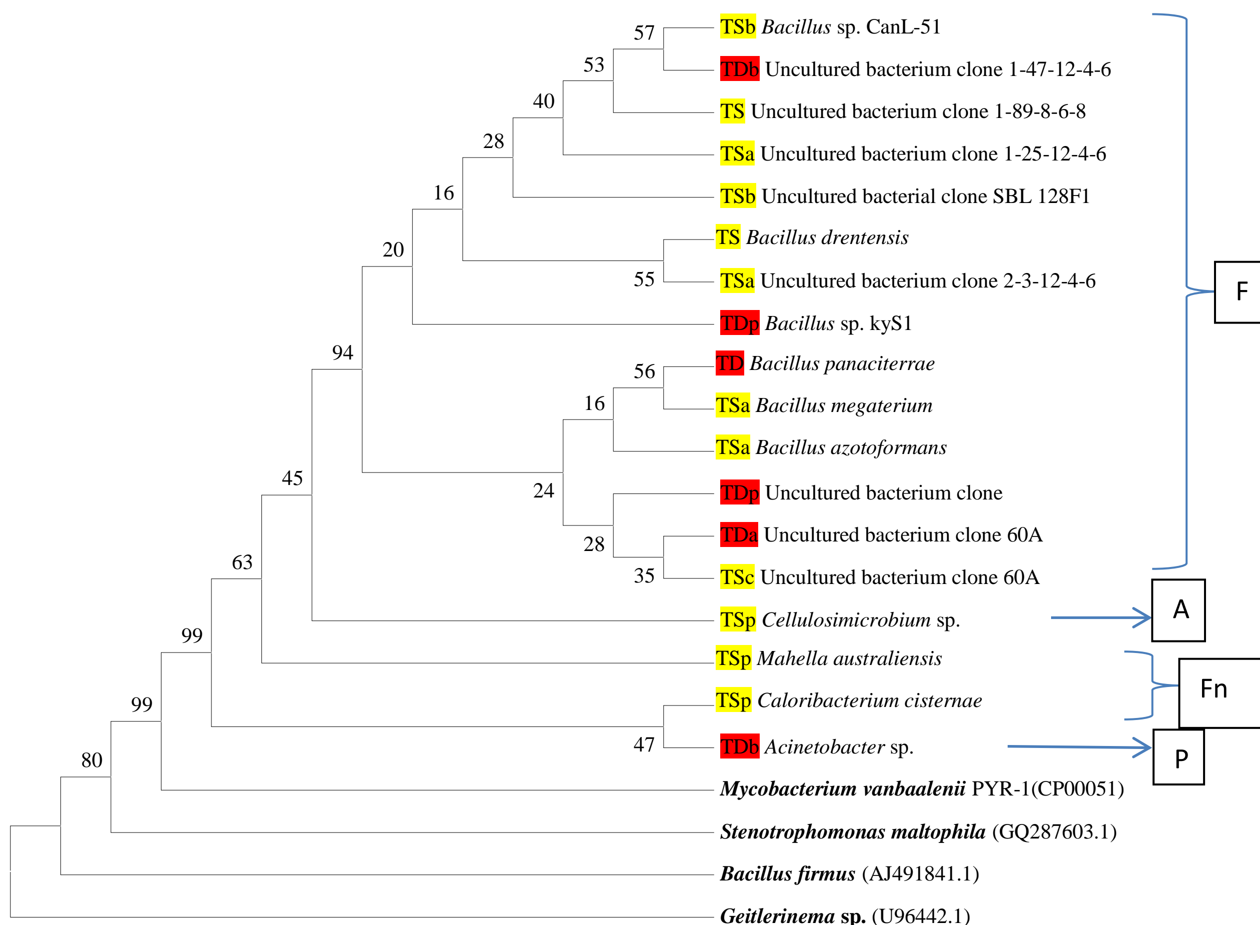



**Figure 4.6: A Maximum Parsimony Tree showing Phylogenetic Affiliation of 16S rRNA sequences obtained from a Trailer park soil in Kaduna State**

**The tree was rooted with a genus from the Cyanobacteria group (*Geitlerinema* sp.); reference strains included in the tree are indicated in bold type. Bootstrap values carried out for 500 replicates; the numbers at the nodes indicate the bootstrap value in percent.**

**KEY:**
M: Molecular marker
RS: Untreated Refinery effluent shallow
RSa: Acetone-treated Refinery effluent shallow
RSp: Phenanthrene-spiked Refinery effluent shallow
RSc: Chrysene-spiked Refinery effluent shallow
RSb: B[a]P-spiked Refinery effluent shallow
RD: Untreated Refinery effluent deep
RDa: Acetone-treated Refinery effluent deep
RDp: Phenanthrene-spiked Refinery effluent deep
RDc: Chrysene-spiked Refinery effluent deep
RDb: B[a]P-spiked Refinery effluent deep
Yellow highlight: Shallow sampling sites (17 – 20 cm) Red highlight: Deep sampling sites (37 – 40 cm)
F: Firmicutes; A: Actinobacteria; Fn: Novel strains of Firmicutes; P: Proteobacteria

**Table 4.15: Phylogenetic affiliations of 16S rRNA sequences obtained from Refinery effluent Soil treated with Polycyclic Aromatic Hydrocarbons (using BLAST$_N$ analysis)**

| S/No. | DNA from soil sample | DGGE Band No. | Closest match from Genbank database (Accession No.) | Sequence Identity (%) | E-value |
|---|---|---|---|---|---|
| 1 | RDb | 2 | *Bacillus megaterium* strain WN603 (DQ275182) | 99 | 7E-93 |
| 2 | RSb | 8 & 10 | **Bacillus subterraneus* strain 2L (KP453777.1) | 100 | 1E-95 |
| 3 | RDc | 11 | *Bacillus* sp. dsH1 (KP126759.1) | 100 | 1E-95 |
| 4 | RDc | 12 | Uncultured Bacillus sp. isolate DGGE gel band Scsm16:13-1 (KP693651.1) | 100 | 1E-95 |
| 5 | RSc | 13 | *Bacillus firmus* strain Ba2 (JF732747.1) | 99 | 2E-92 |
| 6 | RSc | 14 | *Bacillus cereus* strain: R5 (LC076294.1) | 98 | 3E-91 |
| 7 | RSc | 16 | Uncultured bacterium clone JX47 (FR716268.1) | 96 | 7E-72 |
| 8 | RDp | 17 | *Bacillus* sp. dsH1(KP126759.1) | 96 | 5E-84 |
| 9 | RDp | 19 | *Bacillus subterraneus* strain 2L (KP453777.1) | 100 | 1E-95 |
| 10 | RDa | 27 | *Bacillus subterraneus* strain 2L(KP453777.1) | 100 | 7E-63 |

**KEY:**
M: Molecular marker
RS: Untreated Refinery effluent shallow
RSa: Acetone-treated Refinery effluent shallow
RSp: Phenanthrene-spiked Refinery effluent shallow
RSc: Chrysene-spiked Refinery effluent shallow
RSb: B[a]P-spiked Refinery effluent shallow
RD: Untreated Refinery effluent deep
RDa: Acetone-treated Refinery effluent deep
RDp: Phenanthrene-spiked Refinery effluent deep
RDc: Chrysene-spiked Refinery effluent deep
RDb: B[a]P-spiked Refinery effluent deep
**Present in >1of the sites studied**
*The sequence with the higher significant E-value and percentage is shown

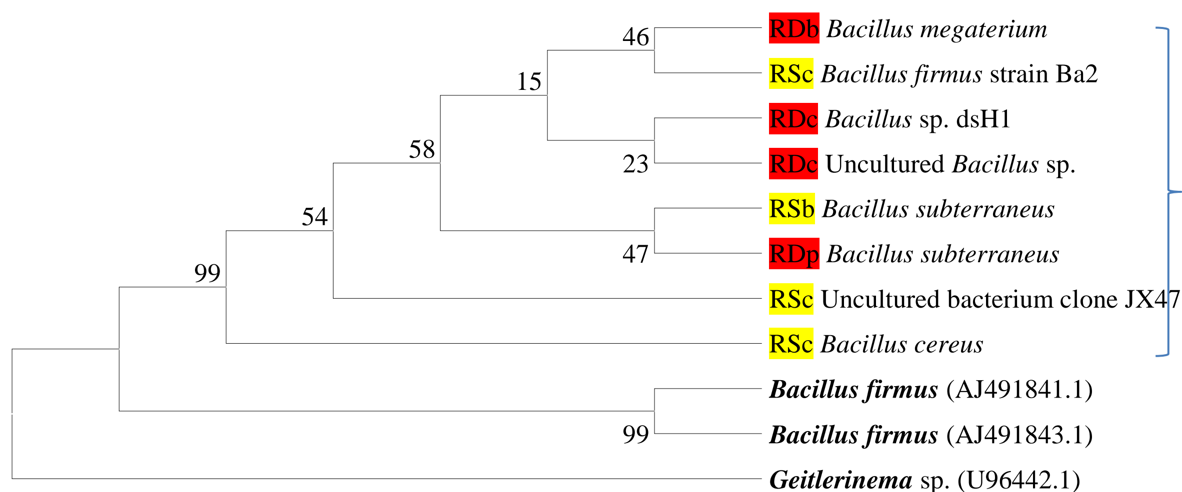



**Figure 4.7: A Maximum Parsimony Tree showing Phylogenetic Affiliation of 16S rRNA sequences obtained from a Refinery effluent soil in Kaduna State**

**The tree was rooted with a genus from the Cyanobacteria group (*Geitlerinema* sp.); reference strains included in the tree are indicated in bold type. Bootstrap values carried out for 500 replicates; the numbers at the nodes indicate the bootstrap value in percent.**

**KEY:**
M: Molecular marker
RS: Untreated Refinery effluent shallow
RSa: Acetone-treated Refinery effluent shallow
RSp: Phenanthrene-spiked Refinery effluent shallow
RSc: Chrysene-spiked Refinery effluent shallow
RSb: B[a]P-spiked Refinery effluent shallow
RD: Untreated Refinery effluent deep
RDa: Acetone-treated Refinery effluent deep
RDp: Phenanthrene-spiked Refinery effluent deep
RDc: Chrysene-spiked Refinery effluent deep
RDb: B[a]P-spiked Refinery effluent deep
Yellow highlight: Shallow sampling sites (17 – 20 cm) Red highlight: Deep sampling sites (37 – 40 cm)
F: Firmicutes

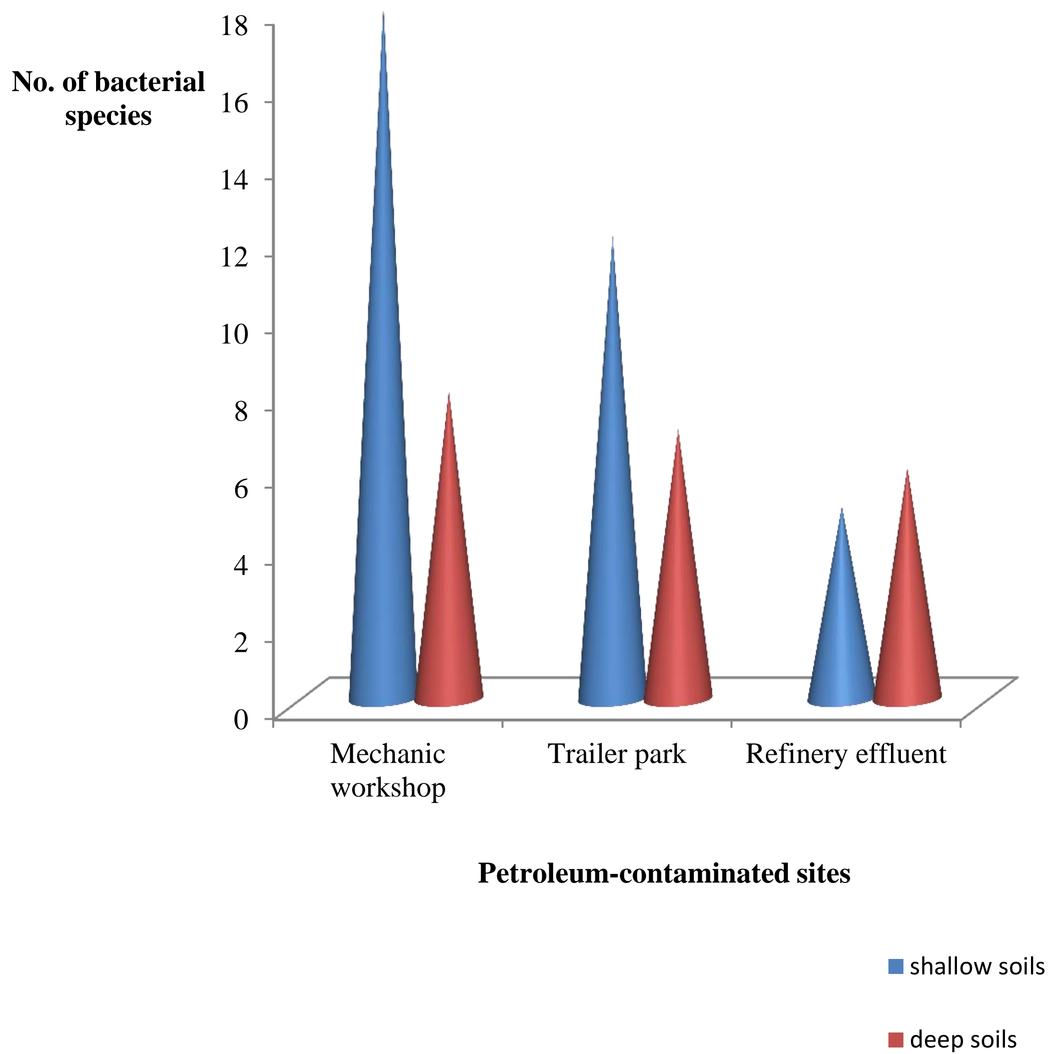



**Figure 4.8: Abundance of bacterial species in the PAH-spiked soils from petroleum-contaminated sites based on depth of sampling**

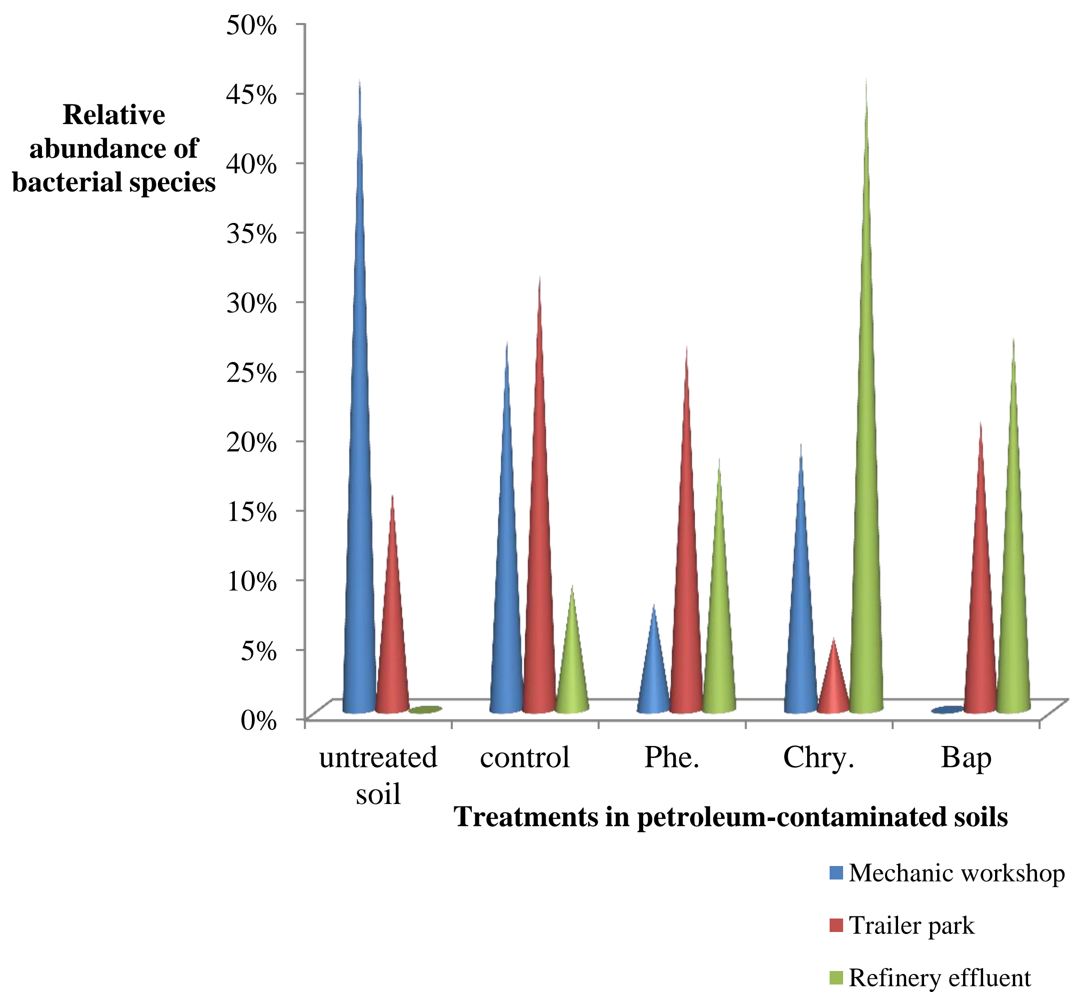



**Figure 4.9: Abundance of bacterial species in the PAH-spiked soils from the petroleum-contaminated sites based on the PAH treatment**

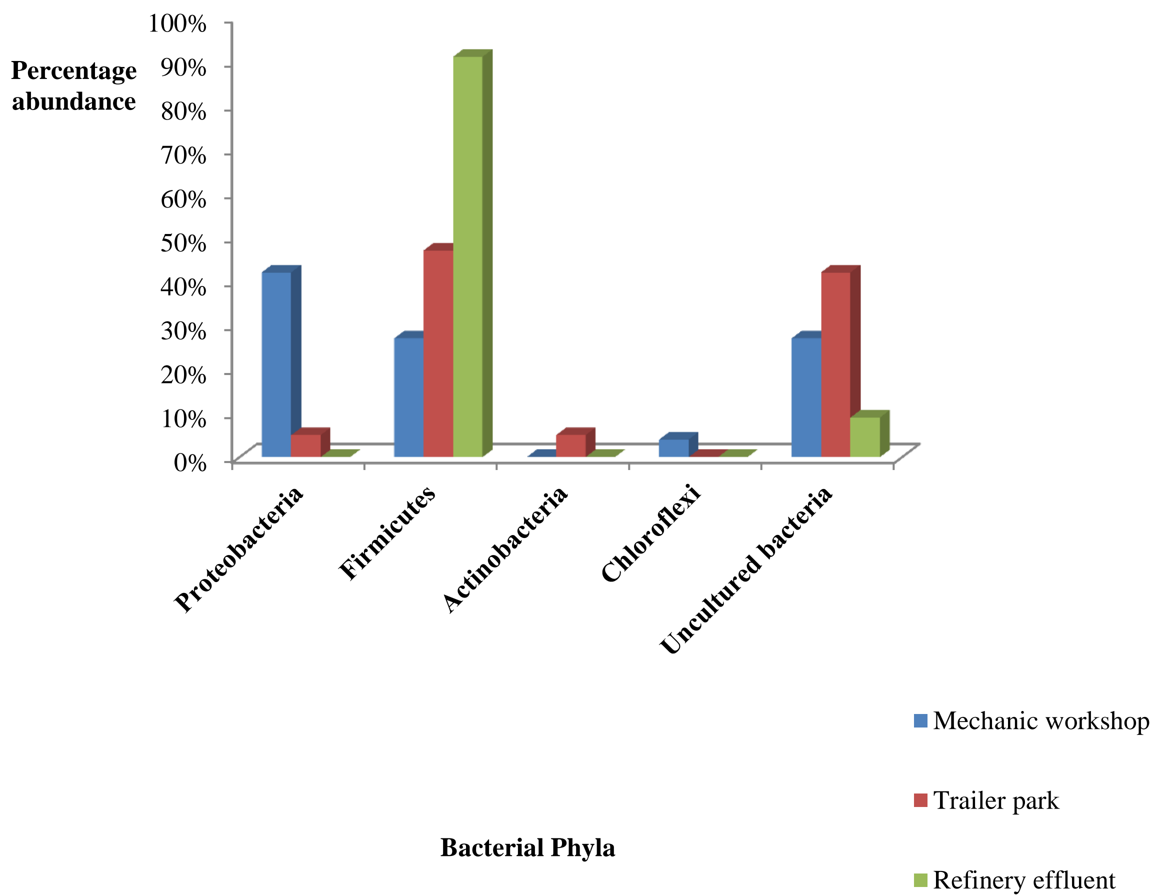



**Figure 4.10: Distribution of bacterial taxonomic groups in PAH-spiked soils from petroleum-contaminated sites**

The site recording the highest number of 16S gene copies is the Mechanic workshop soil, while the refinery effluent site had the least gene copies. In the Mechanic workshop site (Fig. 4.11), the sample treated with chrysene had the highest 16S gene copies (5.20E+10 gene copies per gram of soil), while in the Trailer park (Fig. 4.12) and Refinery effluent (Fig. 4.13) sites the sample treated with phenanthrene had the highest gene copies respectively (4.27E+09 and 7.91E+07 gene copies per gram of soil). Unlike the Mechanic workshop and trailer park sites that had the least 16S gene copies in the untreated samples, the Refinery effluent site had the least number of gene copies in the sample treated with chrysene (Fig. 4.13).

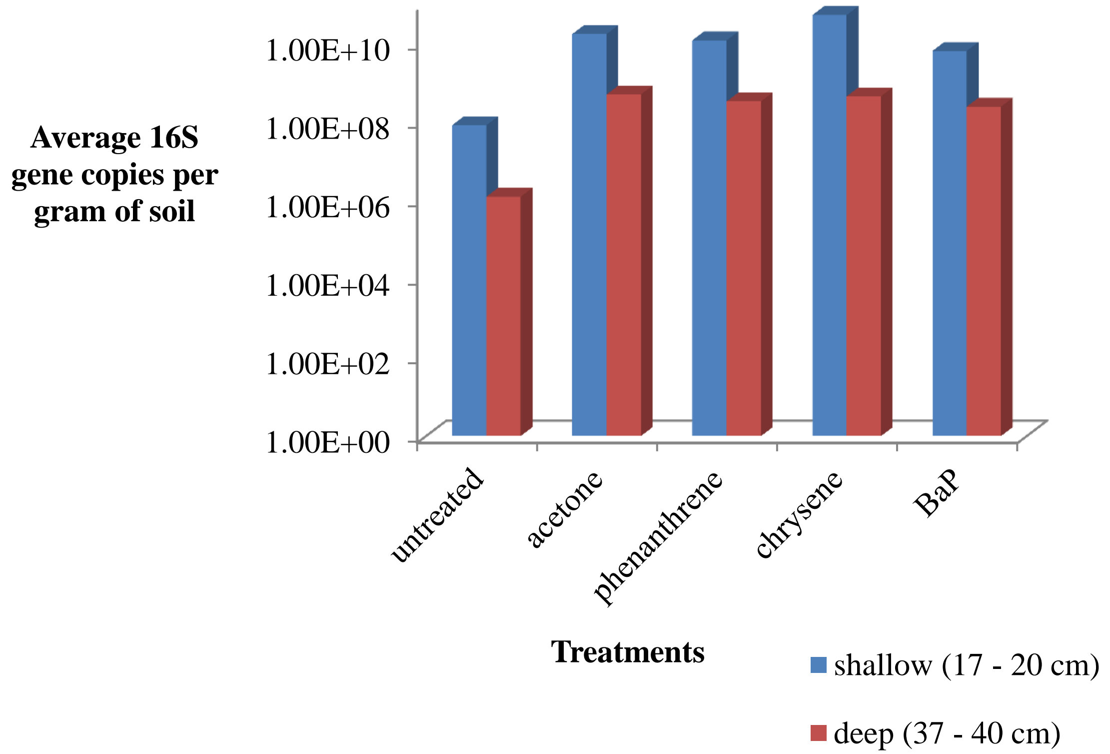



**Figure 4.11: The abundance of bacterial 16S gene in a Mechanic workshop soil treated with polycyclic aromatic hydrocarbons**

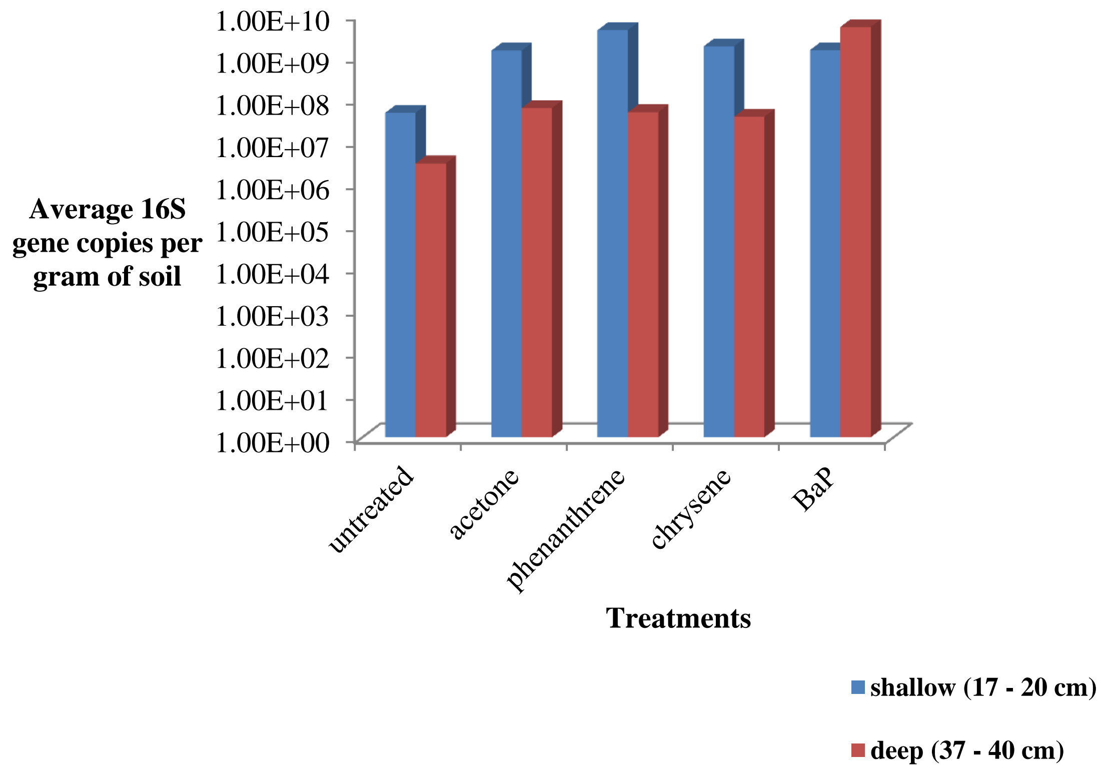



**Figure 4.12: The abundance of bacterial 16S gene in a Trailer park soil treated with polycyclic aromatic hydrocarbons**

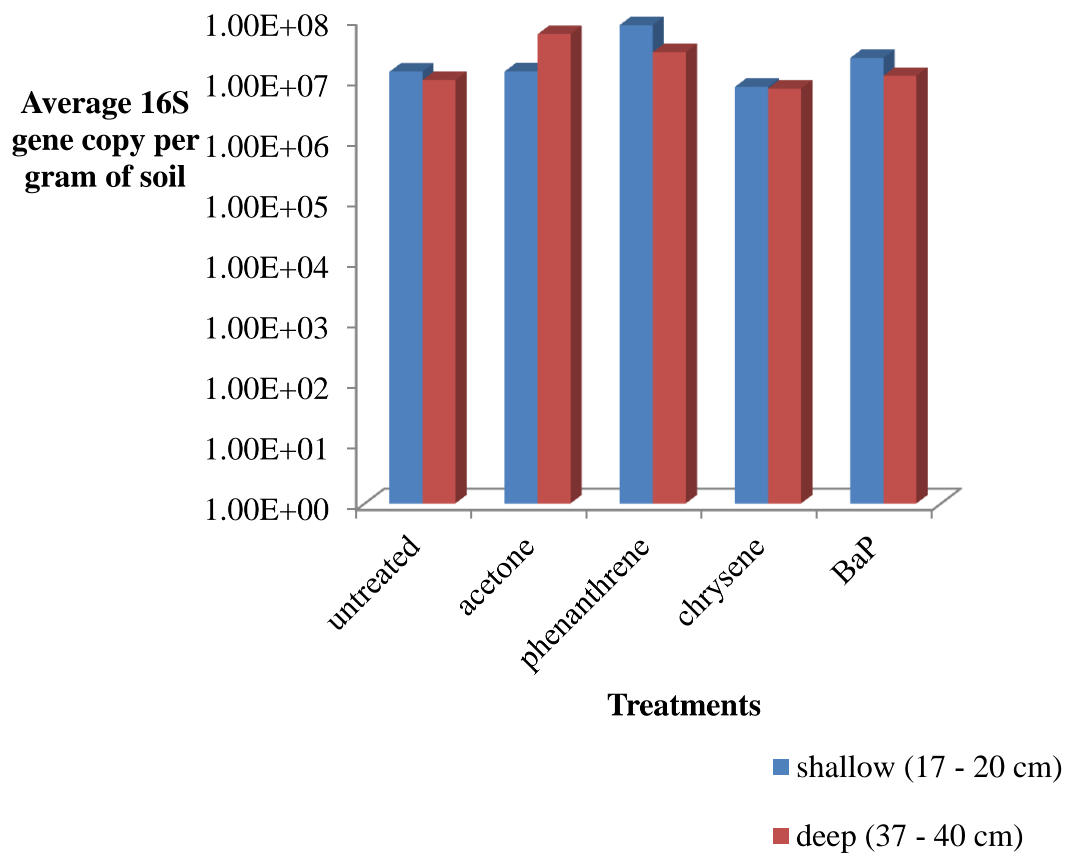



**Figure 4.13: The abundance of bacterial 16S gene in a Refinery effluent soil treated with polycyclic aromatic hydrocarbons**

# CHAPTER FIVE

## 5.0 DISCUSSION

### 5.1 Physico-chemical Characteristics, Macronutrients Content, Microelements Analyses and PAH Analyses of the Petroleum-contaminated Soils in First Sampling:

In this study the soil type in the three sampling sites was the same for both depths (Table 4.1). Thus, the depth of sampling did not affect the texture of the soil in each site. Most of the soils sampled were of loamy texture, and the particle size is smaller and less porous than sandy soil. This feature increases its capacity to retain nutrients for not just plant growth but microbial survival. Soil texture plays a role in the makeup of bacterial community due to factors such as porosity that affect the uptake and assimilation of nutrients by bacteria (Ding *et al*., 2010). In a study about biodegradation of PAHs by bacteria in the rhizosphere, breakdown of petroleum hydrocarbons was higher in the loamy soils than the sandy soils (Afzal *et al*., 2011). In another study by Ding *et al*. (2010), the soil type was found to affect the sorption of phenanthrene, a polycyclic aromatic hydrocarbon associated with petroleum contamination as well as other fossil fuels.

All the samples had an acidic pH (Table 4.1) which could also play a role in the diversity of the prokaryotic population in these locations since it might affect their nutritional requirements.The Trailer park and Mechanic workshop showed relatively higher quantities of oil and grease compared to the Refinery effluent soil because these two sites had similar history of petroleum contamination. Activities such as repair of automobiles exposed the soils in these two sites to gasoline, engine oil and diesel. The

Refinery effluent soil on the other hand, was obtained at a site not having obvious petroleum contamination thus, the oil and grease content was considerably less.

The Carbon to Nitrogen ratio in the Mechanic workshop and Trailer park soils varied greatly between the depths of sampling (Table 4.2). However, the Refinery effluent soil had relatively similar values at both depths. This could be a reason why its DGGE profile in the Refinery effluent soil was almost uniform, reflecting possible similar bacterial community.

Among the microelements assayed in the samples (Table 4.3), Cadmium and Cobalt had markedly lower quantities in the deep samples compared to the shallow samples. The other metals on the other hand, did not show any pattern in the two depths. Zinc (114.3 – 2725.53 mg/kg) and Lead (5.2 – 351.42 mg/kg) had the highest quantity of heavy metal. The Refinery effluent samples had the highest content of all the heavy metals analyzed with the exception of Cadmium which was highest in the Mechanic Workshop. Industrial processes such as petroleum refining has been known to cause the release of heavy metals in the resulting effluent (Junca and Pieper, 2004; Kim *et al*., 2006; Cebron *et al*., 2008) this could be the reason why the Refinery effluent samples had higher heavy metals than the Mechanic workshop and Trailer park samples. Polycyclic aromatic hydrocarbons were not detected in the soil samples (Table 4.4), either because they were not in the samples to begin with, or perhaps they were present in quantities far below the detection limit.

**5.2 PCR-DGGE Analysis of partial 16S rRNA Gene of Bacteria in Petroleum-contaminated Soils in First Samping:**

The forty base pair GC clamp present on the forward primer, 357F-GC enhanced the separation of nucleotides of varying sequences during the DGGE analysis.The Refinery

effluent and Trailer park samples produced relatively clearer bands than the Mechanic workshop samples on the gel (Plates V - VII). A possible reason for this could be the higher oil and grease content of the Mechanic workshop samples thus, affecting the DNA elute. Subsequent DGGE analysis of the samples in triplicates revealed similar profiles in the replicates of the Trailer park and Refinery effluent samples. On the other hand, the Mechanic workshop deep sample had a marked difference in one of its replicates, MWD 2 (Plate V), which might be attributable to slight variations during DNA extraction. This brings to the fore the importance of analysing replicates of a sample in order to check such abnormalities. The DGGE fingerprints of the 16S sequences from the contaminated soils provides valuable information on the different bacterial phyla

### 5.3 Sequencing and phylogenetic analysis of 16S rRNA sequences from petroleum-contaminated soils

The high bootstrap value (100%) of the 16S sequences from the Mechanic workshop (shallow) soils (Fig. 4.1) indicates a very strong relationship between them; the other bootstrap values for the other sequences are relatively good since they are mostly above 90%. Only the Mechanic workshop soil showed the likelihood of the depth of sampling affecting the diversity of the species as the deep and shallow soils was observed to have seperate clades respectively (Fig. 4.1).

The phylogenetic relationship of the 16S rRNA sequences obtained from the Trailer park soils (Table 4.6) revealed them to be Actinobacteria and Gram positive low G+C bacteria (*Bacillus*). The sequences from the shallow sample (TS13: 100% and TS14: 100%) were closely related to *Bacillus*, and one related to *Arthrobacter* (TS16: 98%). The deep samples (TD18: 94%; TD21: 98%) had sequences closely related to

Actinobacteria and one being affiliated to *Bacillus* (TD20: 93%). The phylogenetic affiliation of the sequences in the Trailer park soil revealed that they are all Gram positive bacteria, belonging to the phyla, Actinobacteria and Firmicutes. Perhaps the relatively higher quantities of macronutrients and micronutrients played a role in the multiplication of these bacteria (Table 4.2 and Table 4.3). Gram positive bacteria belonging to both phyla in question have been known to degrade some high molecular weight PAHs (Sho *et al*., 2004; Zhou *et al*., 2008; Kim *et al*., 2012), especially certain species of the genus, *Mycobacterium*. Enzymes important in the degradation of PAHs are present in both Gram negative and Gram positive bacteria; however both groups have specific enzyme systems. Although a study by Margesin *et al*. (2003) on the growth of bacteria in pristine and PAH-contaminated alpine soils, revealed that species of *Rhodococcus* and *Mycobacterium* grew better in conditions of low-nutrient than when there were more resources.

The 16S sequences from the Refinery effluent soils had phylogenetic association with both Gram positive bacteria and Proteobacteria (Fig. 4.1), but more of the former than the latter. Two of the 16S sequences obtained from the shallow sample (RS22 and RS24) formed a cluster; both sequences have a similarity of 93% to Alphaproteobacteria. However, the only other two sequences from the shallow sample RS25 (93%) and RS26 (95% to *Micrococcus endophyticus*) appeared to be Actinobacteria. *Micrococcus endophyticus* is commonly found in soil and is associated with plant tissues (Chen *et al*., 2009), the soil sample having this 16S sequence was obtained from a Refinery effluent site that is cultivated for crop production. This might explain its presence in this site. Soils having the presence of plants usually encourage the growth of a wide diversity of bacteria due to plant exudates especially in the rhizosphere thus, increasing the amount of nutrients in the soil. The same phenomenon

was observed by Cebron *et al*. (2009) in their study on diversity of bacteria in soils with aged PAH contamination. The deep samples RD27 and RD29 (both uncultured) form a cluster and are closely related to Acidobacteria (Fig. 4.1), with RD27 having 92% similarity to an uncultured Acidobacteria. Acidobacteria are a newly devised phylum of bacteria, whose members are physiologically diverse and ubiquitous, especially in soils, but are under-represented in culture (Quaiser *et al*., 2003; Rappe and Giovannoni, 2003; Barns *et al.,* 2007), this explains why the sequences most closely affiliated to be Acidobacteria are uncultured bacteria. The acidic nature of the soils used in this study supports the growth of members of Acidobacteria, since some of them are acidophilic. The other deep sample 16S sequence, RD28 was also an uncultured bacterium having a 92% similarity to an uncultured *Bacillus* sp. Clone (Table 4.7); it forms a cluster with three sequences (TS13; TS14 and TD20) from the Trailer park soils which are all also closely affiliated to *Bacillus*.

Members of the genera *Acinetobacter*, *Burkholderia*, *Rhodopseudomonas*, *Pseudomonas*, *Bacillus*, as well as the Actinobacteria detected in this study, *Arthrobacter* and *Micrococcus* are known hydrocarbon-degrading soil-dwellers (Del Panno *et al*., 2005; Jacques *et al*., 2005; Kim *et al*., 2006; Kim *et al*., 2009; Adetutu *et al*., 2013). Members of the phyla, Proteobacteria and Actinobacteria have been the focus of numerous studies on the metabolic pathway as well as catabolic genes of PAHs (Brennerova *et al*., 2009)

There has been evidence of Acidobacteria in bacterial degradation of polycyclic aromatic hydrocarbons (Ni Chadhain *et al*., 2006).

Bootstrapping was carried out on the Neighbour-Joining tree of 16S rRNA sequences obtained from the petroleum-contaminated soils (Fig. 4.1), in order to test the reliability

of the relationships. The sequences from Mechanic workshop shallow soil (MS1, MS3, MS4, MS5, and MS6) had the highest bootstrap value (100%), signifying a strong possibility that the sequences are very related. However, all the other clades showed very good bootstrap values. This statistic shows that the sequences in the OTUs in the clades have a strong affiliation.

## 5.4 Quantitative PCR (Real-time PCR) Analysis of Bacterial and Archaeal 16S Gene in Petroleum-contaminated Soils:

The quantification of the conserved region of the 16S ribosomal RNA gene in both bacteria and archaea in the petroleum-contaminated soils (Figures 4.2 and 4.3) showed that the soils obtained closer to the surface (17 – 20 cm) had more gene copies than the deeper layers (37 – 40 cm). This is hardly shocking considering the fact that nutrients necessary for growth as well as oxygen are more readily available at the soil surface thus, favouring prokaryotic growth and multiplication.

One of the replicates of the Trailer park shallow soil had the highest number of bacterial 16S gene copies (Fig. 4.2), while the Refinery effluent shallow sample showed the highest number of archaeal 16S gene copies (Fig. 4.3). However, the mean of the three replicates of the Trailer park soil is lower than that of the replicates of the Refinery effluent soil. Thus, the Refinery effluent soil can be said to have the highest number of archaeal and bacterial 16S gene copies, based on the mean of the replicates. The Carbon to Nitrogen ratio (C:N) in the Trailer park soil and Refinery effluent soil were relatively similar (9:1; 10:1 respectively) and might play a role in the proliferation of these prokaryotes. The importance of moisture content in bacterial multiplication can be observed in sample TS which recorded the highest moisture content (3.1%) compared to the other samples. The relatively high quantity of microelements in the Refinery

effluent soil compared to the Mechanic workshop and Trailer park soils might have favoured the growth of the archaea and bacteria (Table 4.3). Archaeal and bacterial populations in soils have been known to utilize metals such as iron,

The standard curves for quantification of the bacterial and archaeal 16S gene were both linear as the $R^2$ value in both cases were observed to be within the acceptable range (Appendix X and Appendix XI). The 16S gene copies of bacteria (3.11E+07 – 1.23E+08) and archaea (8.13E+06 – 5.76E+07) in the Refinery effluent soils is relatively even and having the lower value only an order of magnitude less than the higher value in both prokaryotic groups. This comparatively stable range of bacterial and archaeal numbers in the Refinery effluent soil could be as a result of the effect of cultivation of crops on this site. Soil tillage as well as the release of organic compounds by plant roots all favour microbial growth due to the resultant increase in nutrients in such soils. The amount of nutrients in this soil is also very similar at both depths (Table 4.2 and Table 4.3), further influencing the relatively stable 16S gene copies at both depths. The DGGE profile (Plate V) of the bacterial community in the Refinery effluent sample also showed this rather consistent trend. All these point to the fact that the prevailing conditions at the two sampling depths did not greatly affect the number of prokaryotes in the Refinery effluent soil.

However, the 16S gene copies in the Mechanic workshop soil had a difference of two orders of magnitude in both bacteria, 2.49E+05 – 6.61E+07 gene copies per gram of soil (Fig. 4.2) and archaea, 1.91E+04 -4.53E+06 gene copies per gram of soil (Fig. 4.3). The same trend was also observed in the number of 16S gene copies in the Trailer park soil, between the bacteria (1.21E+06 – 9.28E+08) and archaea (3.59E+04 – 3.02E+06). This occurrence can be explained by the wide difference in the amount of macronutrients between the shallow and deep sampling points (Table 4.2). This

phenomenon might play a role in the number of the prokaryotic populations as the availability of nutrients determines the survival of these microorganisms (Afzal *et al*., 2011).

### 5.5 Physicochemical characteristics of petroleum-contaminated soils spiked with polycyclic aromatic hydrocarbons:

The soil samples collected from the petroleum-contaminated sites had pH values around neutral, only the deep sample (37 – 40 cm) from the Mechanic workshop had an acidic pH (5.8). The texture of the soils varies between both sampling depths in each site as a result of the occurrence of different particle sizes (Table 4.8). The particle size of a soil sample influences the diversity of bacteria in the soil (Kakirde *et al*., 2010), as well as the uptake of such hydrocarbons by indigenous bacteria. In this study, the high clay content in the deep sample of the Trailer park enhanced possible utilization of the PAH, benzo[a]pyrene which was used in treating that sample. This development was not observed in the shallow sample from the same site having the same compound but having much lower clay content. This is why researches centred on pollutant degradation in soil environments lay some emphasis on the soil texture (Ding *et al*., 2010; Afzal *et al*., 2011).

The moisture content was observed to be highest in the Refinery effluent soils. The samples were obtained by the bank of the Romi river which receives effluent released from the petroleum refinery. Moisture content affects the bioavailability of polycyclic aromatic hydrocarbons since they have weak solubility in water (Haritash and Kaushik, 2009). Consequently, the highest 16S gene copy was observed in the soil (MS) with the least moisture content (Figure 4.11).

The organic Carbon and Nitrogen content was higher in the shallow samples than the deep samples in all the sites (Table 4.9). Most metabolic activities are more prominent in soils closer to the surface due to the presence of more oxygen and nutrients. The Carbon-Nitrogen ratio was highest in the Mechanic workshop soil; this is most likely due to the very high organic Carbon content resulting from the high amounts of oil and grease in the soil. The C:N ratio in the Mechanic workshop (34.88 and 10.23) and Trailer park soils (9.46 and 7.61) was higher in the shallow samples than the deep samples. Conversely, the Refinery effluent site had very similar amounts of C:N ratio in both the shallow and deep samples (11.07, 11.42 respectively), consequently leading to similar number of 16S gene copies between the shallow and deep samples in this site.

The soil having the highest quantity of microelements (with the exception of chromium) is the Mechanic workshop shallow soil (Table 4.10). The values of zinc and lead in this sample greatly exceeded the intervention limit set by the Department of Petroleum Resources (DPR, 2002). The high amounts of zinc and lead in this soil may have negatively affected utilization of PAHs by the bacteria in this soil, as most of the bacteria identified in this site were obtained from the soils not spiked with PAHs (Fig. 4.9). The presence of heavy metals in soil affects the uptake and utilization of PAHs by bacteria (Chen *et al*., 2013; Afegbua, 2015).

Analyses of the soil samples from the first and second sampling using GC-MS (Table 4.11) showed that there was no PAH detected. This is however very surprising since the sites studied had close association with petroleum and inevitably polycyclic aromatic hydrocarbons. There is a strong possibility that the hydrocarbons were present but in amounts not detected by the standards used in the chromatography. The concentration of the standards used in the GC-MS procedure was in micro grams ($10^{-6}$), however it has been shown that a lower concentration such as pica gram ($10^{-9}$) might prove more

useful in detecting very low quantities of PAHs in the environment (Personal communication with Afegbua, S.L.).

## 5.6 PCR-DGGE Analysis of Bacterial 16S gene in Petroleum-contaminated Soils Spiked with Phenanthrene, Chrysene and Benzo[a]pyrene:

The identification of soil bacteria using methods based on the amplification of the 16S rRNA gene ensures a comprehensive approach in determining the diversity of indigenous bacteria. The amplicons obtained on amplification of the 16S rRNA in the soils spiked with the various polycyclic aromatic hydrocarbons appeared sharper with the shallow samples compared to the deep samples. This could be related to the fact that the 16S gene was observed to be more abundant in the shallow samples on analysis with Real-time PCR. However, among the three sites, the Refinery effluent soils (Plate XI) produced relatively brighter bands than the Mechanic workshop (Plate XII) and Trailer park soils (Plate XIII) respectively. This could likely be as a result of the lower quantity of oil and grease in the refinery effluent soil thus, reducing the chances of inhibitors during the PCR reaction. The use of universal primers in the amplification of the 16S ribosomal RNA of both Gram negative and Gram positive bacteria in soil has become the basis of molecular studies involving the taxonomy of bacterial communities in the soil (Nubel *et al*., 1997; Marquez-Rocha *et al*., 2005; Klankeo *et al*., 2009; Korotkevych *et al*., 2011). The acetone used as an organic solvent during spiking of the soil samples may have aided the extraction of DNA from the soils as the soils treated with the mixture of PAH + acetone as well as the control soils treated with only acetone had better quality DNA.

The DGGE profile of the bacterial population in the three petroleum-contaminated sites illustrated the diversity based on the various nucleotide sequences. The bulk of the

bands excised for sequencing (Fig. 4.4) were from the Mechanic workshop and Trailer park soils because these two sites had a higher variety in the band patterns. This variety in band patterns is a reflection of the different nucleotide sequences. In all three sites, the samples treated with either acetone or one of the polycyclic aromatic hydrocarbons (phenanthrene, chrysene, or benzo[a]pyrene) had more defined band patterns compared to the samples without any form of treatment. The presence of PAHs in the samples showed some impact on the bacterial population as a number of bands present in the treated samples were not observed in the untreated samples, and vice versa; this is suggestive of the PAH influencing the bacterial community. However, the most profound impact by any of the PAHs on the banding pattern was exhibited in the Trailer park sample treated with benzo[a]pyrene (Plate XII), the profile in this sample was more distinct than the other treatments. Possibly, BaP or one of its metabolites favoured the growth of the bacteria in that soil sample.

DGGE has been used in numerous studies relating to bacterial degradation of PAHs as a means of showing diversity of the bacterial community (Muyzer *et al*., 1993; Nakatsu *et al*. 2000; Del Panno *et al*., 2005; Hong *et al*., 2007). It is a fingerprinting method of obtaining the community profile of a given bacterial population and changes in the number and/or of type of species can be monitored over a period of time and during bioremediation (Korotkevych *et al*., 2011).

The Shannon Index of diversity showed the level of diversity in each of the sites sampled (Table 4.12). The Mechanic workshop (2.8292) and Trailer park sites (2.8319) had similar indices, although the former was slightly higher. Hence, indicating a higher diversity in the Trailer park site. The Refinery effluent site had the lowest index (2.4424) suggestive of the least diversity of bacterial species among the three sites. The C:N ratio in the Trailer park and Mechanic workshop sites might have influenced the

growth and diversity of bacteria in these sites. Both sites had similar C:N ratios and more importantly, the ratio is favourable for the growth of bacteria. In terms of Species richness, the Mechanic workshop site had the highest value (140), while the Refinery effluent site had the least value (108). The evenness of the bacterial community in the three sites was highest in the Trailer Park site and least in the Refinery effluent site. The implication of this is that, there is a more even distribution of the species in the Trailer park site in comparison to the Mechanic workshop and Refinery effluent sites respectively. The Shannon index of diversity, evenness and Specie richness are all valuable indices that provide valuable information on the diversity, total number of species as well as, the number of each species in a particular study area. They have been used in many studies involving microbial ecology and diversity (Boon *et al*., 2002; Andreoni *et al*., 2004; Moura *et al*., 2007), in this study these indices have shown that the presence of PAHs in a soil, physicochemical parameters, and the depth of sampling can affect the diversity of the bacterial population in a soil sample.

### 5.7 Diversity and Phylogenetic Analysis of Bacterial Population in the Petroleum-contaminated Sites:

Sixty one 16S rRNA sequences were successfully analysed using the Chromas lite software for chromatograms and subsequently ran on the NCBI website using the BLAST program (version $BLAST_N$ 2.2.30), the sequences in which optimum amplification were not achieved with the sequencing primers were excluded from the BLAST search. Another reason for excluding some sequences is short fragments during alignment with the MEGA 4 software (Tamura *et al*., 2007). About 50% of the total sequences used in phylogenetic studies belong to the Mechanic workshop site (30), while the least number of sequences was from the Refinery effluent site (12).

The Mechanic workshop site showed considerable bacterial diversity (Fig. 4.5), the majority of the sequences showed close affiliation to Gamma-proteobacteria (*Salmonella*, *Cronobacter*, *Pantoea* and *Acinetobacter*). *Acinetobacter* species are common soil-dwellers and have been known to show ability to degrade hydrocarbons (Kim *et al*., 2006; Adetutu *et al*., 2013). A member of this genus, *Acinetobacter baumanii*, was found to degrade phenanthrene in conjunction with other bacterial species; this ability was thought to be either through surfactants or extracellular polymeric substances (Kim *et al*., 2009). A member of the genus, *Acinetobacter*, *Acinetobacter lwoffi*, was found to achieve a little growth on benzene unlike other bacterial strains which could not grow on the same substrate (Adebusoye *et al*., 2007).

The Gamma-proteobacteria form a diverse group of medically important species, but they are able to utilise a wide range of substrates for growth and several species have shown the ability to degrade hydrocarbons in the soil and marine environment. The Gammaproteobacteria were shown to carry out the highest degradative activity of PAHs in a site contaminated by coal tar (Lors *et al*., 2010). The presence of these bacteria in this petroleum-contaminated site means there is the likelihood of horizontal transfer of genes enabling them to utilise the hydrocarbons in this environment. Interestingly, all the species belonging to this group of bacteria were present in only the shallow soils. However, the samples where they were present did not have any of the PAHs as a treatment, thus it is very likely these bacteria were utilising the simpler hydrocarbons which are also constituents of petroleum. In a study by Guazzaroni *et al*. (2013), a very high percentage of the members of Gammaproteobacteria present in a PAH-contaminated soil belonged to the genus, *Pseudomonas*. The genus, *Pantoea* was recently seperated from the genus, *Enterobacter*, it can be found in the soil, plants, rhizopshere and feculent material. The species can occur as pathogens or commensals

(Cruz *et al*., 2007). In a study by Afzal *et al*. (2010), soils inoculated with *Pantoea* sp. strain BTRH79 were observed to have increased rate of hydrocarbon degradation, the strain possesses the gene, cytochrome P450 alkane hydroxylase gene, which enables it to degrade alkanes. Identification of 16S sequences from a chronically contaminated soil showed two strains that are closely related to *Pantoea* and *Enterobacter aerogenes* (Festa *et al*., 2013), hence supporting the fact that many species belonging to the phylogenetic group, gammaproteobacteria are able to utilize petroleum hydrocarbons.

A species of *Cronobacter*, *Cronobacter sakazakii* has been implicated in diseases affecting adults and a rare cause of invasive infection in babies (Drudy *et al*., 2006). The members of this genus have been known to survive very dry conditions. The presence of this bacterium as well as *Salmonella enterica* and *Pantoea* sp. is indicative of likely faecal contamination in the Mechanic workshop site or in the surrounding area. The other predominant taxonomic group in this site is the Firmicutes, of which species belonging to the genera, *Bacillus* were the only bacteria observed (Fig. 4.5).

There were three uncultured species observed in the Mechanic workshop site (Table 4.13), as such their taxonomic group was not determined. Their closest relatives were all obtained from environmental samples, and one of them possesses a gene for biphenyl degradation (uncultured bacterium clone Biph S-1-35). This is of relevance since that sequence was amplified from a sample treated with chrysene, a four-ring PAH. Despite the fact that the presence of functional dioxygenase genes was not established, a close affiliation to species that actually possess similar genes shows that there is a possibility that the indigenous bacteria in that soil could also harbour such genes that lead to the degradation of PAHs. An uncultured species of *Chloroflexi* was the closest relative to a sequence amplified from the deep soil of the Mechanic workshop. Kumar and Khanna (2010) also identified uncultured species of *Chloroflexi*

during their study on the bacterial community on a coal-tar contaminated site. Species belonging to the genus, *Chloroflexi*, are filamentous green non-sulphur bacteria possessing photosynthetic pigments that enable them utilise light for their metabolic needs. In a study by Cebron *et al*. (2009), the sequences of clone obtained from a cultivated soil revealed the presence of bacteria belonging to the phylum, *Chloroflexi* as well as Proteobacteria and other phylogenetic groups. In the study, the influence of vegetation on the structural and phylogenetic diversity of soil bacteria was analyzed.

Treatment of the soils from the Mechanic workshop site with three PAHs yielded sequences from chrysene and phenanthrene but not benzo[a]pyrene. This could possibly be as a result of the five-ring structure of the hydrocarbon, making it more difficult to utilise by the indigenous bacteria.

The Trailer park site showed the highest bacterial diversity as observed from the Shannon index (Table 4.12), even though the index was only slightly higher than the Mechanic workshop site. The species with closest affiliation to the 16S rRNA sequences belonged to the following taxonomic groups, Firmicutes, Actinobacteria, and Proteobacteria. However, among the Firmicutes were two novel strains, *Mahella australiensis* and *Caloribacterium cisternae*. The former was first isolated from an oil well in Australia (Salinas *et al*., 2004), while the latter was isolated in an underground gas storage reservoir in Russia (Slobodkina *et al*., 2012). They are both anaerobic and moderately thermophilic. In this study, the soil containing the three-ringed PAH, phenanthrene was the treatment in which they were observed, further proving their close association with petroleum hydrocarbons. The other predominant group of bacteria belonging to the Firmicutes are the *Bacillus* species, however, there were also some uncultured species in this site. One genus showed close relation to Gammaproteobacteria (*Acinetobacter* sp.), this group of bacteria was not as abundant as

they were in the Mechanic workshop site, possibly due to the different physical and chemical properties in the soil in the sites. Soil physicochemical properties have been reported to affect the bacterial community in the polluted environments (Ding *et al*., 2010; Lors *et al*., 2010). Most of the 16S rRNA sequences obtained in this site were from the soils treated with PAHs, the untreated soils had very few sequences, and it is possible that the presence of the hydrocarbons favoured the bacteria capable of utilising these compounds thus enhancing their growth. Nevertheless, the DGGE profile of the untreated Trailer park soil was not as prominent as the treated soils, thus it could be inferred that the quality of the DNA might have affected through biases during the extraction process.

The Refinery effluent site exhibited a unique trend of having sequences belonging to only one taxonomic group, Firmicutes (Table 4.15). And even at that, only one genus, *Bacillus*. This distinctive phenomenon may be related to the nature of the soil with regards to location. The site in question is located adjacent to the banks of a stream receiving effluent from a petroleum refinery. The relatively high moisture content in this site seems to be its only distinguishing feature however; other factors such as the presence of certain pollutants (not determined in this study) might play a role in the nature of the bacterial community. Another interesting fact about this site is the fact that most of the 16S ribosomal RNA sequences observed were from the samples treated with PAHs especially the four and five ringed compounds (chrysene and benzo[a]pyrene), implying that the bacteria in the soil from this site have the potential of utilising these PAHs. *Bacillus* species are one of the most predominant bacteria in the soil environment; and many have the ability to degrade high molecular weight PAHs (Marquez-Rocha *et al*., 2005; Isaac *et al*., 2013).

The highest number of bacterial species was found in the Mechanic workshop soil, with the shallow soil having more than twice the number of species in the deep soil. This was the trend in the Trailer park site as well. The nutritional and aeration conditions in the shallow soils (17 – 20 cm) are favourable to the growth of aerobic bacteria important in pollutant degradation, thus aiding the proliferation of diverse genera. However, the Refinery effluent site had slightly more species in the deep soil (about 60%) than the shallow soil (Fig. 4.8), but the least number of species (Fig. 4.9) among the three sites. The amplification of 16S gene in the Refinery effluent site showed even and consistent at both depths, which is not the case with the Mechanic workshop and Trailer park sites, where the 16S amplicons from the shallow soils were brighter than the amplicons from the deep soils. The even brightness of the amplicons in the sampling depths of the refinery effluent site suggests that the number or diversity of bacteria in the site is similar at both depths. The sampling depth was observed to play a role in the number of bacterial species only in the other two sites. The physico-chemical properties of the Refinery effluent soil are not considerably different from the other sites except for its relatively high moisture content. Perhaps this factor may have played a role in the comparatively similar number of species in the site.

Spiking the contaminated soils with polycyclic aromatic hydrocarbons might have affected their bacterial composition as can be seen in Fig. 4.10. The bacterial species identified in the Mechanic workshop site were mostly found in the soils not spiked with PAHs (45%); implying that the indigenous bacteria in this soil thrived better without the presence of PAHs. The control soil from this site (soils containing only acetone) had a considerable percentage of the identified species; the treatment with benzo[a]pyrene yielded no species, thus suggesting that the bacteria in the sample do not have sufficient enzymatic machinery to utilize this five-ringed hydrocarbon for metabolism. Or

perhaps, the bacteria were still adapting to the hydrocarbon in question, thus, existing in a condition of slow growth. The treatment containing chrysene had the highest number of bacterial species among the soils spiked with PAHs from Mechanic workshop. Generally, chrysene appeared to have the most effect on the presence of bacteria in all the sites. Chrysene is a four-ringed PAH and has been found to be utilized by pure isolates of bacteria belonging to the genera, *Bacillus*, *Mycobacterium*, *Pseudomonas* (Demaneche *et al*., 2004). Studies on its degradation have not been extensively studied as its four-ringed counterpart, pyrene, probably due to the arrangement of its rings making it less susceptible to nucleophilic attack (Demaneche *et al*., 2004; Klankeo *et al*., 2009). The soils from the Trailer park had bacteria from all the treatments including the untreated soil and control, possibly signifying that the texture and physico-chemical properties of the soil from this site is suitable for the proliferation of bacteria. Another possibility is that the native bacteria in the soil from this site are functionally diverse thus, equipping them with enhanced metabolic capabilities. However, among the three treatments containing PAHs, those with phenanthrene and benzo[a]pyrene had more bacterial species than those with chrysene (Fig. 4.9). The high clay content in the soil from this site might have played a role in the sorption of phenanthrene and benzo[a]pyrene by the indigenous bacteria (Chang *et al*., 2007). The treatments containing chrysene in the soils from Refinery effluent site had the highest number of bacterial species implying that chrysene had the most favourable impact on the growth of the indigenous bacteria. The similarity search on the NCBI website revealed that the closest identity of all the 16S sequences is *Bacillus* species. Enzymes important in PAH utilization have been studied comprehensively in *Bacillus* species (Kim *et al*., 2006), perhaps the native bacteria in the Refinery effluent soil possess such enzymes, as well as biosurfactants enabling them to degrade chrysene. Other than the relatively high oil

and grease content in the soil from this site, there were no peculiar physico-chemical properties of the soil in this site that will selectively favour the presence of bacteria belonging to the genera, *Bacillus*. The presence of large amount of oil in the soil might have affected the availability of nutrients in the Refinery effluent soil as evidenced by the lower quantities of the following nutrients, Nitrogen, phosphorus, Potassium, Calcium and Magnesium (Table 4.9) compared to the other sites. *Bacillus* spp. have been known to survive in poor-nutrient environments due to spore formation and the possession of many enzymes thus affording them the ability to utilize a wide variety of compounds (Habe and Omori, 2003; Haritash and Kaushik, 2009; Kostka *et al*., 2011).

The phylogenetic affiliation of the bacteria in the petroleum-contaminated sites were analysed in Fig. 4.10, and the most prominent group was Firmicutes, with a good majority of them belonging to *Bacillus* species. Although several bacteria were either unidentified or uncultured based on the BLAST analysis, most of them in the Trailer park and Refinery effluent sites were found to be closely related to *Bacillus* species (Figures 4.6 and 4.7). This point to the fact that some of the bacteria present in the contaminated sites used in this study have not been cultured before. Thus emphasizing the important role of molecular and omic methods in the identification and ecology of bacteria in polluted soils. Bacteria belonging to the group, Proteobacteria were all 100% Gammaproteobacteria. Various genera of this catergory of bacteria were found to be present in the Mechanic workshop and Trailer park, none were found in the Refinery effluent soil. All the other types of Proteobacteria have been found to be involved in PAH degradation in many studies (Vinas *et al*., 2005; Chang *et al*., 2007; Higashioka *et al*., 2009; El Azhari *et al*., 2010; Chikere *et al*., 2012; Isaac *et al*., 2013); genera belonging to Betaproteobacteria and Alphaproteobacteria were identified in the first phase of sampling carried out in this study (Fig. 4.1). Functional genes important in

PAH degradation have been studied in most groups of Proteobacteria (Witzig *et al.*, 2006; Cebron *et al.*, 2008). The most studied enzyme for the degradation of PAHs, naphthalene dioxygenase was first identified in *Pseudomonas* (Habe and Omori, 2003). Among the three sites, the Trailer park has the highest occurrence of uncultured/unidentified bacteria; although this is not determined by the soil physico-chemical properties. The predominant group of bacteria were Gram positive even though most of them belong to the genus, *Bacillus*; a study on the role of plasmids in the degradation of PAHs by soil bacteria also revealed the presence of a higher percentage of Gram positive bacteria as opposed to Gram negative (Igwo-Ezikpe *et al.*, 2010).

**5.8 Quantitative PCR (qPCR) analysis of 16S rRNA genes in spiked soil samples:**

As much as the identity of bacterial species in a particular soil is important in microbial ecology, the number is also vital in metagenomics. In this study, the number of the bacterial 16S rRNA in soil collected from the petroleum-contaminated sites was determined using Real-time PCR. The Standard curves generated from the q-PCR analysis of the 16S rRNA in the petroleum-contaminated soils (Appendices XIII, XV and XVII) were linear ($R^2$ = 0.985, 0.973, 0.997, respectively) on 7 orders of magnitude for all standards ($10^1$ - $10^8$ gene copies per µL). Thus, the Real-time PCR assay could be said to be efficient. The site having the most abundant 16S gene was the Mechanic workshop having 1.23E+06 - 5.20E+10 gene copies per gram of soil (Fig. 4.11), while the site with the least was the Refinery effluent soil with 7.08E+06 – 7.91E+07 gene copies per gram of soil (Fig. 4.13). Generally, the trend observed with respect to depth of sampling is more abundant 16S genes in the shallow depth for all sites, this phenomenon is associated to the availability of more nutrients and oxygen in the samples obtained closer to the surface (17 – 20 cm) as opposed to the samples collected

from deeper sites (37 – 40 cm). Soils closer to the surface have been associated with greater cell density (Kakirde *et al*., 2010). With regards to treatment of the soil samples with PAHs, it was interesting to note that the soils with the highest 16S gene copies for each site were either treated with chrysene (Mechanic workshop) or phenanthrene (Trailer park and Refinery effluent). Thus signifying that the indigenous bacteria in these petroleum-contaminated sites were utilizing these hydrocarbons for their metabolic needs. This is obvious in the fact that in each of these sites, the untreated samples had less 16S gene copies than the samples treated with PAHs (Figures 4.11, 4.12 and 4.13); Cebron *et al*. (2008) also reported a higher number of 16S ribosomal gene in the soil contaminated with PAHs than the uncontaminated soils.

The Mechanic workshop site had more 16S gene copies in the shallow samples than the deep samples; by as much as two orders of magnitude in some treatments (phenanthrene and chrysene). However, the sample with the most number of gene copies is the shallow soil treated with chrysene (5.20E+10 gene copies per gram of soil). The distinguishing physicochemical characteristic of this sample is the relatively low moisture content (5%) compared to the deep sample, and indeed the samples from the other sites (6% – 18%). This occurrence of high 16S gene copies with low moisture content seems to be a contributory factor in the utilization of chrysene by soil bacteria. Since chrysene as with other PAHs has a low solubility in water, it is logical that low moisture content will likely make chrysene more available and susceptible in the soil microenvironment for attack by degradative enzymes. Bioavailability of polycyclic aromatic hydrocarbons to microorganisms influences their diversity (Rathbone *et al*., 1998).

The Trailer park site (Fig. 4.12) interestingly had more 16S gene copies in the soils treated with benzo[a]pyrene in the deep samples than the shallow samples unlike the Mechanic workshop (Fig. 4.11) and Refinery effluent (Fig. 4.13) sites respectively. This

could be attributed to the high content of clay in the deep sample from this site (190 g/kg) compared to the shallow sample having almost half this quantity (90 g/kg). Thus, the high clay content appears to favour the growth of indigenous bacteria on benzo[a]pyrene. The relatively small particle size of the particles in clay soils appear to aid in uptake of nutrients. However, in a study by Chang *et al*. (2007), clay in a soil sample inhibited the biodegradation of naphthalene (a two-ringed PAH). The impact of soil texture in pollutant degradation by soil bacteria has been highlighted in several studies (Chang *et al*., 2007; Al-Turki, 2009; Afzal *et al*., 2011; Tang *et al*., 2013). In a study by Ding *et al.* (2010), phenanthrene sorption was found to be faster in soils with higher clay content.

All the soils treated with PAHs in the Trailer park had higher gene copies than the soils treated with acetone or having no treatment at all (Figure 4.12).

In the Refinery effluent site, the soil treated with chrysene had the least number of 16S gene copies (7.57E+06 and 7.08E+06 gene copies per gram of soil) compared to the soils treated with other PAHs and even the untreated soils. It appears that the high moisture content (Table 4.8) in the soil from this site likely inhibited the sorption of chrysene by the indigenous bacteria, thus slowing their growth. The high moisture content of the soil in this site seems to be the only physicochemical characteristic that is distinct from the other sites. The 16S gene copies in the deep soils in this site did not increase in the soils treated with PAHs as opposed the shallow soils which showed a marked increase in the gene copy numbers with respect to the acetone-treated soils. This goes to show that the high moisture content in the deep samples had a negative impact on the growth of the bacteria in the PAH-treated samples. Polycyclic aromatic hydrocarbons do not dissolve well in water (Skupinska *et al*., 2004), as such high moisture content will affect their uptake and possibly their breakdown by bacteria.

Another important feature of the Refinery effluent soil is the relatively similar gene copies in the shallow and deep soils (Fig. 4.13), while in the Mechanic workshop and Trailer park soils the trend is the shallow soils had more gene copies by an order of magnification of 2 in most instances (Figures 4.11 and 4.12). A possible reason for this occurrence is the similar Carbon to Nitrogen ratio in the shallow and deep soils of the refinery effluent soil hence, similar 16S gene copies at both depths. On the other hand, the Mechanic workshop and Trailer park soils had higher C:N ratios in the shallow soils as opposed to the deep soils. This highlights the impact of nutrients in the growth of soil bacteria.

Quantification of the 16S rRNA gene was not expressed relative to the bacterial cell because of the ability of bacteria to possess multiple copies of the gene (Cebron *et al.*, 2008).

On analysis of the means of the 16S gene copies of the samples using analysis of variance (ANOVA), the P values obtained for each of the sites was less than 0.05; thus statistically significant. The distinct difference in the number of 16S genes in the samples from both sampling depths is probably the reason the data was significantly different. This leads to the strong possibility that the depth of sampling played a role in the number and diversity in soil bacteria. Another reason could be biases arising during the process of DNA extraction which could lead to significant differences in the resulting abundance of the gene during PCR amplification (Cebron *et al.*, 2008).

# CHAPTER SIX

## 6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

### 6.1 Summary:

The soils sampled in this study were mostly of loamy texture, which signifies ideal nutritional status for plants as well as the microorganisms indigenous to those soils. In the first phase of sampling, the Refinery effluent soils had the highest amounts of heavy metals and iron while in the second phase of sampling, the Mechanic workshop soil had the highest amounts of heavy metals. Extraction of DNA from the contaminated soils was achieved using a bead-beating cell lysis method to enhance recovery of the DNA. The extraction kit used (Fast DNA Spin kit) ensured removal of humic substances found in high quantities in petroleum-contaminated soils. PCR amplification of the 16S rRNA gene in the soils was done using the universal primer pair, 357F-GC and 518R; the forward primer was coupled with a forty base pair GC clamp to facilitate separation of the different sequences during denaturing gradient gel electrophoresis (DGGE). DGGE is a fingerprinting method used to separate similar sized amplicons based on the various sequences. It can also be used as a means of analysing the response of bacterial communities to varying nutrient, pollutant or environmental conditions. PCR-DGGE analysis of the contaminated soils treated with various PAHs showed that some sequences were more prominent in certain PAH treatments. The PCR-DGGE fingerprints revealed the dominant bacteria present in soils in the sites studied as belonging to *Bacillus* spp. There were also representatives of genera belonging to Gammaproteobacteria. Other phyla identified were Actinobacteria, Chloroflexi, while the rest were uncultured species. The Refinery effluent and Trailer park soils had a high proportion of bacteria in the soils spiked with PAHs while the reverse was the case for

the Mechanic workshop soil. The Refinery effluent soil had the least species diversity while the Trailer park soil was the most diverse. Quantification of 16S rRNA gene in the soils using Real-time PCR showed the shallow soils having more copies than the deep soils with the exception of a few cases. The soils treated with PAHs as well as those having acetone had higher gene copies than the untreated soils.

**6.2 Conclusion:**

The soils sampled in the first phase of this study were of loamy texture; with acidic pH while the macro- and micro- nutrients were relatively more abundantly in the shallow soils than the deep soils. The Refinery effluent soils had comparatively similar quantities of nutrients in both shallow and deep soils while the Mechanic workshop and Trailer park soils had a noticeable variation between the amount of nutrients in the shallow and deep sampling points. The Refinery effluent soil had the highest quantities of heavy metals, with some exceeding the recommended limit set by DPR (zinc, copper and lead).

The 16S rRNA gene was successfully amplified from DNA extracted from the soil samples using a universal primer pair. Denaturing gradient gel electrophoresis of the 16S amplicons revealed the various sequences in the soils. The DGGE pattern of the amplicons from the Refinery effluent soil showed similar patterns in the bands from shallow and deep sampling points.

Sequence analysis of the 16S sequences revealed their identities to be closely affiliated to the following groups, Betaproteobacteria, Gammaproteobacteria, Alphaproteobacteria, Firmicutes, Actinobacteria and Acidobacteria. An unrooted neighbour joining tree showed that the sampling depth only affected the clustering of the OTUs in the soils from the Mechanic workshop site.

The bacterial 16S gene (2.49E+05 – 9.28E+08 copies per gram of soil) was more abundant than the archaeal 16S gene (1.91E+04 – 5.76E+07 copies per gram of soil) in the soils from the contaminated sites. The Trailer park soil had the highest copies of the bacterial 16S gene; while the Refinery effluent soil had the most abundant copies of the archaeal 16S gene.

The second phase of sampling carried out in this study revealed that the texture of the soil from the contaminated sites as loamy but with different proportions of silt and clay. The pH was in the range, 5.8 – 7.3. Moisture content as well as nutrients such as nitrogen, phosphorus and potassium were present in higher quantities in the shallow soils compared to the deep soils. The Mechanic workshop soil had the highest quantity of heavy metals (with the exception of chromium) compared to the soil from the other sites; with the levels of zinc and lead exceeding the recommended limit set by the Department of Petroleum Resources (2002).

PCR-DGGE analysis of the soils spiked with PAHs showed amplification of the 16S rRNA gene in all the soils. The DGGE fingerprinting revealed more prominent bands in the soils spiked with PAHs while the untreated soils had less intense bands. Some bands were more prominent in soils spiked with one of the PAHs (phenanthrene, chrysene or Bap). The Shannon index of diversity was 2.8319, 2.8292 and 2.4424 for Trailer park, Mechanic workshop and Refinery effluent sites respectively.

Sequence analysis of the 16S gene from the conmtaminated sites showed a strong affiliation to bacteria in the following phyla, Gammaproteobacteria, Firmicutes, Actinobacteria, Chloroflexi. Some of the sequences were closely related to uncultured bacteria. The evolutionary analysis was conducted using a maximum parsimony tree rooted with a specie of Cyanobacteria; the depth of sampling did not appear to have any

effect in the relationships of the bacteria in the Trailer park and Refinery effluent sites. However, the sequences from Mechanic workshop soils showed clustering based on the sampling depth.

Quantification of the 16S rRNA sequences of the bacteria in the sites showed the Mechanic workshop shallow soil treated with chrysene having the highest value, 5.20E+10 copies per gram of soil; while the lowest number of the gene was observed in a soil sampled from the deep point but not treated with any of the PAHs, 1.23E+06 copies per gram of soil.

**6.3 Recommendations:**

1. Bioremediation studies in Nigeria should focus on the simultaneous use of culture techniques and molecular methods in identifying bacteria important in PAH degradation.
2. Detection of functional genes should be conducted in order to determine active PAH degradation, and also to aid the likelihood of discovering novel catabolic genes.
3. Transcriptomics is a relevant tool in the confirmation of expression of catabolic genes, and as such, should be included in PAH degradation studies.
4. Studies related to the molecular identification of soil bacteria important in PAH degradation should include soils from sites having other sources of PAH, such as pyrogenic and biogenic sources.
5. Nigerian government parastatals and relevant industries in the private Oil sector should provide funds for studies aimed at eliminating toxic

compounds associated with petroleum pollution in the oil-producing regions of the country.

6. Concentrations lower than micro gram should be utilized in the determination of PAH content in soils using GC-MS, in order to increase the chances of detecting their presence in very low quantities.

# REFERENCES

Adebusoye, S.A., Ilori., M.O., Amund, O.O., Olakunle, T.D. and Olatope, S.O. (2007). Microbial degradation of petroleum hydrocarbons in a polluted tropical stream. *World Journal of Microbiology and Biotechnology*, **23**:1149-1159. Doi 10.1007/s11274-007 9345-3.

Adetutu, E.M., Smith, R.J., Weber, J., Aleer, S., Mitchell, J.G., Ball, A.S. and Juhasz, A.L. (2013). A polyphasic approach for assessing the suitability of bioremediation for the treatment of hydrocarbon-impacted soil. *Science of the Total Environment*, **450-451**: 51-58.

Adoki, A.A. and Odokuma, L.O. (2007). Bioluminescent hydrocarbonclastic bacteria of the Niger Delta. *African Journal of Biotechnology*, **6**(4):393-399.

Afegbua, S.L. (2015). Importance of plants and microorganisms in the phytoremediation of brownfield sites. Ph.D. Thesis, University of Birmingham, United Kingdom.

Afzal, M., Yousaf, S., Reichenauer, T.G., Kuffner, M., and Sessitsch, A. (2011). Soil type affects plant colonization, activity and catabolic gene expression of inoculated bacterial strains during phytoremediation of diesel. *Journal of Hazardous Materials*, **186**:1568-1575.

Aitken, M.D., Stringfellow, W.T., Nagel, R.D., Kazunga, C. and Chen, S.H. (1998). Characteristics of phenanthrene-degrading bacteria isolated from soils contaminatedwith polycylic aromatic hydrocarbons. *Canadian Journal of Microbiology*, **44**:743-752.

Akpe, A.R., Ekundayo, A.O., Aigere, S.P. and Okwu, G. I. (2015). Bacterial degradation of petroleum hydrocarbons in crude oil polluted soil amended with cassava peels. *American Journal of Research Communication*, **3**(7):99-118.

Alonso-Gutiérrez, J., Figueras, A., Albaigés, J., Jiménez, N., Viñas, M., Solanas, A.M., and Novoa, B. (2009). Bacterial communities from shoreline environments (Costa da Morte, Northwestern Spain) affected by the Prestige oil spill *Applied and Environmental Microbiology*, **75**(11):3407-3418.

Al-Turki, A.I. (2009). Microbial polycyclic aromatic hydrocarbons degradation in soil. *Research Journal of Environmental Toxicology*, **3**(1):1-8. ISSN 1819-3420.

Andreoni, V., Cavalca, L., Rao, M.A., Nocerino, G., Bernasconi, S., Dell-Amico, E., Colombo, M., and Gianfreda, L. (2004). Bacterial communities and enzyme activities of PAHs polluted soils. *Chemosphere*, **57**:401-412.

Ayotamuno, M.J., Kogbara, R.B., Agunwamba, J.C. (2006). Bioremediation of a petroleum hydrocarbon polluted agricultural soil at various levels of soil tillage in Port-Harcourt, Nigeria. *Nigerian Journal of Technology*, **25**(1):44-51.

Baldwin, B.R., Nakatsu, C.H. and Nies, L. (2003). Detection and enumeration of aromatic oxygenase genes by multiplex and real-time PCR *Applied and EnvironmentalMicrobiology*, **69**(6): 3350-3358.

Barns, S.M., Cain, E.C., Sommerville, L. and Kuske, C.R. (2007). Acidobacteria phylum sequences in uranium-contaminated subsurface sediments greatly expand the known diversity within the phylum. *Applied and Environmental Microbiology*, **73**(9):3113-6.

Bengtsson, G., Törneman, N., De Lipthay, J.R. and Sørensen, S.J. (2013). Microbial diversity and PAH catabolic genes tracking spatial heterogeneity of PAH concentrations. *Microbial Ecology*, **65**:91-100. doi 10.1007/s00248-012 0112-0.

Boon, N., Windt, W.D., Verstraete, W. and Top, E.M. (2002). Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. *FEMS Microbiology Ecology*, **39**:101-112.

Boonchan, S. (1998). Biodegradation of polycyclic aromatic hydrocarbons: application of fungal-bacterial co-cultures and surfactants. Ph.D. Thesis, Victoria University of Technology, Melbourne, Australia.

Bordenave, S., Goñi-urriza, M., Vilette, C., Blanchard, S., Caumette, P. and Duran, R. (2008). Diversity of ring-hydroxylating dioxygenases in pristine and oil

contaminated microbial mats at genomic and transcriptomic levels. *Environmental Microbiology*, **10**(12):3201-3211. doi:10.1111/j.1462 2920.2008.01707.

Brennerova, M.V., Josefiova, J., Brenner, V., Pieper, D.H. and Junca, H. (2009). Metagenomics reveals diversity and abundance of *meta*-cleavage pathways in microbial communities from soil highly contaminated with jet fuel under air sparging bioremediation. *Environmental Microbiology*, **11**(9):2216-2227. doi:10.1111/j.1462 2920.2009.01943.

Brezna, B., Khan, A. and Cerniglia, C. (2003). Molecular characterization of dioxygenases from polycyclic aromatic hydrocarbon-degrading *Mycobacterium* spp. *FEMS Microbiology Letters*, **223**(2):177-183. doi:10.1016/S0378 1097(03)00328-8.

Canadian Council of Ministers of the Environment (1999). Canadian Sediment Quality Guidelines for the Protection of Aquatic life: Polycyclic aromatic hydrocarbons (PAHs). In: Canadian environmental quality guidelines, 1999, Canadian Council of Ministers of the Environment, Winnipeg.

Castorena-Torres, F., Bermadez de Leon, M., Cisneros, B., Zapata-Perez, O., Salinas, J.E. and Albores, A. (2008). Changes in gene expression induced by polycyclic aromatic hydrocarbons in the human cell lines HepG2 and A549. *Toxicology In vitro*, **22**:411- 421.

Cebron, A., Norini, M.P., Beguiristain, T. and Leyval, C. (2008). Real-Time PCR quantification of PAH-ring hydroxylating dioxygenase (PAH-$RHD_{\alpha}$) genes from Gram positive and Gram negative bacteria in soil and sediment samples. *Journal of Microbiological Methods*, **73**:148-159.

Cebron, A., Beguiristain, T., Faure, P., Norini, M.P., Masfaraud, J.F. and Leyval, C. (2009). Influence of vegetation on the in situ bacterial community and polycyclic aromatic hydrocarbon (PAH) degraders in aged pah-contaminated or thermal-desorption treated soil. *Applied and Environmental Microbiology*, **75**(19):6322-6330. doi:10.1128/AEM.02862-08.

Cébron, A., Faure, P., Lorgeoux, C., Ouvrard, S and Leyval, C. (2013). Experimental increase in availability of a PAH complex organic contamination from an aged contaminated soil: Consequences on biodegradation *Environmental Pollution*, **177**: 98-105.

Cerniglia, C.E. (1993). Biodegradation of polycyclic aromatic hydrocarbons. *Current Opinions in Biotechnology*, **4**:331–338.

Cerniglia, C.E. (2003). Recent advances in the biodegradation of polycyclic aromatic hydrocarbons by *Mycobacterium* species. *In*: The Utilization of Bioremediation to Reduce Soil Contamination: Problems and Solutions Edited by Sasek *et al*., pp 51-73.

Chang, Y.T., Lee, J.F. and Chao, H.P. (2007). Variability of communities and physiological characteristics between free-living bacteria and attached bacteria during the PAH biodegradation in a soil/water system *European Journal of Soil Biology*, **43**:283-296.

Chauhan, A., Fazlurrahman, Oakeshott, J.G. and Jain, R.K. (2008). Bacterial metabolism of polycyclic aromatic hydrocarbons: strategies for bioremediation. *Indian Journal of Microbiology*, **48**:95‑113.

Chen, S., Yin, H., Ye, J., Peng, H., Zhang, N. and He, B. (2013). Effect of copper(II) on biodegradation of benzo[a]pyrene by *Stenotrophomonas maltophilia*. *Chemosphere*, **90**:1811-1820.

Chikere, C.B., Chikere, B.O. and Okpokwasili, G.C. (2012). Bioreactor-based bioremediation of hydrocarbon-polluted Niger Delta marine sediment, Nigeria. *3 Biotechnology* **2**:53-66. doi 10.1007/s13205-011-0030-8.

Chikere, C.B. (2013). Application of Molecular Microbiology Techniques in Bioremediation of Hydrocarbons and Other Pollutants. *British Biotechnology Journal*, **3**(1):90-115.

Chikere, C.B. and Ekwuabu, C.B. (2014). Culture-dependent characterization of hydrocarbon utilizing bacteria in selected crude oil-impacted sites in Bodo,

Ogoniland, Nigeria. *African Journal of Environmental Science and Technology*, **8**(6):401-406. doi:10.5897/ajest2014.1707.

Cho, O., Choi, K.Y., Zylstra, G.J., Kim, Y.S., Kim, S.K., Leed, J.H., Sohn, H.Y., Kwon, G.S., Kim, Y.M. and Kim, E. (2005). Catabolic role of a three-component salicylate oxygenase from *Sphingomonas yanoikuyae* B1 in polycyclic aromatic hydrocarbon degradation. *Biochemical and Biophysical Research Communications*, **327**:656‑662.

Christensen, E.R. and Bzdusek P.A. (2005). PAHs in sediments of the Black River and the Ashtabula River, Ohio: source apportionment by factor analysis. *Water Resources*, **39**:511-524.

Cruz, A.T., Cazacu, A.C. and Allen, C.H. (2007). *Pantoea agglomerans*, a plant pathogen causing human disease. *Journal Of Clinical Microbiology*, **45**(6):1989 1992. doi:10.1128/JCM.00632-07.

Cui, Z., Lai, Q., Dong, C. and Shao, Z. (2008). Biodiversity of polycyclic aromatic hydrocarbon-degrading bacteria from deep sea sediments of the middle atlantic ridge *Environmental Microbiology*, **10**(8):2138-2149.

Daane, L.L., Harjono, I., Zylstra, G.J., and Haggblom, M.M. (2001). Isolation and characterization of polycyclic aromatic hydrocarbon-degrading bacteria associated with the rhizosphere of salt marsh plants. *Applied and Environmental Microbiology*, **67**:2683-2691.

Del Panno, M.T., Morelli, I.S., Engelen, B. and Berthe-Corti, L. (2005). Effect of petrochemical sludge concentrations on microbial communities during soil bioremediation. *FEMS Microbiology Ecology*, **53**:305-316.

Demaneche, S., Meyer, C., Micoud, J., Louwagie, M., Willison, J.C., and Jouanneau, Y. (2004). Identification and functional analysis of two aromatic-ring-hydroxylating dioxygenases from a *Sphingomonas* strain that degrades various polycyclic aromatic hydrocarbons. *Applied and Environmental Microbiology*, **70**(11):6714-6725.

Dendooven, L., Alvarez-Bernal, D. and Contreras-Ramos, S.M. (2011). Earthworms, a means to accelerate removal of hydrocarbons (PAHs) from soil? A mini-review. 9th International Symposium on Earthworm Ecology. *Pedobiologia*, **54**:187-192. doi:10.1016/j.pedobi.2011.08.006.

Department for Petroleum Resources (2002). Environmental Guidelines and Standards for the Petroleum Industry in Nigeria (DPR-EGASPIN) Department for Petroleum Resources (DPR), Lagos, Nigeria.

Desai, C., Pathak, H. and Madamwar, D. (2010). Advances in molecular and ‘‘-omics” technologies to gauge microbial communities and bioremediation at xenobiotic/anthropogen contaminated sites. *Bioresource Technology*, **101**:1558-1569. doi:10.1016/j.biortech.2009.10.080.

Ding, G.C., Heuer, H., Zuhlke, S., Spiteller, M., Pronk, G.J., Heister, K., Kogel Knabner, I. and Smalla, K. (2010). Soil type-dependent responses to phenanthrene as revealed by determining the diversity and abundance of polycyclic aromatic hydrocarbon ring hydroxylating dioxygenase genes by using a novel PCR detection system. *Applied and Environmental Microbiology*, **76**(14):4765-4771.

Dionisi, H.M., Chewning, C.S., Morgan, K.H., Menn, F.M., Easter, J.P. and Sayler, G.S. (2004). Abundance of dioxygenase genes similar to *Ralstonia* sp. Strain U2 nagAc is correlated with naphthalene concentrations in coal tar contaminated freshwater sediments. *Applied and Environmental Microbiology*, **70**(7):3988-3995. doi: 10.1128/AEM.70.7.3988-3995.2004.

Drudy, D., Mullane, N.R., Quinn, T., Wall, P.G. and Fanning, S. (2006). *Enterobacter sakazakii*: An emerging pathogen in powdered infant formula. *Clinical Infectious Diseases*, **42**:996-1002.

Edlund, J. and Jansson, J.K. (2006). Changes in active bacterial communities before and after dredging of highly polluted Baltic sea sediments *Applied and EnvironmentalMicrobiology*, **72**(10):6800-6807. doi:10.1128/AEM.00971-06.

El-Azhari, N., Devers-Lamrani, M., Chatagnier, G., Rouard, N. and Martin-Laurent, F. (2010). Molecular analysis of the catechol-degrading bacterial community in a

coal wasteland heavily contaminated with PAHs. *Journal of Hazardous Materials*, **177**:593-601. doi:10.1016/j.jhazmat.2009.12.074.

Eziuzor, S.C. and Okpokwasili, G.C. (2013). Effect of mechanical mixing and microbial population dynamics in slurry-phase bioremediation. *JSM Microbiology*, **1**(1):1-6.

Ferrero, M., Llobet-Brossa, E., Lalucat, J., Garcı´a-Valdés, E., Rosselló-Mora, R. and Bosch, R. (2002). Coexistence of two distinct copies of naphthalene degradation genes in *Pseudomonas* strains isolated from the Western Mediterranean region. *Applied and Environmental Microbiology*, **68**(2):957-962.doi: 10.1128/AEM.68.2.957–962.2002.

Festa, S., Coppotelli, B.M. and Morelli, I.S. (2013). Bacterial diversity and functional interactions between bacterial strains from a phenanthrene-degrading consortium obtained from a chronically contaminated-soil. *International Biodeterioration & Biodegradation*, **85**:42-51.

Gerhardt, K.E., Huang, X-D., Glick, B.R. and Greenberg, B.M. (2009). Phytoremediation and rhizoremediation of organic soil contaminants: Potential and challenges. *Plant Science*, **176**:20-30.

Gomes, N.C.M., Kosheleva, I.A., Abraham, W.R. and Smalla, K. (2005). Effects of the inoculant strain Pseudomonas putida KT2442 (pNF142) and of naphthalene contamination on the soil bacterial community. *FEMS Microbiology Ecology*, **54**: 21- 33.

Gomes, N.C.M., Borges, L.R., Paranhos, R., Pinto, F.N., Kro¨gerrecklenfort, E., Mendonc¸a-Hagler, L.C.S., and Smalla, K. (2007). Diversity of *ndo* genes in mangrove sediments exposed to different sources of polycyclic aromatic hydrocarbon pollution. *Applied and Environmental Microbiology*, **73**(22):7392-7399.

González, N., Simarro , R., Molina, M.C., Bautista, L.B., Delgado, L and Villa, J.A. (2011). Effect of surfactants on PAH biodegradation by a bacterial consortium and on the dynamics of the bacterial community during the process. *Bioresource Technology*, **102**:9438-9446.

Guazzaroni, M.E., Herbst, F.A., Lores, I., Tamames, J., Pela´ez, A.I., Lo´pez-Corte´s, N., Alcaide, M., Del Pozo, M.V., Vieites, J.M., von Bergen, M., Gallego, J.L.R., Bargiela, R., Lo´pez-Lo´pez, A., Pieper, D.H., Rossello´-Mo´ra, R., Sa´nchez, J., Seifert, J. and Ferrer, M. (2013). Metaproteogenomic insights beyond bacterial response to naphthalene exposure and bio-stimulation. *The ISME Journal*, **7**:122 136.

Guo, C., Dang, C., Wong, Y. and Tamb, N.F. (2010). Biodegradation ability and dioxgenase genes of PAH-degrading Sphingomonas and Mycobacterium strains isolated from mangrove sediments. *International Biodeterioration & Biodegradation*, **64**:419-426.

Gutierrez, T., Biddle, J.F., Teske, A. and Aitken, M.D. (2015). Cultivation-dependent and cultivation-independent characterization of hydrocarbon-degrading bacteria in Guaymas Basin sediments. *Frontiers in Microbiology*, **6**(695):1-12. doi: 10.3389/fmicb.2015.00695.

Habe, H. and Omori, T. (2003). Genetics of polycyclic aromatic hydrocarbon metabolism in diverse aerobic bacteria. (2003). *Bioscience, Biotechnology, and Biochemistry*, **67**(2):225-243. doi: 10.1271/bbb.67.225.

Hamann, C., Hegemann, J. and Hildebrandt, A. (1999). Detection of polycyclic aromatic hydrocarbon genes in different soil bacteria by polymerase chain reaction and DNA hybridization. *FEMS Microbiology Letters*, **173**:255-263.

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

Higashioka, Y., Kojima, H., Sato, S. and Fukui, M. (2009). Microbial community analysis at crude oil-contaminated soils targeting the 16S ribosomal RNA, xylM, C23O, and bcr genes. *Journal of Applied Microbiology*, **107**: 126-135. doi:10.1111/j.13652672.2009.04198.x.

Hong, H., Pruden, A. and Reardon, K.F. (2007). Comparison of CE-SSCP and DGGE for monitoring a complex microbial community remediating mine drainage.

*Journal of Microbiological Methods*, **69**: 52-64. doi:10.1016/j.mimet.2006.11.016.

Huijie, L., CaiYun, Y., Yun, T., GuangHui, L. and TianLing, Z. (2011). Using population dynamics analysis by DGGE to design the bacterial consortium isolated from mangrove sediments for biodegradation of PAHs. *International Biodeterioration and Biodegradation*, **65**:269-275

Ichor, T., Okerentugba, P.O. and Okpokwasili, G.C. (2015). Biodegradation of total petroleum hydrocarbon by a consortium of Cyanobacteria isolated from crude oil polluted brackish waters of Bodo creeks in Ogoniland, Rivers state. *Research Journal of Environmental Toxicology*, **10**(1):16-27.

Igwo-Ezikpe, M.N., Gbenle, O.G. and Ilori, M.O. (2006). Growth study on chrysene degraders isolated from polycyclic aromatic hydrocarbon polluted soils in Nigeria. *African Journal of Biotechnology*, **5**(10):823-828. DOI: 10.5897/AJB06.147.

Igwo-Ezikpe, M.N., Gbenle, O.G., Ilori, M.O., Okpuzor, J. and Osuntoki, A.A. (2010). High molecular weight polycyclic aromatic hydrocarbons biodegradation by bacteria isolated from contaminated soils in Nigeria. *Research Journal of EnvironmentalSciences*, **4**:127-137. doi: 10.3923/rjes.2010.127.137.

Ijah, U.J.J., Safiyanu, H. and Abioye, O.P. (2008). Comparative study of biodegradation of crude oil in soil amended with chicken droppings and NPK fertilizer. *Science World Journal*, **3**(2):63-67.

Isaac, P., Sánchez, L.A., Bourguignon, N., Cabral, M.E. and Ferrero, M.A. (2013). Indigenous PAH-degrading bacteria from oil-polluted sediments in Caleta Cordova, Patagonia Argentina *International Biodeterioration & Biodegradation*, **82**:207-214.

Iwai, S., Johnson, T.A., Chai, B., Hashsham, S.A. and Tiedje, J.M. (2011). Comparison of the specificities and efficacies of primers for aromatic dioxygenase gene analysis of environmental samples. *Applied Environmental Microbiology*, **77**(11):3551. doi: 10.1128/aem.00331-11.

Jacques, R.J.S., Santos, E.C., Bento, F.M., Peralba, M.C.R., Selbach, P.A., Sa´, E.L.S. and Camargo, F.A.O. (2005). Anthracene biodegradation by Pseudomonas sp. isolated from a petrochemical sludge landfarming site. *International Biodeterioration & Biodegradation*, **56**:143-150.

Johnsen, A.R., Lukas Y. Wick, L.Y. and Harms, H. (2005). Principles of microbial PAH degradation in soil. *Environmental Pollution*, **133**:71-84. doi:10.1016/j.envpol.2004.04.015.

Johnsen, A.R., Delipthay, J.R., Reichenberg, F., Sorenson, S.J., Andersen, O., Christensen, P., Binderup, M. and Jacobsen, C. (2006). Biodegradation, bioaccessibility, and genotoxicity of diffuse polycyclic aromatic hydrocarbon (PAH) pollution at a motorway site. *Environmental Science and Technology*, **40**(10):3293-3298.

Johnsen, A.R., Schmidt, S., Hybholt, T.K., Henriksen, S., Jacobsen, C.S. and Ole Andersen, O. (2007). Strong impact on the polycyclic aromatic hydrocarbon (PAH)-degrading community of a PAH-polluted soil but marginal effect on PAH degradation when priming with bioremediated soil dominated by Mycobacteria. *Applied and Environmental Microbiology*, **73**(5):1474-1480. doi:10.1128/AEM.02236-06.

Jones, M.D., Crandell, D.W., Singleton, D.R. and Aitken, M.D. (2011). Stable-isotope probing of the polycyclic aromatic hydrocarbon-degrading bacterial guild in a contaminated soil. *Environmental Microbiology*, **13**:2623-2632.

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

Junca, H. and Pieper, D.H. (2003). Amplified functional DNA restriction analysis to determine catechol 2,3-dioxygenase gene diversity in soil bacteria. *Journal of Microbiological Methods*, **55**:697-708. doi:10.1016/S0167-7012(03)00214-8.

Junca, H. and Pieper, D.H. (2004). Functional gene diversity analysis in BTEX contaminated soils by means of PCR-SSCP DNA fingerprinting: comparative diversity assessment against bacterial isolates and PCR-DNA clone libraries.

*Environmental Microbiology*, **6**(2):95-110. doi:10.1046/j.1462 2920.2003.00541.

Jurelevicius, D., Alvarez, V.M., Peixoto, R., Rosado, A.S. and Seldin, L. (2012). Bacterial polycyclic aromatic hydrocarbon ring-hydroxylating dioxygenases (PAH-RHD) encoding genes in different soils from King George Bay, Antarctic Peninsula. *Applied Soil Ecology*, **55**:1-9.

Kakirde, K.S., Parsley, L.C. and Liles, M.R. (2010). Size does matter: Application driven approaches for soil metagenomics. *Soil Biology and Biochemistry*, **42**:1911-1923.

Kanaly, R.A. and Bartha, R. (1999). Cometabolic mineralization of benzo[*a*]pyrene caused by hydrocarbon additions to soil. *Environmental Toxicology and Chemistry*, **18**(10): 2186-2190.

Kanaly, R.A. and Harayama, S. (2000). Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria**.** *Journal of Bacteriology,* **182**(8):2059-2067.

Kästner, M., Breuer-Jammali, M. and Mahro, B**.** (1998). Impact of inoculation protocols, salinity, and pH on the degradation of polycyclic aromatic hydrocarbons (PAHs) and survival of PAH-degrading bacteria introduced into soil. *Applied and Environmental Microbiology*, **64:**359-362.

Khan, S., Afzal, M., Iqbal, S., Mirza, M.S. and Khan, Q.M. (2013). Inoculum pretreatment affects bacterial survival, activity and catabolic gene expression during phytoremediation of diesel contaminated soil. *Chemosphere*, **91**:663 668.

Kim, S.I., Kukor, J.J., Oh, K.H., and Kahng, H.Y. (2006). Evaluating the genetic diversity of dioxygenases for initial catabolism of aromatic hydrocarbons in *Pseudomonas rhodesiae* KK1 *Enzyme and Microbial Technology*, **40**(1):71-78.

Kim, Y.M., Ahn, C.K., Woo, S.H., Jung, G.Y and Park, J.M. (2009). Synergic degradation of phenanthrene by consortia of newly isolated bacterial strains *Journal of Biotechnology*, **144:**293-298.

Kim, S.J., Song, J., Kweon, O., Holland, R.D., Kim, D.W., Kim, J., Yu, L.R. and Cerniglia, C.E. (2012). Functional robustness of a polycyclic aromatic hydrocarbon metabolic network examined in a *nidA* aromatic ring hydroxylating oxygenase mutant of *Mycobacterium vanbaalenii* PYR 1. *Applied and Environmental Microbiology*, **78**(10):3715-3723. doi:10.1128/AEM.07798-11.

Klankeo, P., Nopcharoenkul, W., and Pinyakong, O. (2009). Two novel pyrene degrading Diaphorobacter sp. and Pseudoxanthomonas sp. isolated from soil. *Journal of Bioscience and Bioengineering*, **108**(6):488-495.

Korotkevych, O., Josefiova, J., Praveckova, M., Cajthaml, T., Stavelova, M. and Brennerova, M.V. (2011). Functional adaptation of microbial communities from jet fuel contaminated soil under bioremediation treatment: Simulation of pollutant rebound. *FEMS Microbiology Ecology*, **78**:137-149.

Kostka, J.E., Prakash, O., Overholt, W.A., Green, S.J., Freyer, G., Canion, A., Delgardio, J., Norton, N., Hazen, T.C. and Huettel, M. (2011). Hydrocarbon degrading bacteria and the bacterial community response in gulf of Mexico beach sands impacted by the deepwater horizon oil spill. *Applied and Environmental Microbiology*, **77**(22):7962-7974. doi:10.1128/AEM.05402-11.

Krivobok, S., Kuony, S., Meyer, C., Louwagie, M., Willison, J.C. and Jouanneau, Y. (2003). Identification of pyrene-induced proteins in *Mycobacterium* sp. Strain 6PY1: Evidence for two-Ring Hydroxylating Dioxygenases *Journal of Bacteriology*, **185**(13):3828-3841.

Kumar, M. and Khanna, S. (2010). Diversity of 16S rRNA and dioxygenase genes detected in coal-tar-contaminated site undergoing active bioremediation. *Journal of Applied Microbiology*, **108**:1252-1262.

Kweon, O., Kim, S-J., Holland, R.D., Chen, H., Kim, D-W. and Gao, Y. (2011). Polycyclic aromatic hydrocarbon metabolic network in *Mycobacterium vanbaalenii* PYR-1. *Journal of Bacteriology*, **193**(17):4326-4337. doi:10.1128/JB.00215-11.

Larkin, M.J., Allen, C.C.R., Kulakov, L.A. and Lipscomb, D.A. (1999). Purification and Characterization of a novel naphthalene dioxygenase from *Rhodococcus* sp. strain NCIMB12038. *Journal of Bacteriology*, **181**(19):6200-6204.

Laurie, A.D. and Lloyd-Jones, G. (1999). The *phn* genes of *Burkholderia* sp. Strain RP007 constitute a divergent gene cluster for polycyclic aromatic hydrocarbon catabolism. *Journal of Bacteriology*, **181**(2):531-540.

Lee, B.K. and Vu, V.T. (2010). Sources, distribution and toxicity of Polycyclic Aromatic Hydrocarbons (PAHs) in particulate matter. In: Air Pollution, Edited by Vanda Villanyi. Pp 99-123.

Lloyd-Jones, G., Laurie, A.D., Hunter, D.W.F. and Fraser, R. (1999). Analysis of catabolic genes for naphthalene and phenanthrene degradation in contaminated New Zealand soils. *FEMS Microbiology Ecology*, **29**:69-79.

Loganathan, B.G. and Lam, P.K. (2011). Global contamination trends of persistent organiccontaminants. Retrieved from, books.google.com.ng/books. Date retrieved: December 9th, 2015

Lors, C., Ryngaert, A., Périé, F., Diels, L. and Damidot, D. (2010). Evolution of bacterial community during bioremediation of PAHs in a coal tar contaminated soil. *Chemosphere*, **81**: 1263-1271. doi:10.1016/j.chemosphere.2010.09.021.

Louvel, B., Cébron, A. and Leyval, C. (2011). Root exudates affect phenanthrene biodegradation, bacterial community and functional gene expression in sand microcosms. *International Biodeterioration & Biodegradation*, **65**:947-953.

Lu, X.Y., Li, B., Zhang, T. and Fang, H.H.P. (2012). Enhanced anoxic bioremediation of PAHs-contaminated sediment. *Bioresource Technology*, **104:**51-58.

Luo, Y.R., Tian, Y., Huang, X., Yan, C.L., Hong, H.S., Lin, G.H. and Zheng, T.L. (2009). Analysis of community structure of a microbial consortium capable of degrading benzo(a)pyrene by DGGE. *Marine Pollution Bulletin*, **58**:1159-1163. doi:10.1016/j.marpolbul.2009.03.024.

Maigari, A.U. and Maigari, M.U. (2015). Microbial metabolism of polycyclic aromatic hydrocarbons (PAHs): A Review. *International Journal of Scientific & Engineering Research*, **6**(4):1449-1459. ISSN 2229-5518.

Margesin, R., Labbe´, D., Schinner, F., Greer, C.W. and Whyte, L.G. (2003). Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine alpine soils. *Applied and Environmental Microbiology*, **69**(6):3085-3092.

Ma´rquez-Rocha, F.J., Olmos-Soto, J., Rosano-Herna´ndez, M.C. and Muriel-Garcia, M. (2005). Determination of the hydrocarbon-degrading metabolic capabilities of tropical bacterial isolates. *International Biodeterioration & Biodegradation*, **55**:17-23. doi:10.1016/j.ibiod.2004.05.007.

Massachusetts Department of Environmental Protection (2004). Method for the determination of extractable petroleum hydrocarbons. Division of Environmental Analysis, Office of Research and Standards, Bureau of Waste site Clean-up. Revision 1.1.

Moura, A., Taca˜o, M., Henriques, I., Dias, J., Ferreira, P. and Correia, A. (2009). Characterization of bacterial diversity in two aerated lagoons of a wastewater treatment plant using PCR–DGGE analysis. *Microbiological Research*, **164**:560 569. doi:10.1016/j.micres.2007.06.005.

Mrozik, A., Piotrowska-Seget, Z. and Labuzek, S. (2003). Bacterial degradation and bioremediation of polycyclic aromatic hydrocarbons. *Polish Journal of Environmental Studies*, **12**(1):15-25.

Muyzer, G., De Waal, E.C. and Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and EnvironmentalMicrobiology*, **59**:695-700.

Muyzer, G. and Smalla, K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek*, **73**:127-141.

Nakatsu, C.H., Torsvik, V. and Øvreas, L. (2000). Soil community analysis using DGGE of 16S rDNA polymerase chain reaction products. *Soil Science Society of America Journal*, **64**:1382-1388.

Nayak, A.S., Sanganal, S.K., Mudde, S.K., Oblesha, A. and Karegoudar, T.B. (2011). A catabolic pathway for the degradation of chrysene by *Pseudoxanthomonas* sp.PNK 04. *FEMS Microbiology Letters*, **320**:128-134. doi:10.1111/j.1574 6968.2011.02301.x.

Ni Chadhain, S.M.N., Norman, R.S., Pesce, K.V., Kukor, J.J. and Zylstra, G.J. (2006). Microbial dioxygenase gene population shifts during polycyclic aromatic hydrocarbon biodegradation. *Applied and Environmental Microbiology*, **72**(6):4078-4087. doi 10.1128/AEM.02969-05.

Nkpaa, K.W., Wegwu, M.O. and Essien, E.B. (2013). Assessment of polycyclic aromatic hydrocarbons (PAHs) levels in two commercially important fish species from crude oil polluted waters of Ogoniland and their carcinogenic health risks. *Journal of Environment and Earth Science*, **3**(8):128-138.

Nubel, U., Garcia-Pichel, F. and Muyzer, G. (1997). PCR primers to amplify 16S rRNA genes from Cyanobacteria. *Applied and Environmental Microbiology*, **63**(8):3327-3332.

Nwinyi, O.C., Kanu, I.A., Tunde, A. and Ajanaku, K.O. (2014). Characterization of diesel degrading bacterial species from contaminated tropical ecosystem. *Brazilian Archives of Biology and Technology*, **57**(5):789-796.

Onifade, A.K. and Abubakar, F.A. (2007). Characterization of hydrocarbon-degrading microorganisms isolated from crude oil-contaminated soil and remediation of the soil by enhanced natural attenuation. *Research Journal of Microbiology*, **2**(2):145-159.

O'Sullivan, L.A., Webster, G., Fry, J.C., Parkes, R.J. and Weightman, A.J. (2008). Modified linker-PCR primers facilitate complete sequencing of DGGE DNA fragments. *Journal of Microbiological Methods*, **75**:579-581. doi:10.1016/j.mimet.2008.08.006.

Ouyang, J. (2013). Phenanthrene pathway map. Retrieved from, http://eawag-bbd.ethz.ch/pha/pha_image_map_1.html. Date retrieved: December 9th, 2015.

Patel, N. and Ali, A. (2014). Chrysene: a carcinogen and its microbial degradation. *Journal of Innovative Biology*, **1**(3):155-162.

Peng, R.H., Xiong, A.S., Xue, Y., Fu, X.Y., Gao, F., Zhao, W., Tian, Y.S. and Yao, Q.H. (2008). Microbial biodegradation of polyaromatic hydrocarbons. *FEMS Microbiology Review*, **32**:927-955. doi:10.1111/j.1574-6976.2008.00127.

Phillips, L.A., Greer, C.W., Farrell, R.E. and Germida, J.J. (2012). Plant root exudates impact the hydrocarbon degradation potential of a weathered-hydrocarbon contaminated soil. *Applied Soil Ecology*, **52**:56-64.

Quaiser, A., Ochsenreiter, T. and Lanz, C. (2003). Acidobacteria form a coherent but highly diverse group within the bacterial domain: evidence from environmental genomics. *Molecular Microbiology*, **50**(2):563-75.

Raji, H.M., Ameh, J.B. and Ado, S.A. (2012). The use of fertilizer and hydrogen peroxide in the biodegradation of crude oil. *Continental Journal of Microbiology*, **6**(1):33-39.

Rappe, M.S. and Giovannoni, S.J. (2003). The uncultured microbial majority. *Annual Review of Microbiology*, **57**:369-394.

Rathbone, K., Fuchs, J., Anderson, K., Karthikeyan, R. and Nurhidayat, N. (1998). Effects of PAHs on microbial activity and diversity in freshly contaminated and weathered soils. Proceedings of the 1998 Conference on Hazardous Waste Research, pp. 383-402.

Rentz, J.A., Alvarez, P.J.J. and Schnoor, J.L. (2005). Benzo[a]pyrene co-metabolism in the presence of plant root extracts and exudates: Implications for phytoremediation. *Environmental Pollution*, **136**:477-484. doi:10.1016/j.envpol.2004.12.034.

Salinas, M.B., Fardeau, M.L., Thomas, P., Cayol, J.L., Patel, B.K.C. and Ollivier, B. (2005). *Mahella australiensis* gen. nov., sp. nov., a moderately thermophilic anaerobic bacterium isolated from an Australian oil well. *International Journal of Systematic and Evolutionary Microbiology*, **54**:2169-2173.

Schadt, C.W., Liebich, J., Chong, S.C., Gentry, T.J., He, Z., Pan, H. and Zhou, J. (2005). Design and use of Functional Gene Microarrays (FGAs) for the characterization of microbial communities. *In*: Methods in Microbiology, Volume 34, pp 331-368. doi:10.1016/S0580-9517(04)34011-0.

Schadt, C.W. and Zhou, J. (2006). Advances in microarray-based technologies for soil microbial community analyses. *In*: Soil Biology, Nucleic acids and Proteins in Soils. Edited by Nannipieri, P. and Smalla, K. Vol. 8 pp. 189-203.

Sei, K., Asano, K-I., Tateishi, N., Mori, K., Ike, M. and Fujita, M. (1999). Design of PCR primers and gene probes for the general detection of bacterial populations capable of degrading aromatic compounds via catechol cleavage pathways. *Journal of Bioscience and Bioengineering*, **88**(5):542-550.

Seo, J.S., Keum, Y.S. and Qing, X. L. (2009). Bacterial Degradation of Aromatic Compounds. *International Journal of Environmental Research and Public Health*, **6**:278- 309. doi:10.3390/ijerph6010278.

Sho, M., Hamel, C. and Greer, C.W. (2004). Two distinct gene clusters encode pyrene degradation in *Mycobacterium* sp. strain S65. *FEMS Microbiology Ecology*, **48**:209-220.

Sipila, T. P., Riisiö, H. and Yrjälä, K. (2006). Novel upper meta-pathway extradiol dioxygenase gene diversity in polluted soil. *FEMS Microbiology Ecology*, **58**:134 144.

Sipila, T.P., Keskinen, A.K., Akerman, M.L., Fortelius, C., Haahtela, K. and Yrjala, K. (2008). High aromatic ring-cleavage diversity in birch rhizosphere: PAH treatment specific changes of I.E.3 group extradiol dioxygenases and 16S rRNA bacterial communities in soil. *The International Society for Microbial Ecology Journal*, **2**:968-981.

Skupinska, K., Misiewicz, I., Kasprzycka-Guttman, T. (2004) Polycylic aromatic hydrocarbons: Physicochemical properties, environmental appearance and impact on living organisms. *Acta Poloniae Pharmaceutica – Drug Research*, **61**(3):233-240.

Slobodkina, G.B., Kolganova, T.V., Kostrikina, N.A., Bonch-Osmolovskaya, E.A. and Slobodkina, A. I. (2012). *Caloribacterium cisternae* gen. nov., sp. nov., an anaerobic thermophilic bacterium from an underground gas storage reservoir. *International Journal of Systematic and Evolutionary Microbiology*, **62**:1543-1547.

Sun, R., Jin, J., Sun, G., Liu, Y. and Liu, Z. (2010). Screening and degrading characteristics and community structure of a high molecular weight polycyclic aromatic hydrocarbon-degrading bacterial consortium from contaminated soil. *Journal of Environmental Sciences*, **22**(10):576-1585. doi: 10.1016/s1001 0742(09)60292-8.

Sutherland, J.B., Rafii, F., Khan, A.A.and Cerniglia. C.E. (1995). Mechanisms of polycyclic aromatic hydrocarbon degradation. *In*: L. Y. Young, and C.E. Cerniglia (ed.), Microbial transformation and degradation of toxic organic chemicals. Wiley-Liss, New York, NY. 269-306.

Takizawa, N., Kaida, N., Torigoe, S., Moritani, T., Sawada, T., Satoh, S. and Kiyohara, H. (1994). Identification and characterization of genes encoding polycyclic aromatic hydrocarbon dioxygenase and polycyclic aromatic hydrocarbon dihydrodiol dehydrogenase in *Pseudomonas putida* OUS82. *Journal of Bacteriology*, **176**(8): 2444-2449.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, **24**:1596-1599.

Tang, C., Madigan, M.T. and Lanoil, B. (2012). Bacterial and archaeal diversity in sediments of West Lake Bonney, Mcmurdo dry valleys, Antarctica. *Applied and EnvironmentalMicrobiology*, 79(3):1034–1038. doi:10.1128/AEM.02336-12.

Ukiwe, L.N., Egereonu, U.U., Njoku, P.C., Nwoko, C.I.A. and Allinor, J.I. (2013). Polycyclic Aromatic Hydrocarbons Degradation Techniques: A Review. *International Journal of Chemistry*, **5**(4): 43-55. doi:10.5539/ijc.v5n4p43.

United States Environmental Protection Agency (USEPA) Report (1996). Recent developments for *In Situ* treatment of metals contaminated soils, USEPA, Office of Solid Waste and Emergency Response.

USEPA (2000). Guidance for assessing chemical contaminant data for use in fish advisories, EPA/823/B- 00/007, United States Environmental Protection Agency.

Uyovbisere, E.O., Ogunwole, J.O., Odigie, V.O. and Abdu, N. (2013). Laboratory manual of routine soil, plant and fertilizer analyses for teaching and research. A compilation of the Department of Soil Science, Faculty of Agriculture, Institute for Agricultural Reasearch, Ahmadu Bello University, Zaria, Nigeria.

Van Hamme, J.D., Singh, A. and Ward, O.P. (2003). Recent Advances in Petroleum Microbiology. *Microbiology Molecular Biology Review*, **67**(4):503-549.

Vázquez, S., Nogales, B., Ruberto, L., Mestre, C., Christie-Oleza, J., Ferrero, M., Bosch, R. and Mac. W.P. (2013). Cormack Characterization of bacterial consortia from diesel contaminated Antarctic soils: Towards the design of tailored formulas for bioaugmentation. *International Biodeterioration & Biodegradation*, **77**: 22-30.

Vetriani, C., Jannasch, H.W., MacGregor, B.J., Stahl, D.A. and Reysenbach, A.L. (1999). Population tructure and phylogenetic characterization of marine benthic archaea in deep-sea sediments. *Applied and Environmental Microbiology,* **65**(10):4375-4384.

Vinas, M., Sabate´, J., Jose´ Espuny, M. and Solanas, A.M. (2005). Bacterial community dynamics and polycyclic aromatic hydrocarbon degradation during bioremediation of heavily creosote-contaminated soil. *Applied and Environmental Microbiology*, **71**(11):7008-7018. doi:10.1128/aem.71.11.7008 7018.2005.

Walter, U., Beyer, M., Klein, J. and Rehm, H.J. (1991). Degradation of pyrene by *Rhodococcus* sp. UW1. *Applied Microbiology and Biotechnology*, **34:**671-676.

Wang. Z., Li. J., Hesham, A.L., He, S., Zhang, Y. Wang, Z. and Yang, M. (2007). Co variations of bacterial composition and catabolic genes related to PAH degradation in a produced water treatment system consisting of successive anoxic and aerobic units. *Science of the total environment*, **373**:356-362.

Wang, Z., Ma, X., Na, G., Lin, Z., Ding, Q. and Yao, Z. (2009). Correlations between physicochemical properties of PAHs and their distribution in soil, moss and reindeer dung at Ny-Ålesund of the Arctic. *Environmental Pollution*, **157**:3132 3136. doi:10.1016/j.envpol.2009.05.014.

Wang, S., Nomura, N., Nakajima, T. and Uchiyama, H. (2012). Case study of the relationship between fungi and bacteria associated with high-molecular-weight polycyclic aromatic hydrocarbon degradation. *Journal of Bioscience and Bioengineering*, **113**(5):624-630.

Watanube, K. and Hamamura, N. (2003). Molecular and physiological approaches to understanding the ecology of pollutant degradation. *Current Opinion in Biotechnology*, **14**:289-295. doi 10.1016/S0958-1669(03)00059-4.

Whyte, L.G., Greer, C.W. and Inniss, W.E. (1996). Assessment of the biodegradation potential of psychrotrophic microorganisms. *Canadian Journal of Microbiology*, **42**:99-106.

Wikstrom, P., Wiklund, A., Andersson, A.C. and Forsman, M. (1996). DNA recovery and PCR quantification of catechol 2,3-dioxygenase genes from different soil types. *Journal of Biotechnology*, **52**: 107-120.

Wilcke, W. (2007). Global patterns of polycyclic aromatic hydrocarbons (PAHs) in *Geoderma*, **141**: 157-166. doi:10.1016/j.geoderma.2007.07.007.

Wilms, R., Sass, H., Kopke, B., Cypionka, H., and Engelen, B. (2007). Methane and sulphate profiles within the subsurface of a tidal flat are reflected by the distribution of sulphate-reducing bacteria and methanogenic archaea. *FEMS Microbial Ecology*, **59**(3):611-621.

Wilson, M.S., Bakermans, C. and Madsen, E.L. (1999). In situ, real-time catabolic gene expression: extraction and characterization of naphthalene dioxygenase mRNA transcripts from groundwater. *Applied and Environmental Microbiology*, **65**(1):80-87.

Witzig, R., Junca, H., Hecht, H.J. and Pieper, D.H. (2006). Assessment of toluene/biphenyl dioxygenase gene diversity in benzene-polluted soils: links between benzene biodegradation and genes similar to those encoding isopropylbenzene dioxygenases. *Applied and Environmental Microbiology*, **72**(5):3504-3514.

Wong, J.W.C., Lai, K.M., Wan, C.K., Ma, K.K. and Fang, M. (2001). Isolation and optimisation of polycyclic aromatic hydrocarbons (pah)-degradative bacteria from contaminated soil for pahs bioremediation. *Water, Air and Soil Pollution*, **139**: 1-13.

Wuana, R.A. and Okieimen, F.E. (2011). Heavy metals in contaminated soils: A Review of sources, chemistry, risks and best available strategies for remediation. International Scholarly Research Network Ecology Pages 1-20.

Xue, W. and Warshawsky, D. (2005). Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: a review. *Toxicology and Applied Pharmacology*, **206**:73-93.

Yagi, J.M. and Madsen, E.L. (2009). Diversity, abundance, and consistency of microbial oxygenase expression and biodegradation in a shallow contaminated aquifer. *Applied and Environmental Microbiology*, **75**(20):6478-6487.

Ye, B., Siddiqi, M.A., Maccubbin, A.E., Kumar, S. and Sikka, H.C. (1996). Degradation of polynuclear aromatic hydrocarbons by *Sphingomonas paucimobilis*. *Environmental Science and Technology*, **30**:136-142.

Yergeau, E., Arbour, M., Brousseau, R., Juck, D., Lawrence, J.R., Masson, L., Whyte, L.G and Greer, C.W (2009). Microarray and Real-time PCR analyses of the responses of high-arctic soil bacteria to hydrocarbon pollution and bioremediation treatments. *Applied Environmental Microbiology*, **75**(19):6258-6267.

Yrjälä, K., Keskinen, A.K., Åkerman, M.L., Fortelius, C and Sipilä, T.P. (2010). The rhizosphere and PAH amendment mediate impacts on functional and structural bacterial diversity in sandy peat soil. *Environmental Pollution*, **158**(5):1680-1688. doi:10.1016/j.envpol.2009.11.026.

Zhang, S.Y., Wang, Q.F. and Xie, S.G. (2011). Microbial community changes in contaminated soils in response to phenanthrene amendment. *International Journal of Environmental Science and Technology*, **8**(2):321-330.

Zhou, H.W., Luan, T.G., Zou, F., Fung, N. and Tam, Y. (2008). Different bacterial groups for biodegradation of three- and four-ring PAHs isolated from a Hong Kong mangrove sediment. *Journal of Hazardous Materials*, **152**(3):1179-1185.

# APPENDIX

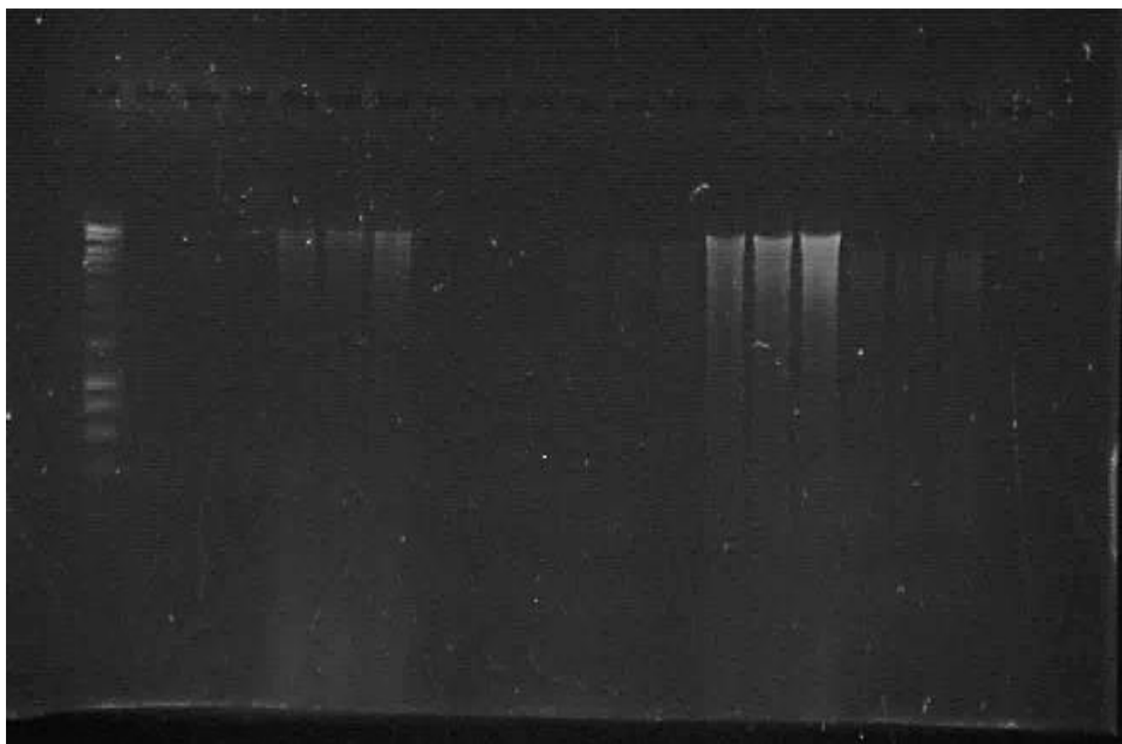
**Appendix I: Mechanic workshop site and the soils sampled. (MS and MD signify the shallow and deep soils respectively)**

**Appendix II: Trailer park site and the soils sampled. (TS and TD signify the shallow and deep soils respectively)**

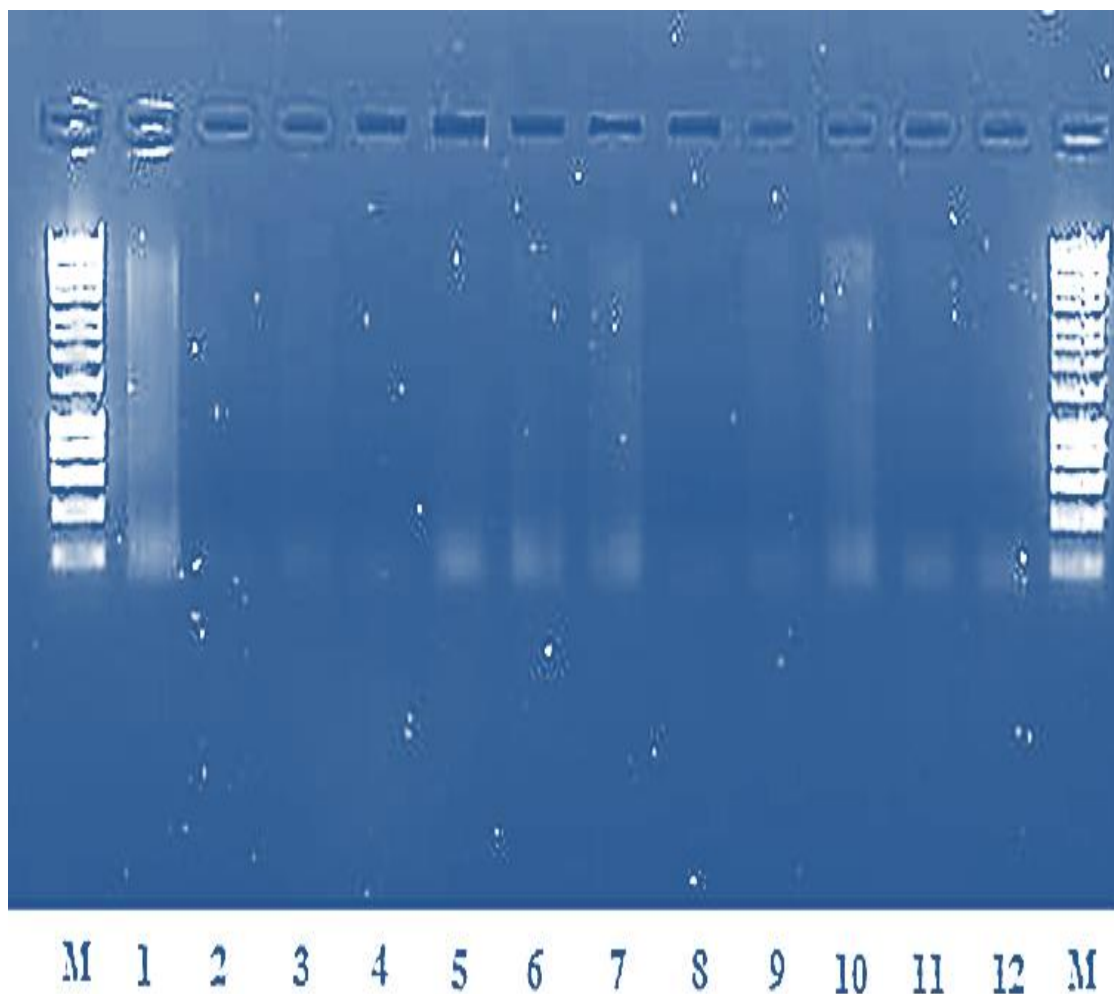
**Appendix III: Refinery effluent site and the soils sampled. (X marks one of the sampling spots at the site; RS and RD signify the shallow and deep soils respectively)**

**Appendix IV: A Sampling point from the Mechanic workshop site showing the core from which soil was obtained**

**Appendix V: Electrophoregram of genomic DNA extracted from petroleum-contaminated soils in first sampling**

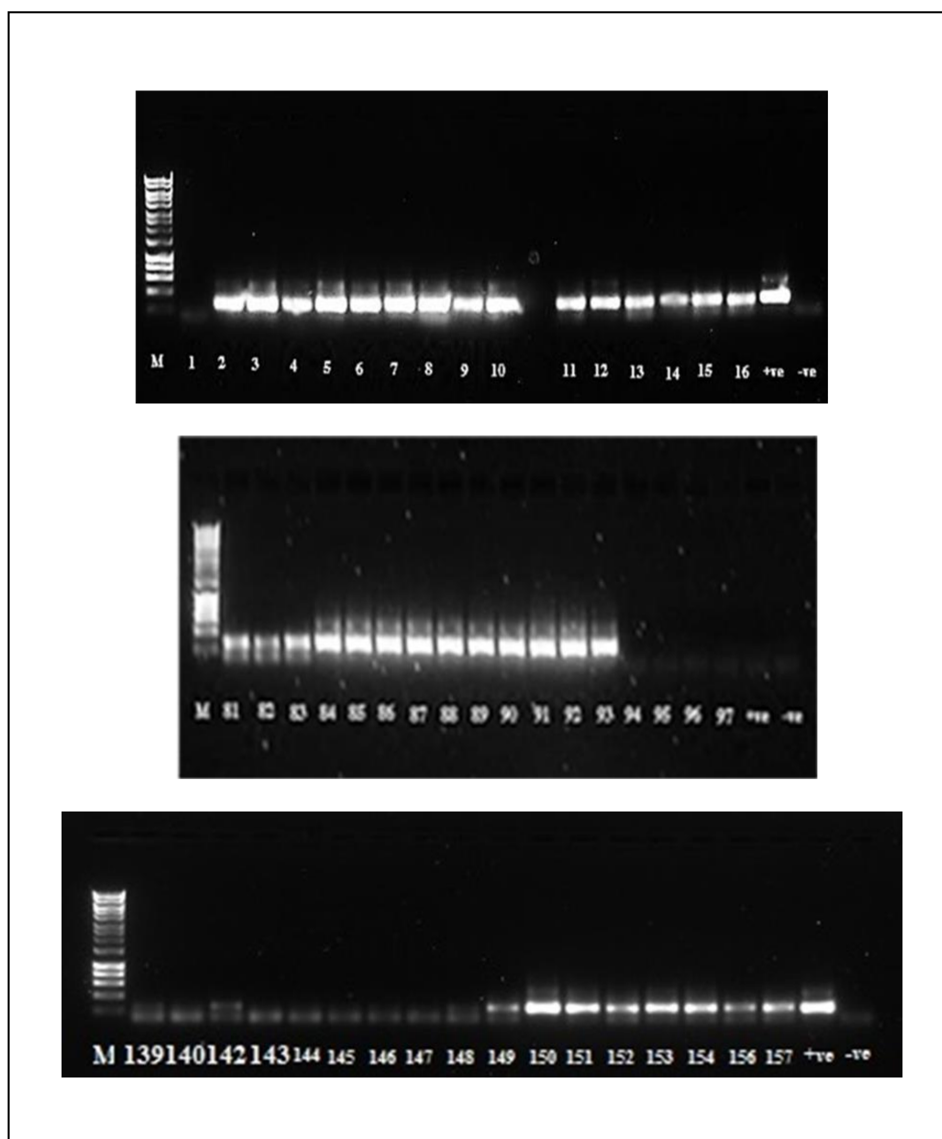


**Appendix VI: Electrophoregram of genomic DNA extracted from petroleum-contaminated soils spiked with PAHs.**

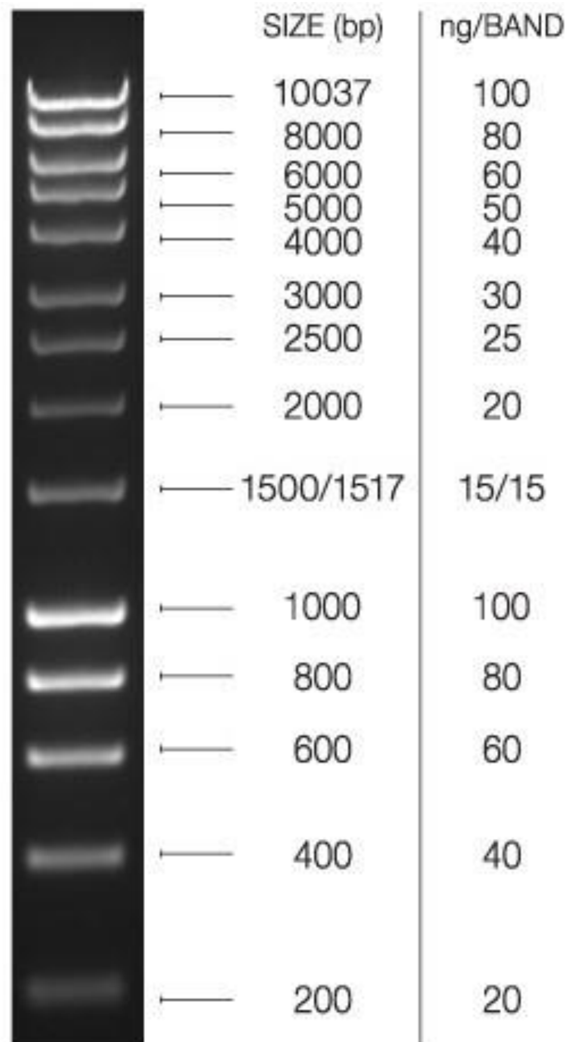



KEY:
M: Molecular marker
1: Chrysene-spiked Mechanic workshop shallow
2: Chrysene-spiked Mechanic workshop deep
3: Chrysene-spiked Trailer park shallow
4: Chrysene-spiked Trailer park deep
5: Chrysene-spiked Refinery effluent shallow
6: Chrysene-spiked Refinery effluent deep
7: B[a]P-spiked Mechanic workshop shallow
8: B[a]P-spiked Mechanic workshop deep
9: B[a]P-spiked Trailer park shallow
10: B[a]P-spiked Trailer park deep
11: B[a]P-spiked Refinery effluent shallow
12: B[a]P-spiked Refinery effluent deep

**Appendix VII: Reamplification of some of the excised DGGE bands using sequencing primers, 357F-GC and 518R-M13 (M: Marker; numbers shown on gels indicate the band designation)**

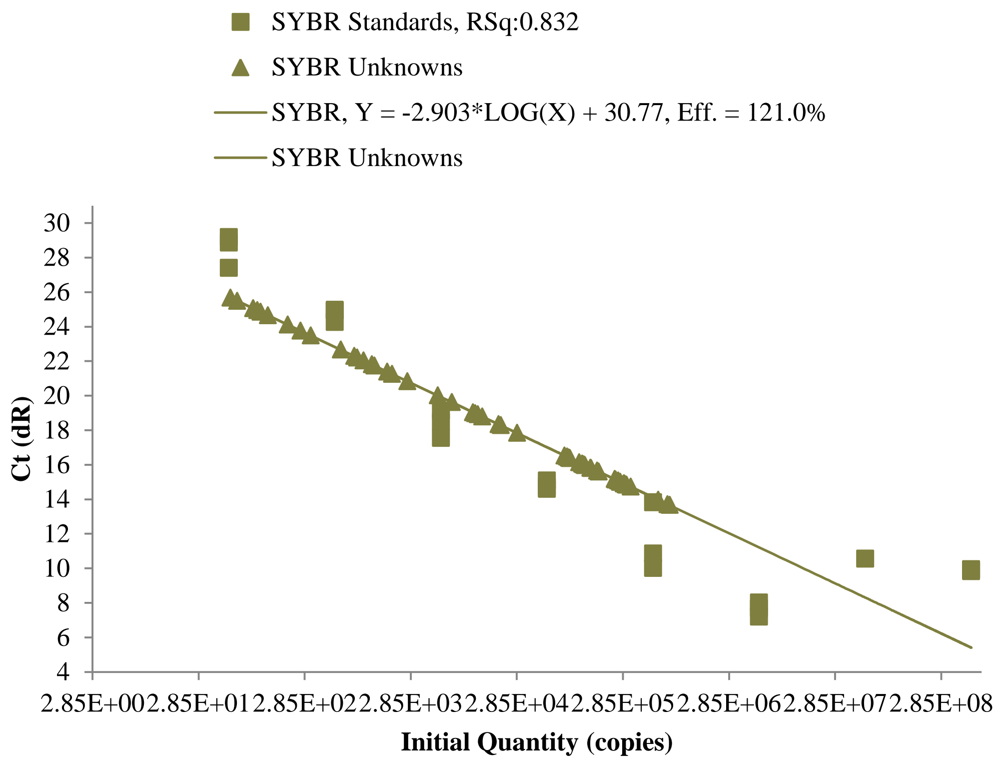


**Appendix VIII: Molecular marker used during research (Bioline, U.S.A)**



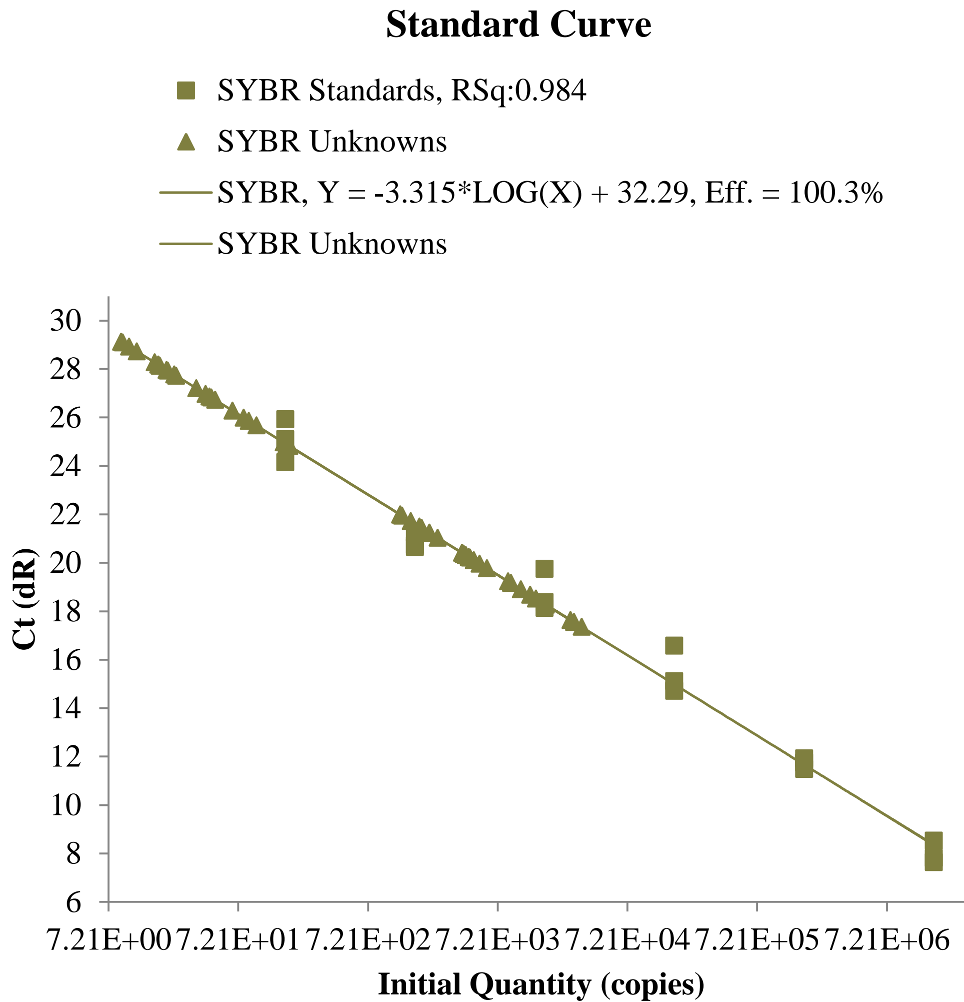
## Appendix IX: DGGE Gel in a tank containing TAE Buffer undergoing electrophoresis

**Appendix X: Standard curve of bacterial 16S gene copies in petroleum-contaminated soils from Real-Time amplification assays**




Ct: Threshold cycle; concentration of DNA in standard: 0.88ng/μL

**Appendix XI: Standard curve of archaeal 16S gene copies in petroleum-contaminated soils from Real-Time amplification assays**




Ct: Threshold cycle; concentration of DNA in standard: 2.66ng/µL

**Appendix XII: Quantification of bacterial 16S rRNA in soil collected from a Mechanic workshop site.**

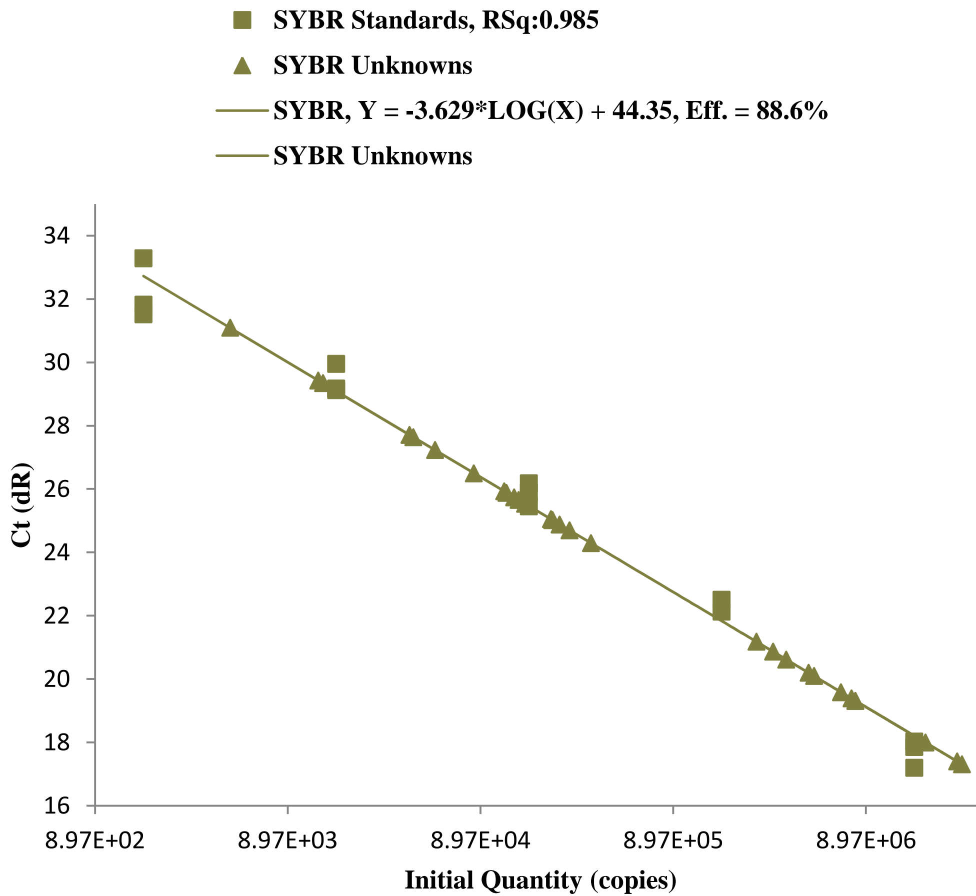
| Sample | Quantity average | Average gene copies | 1:10 dilution | in 100µL water | In 1 gram of soil (X2) |
|---|---|---|---|---|---|
| MS | 4.47E+04 | 4.04E+04 | 4.04E+05 | 4.04E+07 | 8.08E+07 |
| MD | 1.05E+04 | 6.14E+03 | 6.14E+03 | 6.14E+05 | 1.23E+06 |
| MSA | 8.48E+06 | 8.47E+06 | 8.47E+07 | 8.47E+09 | 1.69E+10 |
| MDA | 2.51E+05 | 2.46E+05 | 2.46E+06 | 2.46E+08 | 4.92E+08 |
| MSP | 5.93E+06 | 5.92E+06 | 5.92E+07 | 5.92E+09 | 1.18E+10 |
| MDP | 1.69E+05 | 1.65E+05 | 1.65E+06 | 1.65E+08 | 3.30E+08 |
| MSC | 2.61E+07 | 2.60E+07 | 2.60E+08 | 2.60E+10 | 5.20E+10 |
| MDC | 2.26E+05 | 2.22E+05 | 2.22E+06 | 2.22E+08 | 4.44E+08 |
| MSB | 3.18E+06 | 3.17E+06 | 3.17E+07 | 3.17E+09 | 6.34E+09 |
| MDB | 1.26E+05 | 1.21E+05 | 1.21E+06 | 1.21E+08 | 2.42E+08 |

**KEY:**

MS: Untreated Mechanic workshop shallow
MSa: Acetone-treated Mechanic workshop shallow
MSp: Phenanthrene-spiked Mechanic workshop shallow
MSc: Chrysene-spiked Mechanic workshop shallow

MD: Untreated Mechanic workshop deep
MDa: Acetone-treated Mechanic workshop deep
MDp: Phenanthrene-spiked Mechanic workshop deep
MDc: Chrysene-spiked Mechanic workshop deep

**Appendix XIII: Standard curve for Real-time PCR of DNA extracted from Mechanic workshop soils treated with PAHs**

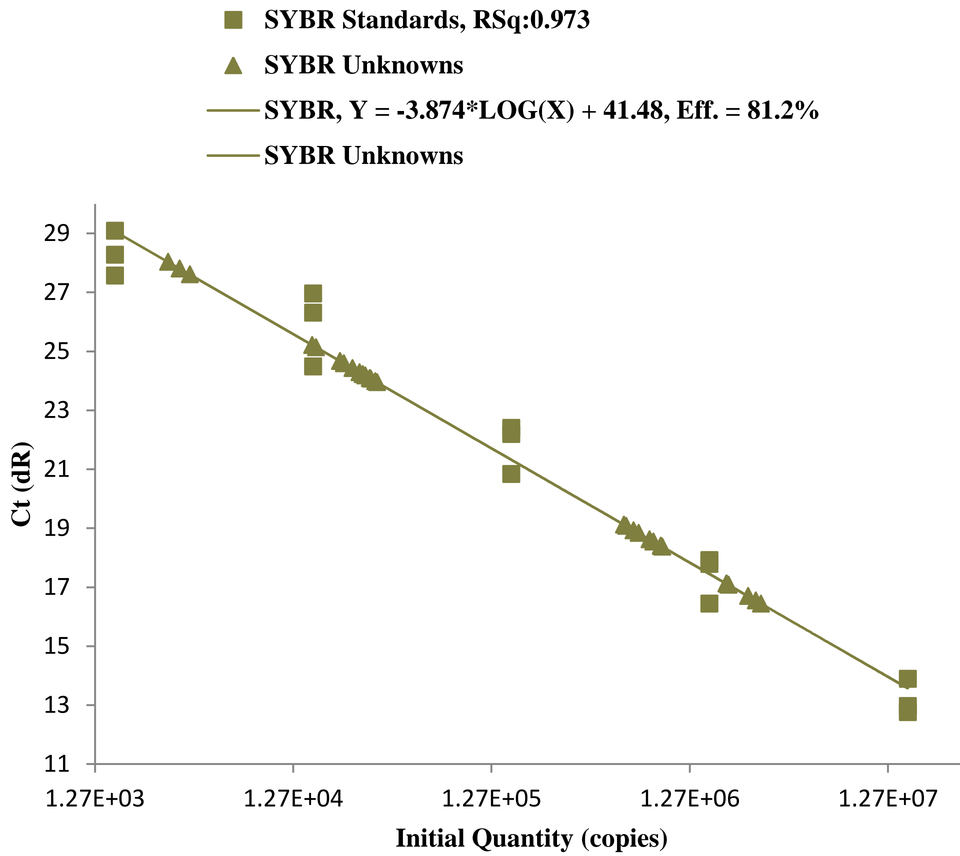


**Appendix XIV: Quantification of bacterial 16S rRNA in soil collected from a Trailer park site.**

| Sample | Quantity average | Average gene copies - NTC | 1:10 Dilution | In 100µL water | In 1 gram of soil (X2) |
|---|---|---|---|---|---|
| TS | 2.56E+04 | 2.37E+04 | 2.37E+05 | 2.37E+07 | 4.74E+07 |
| TD | 3.40E+03 | 1.51E+03 | 1.51E+04 | 1.51E+06 | 3.02E+06 |
| TSa | 7.02E+05 | 7.00E+05 | 7.00E+06 | 7.00E+08 | 1.40E+09 |
| TDa | 3.26E+04 | 3.07E+04 | 3.07E+05 | 3.07E+07 | 6.14E+07 |
| TSp | 2.14E+06 | 2.13E+06 | 2.13E+07 | 2.13E+09 | 4.27E+09 |
| TDp | 2.62E+04 | 2.44E+04 | 2.44E+05 | 2.44E+07 | 4.87E+07 |
| TSc | 8.89E+05 | 8.87E+05 | 8.87E+06 | 8.87E+08 | 1.77E+09 |
| TDc | 2.11E+04 | 1.92E+04 | 1.92E+05 | 1.92E+07 | 3.84E+07 |
| TSb | 7.25E+05 | 7.23E+05 | 7.23E+06 | 7.23E+08 | 1.45E+09 |
| TDb | 2.55E+06 | 2.55E+06 | 2.55E+07 | 2.55E+09 | 5.09E+09 |

**KEY:**
M: Molecular marker (200bp)
TS: Untreated Trailer park shallow
TD: Untreated Trailer park deep
TSa: Acetone-treated Trailer park shallow
TDa: Acetone-treated Trailer park deep
TSp: Phenanthrene-spiked Trailer park shallow
TDp: Phenanthrene-spiked Trailer park deep
TSc: Chrysene-spiked Trailer park shallow
TDc: Chrysene-spiked Trailer park deep
TSb: B[a]P-spiked Trailer park shallow
TDb: B[a]P-spiked Trailer park deep

**Appendix XV: Standard curve for Real-time PCR of DNA extracted from Trailer park soils treated with PAHs**

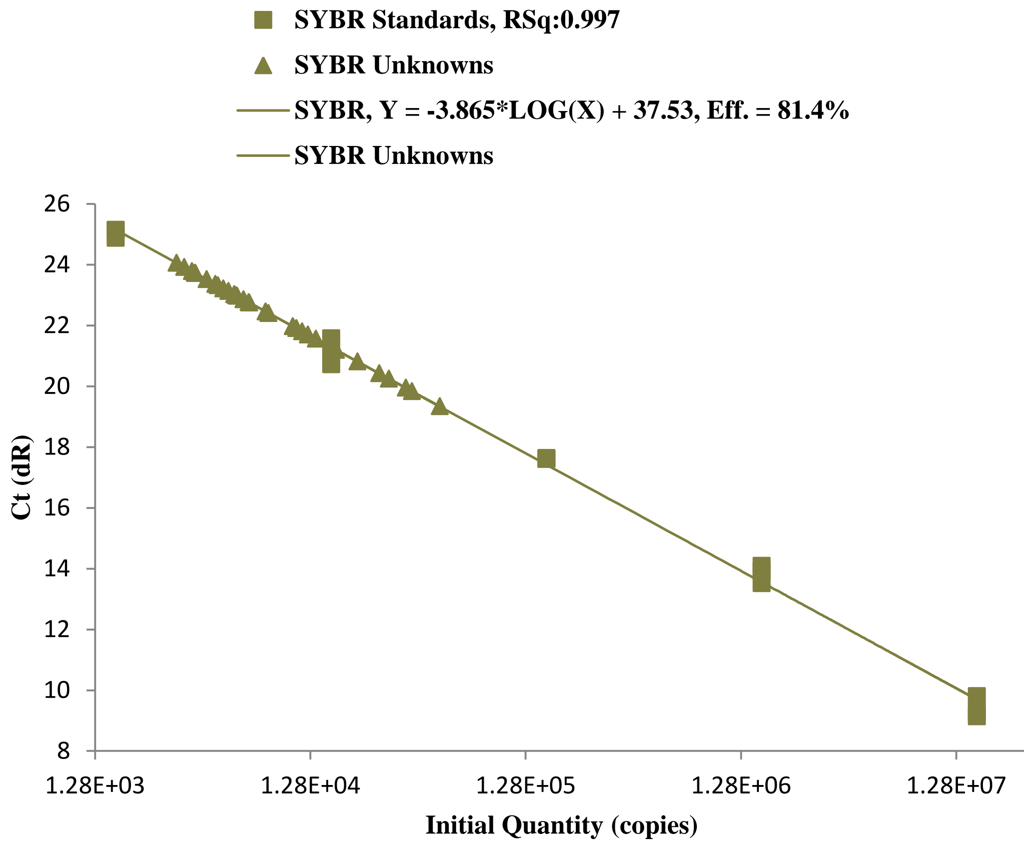


**Appendix XVI: Quantification of bacterial 16S rRNA in soil collected from a Refinery effluent site.**

| Sample | Quantity average | Average gene copies - NTC | 1:10 Dilution | In 100µL elute | In 1 gram of soil (X2) |
|---|---|---|---|---|---|
| RS | 6.72E+03 | 6.69E+03 | 6.69E+04 | 6.69E+06 | 1.34E+07 |
| RD | 4.87E+03 | 4.84E+03 | 4.84E+04 | 4.84E+06 | 9.68E+06 |
| RSa | 6.78E+03 | 6.75E+03 | 6.75E+04 | 6.75E+06 | 1.35E+07 |
| RDa | 2.78E+04 | 2.78E+04 | 2.78E+05 | 2.78E+07 | 5.56E+07 |
| RSp | 3.96E+04 | 3.96E+04 | 3.96E+05 | 3.96E+07 | 7.91E+07 |
| RDp | 1.41E+04 | 1.40E+04 | 1.40E+05 | 1.40E+07 | 2.81E+07 |
| RSc | 3.81E+03 | 3.78E+03 | 3.78E+04 | 3.78E+06 | 7.57E+06 |
| RDc | 3.57E+03 | 3.54E+03 | 3.54E+04 | 3.54E+06 | 7.08E+06 |
| RSb | 1.14E+04 | 1.14E+04 | 1.14E+05 | 1.14E+07 | 2.27E+07 |
| RDb | 5.76E+03 | 5.73E+03 | 5.73E+04 | 5.73E+06 | 1.15E+07 |

**KEY:**
RS: Untreated Refinery effluent shallow
RSa: Acetone-treated Refinery effluent shallow
RSp: Phenanthrene-spiked Refinery effluent shallow
RSc: Chrysene-spiked Refinery effluent shallow
RSb: B[a]P-spiked Refinery effluent shallow
RD: Untreated Refinery effluent deep
RDa: Acetone-treated Refinery effluent deep
RDp: Phenanthrene-spiked Refinery effluent deep
RDc: Chrysene-spiked Refinery effluent deep
RDb: B[a]P-spiked Refinery effluent deep

**Appendix XVII: Standard curve for Real-time PCR of DNA extracted from Refinery effluent soils treated with PAHs**

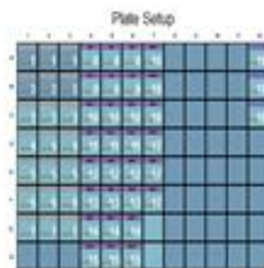


**Appendix XVIII: Consolidated report of Q-PCR run during quantification of bacterial 16S gene in Mechanic workshop soil**

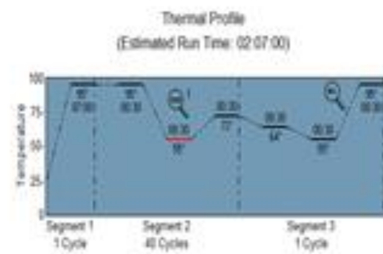
## Consolidated Report

*mechanic workshop samples Bact.mxp*

Plate Setup

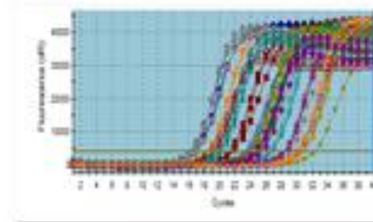


Thermal Profile
(Estimated Run Time: 02:07:00)




Analysis Set./Setup-Term Settings View

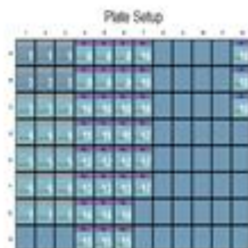
Amplification Plots

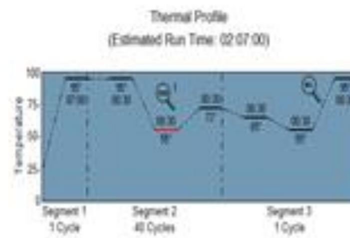


**Appendix XIX: Consolidated report of Q-PCR run during quantification of bacterial 16S gene in Trailer park soil**

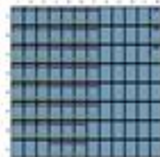
# Consolidated Report

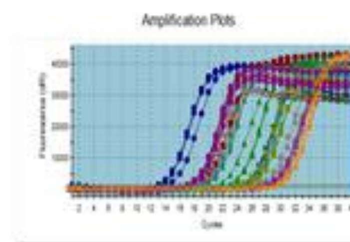
*trailer park samples Bact 11-06-2015.mxp*




Analysis Sel./Setup-Term Settings View

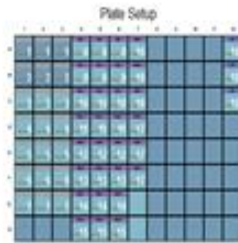


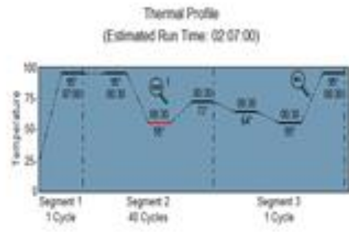


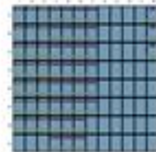
**Appendix XX: Consolidated report of Q-PCR run during quantification of bacterial 16S gene in Refinery effluent soil**

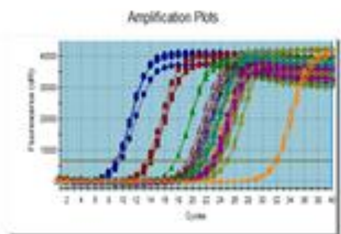
## Consolidated Report

*refinery eff samples Bact 10-06-2015.mxp*



**Appendix XXI: Publications from Conference proceedings of Nigerian Society for Microbiology (NSM), 2014**

**EM097**

**DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) ANALYSIS OF PARTIAL 16S RRNA GENES IN SOILS FROM PETROLEUM-CONTAMINATED SITES IN KADUNA STATE, NIGERIA**

Raji[1]*, H. M., Ameh[1], J. B., Ado[1], S. A., Yakubu[1], S. E. and Weightman[2], A.J
[1]Dept. of Microbiology, Ahmadu Bello University, Zaria, [2]School of Biosciences, Cardiff University, Wales, United Kingdom
habibasalam19@yahoo.com

**ABSTRACT**

Denaturing gradient gel electrophoresis (DGGE) is a technique that employs the use of two gradients of denaturant (urea and formamide) increasing in the direction of electrophoresis thus, separating PCR products into different bands on polyacrylamide gel. The resulting bands represent different gene sequences. In this study, the diversity of bacterial community in soil samples obtained from three petroleum-contaminated sites was analysed using DGGE. High-molecular-weight DNA was isolated from the soil samples, PCR amplification was carried out on the DNA samples using the primers, 357F (a GC clamp comprising 40-bp was attached to the forward primer) and 518R. The PCR amplicons obtained were separated with a parallel 30% and 60% urea-formamide denaturing gradient; after electrophoresis, the gel was viewed using a Geldocu.v illuminator.All replicates of soils from trailer park and refinery effluent sites showed similar profiles based on depth of sampling (20cm and 40cm, respectively). However, the mechanic's workshop soil revealed a different profile in one of the replicates of the deep soil sample (40cm), while replicates of the shallow (20cm) soil had identical bands.

**EM098**

**ANALYSIS OF 16SrRNA BACTERIAL GENE IN A PETROLEUM-CONTAMINATED SOIL USING QUANTITATIVE POLYMERASE CHAIN REACTION (q-PCR)**

Raji[1*], H.M., Ameh[1], J.B., Ado[1], S.A., Yakubu[1], S.E. and Weightman[2], A.J.
[1]Dept. of Microbiology, Ahmadu Bello University, Zaria, Nigeria[2]School of Biosciences, Cardiff University, Cardiff, Wales, United Kingdom
habibasalam19@yahoo.com

**ABSTRACT**

The bacterial community inpetroleum-contaminated soils adapt to the presence of petroleum hydrocarbons using them as a source of Carbon to meet their metabolic needs. Thus, in this study, a soil sample was obtained from an auto-workshop in Zaria metropolis and the q-PCR amplification of 16S bacterial gene was determined usingthe bacterial primers, 518F and907R. Tenfold dilution($10^{-1}$– $10^{-8}$) of the standard, *Anaerolineaethermpophila*DSM 14523 was prepared.Reaction mixtures (20$\mu$L) containing1 $\mu$L of soil DNA, 20pmol.$\mu$L of each primer, 10 $\mu$L 5 X SYBR Green qPCRSuperMix and BSA 0.5$\mu$L, were used for the amplification. Also included in the run is a no-template control (NTC) which is amixture of all the PCR reagents without any DNA. The resulting data after the qPCR run was analysed using MxPro, QPCR

179

**Appendix XXII: Publication from Conference proceedings of Biotechnology Society of Nigeria (BSN), 2014**

**ABBPE 10. ANALYSIS OF BACTERIAL 16S rRNA GENE IN SOIL OBTAINED FROM REFINERY EFFLUENT SITE IN KADUNA USING QUANTITATIVE POLYMERASE CHAIN REACTION (Q-PCR)**

Raji, H. M. [1*], Ameh, J. B.[1], Ado, S. A.[1], Yakubu, S. E.[1], Webster, G.[2] and Weightman, A. J.[2]
[1]Department of Microbiology, Ahmadu Bello University, Zaria.
[2]School of Biosciences, Cardiff University, Wales, United Kingdom.
[*]Corresponding author: *E-mail habibasalam19@yahoo.com*

**ABSTRACT**

The microbial community in environments exposed to effluents released from industrial processes such as petroleum refining are usually adapted to utilising and degrading these bye-products. Soil was sampled in an area adjacent to the water body containing refinery effluent released from the refinery in Nigerian National Petroleum Company (N.N.P.C), Kaduna. The samples were obtained at two depths, 17 – 20 cm and 37 – 40 cm respectively. Genomic DNA was extracted from these samples in triplicates and the 16S rRNA gene was amplified using the primers, 518F and 907R in 20 µL reaction mixtures. The data obtained after the Q-PCR run was analysed using MxPro, Q-PCR software. The final number of target genes was an average of triplicate measurements from three independent DNA extractions from each soil sample. The average 16S gene copy number in the samples was in the range, 1.55E+07 – 6.17E+07. Sampling depths of 17 – 20 cm had relatively higher gene copy number as opposed to depths of 37 - 40 cm. Soils closer to the surface are typically richer in nutrients and oxygen thus favouring bacterial growth. The 16S rRNA gene is highly conserved and very useful in taxonomic studies of bacterial populations. However, in order to screen for specific activities such as degradation of toxic compounds by bacteria in soil, detection of functional genes is necessary.

**Keywords:** Microbial community, industrial effluents, soil samples, 16S rRNA gene, PCR.

**Appendix XXIII: Details of Poster presented during the Conference of American Society for Microbiology (ASM), 2015 at New Orleans, Louisiana, United States.**