Biodegradation of petroleum hydrocarbons in soils co-contaminated with petroleum hydrocarbons and heavy metals derived from petroleum

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ABSTRACT

The biodegradation of sites co-contaminated by organic pollutants and Heavy Metals is often a challenge due to the inhibition of microbial activities. Microbes play important role in the mineralization of petroleum hydrocarbons to CO_2 by utilizing petroleum hydrocarbons as a carbon/ energy source. Heavy metals are often constituents of petroleum. Petroleum spills may result to the release associated metals (e.g. Ni, Cd, Pb, As) into the environment. Subsequent spills may cause an increase in metal concentrations in soils that may build to concentrations above intervention values. This may result to the inhibition of important biological activities such as the biodegradation of organic contaminants. This research investigates the effects of Ni, Cd and Pb contamination on biodegradation of petroleum hydrocarbons in complex soil system using a microbiological approach combined with geochemical approach. Such an approach will provide a more detailed understanding of the patterns of oil degradation under different and increasing metal stresses and how microbial communities change in such environments. Results indicated that Ni has stimulatory or no effects on biodegradation of petroleum hydrocarbons in soils depending on the chemical form of added Ni. The stimulatory effect was observed in Ni-Porphyrin contaminated soils and declined with increasing Ni concentration. In NiO soils, no effects occurred at low concentrations and increased concentration of Ni resulted to increased inhibition of biodegradation. This is unlike $NiCl_2$ amended soils where Ni effects on biodegradation were neutral irrespective of Ni concentration. The microbial diversity study of the microbial soil community indicated that there was a selective enrichment of species in the soil communities. Phylogenetic study indicated that the dominant microorganism in the community is a strain of *Rhodococcus* (100%), which was closely related to most *Rhodococcus* strains isolated from hydrocarbon-contaminated environments, metal contaminated environments and extreme environments. Results indicated that Cd inhibited biodegradation of crude oil in soils, irrespective of Cd form or concentrations. The inhibitory effect increased with increasing concentrations. Also, the microbial diversity study of the microbial soil community indicated that there was a selective enrichment of species in the soil communities. Similar to Ni, Phylogenetic study indicated that the dominant microorganism in the community is a strain of *Rhodococcus.* Also biodegradation of

petroleum was significantly reduced in crude oil degrading short-term Pb contaminated soils, irrespective of Pb form or concentration. However, in long-term Pb contaminated soils, while maximal rate of petroleum degradation reduced at high-Pb concentration, no effect was observed at low lead concentration. Also, the microbial diversity study of the microbial soil community indicated that there was a selective enrichment of species in the soil communities. Two dominating specie were identified in Pb-soils depending on soil. Both are closely related to a strain belonging to *Bacillales* that were originally isolated Rock, *Scopulibacillus darangshiensis* strain (98%) and oil contaminated soil *Bacillus circulans* (99%). While the former dominated in Pb -short-term contaminated soils as well as Pb-long term contaminated soil at high concentration, the later dominated long-term-Pb contaminated soil at low concentration.

Table of content

Title page		
Acknowledgment		i
Abstract		iii
Table of content		v
List of figures		xiv
List of tables		xxiv
Chapter 1: Literature review		
1.1	Introduction	1
1.2	Research Justifications	2
1.3.	Aim	4
1.4.	Research questions	4
1.5.	Research hypotheses	5
1.6.	Objectives	5
1.7	Thesis structure	6
1.8	Components and classification of Petroleum	6
1.9	Heavy metals as components of petroleum	7
1.10	Remediation strategies for polluted soils	9
1.11.	Microbiology of Petroleum Biodegradation	13
1.12.	Petroleum Hydrocarbon degraders	13
1.13.	The metabolic pathway of hydrocarbon degradation	16
1.14.	Metals - Microorganisms interaction	21
1.15.	Mechanism of Metal toxicity to microbes	22
1.16.	Mechanism of metal resistance	26
Chapter 2: Methods and Method Development		
2.1	Sampling Sites Description	30
2.2	Soil Sample collection and Handling Methods	31
2.3	Soil Preliminary Analysis	32
2.3.1	Soil pH	32
2.3.2	Soil moisture content	32
2.3.2	Soil water holding capacity	34
2.3.4	Soil total organic carbon	35

2.3.5 Soil heavy metal content 35
2.4 Experimental design 36
2. 4. 1 Soil Microcosm 37
2.5. Soil Microbial Activities 39
2.6 Compositional changes in petroleum hydrocarbon 40
2.6.1 Solvent- Solvent extraction 41
2.6.2. Solid Phase extraction 41
2.7 Heavy metal partitioning in petroleum soil 43
2.8. Soil Microbiology 46
2.8.1 DNA extraction 46
2.8.2. Polymerase chain reaction (PCR) 46
2.8.3. Agarose gel electrophoresis (AGE) 49
2.8.4. Denaturing gradient gel electrophoresis (DGGE) 49
2.8.5 Statistical Analysis DGGE Gels 50
2.8.6. Ion torrent -PGM sequencing of tagged 16S rRNA PCR products 51
2.8.6.1. DNA Library Construction 53
2.8.6.2. DNA Template preparation for sequencing 53
2.8.6.3. Personal Genomic Machine (PGM) sequencing 53
2.8.7. Primer validation and Optimisation of Real time-qPCR assay 55

Chapter 3: Development and optimization of qPCR assays for Hydrocarbon degrading taxa enriched in the oil degrading experiments
3.1. Introduction 58
3.2. qPCR assay Target 58
3.3. Primer Design 59
3.4. Primer pair selection and validation 60
3.5. Selected Primer sets employed in the qPCR assay optimization for bacterial 16S gene abundance determination 65
3.7. Application of 16Sr RNA gene abundance in petroleum Degrading-heavy metal contaminated aerobic soil microcosms 74
Conclusions

Chapter 4: The Effects of Nickel on the Biodegradation of Petroleum Hydrocarbon in the Soil

4.1. Introduction 78
4.2. Aim and objective 80
4.3. Methods 80
4.4. Results 85
4.4.1. Chemical properties of soil 86
4.4.2. Soil microbial activities
4.4.2.1. Lag and linear phase time comparison of oil with no-oil controls indicating the presence of HC degrading bacteria 86
4.4.2.2. The effect of Nickel concentrations and chemical forms on the Lag and Linear phase time during petroleum degradation 88
4.4.2.3. The Effect of Ni on the maximum cumulative CO2 during the degradation of petroleum Hydrocarbon 90
4.4.2.4. Effect of Ni on the maximal rate of CO2 production during the degradation of petroleum hydrocarbon 91
4.4.2.5. Influence of porphyrin ligand of Ni-porph on the stimulation of petroleum hydrocarbon degradation 94
4.4.3. The fate and chemical association of Ni amendments after addition to the soil microcosms 95
4.4.4. Geochemistry of degraded soil microcosms
4.4.4 .1. Degradation of n-Alkanes in microcosms 97
4.4.4.2. Pattern of n-alkane degradation in microcosms 99
4.4.5. Effect of Ni on Microbial community diversity 101
4.4.5.1. Denaturing gel electrophoresis indicating the microbial communities influenced by Ni-amendment and the reproducibility of communities 101
4.4.5.2. Qualitative analysis of sequencing data set used for the biodiversity study of microbial communities of Ni amended crude oil degraded soil 104
4.4.5.3. OTU-base studies on the influence of Ni on the community diversity of oil degraded soil microcosms 106
4.4.5.4. Community diversity study; Ni effects on the diversity of hydrocarbon degraders in oil degraded soil microcosms based on phylogenetic analysis 110
4.4.5.5. Comparative phylogenetic analysis of microbial communities of Ni-Crude oil amended soils relative

to oil only amended soils and unamended soils 110
4.4.5.6. Evidence of Ni-influence on selective enrichment of hydrocarbon degraders in soil 113
4.4.6. Absolute 16S rRNA gene abundances of general bacteria and *Rhodococcus* in the microbial communities of degraded soil microcosms 117
4.4.6.1. Influence of oil amendment on Bacterial abundance in oil degraded soil
4.4.6.2. Influence of Ni on the bacterial abundance in oil amended soil systems 117
4.4.6.3. The influence of oil amendment on the absolute abundance of Rhodococcus in soils 118
4.4.6.4. The influence of Ni on absolute abundance of Rhodococcus in oil degraded soil microcosms 118
4.4.7. Numerical relationships between cumulative CO2, maximal CO2 production rates, Total petroleum hydrocarbons recovered after degradation period, Bacterial 16S rRNA Gene abundances and Rhodococcus 16S rRNA Gene abundances 121
4.4.7.1. Correlation of 16S rRNA gene abundance (of bacteria and Rhodococcus) relative to maximal CO2 produced during the degradation of petroeleum hydrocarbon in soil microcosms 121
4.4.7.2. Correlation between 16S rRNA gene (bacterial and Rhodococcal) and maximal rate of CO2 production during the degradation of petroeleum hydrocarbon in soil microcosms 123
4.4.7.3. Correlation of 16S rRNA gene (bacterial and Rhodococcal) relative to Total n-alkanes recovered after the degradation of petroleum hydrocarbon in soil microcosms 123
4.5. Discussions 127
4.5.1. Microbial metabolic activities in oil amended soil during a short-term degradation period 127
4.5.2. The influence of Ni on the microbial metabolic activities during oil degradation in soils 127
4.5.3. Bioavailability of Ni and its effect on the biodegradation of petroleum hydrocarbons 131

4.5.4. Mechanism of Ni Uptake 132
4.5.5. Total petroleum hydrocarbon and degradation pattern in degraded Ni-Petroleum hydrocarbon contaminated soil 133
4.5.6. Community diversity and phylogeny 134
Conclusions

Chapter 5: The effect of Cadmium on the biodegradation of petroleum hydrocarbon in the soil environment
5.1. Introduction 140
5.2. Aims and objective 141
5.3. Methods 142
5.4. Results 143
5.4.1. Background concentrations of Cd in Nafferton soil 143
5.4.2. Effect of Cd on soil oil biodegradation microbial activities 144
5.4.2.1. The effect of Cd concentrations and chemical forms on CO2 production profiles during petroleum degradation 152
5.4.2.2. Effect of Cd on the maximum cumulative CO2 during degradation 147
5.4.2.3. Effect of Cd on the maximal rate of CO2 production during the degradation of petroleum Hydrocarbon 145
5.4.3. The fate and chemical association of Cd amendments after addition to the soil microcosms 147
5.4.4. Degradation of n-alkanes in soil microcosms; total n-alkanes recovered from Cd-crude oil degraded soils 153
5.4.5. Microbiological diversity and phylogeny 156
5.4.5.1. the effect of Cd concentration on microcosm bacterial communities identified by end point PCR of 16S rRNA genes. 156
5.4.5.2. Denaturing gel electrophoresis indicating the microbial communities enrichment influenced by Cd-amendment and the reproducibility of communities 158
5.4.5.3. Qualitative analysis of sequencing data set used for the biodiversity study of microbial communities of Cd amended crude oil degraded soil 161
5.4.5.4. OTU-base studies on the influence of Cd on the community diversity of oil degraded soil microcosms 163

5.4.5.5. Community diversity study; Cd effects on the diversity of hydrocarbon degraders in oil degraded soil microcosms based on phylogenetic analysis 165
5.4.5.6. Comparative phylogenetic analysis of microbial communities of Cd-Crude oil amended soils relative to oil only amended soils and unamended soils 166
5.4.5.7. Evidence of Cd-influence on selective enrichment of hydrocarbon degraders in soil 168
5.4.6. Absolute 16S rRNA gene abundances of general bacteria and Rhodococcus in the microbial communities of Cd-crude oil amended degraded soil microcosms 171
5.4.6.1. Influence of crude oil amendment of soil on the abundance of bacteria and Rhodococcus 172
5.4.6.2 Influence of Cd amendment on the abundance of bacteria and Rhodococcus in crude oil degrading soil 176
5.4.7. Numerical relationships between cumulative CO2, maximal CO2 production rates, recovered petroleum hydrocarbons after degradation period, Bacterial 16S rRNA Gene abundances and Rhodococcus 16S rRNA Gene abundances 176
5.4.7.1.Correlation of 16S rRNA gene abundance of bacteria relative Rhodococcal 16S rRNA gene abundance in soil communities 176
5.4.7.2 Correlation of 16S rRNA gene abundances (Bacteria and Rhodococcus) relative to maximal cumulative CO2 during the degradation of petroleum hydrocarbons in soil microcosms 177
5.4.7.3 Correlation between 16S rRNA gene abundance (of bacterial and Rhodococcus) and maximal rate of CO2 production during the degradation of petroleum hydrocarbon in soil microcosms 178
5.4.7.4. Correlation of 16S rRNA gene (bacterial and Rhodococcal) relative to Total petroleum hydrocarbon recovered after the degradation of petroleum hydrocarbon in soil microcosms 179
5.5. Discussions 183
5.5.1. Inhibitory effect of Cd on microbial activities and

biodegradation of petroleum in the soils 183
5.5.2. Bioavailability of Cd and uptake by microbes 185
5.5.3. Microbial community diversity and phylogeny 187
5.5.4 Cd resistant mechanism in Microcosms 189
Conclusions

Chapter 6: Effects of Lead on the bioremediation of petroleum hydrocarbon
6.1 Introduction 193
6.2. Aims and objective 194
6.3. Methods 194
6.4. Results 196
6.4.1. Physicochemical properties of soils 197
6.4.2. Effect of Pb on soil Microbial activities 197
6.4.2.1. Effect of short-term Pb contamination on oil degradation in the Nafferton farm soils 196
6.4.2.2. Effect of long-term Pb contamination on petroleum degradation in soils 204
6.4.3. The fate and chemical association of Pb on addition to the farm soil microcosms 210
6.4.4 Degradation of n-alkane soil microcosms 211
6.4.4.1. Comparative analysis of total n-alkanes recovered from Pb-crude oil degraded soils relative to oil only amended soils and undegraded crude oil 209
6.4.5. Microbial diversity and Phylogeny 215
6.4.5.1. Denaturing gel electrophoresis indicating the microbial communities enrichment influenced by Pb contamination and the reproducibility of communities 215
6.4.5.2. Qualitative analysis of sequencing data set used for the biodiversity study of microbial communities of Pb-contaminated crude oil degraded soil 218
6.4.5.3. OTU-base studies on the influence of Cd on the community diversity of oil degraded soil microcosms 219
6.4.5.4. Community diversity study; Pb effects on the diversity of hydrocarbon degraders in oil degraded soil microcosms based on phylogenetic analysis 223

6.4.5.5. Comparative phylogenetic analysis of microbial communities of Pb- contaminated soils amended with crude oil relative to oil only amended soils and unamended soils 223
6.4.5.6. Evidence of Pb-influence on selective enrichment of hydrocarbon degraders in soil 225
6.4.6. Absolute 16S rRNA gene abundances of general bacteria and Bacillales in the microbial communities of Pb contaminated crude oil degraded soil microcosms 230
6.4.6.1. The influence of short-term Pb-contamination on general soil bacterial abundance in communities 230
6.4.6.2 The influence of short-term Pb-contamination on Bacillales abundance in soil communities 231
6.4.6.3. The influence of long-term Pb-contamination on bacterial abundance in soil communities 231
6.4.6.4. The influence of long-term Pb-contamination on Bacillales abundance in soil communities 234
6.4.7. Numerical relationships between cumulative CO2, maximal CO2 production rates, recovered n-alkanes after degradation period, Bacterial 16S rRNA Gene abundances and Bacillales 16S rRNA Gene abundances of soils co-contaminated with Pb and crude oil 235
6.4.7.1. Correlation analysis of 16S rRNA gene abundance of bacteria relative Bacillales 16S rRNA gene abundance of soils co-contaminated with Pb and crude oil 236
6.4.7.2. Correlation of 16S rRNA gene abundances (Bacteria and Bacillales) relative to maximum cumulative CO2 during the degradation of petroleum hydrocarbons in Pb contaminated soils 236
6.4.7.3. Correlation of 16S rRNA gene abundances (Bacteria and Bacillales) relative to maximal rate of CO2 production in soil microcosms during the degradation of petroleum hydrocarbons in Pb contaminated soils 237
6.4.7.4. Correlation of 16S rRNA gene abundances (Bacteria and Bacillales) relative to n-alkanes during the degradation of petroleum hydrocarbons in Pb

contaminated soils 240
6.5. Discussion 241
6.5.1 the inhibitory effect of Pb on the microbial activities and biodegradation of petrol hydrocarbon 241
6.5.2. Bioavailability of Pb and uptake by microbes 243
6.5.3 Microbial community diversity of Pb-crude oil mixed contaminated soil 244
6.5.4. Microbial community phylogeny of Pb-crude oil mixed contaminated soils 246
6.5.5. Pb resistance mechanisms 247
Conclusions

Chapter 7: Conclusions and recommendations
7.1 Effects of heavy metals on the petroleum biodegradation in soil microcosms 251
7.2. Effects of heavy metals on soil microbial community diversity 253
7.3. Effects of heavy metals on the phylogeny of microbial community 253
7.4. Effects of heavy metals on the abundance of bacteria and dominant hydrocarbon degraders in the soil communities 254
7.5. Recommendations 255
References
Appendix

List of figures

Chapter 1

Figure 1.1: The effects of biodegradation on oil composition …… 15

Figure 1.2: A microbial degradation network……16

Figure 1.3: The Process of microbial aerobic degradation of n-alkanes……

Figure 1.4: pathways for the aerobic microbial degradation of n-alkanes initiated by terminal and sub-terminal oxidation. The arrows indicate the sequence of the biological process……19

Figure 1.5: the metabolic pathway for the aerobic degradation of cyclohexane…. 20

Figure 1.6: Protein families involved in bacterial heavy-metal metabolism…… 28

Chapter 2

Figure 2.1: location of the first soil sampling site, Nafferton ecological farm (a) a google map showing the location of Nafferton farm ……32

Figure 2.2: Google map showing location of the second soil sampling site, the former St. Anthony lead works, walker, Newcastle upon Tyne, the site is contaminated with Pb and other heavy metals……..33

Figure 2.3: diagrammatic representation of the project's experimental design. The methods used in this project include geochemical techniques and molecular microbiological techniques…….38

Figure 2.4: A diagrammatic representation of soil microcosm set up. Each heavy metals studied in this project have a triplicate of 5 concentration levels and different mineral forms…….38

Chapter 3

Figure 3.1: the determination of optimal Annealing Temperature (T_a) for the optimization of 16S rRNA gene amplification using the primer set Rho53F-Rho189R by thermal gradient qPCR…….....68

Figure 3.2: amplification of 16S rRNA gene at different using Rho53F-Rho189R primer set at different annealing Temperatures (T_a)…….69

Figure 3.3: the determination of optimal Annealing Temperature (T_a) for the optimization of 16S rRNA gene amplification using the primer set Rho1390F-Rho1454R by thermal gradient qPCR……..70

Figure 3.4: the Validation analysis of annealing temperatures (T_a) for primer set Rho1390F-Rho1454R targeting the 16S rRNA gene of *Rhodococcus*…….71

Figure 3.5: specific amplification of 16S rRNA genes of *Rhodococcus* using the primer sets Rho1390F-Rho1454R at annealing temperature of 62 ºC…….72

Figure 3.6: the determination of optimal Annealing Temperature (T_a) for the optimization of 16S rRNA gene amplification using the primer set Bac537F-Bac720R by thermal gradient qPCR. …….73

Figure 3.7: the Validation analysis of annealing temperatures (T_a) for primer set Bac537F-Bac720R targeting the 16S rRNA gene of *Bacillales*……..74

Figure 3.8: specific amplification of 16S rRNA genes of Bacillales using the primer sets Bac537F-Bac720R at annealing temperature of 65 ºC. ……75

Chapter 4

Figure 4.1: the experimental design for the study of crude oil biodegradation in Ni-crude oil amended soil microcosms at increasing Ni-concentrations ranging from 12.5 ppm to 350 ppm relative to oil only amended and no oil amended (soil only) controls in triplicates. …… 84

Figure 4.2: CO_2 production profiles in oil amended (oil only) and unamended (soil only) controls. …….86

Figure 4.3: CO_2 production profiles of Ni and petroleum hydrocarbon co-contaminated soil microcosms relative to oil amended and unamended controls…… 89

Figure 4.4: CO_2 production profiles of Ni and petroleum hydrocarbon co-contaminated soil microcosms relative to oil amended and unamended controls…… 89

Figure 4.5: CO_2 production profiles of Ni and petroleum hydrocarbon co-contaminated soil microcosms relative to oil amended and unamended controls…….90

Figure 4.6: The maximal rate of CO_2 production in Ni-oil amended soils relative to oil amended and unamended controls…… **91**

Figure 4.7: comparative analysis of maximal CO_2 production rates in Ni-porph amended oil degraded soil microcosms at Ni concentrations of 12.5, 25, 50, 200 and 350 ppm relative to oil only amended control. **……..92**

Figure 4.8: comparative analysis of maximal CO_2 production rates in $NiCl_2$ amended oil degraded soil microcosms at Ni concentrations of 12.5, 25, 50, 200 and 350 ppm relative to oil only amended control……93

Figure 4.9: comparative analysis of maximal CO2 production rates in NiO amended oil degraded soil microcosms at Ni concentrations of 12.5, 25, 50, 200 and 350 ppm relative to oil only amended control…… .93

Figure 4.10: the effect of porphyrin on the maximal rate of CO_2 production in soils during the degradation of petroleum hydrocarbon…… 95

Figure 4.11: Comparative analysis of the fate of added Ni in soil by sequential extraction of Ni fractions associated with exchangeable ions, carbonates, oxides of Fe and Mn, organics and residuals…… 97

Figure 4.12: the n-alkane profile indicating the degree of degradation of whole petroleum in soil microcosms studied…… 100

Figure 4.13: recovered Total n-alkanes identified in microcosms contaminated with Ni and/or 100 mg oil after 15 days degradation period…… 101

Figure 4.14: PCR amplified 16S rRNA gene fragment DGGE profiles indicating the diversity of bacterial communities and reproducibility of experimental triplicates of soil microcosms contaminated with Petroleum hydrocarbon and Ni of different forms (NiO and Ni-Porph) and at Ni concentrations of 12.5, 50, and 350 ppm…….. 103

Figure 4.15: Non-metric MDS analysis of the bacterial communities present in Ni-crude oil amended soil microcosms, oil only amended microcosms and unamended soils (soil only) after 15 days of incubation period…… 104

Figure 4.16: Comparative analysis of relative abundances of phylogenic group of 16S rRNA sequences recovered from representative Ni- crude oil amended soil microcosms relative to oil only amended soil and unamended soil…….113

Figure 4.17: Comparative analysis of relative abundances of genera of 16S rRNA sequences recovered from representative Ni- crude oil amended soil microcosms relative to oil only amended soil and unamended soil…… 114

Figure 4.18: Comparison of the dominant sequence recovered from Ni-crude oil amended and oil only amended soil microcosms with closely related bacteria (mostly bacteria belonging to the phylum *Corynebacterium)* on phylogenetic distance tree based on the Neighbour-joining method…… 115

Figure 4.19: the 16S rRNA gene abundance of bacteria and *Rhodococcus* in Ni-crude oil amended soil microcosms after degradation for 15 days. ……119

Figure 4.20: comparative analysis of the mean abundance of the bacterial 16S rRNA gene in soils amended with Ni and Crude oil at Ni-concentrations of 12.5, 50 and 350 ppm relative to oil only amended soils and unamended soils……..120

Figure 4.21: comparative analysis of the mean abundance of the *Rhodococcal* 16S rRNA gene in soils amended with Ni and Crude oil at Ni-concentrations of 12.5, 50 and 350 ppm relative to oil only amended soils and unamended soils……120

Figure 4.22: correlation analysis between maximum cumulative CO and the bacterial 16S rRNA gene abundance in Ni-crude oil amended soil microcosms……. ……122

Figure 4.23: correlation analysis between maximum cumulative CO and the *Rhodococcal* 16S rRNA gene abundance in Ni-crude oil amended soil microcosms……122

Figure 4.24: correlation analysis between recovered total n-alkanes and the bacterial 16S rRNA gene abundance in Ni-crude oil amended soil microcosms.……..124

Figure 4.25: correlation analysis between recovered total petroleum hydrocarbon and the *Rhodococcal* 16S rRNA gene abundance in Ni-crude oil amended soil microcosms…… 125

Chapter 5

Figure 5.1: the experimental design for the study of crude oil biodegradation in Cd-crude oil amended soil microcosms at Cd-concentrations of 0.1, 1, 10, 100 and 1000 ppm relative to oil only amended (PHC) and no oil amended (soil only) controls in triplicates. ……. 144

Figure 5.2: CO_2 production profiles of Cd and petroleum hydrocarbon co-contaminated soil microcosms relative to oil amended and unamended controls…… 146

Figure 5.3: CO_2 production profiles of Cd and petroleum hydrocarbon co-contaminated soil microcosms relative to oil amended and unamended controls…… 146

Figure 5.4: Comparative analysis (Dunnets's multiple comparison) of cumulative CO_2 values measured after 16 days in $CdCl_2$ and CdO amended oil degraded soil microcosms at Cd concentrations of 0.1, 1, 10, 100 1nd 1000 ppm relative to oil only amended control……. 148

Figure 5.5: The maximal rate of CO_2 production in Cd-oil amended soils relative to oil amended and unamended controls……..150

Figure 5.6: comparative analysis of maximal CO_2 production rates in $CdCl_2$ amended oil degraded soil microcosms at Cd concentrations of 0.1, 1, 10, 100 1nd 1000 ppm relative to oil only amended control……..150

Figure 5.7: comparative analysis of maximal CO_2 production rates in CdO amended oil degraded soil microcosms at Cd concentrations of 0.1, 1, 10, 100 1nd 1000 ppm relative to oil only amended control……151

Figure 5.8: comparative analysis of maximal CO_2 production rates in $CdCl_2$ and CdO amended oil degraded soil microcosms at Cd concentrations of 10, 100 1nd 1000 ppm relative to soil only control……151

Figure 5.9: comparative analysis of the fate of added Cd in soil by sequential extraction of Cd fractions associated with exchangeable ions, carbonates, oxides of Fe and Mn, organics and residuals. The graph legend indicates the different chemical forms of Cd used for this study……153

Figure 5.10: comparison of recovered total n-alkanes (nC_{12}-nC_{32}), pristane and phytane, of microcosms contaminated with Cd and/or 100 mg oil after 15 days incubation…… 154

Figure 5.11: comparison of n-alkane profile of petroleum crude oil recovered from Cd-oil amended soil microcosms at low ($CdCl_2$ 0.1 ppm) and high (CdO 1000 ppm) Cd concentrations relative to oil only amended soil and undegraded oil…….. 155

Figure 5.12: Agarose Gel electrophoresis of amplified 16S rRNA genes from Cd-petroleum co-contaminated soil microcosms……. 157

Figure 5.13: the 16S rRNA gene profile on a Denaturing gradient gel indicating the diversity of microbial communities and reproducibility of communities in triplicates of soil Microcosms contaminated with petroleum hydrocarbons and Cd in different forms ($CdCl_2$ and CdO) and at different Cd concentrations (0.1, 10, and 1000 ppm)…… ……...159

Figure 5.14: Non-metric MDS analysis of the bacterial communities present in Cd-crude oil amended soil microcosms, crude oil only amended microcosms and unamended soils (soil only) after 16 days of degradation period……..159

Figure 5.15: Comparative analysis of relative abundances of phylogenic group of 16S rRNA sequences recovered from representative Cd-crude oil amended soil microcosms relative to oil only amended soil and unamended soil……...169

Figure 5.16: Comparative analysis of relative abundances of genera of 16S rRNA sequences recovered from representative Cd- crude oil amended soil microcosms relative to oil only amended soil and unamended soil……...170

Figure 5.17: the 16S rRNA gene abundance of bacteria and *Rhodococcus* in Cd-crude oil amended soil microcosms after degradation for 15 days. The 16S rRNA genes were quantified by specific qPCR assays that target 16S rRNA genes of general bacteria and *Rhodococcus*…….174

Figure 5.18: comparative analysis of the mean abundance of the *Rhodococcal* 16S rRNA gene copies in soils amended with Cd (CdO and $CdCl_2$) and Crude oil at Cd-concentrations of 0.1, 10 and 1000 ppm relative to oil only amended soils…….175

Figure 5.19: comparative analysis of the mean abundance of the *Rhodococcal* 16S rRNA gene copies of soils amended with Cd (CdO and $CdCl_2$) and Crude oil at Cd-concentrations of 0.1, 10 and 1000 ppm relative to unamended soils……..175

Figure 5.20: the logarithmic relationship between concentration and abundance of *Rhodococcal* 16S rRNA genes copies in degrading soil amended with a combination of Cd and crude oil……176

Figure 5.21: correlation analysis between maximum cumulative the bacterial and *Rhodococcal* 16S rRNA gene abundance in Cd-crude oil amended soil microcosms. The coefficient of determination (R^2) is indicated. ……..…180

Figure 5.22: correlation analysis between maximum cumulative CO_2 and the bacterial 16S rRNA gene abundance in Cd-crude oil amended soil microcosms. The coefficient of determination (R^2) is indicated. ……180

Figure 5.23: correlation analysis between maximum cumulative CO_2 and the *Rhodococcal* 16S rRNA gene abundance in Cd-crude oil amended soil microcosms. The coefficient of determination (R^2) is indicated……..…181

Figure 5.24: correlation analysis between maximal rate of CO_2 production and the bacterial 16S rRNA gene abundance in Cd-crude oil amended soil microcosms……...……..181

Figure 5.25: correlation analysis between maximal rate of CO_2 production and the *Rhodococcal* 16S rRNA gene abundance in Cd-crude oil amended soil microcosms.

Figure 5.26: correlation analysis between total n-alkanes recovered from degrading soil microcosms and the Bacterial 16S rRNA gene abundance in Cd-crude oil amended soil microcosms. …… 182

Figure 5.27: correlation analysis between total n-alkanes recovered from the degrading soil microcosms and the *Rhodococcal* 16S rRNA gene abundances in oil amended and Cd-crude oil amended soil microcosms. ……183

Chapter 6

Figure 6.1: the experimental design for the study of crude oil biodegradation in short-term contaminated Pb- soil microcosms and long-term contaminated Pb soils relative to oil only amended and no oil amended (soil only) controls in triplicates……..196

Figure 6.2: CO_2 production profiles of Pb and petroleum hydrocarbon co-contaminated soil microcosms relative to oil amended and unamended controls. Microcosms were amended with $PbCl_2$……..200

Figure 6.3: CO_2 production profiles of Pb and petroleum hydrocarbon co-contaminated soil microcosms relative to oil amended and unamended controls. Microcosms were amended with PbS……..... 201

Figure 6.4: comparative analysis of cumulative CO_2 in $PbCl_2$ and PbS amended oil degraded soil microcosms at Pb concentrations of 150, 1500 and 15000 ppm relative to oil only amended control……..… 202

Figure 6.5: comparative analysis of cumulative CO2 in PbCl2 and PbS amended oil degraded soil microcosms at Pb concentrations of 150, 1500 and 15000 ppm relative to soil only (i.e unamended soil) controls…… 202

Figure 6.6: The maximal rate of CO_2 production in Pb-oil amended soils relative to oil amended and unamended controls. Each bar represents the mean maximal rate of triplicate sample. …… 203

Figure 6.7: comparative analysis of maximal CO_2 production rates in $PbCl_2$ amended oil degraded soil microcosms at Cd concentrations of 150, 1500 and 15000 ppm relative to oil only amended control. ……..203

Figure 6.8: Comparative analysis of maximal CO_2 production rates in PbS amended oil degraded soil microcosms at Pb concentrations of 150, 1500 and 15000 ppm relative to oil only amended control. ……..……204

Figure 6.9: CO_2 production profiles of historically Pb contaminated soil amended with crude oil, historically Pb contaminated soils (at high and lower Pb concentrations) not amended with crude oil and oil only amended and unamended Nafferton Farm soil controls. …….205

Figure 6.10: The maximal rate of CO2 production in historically Pb contaminated soils (high and low) amended with oil relative to oil amended and unamended pristine Nafferton Farm soils and historically Pb contaminated soils (high and low) also not amended with oil…….208

Figure 6.11: Comparative analysis of maximal CO2 production rates in historically Pb contaminated soil amended and unamended with oil at low and high Pb concentrations relative to the oil only amended Nafferton soil control…**…… 209**

Figure 6.12: comparative analysis of the fate of added Pb in soil by sequential extraction of Cd fractions associated with exchangeable ions, carbonates, oxides of Fe and Mn, organics and residuals……...…211

Figure 6.13: the profile of n-Alkane recovered after degradation of crude oil-Pb mixed contaminated soil microcosms indicating the degree of degradation of whole petroleum in long-term and short-term Pb contamination obtained from GCFID analysis of recovered oil. ……....213

Figure 6.14: comparison of total n-alkanes (nC_{12}-nC_{32}) of microcosms contaminated with Pb and/or 100 mg oil after 15 days incubation……214

Figure 6.15: the 16S rRNA gene profile on Denaturing gradient gel indicating the diversity of microbial communities and reproducibility of communities in triplicates of soil microcosms contaminated with petroleum crude oil and Pb……..……..217

Figure 6.16: Non-metric MDS analysis of the bacterial communities present in Pb-crude oil contaminated soil communities, crude oil only amended, high and low Pb soils not contaminated with oil and unamended soils (soil only) after 15 days of degradation period……..218

Figure 6.17: Comparative analysis of relative abundances of phylogenic groups of 16S rRNA sequences recovered from representative Pb-crude oil contaminated soil microcosms relative to oil only amended soil and unamended soil…… 227

Figure 6.18: Comparative analysis of relative abundances of genera of 16S rRNA sequences recovered from representative Cd- crude oil amended soil microcosms relative to oil only amended soil and unamended soil…….228

Figure 6.19: comparison of abundances of enriched strains in oil amended Pb contaminated soil communities relative to oil only amended and unamended soils. Bacterial strains represented were the most enriched stains in communities……228

Figure 6.20: Comparison of the most dominant sequences recovered from Pb-crude oil contaminated soil community with closely related bacteria (mostly bacteria belonging to the phylum *Firmicute)* on phylogenetic distance tree based on the Neighbour-joining method……229

Figure 6.21: the 16S rRNA gene abundance of bacteria and *Bacillales* in short-term Pb contaminated crude oil amended soil microcosms after degradation for 15 days…… 233

Figure 6.22: comparative analysis of the mean abundance of the Bacterial 16S rRNA gene of soils amended with Pb ($PbCl_2$ and PbS) and Crude oil at Pb-concentrations of 150, 1500 and 15000 ppm relative to oil only amended soils……..233

Figure 6.23: the 16S rRNA gene abundance of bacteria and *Bacillales* in historically Pb contaminated soil microcosms amended with crude oil after degradation for 15 days. The 16S rRNA genes were quantified by specific qPCR assays that target 16Sr RNA gene of general bacteria and *Bacillales*……...235

Figure 6.24: relationship between Bacterial and the *Bacillales* 16S rRNA gene abundances in Pb-crude oil co-contaminated soil microcosms……237

Figure 6.25: relationship between maximum cumulative CO_2 and the *Bacillales* 16S rRNA gene abundance in Pb-crude oil co-contaminated soil microcosms……239

Figure 6.26: relationship between maximal CO_2 production rate and the *Bacillales* 16S rRNA gene abundance in Pb-crude oil co-contaminated soil microcosms……239

Figure 6.27: relationship between total n-alkanes recovered after a period of petroleum degradation and the bacterial 16S rRNA gene abundance in Pb-crude oil co-contaminated soil microcosms. ……..240

Figure 6.28: relationship between total n-alkanes and the *Bacillales* 16S rRNA gene abundance in Pb-crude oil amended soil microcosms. ……241

List of tables

Chapter 1

Table 1.1: Petroleum-Heavy metal co-contaminated sites across the Globe……3

Table 1.2: Concentrations (ppb) of Ni, Cd, and Pb in crude oil samples from around the world ……8

Table 1.3: Effects of Remediation Methods on Soil Characteristics and the Estimated Costs of Treatment……11

Table 1.4: Real field case studies of bioremediation strategies……..12

Chapter 2

Table 2.1: forms and concentrations of metals studied to determine metal effects on biodegradation of petroleum hydrocarbon in soil and metal UK environmental agency's soil guide line value and Dutch list recommendation value……39

Table 2.2: Experimental design for the sequential extraction of heavy metal from soils …… 44

Table 2.3: the PGM linker primer/ adapter, a Unique Golay barcode DNA and barcode spacer used as Tags for ion torrent sequence forward primer F515…… 48

Chapter 3

Table 3.1: Rhodococcus strains that are closely related to OTU 1 (most dominant OTU in Cd and Ni crude oin degraded soils) used to design primers that targeted OTU 1 sequences in Microcosms…… 61

Table 3.2: Bacillales strains that are closely related to OTU 5 the most Dominant OTU in Pb-crude oil degraded soil used to design primers that targeted OTU 5 sequences in Microcosms…… …… 62

Table 3.3: Prospective primers for *Rhodococcus* and *Bacillales* target group (see table 3.1 and 3.2) designed with PRIMROSE ……...……63

Table 3.4: Specificity of Primer pairs for *Rhodococcus* and *Bacillales* generated from the ProbeMatch platform on Ribosomal Database Project (RDP)…… 64

Chapter 4

Table 4.1: Metal Concentration of Nafferton ecological farm soil sample…… 84

Table 4.2: Number of sequences recovered from microbial communities of crude oil – Ni amended soils before and after trimming of sequence data……104

Table 4.3: Community species richness and diversity indices evaluated with 8000 16S rRNA sequences of Ni-PHC contaminated soil microcosms……. 108

Table 4.4: Comparison of microbial community structure in soil microcosms based on 8000 16S rRNA gene sequences ……108

Table 4.5: Close relatives of the most abundant sequence recovered from oil degraded microcosms with or without Ni……..114

Table 4.6: the Pearson's correlation analysis describing the interaction between microbiological variables and geochemical variables determined from petroleum-nickel contaminated soil microcosms ……... 125

Table 4.7: the spearman's correlation analysis describing the interaction between microbiological variables and geochemical variables determined from petroleum-nickel contaminated soil microcosms ……... 125

Chapter 5

Table 5.1: the qualitative estimation of the 16S rRNA sequence sampling for the diversity and phylogenetic study of communities of Cd-oil amended soils…….161

Table 5.2: Community specie richness and diversity indices evaluated with 8000 16S rRNA sequences of Cd-oil amended soil microcosms……162

Table 5.3: Clayton's dissimilarity coefficient comparison of microbial community structure in soil microcosms based on 8000 16S rRNA gene sequences **……**164

Table 5.4: Pearson's correlation analysis describing the interaction between microbiological variables and geochemical variables determined from petroleum-Cd contaminated soil microcosms……...…….177

Chapter 6

Table 6. 1: Concentrations of Metal in Nafferton Ecological Farm and St. Anthony w

Table 6.2: the analysis of the 16S rRNA sequence sampling for the diversity and phylogenetic study of communities of Cd-oil amended soilsorks soils…….196

Table 6.3: Community specie richness and diversity indices evaluated with 8000 16S rRNA sequences of Pb-crude oil contaminated soil microcosms……..221

Table 6.4: Comparison of microbial community structure in soil microcosms based on 8000 16S rRNA gene sequences……223

Table 6.5: Pearson's correlation analysis describing the interaction between microbiological variables and geochemical variables determined from petroleum crude oil -Pb contaminated soils……...……237

Appendix

Appendix 1: Reagents for the preparation of DNA Denaturing Gels used for the ecological diversity study of the microbial communities……..299

Appendix II: Enlarged diagram of St. Anthony's Lead works map indicating the concentrations of Lead at the surface (mg/kg) …….300

Chapter 1: Literature review

1.1 Introduction

The petroleum industry is a major contributor of pollutants in the world. This contribution is due to the increased global demand for petroleum as a major source of energy and the diverse locations that oil is recovered from and transported too. In fact, the estimated global demand for crude oil is about 83.1 million barrels per day and is expected to increase to 106 million barrels per day by 2030 (OPEC 2009). To meet this demand, there is increased activity in the upstream, midstream and downstream sectors of the petroleum industry with associated deliberate, and accidental oil spills, tanker and pipeline leakages that release crude oil into the environment across the globe. The clean up of the released hydrocarbon becomes essential to avoid environmental and health hazards that can be caused by the petroleum constituents.

Over the years, different physical, chemical, and biological techniques have been established for the remediation of polluted sites. Bioremediation has been of great interest as it is cost effective, efficient, and eco-friendly. The indigenous microorganisms of the polluted sites are harnessed during the bioremediation of the polluted sites. This is achieved by improving the nutrient status to stimulate these organisms or improve the abiotic conditions (biostimulation) (Rothwell and Cooke 2015). However, the addition laboratory-cultured microbes that are commercially available at an appropriate scale to increase the population of hydrocarbon degrading microbes enhances has been considered (bioaugmentation) (Rothwell and Cooke 2015).

In polluted soils, different factors influence the microbial degradation of petroleum hydrocarbons. The key factors that affect biodegradation of petroleum are the presence of a microbial population (the species and the population size) capable of degrading the oil components and the ability of the environment to sustain their activity. In addition to these key environmental factors the composition of the oil itself is important and the presence of co-contaminants. For instance, heavy metals are one the numerous constituents of complex crude petroleum. Some of the identified heavy metals associated with Crude oil include Nickel, Iron, copper, Zinc, Lead, Vanadium, Cobalt, Cadmium, Chromium etc. (Abioye 2011; Coleman 2002; Freije 2014). These heavy metals can be toxic to living organisms. However, some of them

such as iron, zinc and selenium are essential to metabolic activities. Critically, these elements cannot be degraded by microbes and as a result, tend to accumulate in chronic petroleum polluted soils (Freije 2014; Fu and Zang 2014; Muniz et al. 2004).

Investigations to determine the effect of heavy metals on petroleum degradation by microbes are necessary to enhance the bioremediation of sites co-contaminated by heavy metals and petroleum hydrocarbon. The existences of co-contaminated sites have been reported in previous studies carried out on petroleum-contaminated sites across the globe (see table 1.1). For instance, studies by De Mora et al. (2004) identified contamination of sediments by Cu, Hg, Pb, Zn in Bahrain and Co, Cr and Ni in United Arab Emirate (UAE) due to the chronic oil spills (they also identified bioaccumulation of metals in aquatic life). This thesis describes a series of systematic experiments, which have rigorously investigated the effect of the heavy metals Ni, Cd and Pb in terms of their concentration and chemical association, on the biodegradation of petroleum hydrocarbon in soil. This systematic approach has been coupled to a rigorous analysis of the experimental system from the multiple perspectives of the diversity and abundance of the soil microbial community (specifically the hydrocarbon degraders), the chemical fate of the added metals and the compositional change of the crude petroleum pollutant.

1. 2. Research Justification

The importance of oil spills and its impact have been well acknowledged globally. Investigations on the impact to human health, terrestrial and aquatic environments are well documented Taylor and Rasheed 2011; Dubinsky et al. 2013; Arias et al. 2010; UNEP 2011; Ite et al. 2013). However, the emphasis of these studies is mostly on the major components of the oils. Little is known about the impact of the minute but effective heavy metal concentrations in the environments. Investigating the impact of metals on biodegradation of petroleum may result to the development of more effective and less time-consuming remediation methods for the restoration of contaminated environments. Hence, establishing the thresholds that can be tolerated in soil systems with respect to metal types, mineral form, and levels, where bioremediation can still be relied on, becomes important in predicting effective remediation in regions where the chronic repeat of oil spills result in metal accumulation in the soils. Also, the results of this work could be helpful in making decisions on the reliability of the indigenous community in a typical soil to provide a

hydrocarbon-degrading community, which is adaptable to the different metal concentrations that might occur or the need to augment the hydrocarbon degraders with those that have metal tolerances (engineered or obtained from somewhere) in cases where indigenous community of soil lacks metal tolerating hydrocarbon degraders

Table 1.1: Petroleum-Heavy metal co-contaminated sites across the Globe

Country	Sample Type	Metal	Concentration (ppm)	Reference
Lake Yaounde, Cameroun	Sediment	Cd	4.31	Leopold et al 2012
		Ni	63.49	
		Pb	23.35	
Niger Delta, Nigeria	Soil	Pb	65.82±0.42	Udosen et al 2001
		Ni	41.72±0.53	
Niger Delta, Nigeria	Soil	Ni	76±3.9	Obiajunwa et al 2002
Niger Delta, Nigeria	Soil	Cd	1.3±1	Olawoyin et al 2012
		Ni	42.7±20.3	
		Pb	895.1±423.9	
Bahrain	Soil	Pb	697.2	Akhter and Madany, 1993
		Cd	72	
		Ni	125.6	
Bahrain	Soil	Ni	122	Mandany et al., 1994
		Pb	2550	
Bahrain	Soil	Pb	99	de Mora et al 2004
		Ni	195	
		Cd	109	
Bahrain	Sediment	Cd	19.14	Naser, 2010
China	Soil	Pb	69.4±37.4	Li et al 2008
		Cd	0.23±0.21	
China	Soil	Pb	450	Dong et al., 2013
China	Soil	Cd	5.4	Zhang et al 2014
		Ni	134	
		Pb	180	
Kuwait	Soil	Ni	80	Al-Abdali et al 1996
		Pb	30	
		Cd	2	
Kuwait	Soil	NI	89.30±0.05	Al-Saleh and Abrar Akbar, 2014
		Cd	4.53±0.04	
		Pb	111.40±0.06	
Kuwait	Soil	NI	81.90±0.04	Al-Saleh and Abrar Akbar, 2014
		Cd	3.80±0.05	
		Pb	113.80±0.06	
Kuwait	Soil	NI	87.60±0.06	Al-Saleh and Abrar Akbar, 2014
		Cd	4.71±0.05	
		Pb	116.10±0.06	
Oman	Sediment	Ni	1010	de Mora et al 2004
Mexico	Sediment	Cd	9.6	Brito et al 2015
		Ni	40	
		Pb	109	
montevideo habour Uruguay	Sediment	Pb	85±31	Muniz et al 2004
		Ni	30±2	
Portugal	Sediment	Pb	61	Almeida et al 2013
		Cd	251	
Harghita, Romanian	Soil	Pb	67	Máthé et al 2012

Note: the heavy metals reported here are Pb, Cd and Ni, which are the metals of interest in the project

Furthermore, investigations on the impact of heavy metals and the biodegradation of petroleum hydrocarbons based on individual microorganisms and individual hydrocarbon components are well established (Sokhn et al. 2001; Nakatsu et al. 2005; Olaniran et al. 2013; Riis et al. 2002). Nakatsu et al. (2005) investigated the impact of heavy metals (Pb and Cr) and polyaromatic hydrocarbons addition to soil on the soil microbial community structure in soil microcosms. Studies on a simulated environment in microcosms are more likely to determine a real world scenario resulting to the development of a more realistic approach for effective petroleum hydrocarbon removal in the environment. This provides an avenue to understanding the complex environment of mixed pollutants better by assessing the fate of both hydrocarbon and heavy metals as well as the dynamics of the microbial community. Also, Simulation of environments in microcosms were used in the past by researchers to investigate biodegradation of crude oil in various environments including soils, sediments, aquatic environments e. t. c. (Röling et al. 2002; Taylor et al. 2008; Okoh 2003; ChaIneau et al. 1995; Singh et al. 2014; Hubert et al. 2012; Gray et al. 2011; Sherry et al. 2013; Sung et al. 2013; Salanitro et al. 1997) and this has provided a more realistic understanding of crude oil degradation in complex environments.

1.3. Aim

The general aim of this project is to investigate metal effects on hydrocarbon degradation in complex natural systems using microbial ecological approach combined with rigorous analysis of the fate of individual hydrocarbon components and heavy metals during the degradation of whole oils. Such an approach will provide a more detailed understanding of the patterns of oil degradation under different and increasing metal stresses and how microbial communities progressively change in such environments.

1.4 Research Questions

1. Do the concentrations of heavy metals in the soils contaminated with petroleum hydrocarbon influence the biodegradability of the hydrocarbon pollutants and critically at what threshold concentrations are these effects observable?
2. How do the chemical forms of the metals as added to the soil influence the response of hydrocarbon biodegrading community?
3. How does the pollutant aging i.e. the ultimate chemical fate of the added metal correlate with impacts on hydrocarbon degradation?

4. Do the dominant hydrocarbon degraders within a typical soil vary as a function metal type and concentration or do the dominant hydrocarbon degraders display a broad tolerance to metal concentrations
5. Will biodegradation of crude of petroleum hydrocarbons occur in an environment that has experienced a long-term exposure to heavy metals and if so are the resultant microbial communities similar or distinct to those of pristine soils incubated with similar levels of metal and hydrocarbon?

1.5. Hypotheses

1. Heavy metals, irrespective of concentration and added forms, will inhibit the degradation of petroleum hydrocarbon.

2. The presence of heavy metals in soil systems will limit the proliferation of hydrocarbon degraders to only heavy metal resistance strains of the microbes and this will change the microbial diversity of the soil systems in relation to oil only amended soil and unamended soil controls.

1.6. Objectives

1. To determine the effect of different concentrations of heavy metals (Ni, Cd and Pb) on the rate of biodegradation of petroleum hydrocarbons in soil microcosms through the monitoring of CO_2 production.
2. To determine the effect of metals on the biodegradation of petroleum hydrocarbon as a function of their chemical form (i.e. organically complexed and soluble and insoluble inorganic forms by monitoring the CO_2 production in soil microcosms.
3. To determine the chemical fate of the added metals in a petroleum hydrocarbon contaminated soil system by sequential extraction analysis of metal partitioning into different soil fractions.
4. To corroborate estimates of degradation by CO_2 production by identifying and quantifying individual n-alkanes recovered from microcosms by solvent extraction after incubation.

To determine (using molecular biological techniques) microbial community dynamics and, more specifically, hydrocarbon degrader abundances in the experimental system as a function of metal and oil addition.

1.7. Thesis structure

There are 7 chapters in this thesis. Chapter 1 introduces the project by outlining the aims, objectives, and scope of the project as well as providing a review of the relevant literature. Chapter 2 describes the methods used in this project, which include both the varied molecular microbiological techniques employed and the inorganic and organic geochemical analytical techniques. The multidisciplinary approach described in this second chapter was chosen to provide a broader understanding of biodegradation in metal-petroleum hydrocarbon contaminated soil systems. In Chapters 3 the design and application of specific quantitative Polymerase chain reaction (qPCR) assays are described, which the 16S rRNA gene sequences of important taxonomic groups identified in community diversity studies. The main results chapters for this study encompass chapters 4, 5 and 6. In these chapters, the effects of the heavy metals Ni, Cd and Pb on the biodegradation of petroleum hydrocarbon are respectively discussed. Chapter 7 concludes the project thesis outlining the important deductions, general themes and key differences and similarities between the respective study systems with recommendations for future work.

1.8. Components and classification of Petroleum

Petroleum is a complex mixture of hydrocarbon compounds. Previous studies have established that petroleum consists of more than 17,000 distinct chemical compounds (Head et al. 2006). These chemical compounds are dominated by hydrocarbons classified as either saturates, aromatics, resins and asphaltenes collectively described as the SARAs (Fingas 2015; Peters et al. 2007a; Jokuty et al. 1995; B.P. Tissot and Welte 2013a). In addition to these bulk hydrocarbon components, there are other minor constituents. These include sulphur, nitrogen, oxygen and metals (Peters et al. 2007; Tissot and Welte 2013a). The variety of compounds comes from the complex biological structures from which oil originally originates (B.P. Tissot and Welte 2013b). Also, a variation of crude oil components is determined by the different depositional environments, in which organic matter is preserved and altered to form oil (B.P. Tissot and Welte 2013b). Oil may be further altered during its generation and migration from the source rocks and can even be altered by biological activity once emplaced within an oil reservoir (B.P. Tissot and Welte 2013b). Geologists and geochemists classify crude oil base in the chemical composition (B P Tissot and Welte 2013c). For instance based on hydrocarbon composition, petroleum is classified as paraffinic or naphthenic crude oils (Simanzhenkov and Idem 2003; Sivasankar

2008; B P Tissot and Welte 2013c). Paraffinic crude oils consist of a high percentage of saturates while naphthenic oils consist of the high percentage of aromatics and cyclic aliphatic (Simanzhenkov and Idem 2003). Apart from this base classification, the non-hydrocarbon components of petroleum are also considered while classifying petroleum. Thus, the sulphur, nitrogen content and the ratios of the heavy metals Vanadium and Nickel (the most abundant trace metals in crude oil) have been used to classify petroleum (Peters et al. 2007a). The sulphur content of petroleum classifies petroleum as sweet of sour oil (Carrales and Martin 1975; Peters et al. 2007b; International Council on Clean Transportion 2011). Sweet oils have sulphur contents less than 1% of the whole petroleum by weight and sour oils have sulphur contents above this value. The physical attribute of petroleum that has been most exploited from an economic perspective is its classification in terms of lightness and heaviness called the American petroleum institute (API) gravity (Barwise 1990a; Speight and Ozum 2001). API gravity is related to density and specific gravity and usually, the API of oils range from 10o API (for heavy oil and bitumen with a specific gravity of over 1.0) to 45.3.3o API (for light oils with the specific gravity of about 0.8) (Speight and Ozum 2001). API values and the chemical properties have been correlated in the past in an attempt to classify petroleum based on the source rocks from which they were generated (Barwise 1990a).

1.9 **Heavy metals as components of** petroleum

It has been established in the section above that crude oils vary based on their constituents. All crude oils also consist of trace metals. As described above for the variance of bulk oil compositions the existence of metals in petroleum has been attributed to naturally occurring geochemical processes involved in the generation (in source rock), migration (to the reservoir) and maturation (in the reservoir) of petroleum (Filby 1994). These processes expose the organic material to metal-rich surfaces and fluids (e.g. formation waters). As a result, these processes give rise to differences in abundances of the total and specific metals which occur in different crude oils and the abundance of metals in the oils depends the depositional environment, the nature of the organic material in the source rock, the geological settings, the maturity of oil, the digenetic history of the rock and the chemical composition it the oil (B.P. Tissot and Welte 2013b). As an example table, 1.2 indicates the differences in the average concentrations of Ni, Cd, and Pb in crude oil

samples from around the world. This data emphasises the highly varied compositions that occur.

Metals in petroleum exist in different physical states and chemical forms. In the physical state, they exist as suspended solid particles, colloidal and liquid states (Karchmer and Gunn 1952). In chemical forms, they exist as organometallic complexes and inorganic forms including elemental form and as carbonates, sulphides, oxides, phosphates and chlorides (Filby 1994; Barwise 1990b; Anon n.d.; B.P. Tissot and Welte 2013b).

Table 1.2: Concentrations (ppb) of Ni, Cd, and Pb in crude oil samples from around the world

Oil source	Ni	Cd	Pb	Number samples analysed
Abu Dhabi	25686.4±11454.8	0	7462.1±1775.1	3
Angola	19916.23±5920.7	99.03±55.1	1712.6±1206.1	3
Brent oil North sea	3851.3±830.1	0	1017.2±1005.7	3
Columbia	205312.3	0	4796.9	1
Gulf of Mexico	9761.7±8535.2	20.6±5.6	2701.48±1630.5	4
Iran	183263.5±30257.6	0	13889.6±2229.7	2
Liaohe China	129838.3±71926.3	0	0	3
Mexico	113432.9	231	6058.8	1
Monterey, California	70311.7	2.5	234.8	1
N. Slope, Alaska, USA	36339.4±9956.7	271.4±5.3	864.4±329.9	6
Nigeria	35727.38±14871.7	7.4±6.1	6502.3±4479.4	15
Onshore England	8433.5±3221.3	21.3±10.4	437.3±281.4	9
Santa Maria, California, USA	172562.2±33845.5	306.3±251.3	10449.56±9093.1	5
Shengli China	88950.7±17356.4	39050.4±8483.3	486.5±1318	22
Venezuela	115688.55±34264.35	79.4	1639.7±1425.3	2

Source: Olsen, 1998

The organometallics in petroleum are considered as either metalloporphyrins or non-porphyrins (Filby 1994; Peters et al. 2007a). Metalloporphyrins are the most common form in which metals are known to exist in petroleum and they occur mostly in the asphaltic fraction of the petroleum. These compounds are believed to have originated biologically with chlorophyll and bacteriochlorophyll as the precursor (Peters et al. 2007a; B.P. Tissot and Welte 2013b). Just like chlorophyll and other naturally occurring porphyrins, the structure consists of four pyrrole molecules but the magnesium in chlorophyll is substituted mostly by nickel and vanadium among other metals during the early diagenesis of source rock (Filby 1994; Peters et al. 2007a). Because of their frequent occurrence, Nickel and Vanadyl porphyrins have been used as biomarkers in the geochemistry of petroleum for oil-oil or oil-source rock correlation, identification of depositional environment of source rocks and in the determination of thermal maturity levels of oils or source rocks (Filby 1994; Peters et al. 2007).

1.10 Remediation strategies for hydrocarbon polluted soils

Ensuring environmental sustainability is one of the priorities of the international community for over a decade (UNEP 2010). Pollution control and management has become a preeminent issue in many countries around the world. Consequently, various remediation strategies have been considered by academic studies, which include physical, chemical and biological methods or combinations of these (Semple 2001; Riser-Roberts 1998).

Physical and chemical techniques for soil remediation are strategies developed based on the physical and chemical properties of the contaminants and the contaminated media. The density, viscosity, physical state, solubility and volatility of the contaminants and contaminated media are exploited for physical remediation techniques while chemicals are often used to convert contaminants into a less harmful form. The techniques could be *in-situ* or *ex-situ*. Some of these methods have been used successfully in the fields to decontaminate the environment (Abioye 2011; Riser-Roberts 1998; U.S. EPA 2006; Stegmann et al. 2013). Some of the physical remediation methods include thermal treatment (involves the heating of soil to temperature less than 600 ^{o}C (Jensen and Miller, 1994 to increase the vapour pressure of organic contaminant thereby changing the contaminant to its gaseous form), air sparging, soil washing, chemical extraction (surfactants or solvent washing),

volatilization, steam extraction, solidification/stabilization and encapsulation (Riser-Roberts 1998; Soleimani 2014; Abioye 2011). Usually, the remediation of contaminants, *in-situ* or *ex-situ*, using physical methods requires the containment of the contaminants thereby limiting their further migration (Wong et al. 1997; Friend 1996; Naidu et al. 1996). This means that the contaminants are not quite removed completely from the environment. In the same vain, the chemical techniques that involve the addition of chemicals to convert the contaminant to a less harmful form might generate a more toxic contaminant that could be harmful to the environment. Also, the chemicals usually used to achieve decontamination may become more toxic to the environment (Mohanty et al. 2013; Makkar and Rockne 2003; Holly K. Ober 2010; U.S. EPA 2010a; U.S. EPA 2010b). For instance, US EPA (2010a; 2010b) reported that the combination of Louisiana sweet crude oil with 8 chemical dispersants used for the remediation of Deep Water Horizon oil spill in the Gulf of Mexico was more toxic to the aquatic wide life than the crude oil alone.

Bioremediation is a biological technique that exploits biological systems such as plants and microorganisms and their products to remediate the contaminated environment. Usually, microorganisms utilise organic contaminants as carbon sources and energy (Atlas, 1995; Thapa et al., 2012) and thus a complete mineralization of such organic compounds to the simplest compound CO_2 is achieved. This process therefore effectively removes the organic contaminants from the environment. For heavy metal contaminated environments, the most successful strategy of bioremediation is the use of plants and phytoplanktons that hyperaccumulate the metals within thereby removing them completely from the media of contamination (Rengifo-Gallego and Salamanca 2015; Göhre and Paszkowski 2006; Pulford and Watson 2003; Chekroun and Baghour 2013; Tangahu et al. 2011; Pilon-Smits 2005). Apart from its effective removal of contaminants (R.M. Atlas 1995; Vidali 2001; Das and Chandran 2011). Atlas (1995) pointed out that it required less than $1 million to clean up the shorelines of Prince William Sound, Alaska using a bioremediation technique which is in contrast to the use of physical sand washing which required $1 million a day. Most importantly, bioremediation is considered to be the most environmentally friendly strategy for the remediation of contaminated environments.

In contrast to the physical and chemical methods where the environment is destructively altered or harmful chemicals used for bioremediation the growth of

indigenous microorganisms is enhanced by careful consideration of favourable growth conditions such as the pH, aeration, moisture, CNP ratios etc. to favour the most important microbial species that possesses the enzyme systems useful for the transformation of the contaminants. This concept is called biostimulation. Biostimulation has been used in remediation fieldwork in the past successfully. For instance, the addition of fertilizer to the Exxon Valdez spill site improved the rates of degradation of the petroleum hydrocarbons by 3-5 times Atlas (1995). Some microbes enhance the degradation of hydrocarbons by secreting biosurfactants (such as Rhamnolipids by *Pseudomonas* species) that emulsify the hydrocarbons, breaking them into micelles. The exploitation of this fact was reported to be successful in the case of the Exxon Valdez spill (Bragg et al. 1994; Ronald M. Atlas 1995) and Deep Water Horizon Gulf of Mexico spill (Atlas and Hazen 2011).

Table 1.3: Effects of Remediation Methods on Soil Characteristics and the Estimated Costs of Treatment

Treatment	Effects on soil chemistry	Effects on physical structure	Effects on microorganisms	Approximate remediation cost (£/tonne)
Removal to landfill	?	?	?	Up to 100
Solidification				
Cement and Pozzolan based	N	N	N	25–175
Lime based	N	N	N	25–50
Vitrification	N	N	N	50–525
Physical processes				
Soil washing	Y	N	N	25–150
Physico-chemical washing	Y	N	N	50–175
Vapour extraction	Y	Y	Y	75
Chemical processes				
Solvent extraction	Y	N	?	50–600
Chemical dehalogenation	Y	N	?	175–450
In situ flushing	Y	Y	?	25–80
Surface amendments	Y	Y	Y	10–25
Thermal treatment				
Thermal desorption	Y	N	N	25–225
Incineration	N	N	N	50–1200
Biological treatments				
Windrow turning	Y	N	Y	10–50
Land farming	Y	N	Y	10–90
Bioventing	Y	Y	Y	15–75
Bioslurry	Y	N	Y	50–85
Biopiles	Y	N	Y	15–35
In situ bioremediation	Y	Y	Y	175

[a] N indicates that the above factors will not generally survive in a particular treatment method and Y indicates that they will generally survive. ? indicates that the effects are unclear.

Source: (Semple 2001)

Some microbes enhance the degradation of hydrocarbons by secreting biosurfactants (such as Rhamnolipids by Pseudomonas species) that emulsify the hydrocarbons, breaking them into micelles. The exploitation of this fact was reported to be successful in the case of the Exxon Valdez spill (Bragg et al. 1994; Ronald M. Atlas

1995) and Deep Water Horizon gulf of Mexico spill (Atlas and Hazen 2011). In addition, the inoculation of appropriate microorganisms to the environment can enhance the remediation of the contaminated environment. This strategy is called Bioaugmentation and has been used, alone or in combination with Biostimulation strategy, successful in bioremediation of petroleum-contaminated soils (Zhang et al. 2008; Fan et al. 2013; Hua et al. 2010). These concepts of bioremediation are eco-friendly, as they do not involve destructive alteration of the environment and addition harmful substances to the environment (Head and Swannell 1999; Divya et al. 2015; Atlas and Bragg 2009). Table 1.3 compares the physical, chemical and biological strategies of remediation of contaminated sites.

Table 1.4: Real field case studies of bioremediation strategies

Technique Used	Contaminants/ Remarks	Location	Reference
Field trials			
Bioaugumentation and biostimulation with inorganic mineral nutrients and/microbial inoculaion	Light crude oil contaminating a sandy beach	Delware, USA	Venosa et al (1996)
Biostilation using forced aeration and nutrient supplementation	Spill simulation (light crude oil and fuel oil) at mature mangrove	Gladstone, Australia	Duke et al (2000)
Stimulation by slow release of fertilzer, inipol EAP-22 and 3 different fish compost	Crude oil degadation under severe sub-antarctic conditions	The Grande Terre, Kerguelen Islands	Delille et al (2002)
Biostimulation using N and P fertilizer	Oil degradation on coastal salt marsh	Nova Scotia, Canada	Garcia-Blanco et al (2007)
Biostimulation using fertilizers (urea and diammonium phosphate) and temperatture amendments	Petroleum hydrocarbon degradation in polar desert	Tanquary Fiord, High Arctic	Sanscartier et al (2009)
Biostimulation using manure	Oil sludge contaminated soil with total petroelum hydrocarbon and pahs	China	Lui et al (2010)
Oil spills and bioremediation strategy applied			
Bioaugumentaiton using commercial microbial culture	Heavy crude oil degradation	Nakhodka oil spill, Japan	Tsutsumi et al (2000)
Bioaugumentaiton and biostimulation using hydrophobic fertilizer (F-1) as N and P source along with enriched microbial culture	Cude oil degradation	Zvulon beach oil spill, isreal	rosenberg et al (1992)
Biostimulation using oleophilic fertilizer	Oil biodegradation	Exxon Valdez oil spill, Alaska, USA	Pritchard et al (1992)
Biostimulation using liquid inorganic and slow relaese mineral fertilizer	Crude oil and degradation	Heavy fuel oil	Swannell et al. (1999)
Biostimulation using oleophilic fertilizer	High molecular weight n-alkanes, alkylcyclohexanes and benzenes and alkylated pahs	Prestige oil spill, spain (2006)	Jimenez et al. (2006)

Source: Tyagi et al., 2011

The benefits assured by bioremediation techniques have heightened the interest of researchers in the field over the decades. Research work on the remediation of

contaminated sites by these strategies has been well documented. Some of the cases where bioremediation techniques were utilized is highlighted on table 1.4.

1.11. Microbiology of Petroleum Biodegradation

The hydrocarbon components of petroleum crude oils are readily degradable by microbes (Prince 1997), however, the degradability of the individual components depends on the chemical structure and the chemical state of the component (Tyagi et al. 2011). The biodegradability of petroleum components decreases in the order n-alkanes> branched alkanes > branched alkenes> low-molecular weight n-alkyl aromatic > monoaromatics > cyclic alkanes > polycyclic aromatic hydrocarbons >> asphaltenes (Tyagi et al., 2010; Das and Chandran, 2011). Though the n-alkanes are the most degradable, those with 5-10 carbon atoms are inhibitory to degradation by most hydrocarbon degraders because they disrupt the lipid membrane of microorganisms (Bartha, 1986; Tyagi et al., 2010). Also, the hydrocarbons of 20 carbon atoms to 40 carbon atoms (known as the waxes) have low degradability due to their hydrophobic nature (Bartha and Atlas, 1977, Tyagi et al., 2010).

1.12. Petroleum Hydrocarbon-degrading aerobic microorganisms

Generally, petroleum hydrocarbon degraders possess the special ability to degrade hydrocarbons components of petroleum by utilizing the hydrocarbons as the sole carbon sources releasing energy in the subsequent cleavage of the hydrocarbon chains. These organisms are found in all the domains of life including the Archaea, the bacteria and some eukaryote such as fungi and algae. So far, the recorded hydrocarbon degrading/transforming microorganisms include 79 bacterial genera, 9 cyanobacterial genera, 103 fungal genera and 14 algal genera (Prince, 2005; Head et al., 2006). In fact, the ubiquity of their distribution in the terrestrial and aquatic ecosystems is such that they consist of approximately 1% the total microbial population (R.M. Atlas 1995). However, in the case of hydrocarbon release in the environment, there is usually a significant selective enrichment of the hydrocarbon degraders (Leahy and Colwell 1990).

Members of the bacterial phylogenetic groups *Actinobacteria, Bacteriodetes, Firmicutes* and *Proteobacteria* are frequently isolated in petroleum hydrocarbon

contaminated sites. Some of them include species of *Rhodococcus*, *Nocardia*, *Gordonia*, *Flavobacterium*, *Bacillus*, *Rhodobacter*, *Pseudomonas*, *Achromobacter*, *Alcaligenes*, *Sphingobaterium* and *Burkholderia*. Others, which are significantly enriched in the marine environment, include *Alcanivorax* and *Cycloclasticus* (Singh et al., 2009; Head et al., 2006). The table below documents some of the microbial isolates from petroleum hydrocarbon contaminated environments. Apart from bacterial, species, fungal species such as *Aspergillus*, *Fusarium*, *Trichoderma* and *Penicillium* (Arutchelvi and Doble 2010; John et al. 2012; Michalcewicz 1995; Reyes-César et al. 2014; Elshafie et al. 2007) as well as acidophilic yeast species, *Candida sp.* (Palittapongarnpim et al. 1998; Sood and Lal 2009; Prasad et al. 2005) have also been isolated from Petroleum contaminated sites.

The dominance of the hydrocarbon degrading microbial population in petroleum-contaminated ecosystems signifies their importance in the removal of organic pollutants in such environments. It has been reported that hydrocarbon-degrading microbes interact, directly or indirectly, with each other and non-hydrocarbon degraders in the communities as well as the environmental factors in the petroleum degrading system (Head et al. 2006). This is elaborated in figure 1.2. A good example is the production of biosurfactants by microbes such as certain strains of *Rhodococcus*, *Bacillus* and *Pseudomonas* species, which emulsify the petroleum hydrocarbons, thereby reducing the surface tension of hydrocarbons and subsequently improving the bioavailability the hydrocarbon compounds for easy accessibility to members of the microbial communities. The importance of biosurfactant producers in bioremediation processes has been well documented (Iwabuchi et al. 2000; Ron and Rosenberg 2002; Gudiña et al. 2013; Ronald M. Atlas 1995; Colores et al. 2000). Another interactive relationship observed by Kanaly et al (2002) involves the role played by *Rhodanobacter* isolated from a soil contaminated with benzo[a]pyrene. The microbe was observed to be able to grow on the metabolites produced by other members of the consortium and could not thrive individually in the presence of the hydrocarbon. Apart from interactive mutualistic cooperation, other ecological interactions that could be observed amongst members of the microbial community in a contaminated ecosystem may include competition for limiting nutrients, predation by protozoa and lysis by phage (See figure 1.2) (Head et al., 2006). In addition, all these microbes interact with the environment as well, hence, environmental factors

such as the physicochemical properties as well as chemical properties of the environment could contribute to biodegradation in the environment.

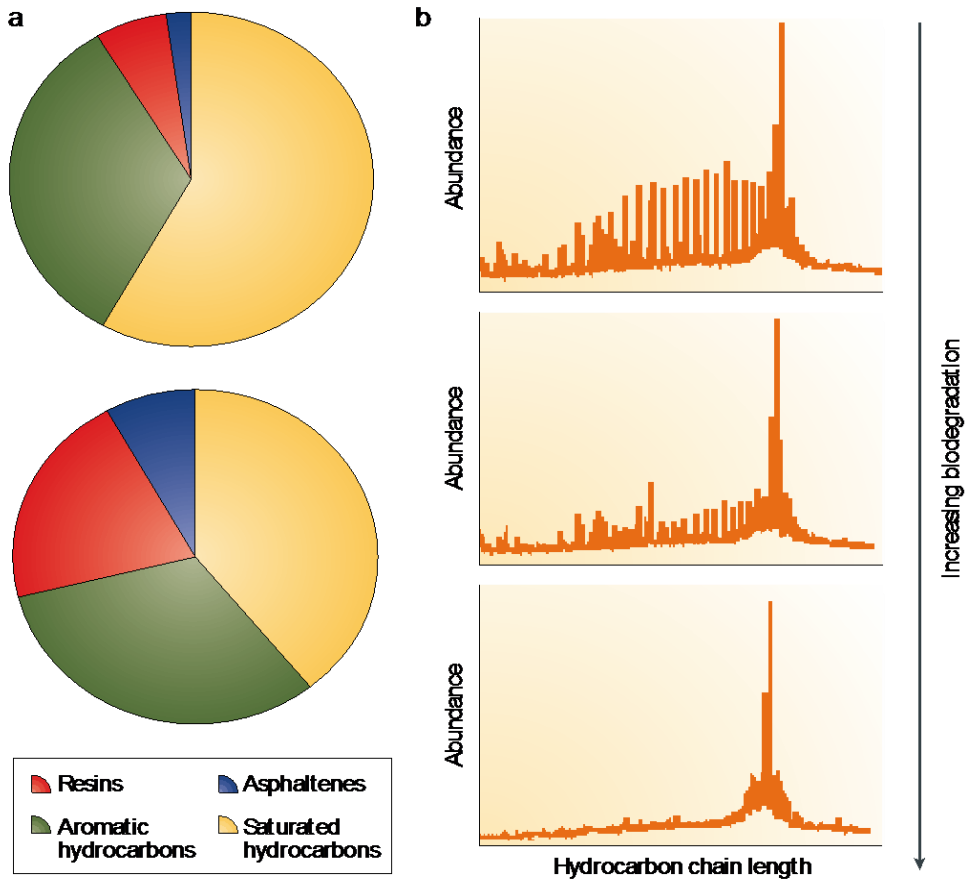


Figure 1.1: The effects of aerobic biodegradation on oil composition. a) Composition of a light North Sea crude oil (top panel) and slightly biodegraded (heavy) oil (bottom panel). The resins and asphaltenes are a complex mixtures of polar compounds. The degraded oil is characterized as being slightly biodegraded on the basis of its detailed molecular composition. Most resolvable saturated hydrocarbons have been biodegraded, as have the non-cyclic terpenoids, pristane and phytane. The cyclic terpenoids, however, are intact, and only the two- and three-ring aromatic hydrocarbons have been extensively degraded. b) Gas-chromatogram traces showing separation of the components of a whole oil that is increasingly biodegraded (from top to bottom). The main peaks that are lost are the resolvable saturated hydrocarbons. The large peaks on the right that do not decrease with biodegradation are the internal standards that are added to the oil before analysis for quantification of individual components of the oil.
Source: (Head et al. 2006)

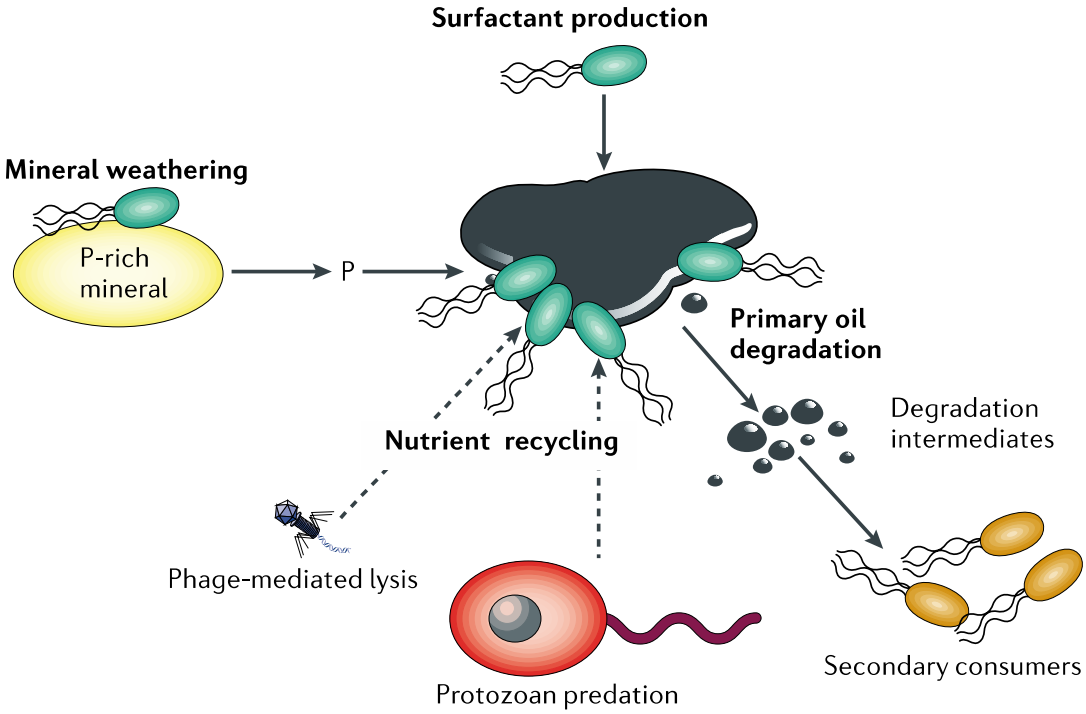


Figure 1.2: A microbial degradation network. The network indicates that oil biodegradation involves more biological components than just the microorganisms that directly attack the oil (the primary oil degraders) and shows that the primary oil degraders interact with these components. Oil-degrading bacteria are shown in green. Solid arrows indicate material fluxes, and broken arrows indicate direct interactions (for example, lysis by phage and predation by protozoa). For simplicity, only one function is assigned to a microorganism in this schema. However, it should be noted that a microorganism can have more than one function or ability (for example, to weather minerals to release phosphate (P), and to degrade oil). It should also be noted that primary oil degraders need to compete with other microorganisms for limiting nutrients (such as P) and that non-oil-degrading microorganisms (shown in yellow) can be affected by metabolites and other compounds that are released by oil-degrading bacteria and vice versa
Source: (Head et al. 2006)

1.13. The metabolic pathway of aerobic hydrocarbon degradation

Due to its complex nature, the degradation of petroleum is a complex process that employs a series of enzymes systems. The mechanisms and enzyme systems may vary according to the organism and petroleum component. However, the general degradation pathway for petroleum hydrocarbon degradation involves initial oxidation, which triggers the series of the various hydrocarbon conversions to Acetyl-CoA. Acetyl-CoA is then channelled to the Kreb's Cycle for energy generation (see figure 1.3).

The biodegradation of n-alkanes in bacteria starts with an initial terminal oxidation of the hydrocarbon by the alkane hydroxylase system to primary alcohols (Rojo 2010; Singh and Kumari 2012; Atlas 1981; Fritsche and Hofrichter 2008; Atlas and Bragg 2009; Atlas and Hazen 2011; Ronald M. Atlas 1995; Atlas and Atlas 1991; Atlas 1991; R.M. Atlas 1995). The primary alcohols are subsequently converted to the corresponding aldehyde, which is then converted to fatty acids (see figure 1.4). In addition contrast to this terminal oxidation mechanism, alkane degradation in some organisms may involve the initial sub-terminal oxidation of an alkane to a secondary alcohol (see figure 1.4) (Rojo 2010; Singh and Kumari 2012; Atlas 1981; Fritsche and Hofrichter 2008). Subsequently, the secondary alcohol is converted to a ketone, followed by oxidation of the ketone to an ester, a reaction catalysed by the Baeyer-Villiger monooxygenase. The ester is subsequently metabolised by esterase to primary alcohol and then to fatty acids. Generally, fatty acids are metabolised to Acetyl CoA through theβ-oxidation pathway and subsequently converted to CO_2 through the Kreb's-cycle (Rojo 2010; Singh and Kumari 2012; Atlas 1981; Fritsche and Hofrichter 2008). The metabolism of fatty acids may involve terminal ω-hydroxylation at the ω-position of the terminal methyl group resulting in the formation of dicarboxylic acid and subsequent conversion to Acetyl-CoA by β-oxidation (Atlas 1981).

Branched alkanes are more complex than the straight-chained n-alkanes. Consequently, the degradation of branched alkanes are more difficult and may involve the initial ω- and β-oxidation of hydrocarbon molecule (Kester and Foster 1963; Atlas 1981).

Cyclic alkanes, which are minor constituents of crude petroleum are less susceptible to microbial attack, basically due to the lack of exposed terminal methyl group. The degradation pathway of cyclohexane is illustrated in figure 1.5. Generally, the degradation of cycloalkanes starts with the hydroxylation of cycloalkane by a monooxygenase. This activation step is sequentially followed by the dehydroxylation to a ketone by a dehydrogenase, conversion of the ketone to a lactone by insertion of oxygen into the ring by monooxygenase, hydrolysis of lactone catalysed by hydrolase and dehydrogenation to α- ω-dicarboxylate (Neilson and Allard 2007). The dicarboxylate undergoes further degradation by β-oxidation to acetyl-CoA, which is a

precursor for Kreb's cycle for the complete degradation to CO_2 and H_2O. Substituted or unsubstituted Cycloalkanes are degraded by oxidative and co-oxidative degradation (Atlas 1981). Microbes appear to attack substituted cycloalkanes, especially those with alkane substitutes, more readily than unsubstituted cycloalkanes (Atlas 1981). The substitute groups are first degraded leading to the formation of cyclohexane carboxylic acid or related compound (Atlas 1981).

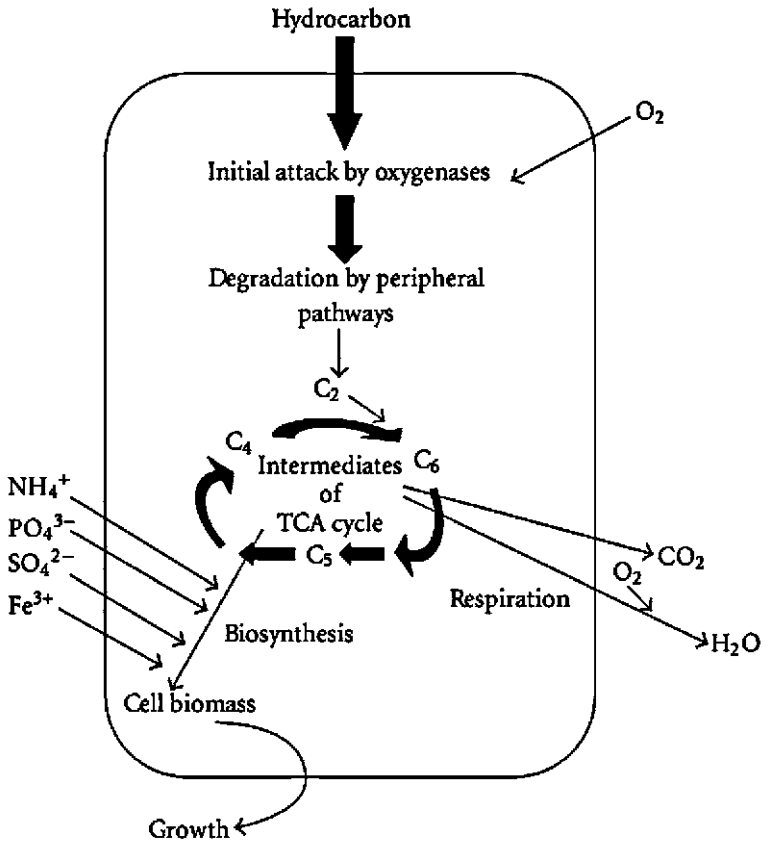


Figure 1.3: The Process of microbial aerobic degradation of n-alkanes
(Source: Singh and Kumari 2012)

Aromatic hydrocarbons are the other major components of petroleum hydrocarbon but they are the most resistant to degradation due to their limited chemical reactivity (Gibson and Chapman 1971; Atlas 1981; Smith 1990; Fuchs et al. 2011). The classical aerobic degradation pathway of aromatics involves the initial attack by oxygenases to form compounds such as Catechol (1, 2-dihydroxybenzene) in bacteria and Protocatechuate (3, 4 -dihydroxybenzoate) in most fungi and some bacteria as central intermediates (Fuchs et al. 2011). Subsequently, central-ring cleavage occurs which is catalysed by ring-cleaving dioxygenases. The cleaved ring compounds are

then further degraded to intermediaries such as acetyl-CoA, succinyl-CoA and pyruvate, which are metabolised to CO_2 or for biomass build up (Fuchs et al. 2011).

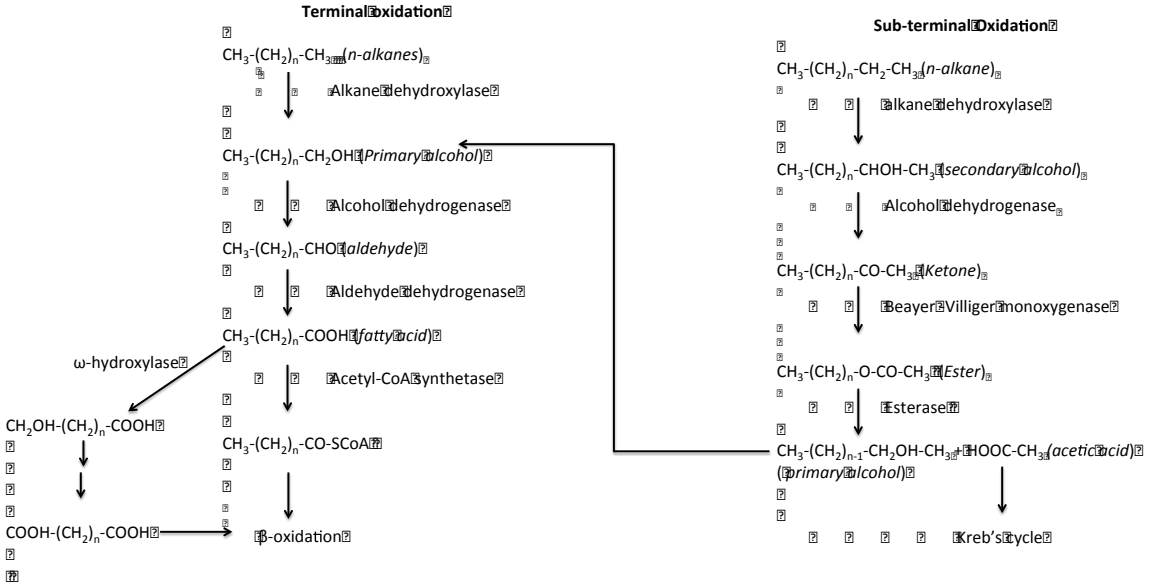


Figure 1.4: pathways for the aerobic microbial degradation of n-alkanes initiated by terminal and sub-terminal oxidation. The arrows indicate the sequence of the biological process.

Biodegradation of petroleum hydrocarbons only occurs if microorganisms possess the appropriate enzyme systems (Atlas 1981; Singh and Kumari 2012; Fritsche and Hofrichter 2008). For the degradation of n-alkanes, depending on the carbon chain length, enzymes belonging to different families catalyse the initial hydroxylation (Rojo 2010; Singh and Kumari 2012). Microorganisms degrading short-chain-length alkanes (C_2–C_4) have enzymes related to methane monooxygenases, such as the butane monooxygenase found in *Pseudomonas butanovora* (Arp 1999; Rojo 2010) and propane monooxygenase in *Gordonia sp.* TY-5 (Kotani et al. 2003; Rojo 2010). Strains degrading medium-chain-length alkanes (C_5–C_{11}), or long-chain-length alkanes (>C_{12}), frequently contain integral membrane non-heme iron monooxygenases related to those (*AlkB* alkane hydroxylase) found in the well-characterized hydrocarbon degrader *Pseudomonas putida* GPo1. For instance, *AlkMa* and *AlkMb are* found in *Acinetobacter* sp. M1 (Tani et al. 2001; Rojo 2010). Some strains contain alkane hydroxylating enzymes that belong to a family of soluble cytochrome P-450s and that are active against C_5–C_{11} alkanes (Singh and Kumari 2012; Rojo 2010). Recently, n-alkane hydroxylases that can catalyse the

hydroxylation of long-chain n-alkanes and which are not related to the ones mentioned above have been identified and are currently being characterised (Rojo 2010). An example of

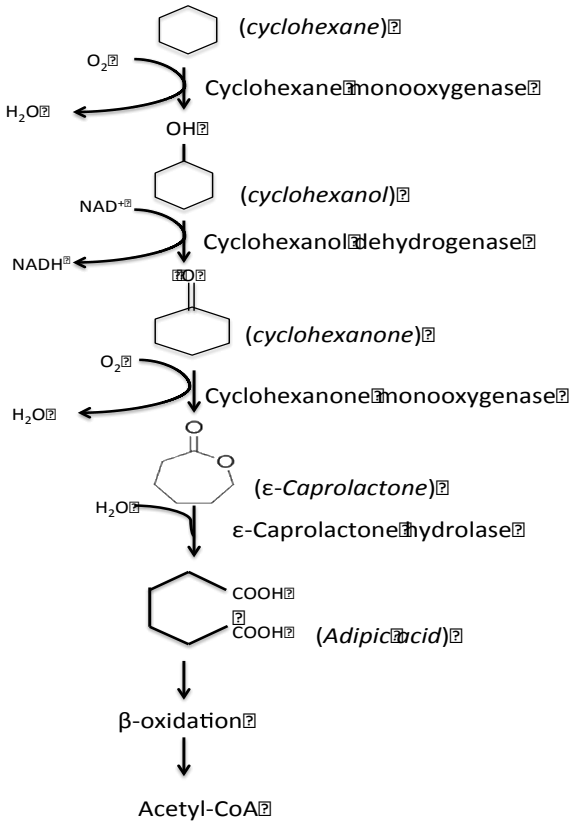


Figure 1.5: the metabolic pathway for the aerobic degradation of cyclohexane

such is the flavin binding monooxygenase, which was identified in long-chain n-alkane assimilating *Acinetobacter* strain DSM 17874 (Throne-Holst et al. 2007). *Acinetobacter* strain DSM 17874 was able to assimilate n-alkanes C_{20}-C_{32} (Throne-Holst et al. 2007). Interestingly, while some alkane degrading microbes possess one hydroxylase enzyme system e.g. GPo1 AlkB in *P. putida*, others possess multiple alkane hydroxylase enzyme systems, e. g in *Acinetobacter* M1, three enzyme systems, *AlkMa* and *AlkMb* that hydrolyses n-alkanes and a dioxygenase which hydrolyses long chain n-alkanes C10-C30 (Rojo 2010; Maeng et al. 1996). As described above, the alcohol generated from the terminal oxidation of these n-alkanes is then converted to aldehydes (or ketones in the case of sub-terminal oxidation) by alcohol dehydrogenases (ADHs) (Rojo 2010; Singh and Kumari 2012; Atlas 1981). There are NADP+ dependent (Rojo 2010; M E Singer and Finnerty 1985; M. E. Singer and Finnerty 1985; Singer et al. 1985) and independent ADHs (Vangnai and Arp 2001; Sayavedra-Soto et al. 2001; Vangnai, Sayavedra-Soto, et al. 2002; Vangnai, Arp, et al. 2002; Rojo 2010). The NADP+ dependent ADHs use NADP+ as an electron acceptor,

while NADP+ independent ADHs depend on other electron acceptors such as cytochromes and ubiquinones and most contain pyrroloquinoline quinone (PQQ) as the prosthetic group (Rojo 2010). The aldehydes generated are usually metabolised to fatty acids by aldehyde dehydrogenases.

1.14. Metals - Microorganisms interaction

Metals are ubiquitous in nature existing in different environments including the lithosphere, hydrosphere and atmosphere. Due to this ubiquity, metals and microbes interact with each other. While some metals are essential to microbial metabolic activities, others are classified as toxic. Microbes cannot degrade metals but they can biotransform them as survival mechanisms in metal-rich environments. Biotransformation processes often involve a variety of redox reactions (Borch et al. 2010; Dynes et al. 2006; Boult et al. 2006; Gadd 2004). These mechanisms affect and are affected by important redox-driven biogeochemical cycles such as the C-, N- , P- and S-cycles (Gadd 2004). For instance: the oxidation and reduction of Fe by Fe-oxidising and reducing bacteria, the oxidation and reduction of Mn by Mn-oxidising and reducing bacteria, sulphate reduction by sulphate reducing bacteria and sulphur oxidation by sulphur oxidising bacteria (Gadd, 2004). These transformations could influence the speciation (Duarte et al. 2008; Dynes et al. 2006), mobility (Gadd 2004; Boult et al. 2006), solubility (Gadd, 2004), bioavailability and toxicity of metals in the environment (Gadd, 2010). As a consequence, metals are either mobilised or immobilised. Mobilisation of metals can be achieved by protonation, chelation, and chemical transformation while immobilisation can occur by precipitation or crystallisation of insoluble organic or inorganic compounds or by sorption, uptake and intracellular sequestration (Gadd 2004). The importance of studying these interactions between microbes and metals has been acknowledged and there has been growing awareness of the natural process. In addition, the concept of metal microbial interaction has been incorporated into industrial strategies such as mining, remediation, and waste treatment e.t.c.

The interaction between metal and microbes depends largely on the environmental factors such as metal-binding agents in the environment and presence of free ions. The pH of the environment determines the ability of heavy metals to engagement in redox processes. Hence, pH largely influences the redox potential of metals. Metals are usually in their free ionic form in acidic conditions and can easily interact with

microbes. Most times, this interaction could result in a greater toxic effect of metals on the microbes (The mechanism of metal toxicity to microbes is discussed in the next section). However, at high pH, the precipitation of such metals as oxides or hydroxides occurs. In this mineral form, metal bioavailability to microbes and mobility is reduced. The influence of pH on microbial metal interaction is applied in biomining activities, which involves the leaching of metals such as Fe in acidic condition facilitated by acidophilic microbes such as *Sulfolobus* and the oxidation of metals equally facilitated by bacteria such as *Acidithiobacillus ferroxidans* (Brune and Bayer 2012; Rawlings and Johnson 2007; Valenzuela et al. 2006; Jerez 2009). Furthermore, certain components of the environment bind strongly to metals immobilising the metals. For instance, in soil environments, organic materials such as Humic, acid and fulvic acid bind strongly to heavy metals reducing metal interaction with the biosphere including microbes (Gadd and Griffiths 1977). In addition to organics, clays such as Kaolinite and Montmorillonite also absorb heavy metals thereby reducing the bioavailability to microbes. Binding agents also include chelating agents such as EDTA, citrates and amino acids such as cysteine and glutamate (Gadd and Griffiths 1977; Borch et al. 2010; Dynes et al. 2006; Poirier et al. 2013; Harrison et al. 2007; Lemire et al. 2013). The secretion of amino-acids by microbes to immobilise metals is a mechanism of survival in a metal rich environment (Nies 2003; Dynes et al. 2006; Huang and Liu 2013; Boult et al. 2006; Lemire et al. 2013; Gadd 2004) and this is discussed in the next section. Sometimes, metals bound to organics are not entirely inaccessible to microbes. For instance, siderophores, which are organic chelates that have high affinity to Fe2+ facilitate the uptake of Fe2+ by microbes. Fe2+ is one of the essential metals for microbial metabolism, as a cofactor for enzymes. Sometimes, transportation of toxic metals, such as Cd, is facilitated (Lemire et al. 2013; Nies 2003; Gadd and Griffiths 1977) by such chelation reactions. Apart from pH and binding agents, the free cations and anions in the environment can influence metal microbes interaction. The presence of anions such as OH-, PO42-, S2O32-, HCO3- and CO32- facilitate the immobilisation of metals by precipitation, e.g. the immobilisation of uranium through the formation of phosphates (Jerden et al. 2003)

1.15. Mechanism of Metal toxicity to microbes

Most heavy metals have no essential positive purpose to microbial metabolic activities. However, certain metals are known to be essential for microbial

metabolism. Some of the essential metals include Mn, Fe, Co, Zn, Ni, Mo, V, Cr. Li and B may be essential (Hughes and Poole, 1989, Bruins et al., 1999). It should be noted here that Ni listed here as being essential is one of those heavy metals which is found in oil and which is investigated in this study (See chapter 4). These heavy metals although essential, could, however, become toxic at high concentration. As, Ag, Cd, Hg, Pb, Ti, Al, Sn are considered non-essential and toxic to microbiological activities (Hughes and Poole, 1989, Bruins et al., 1999). The essential and nonessential metals interact with each other and other essential cell components by chemical bonding, primarily ionic and covalent bonding (Bruins et al., 1999). At toxic concentration levels, metals could cause protein dysfunction, damage to the cell membrane and even DNA damage (Lemire et al; 2013(Bruins et al. 2000).

Usually, metal toxicity involves the accumulation of non-essential metals in the cells. Non-essential metals are usually similar in terms of their basic chemistry of the essential metals and thus, behave similarly. Most heavy metals exist in a divalent form and have ionic diameters of between 138 and 160 pm (Nies, 1999). Due to this close similarity, non-essential metals are transported into the cells using the same transport systems as the essential nutrients. These systems are described as 'open gate' systems due to their non-specificity for ions (Nies 1999; Nies 2003; Nies 2000; Nies 1992; Gadd 2010; Bruins et al. 2000; Lemire et al. 2013; Harrison et al. 2007). Metals are either transported directly or indirectly into the cells. The direct transportation of metals engages the transport systems that do not require the alteration of metal cations. There is the energy independent General Bacterial Porin (GBP) transport system, which involves the direct transportation of metals in their free state across the cell envelopes (Nies 1999). It has been documented that non-essential metals are transported in certain bacterial strains and *Saccharomyces cerevisiae* by this transport system too. Furthermore, energy-dependent transport systems such as Zrt-and Irt-like protein (ZIP), Natural resistance associated macrophage protein (NRAMP), the ATP- binding Cassette (ABC)- type protein family and P-type ATPase transport families have been implicated in the bioaccumulation of non-essential metals in microbial (Nies, 1999, Nies 2000). The indirect transport systems involve the transport of metals bound to low-molecular-mass ligands. Metals are usually co-transported binding to molecules such as organic acids, peptides, phosphates, amino acids, siderophores e.tc. (Lemire et al. 2013; Nies 2003; Grass et al. 2005; Anton et al. 1999; Harrison et al. 2007). Some of the

transport systems are: the citrate-dependent FeCA transporter system, phosphates dependent transporters such as Pho84 in Saccharomyces cerevisiae (Fristedt et al. 1999; Bun-Ya et al. 1991) and PitA in E. coli (Harris et al. 2001; Beard et al. 2000).

Once the metals are transported into the cells, they incur toxicity resulting in cell malfunction, growth inhibition or even death (Lemire et al. 2013). Mechanisms of metal toxicity have been proposed. One mechanism of toxicity of metals to microbial cells is to incur oxidative stress by causing increased levels of reactive oxygen species (ROS) such as H_2O_2 and superoxides (O^{2-}) in cells. ROS are produced in cells through the process of autoxidation. Autoxidation is the transfer of electrons from a reduced enzyme or cofactor to oxygen. This reaction is normally encountered in aerobic environments. Obligate and facultative microbes possess the enzyme systems; Catalase and superoxide dismutase; that eliminates ROS in cells. However, the excessive production of ROS due to the excessive presence of electrons from donors may lead to excessive production of ROS due to the fact that the rate of production may supersede the elimination rate. This, therefore, incurs oxidative stress on the cell. Essential metals, e.g. Ni and Fe, can be important in dealing with oxidative stress at low concentrations as cofactors for the enzymes catalase and superoxide dismutase but at high concentrations, these metals are available to engage in Fenton chemistry where they may lead to oxidative stress. Also, non-essential metals such as Cd and Pb have been reported to instigate oxidative stress in cells (Lushchak 2001; Cao et al. 2012; Srivastava et al. 2014; Cabiscol et al. 2000; Dimkpa et al. 2009; Bussche and Soares 2011; Lushchak 2011; Lemire et al. 2013; Macomber and Hausinger 2011). Excessive ROS due to metals has been implicated in microbial cell DNA, protein and lipid damage (Lemire et al. 2013). Possibly, ROS accumulates in cells due to direct catalysis of autoxidation by metals, by indirect effect whereby certain metals disrupt the cellular donor ligand that coordinates Fe leading to the release of Fe into the cell cytoplasm thereby triggering off autoxidation or by the oxidation of thiols resulting in protein malfunction (Cabiscol et al. 2000; Harrison et al. 2007; Lemire et al. 2013). The oxidation of thiols, as a result of metal poisoning, results to the favourable covalent binding of proteins with metals and the formation of disulphides. The consequence is the depletion of the antioxidants pool in the cell, particularly glutathione, and therefore increased ROS. Metals such as Cd, Cr, and As have been reported to exert toxicity by this mechanism (Cao et al. 2012; Srivastava et al. 2014;

Lemire et al. 2013; Bussche and Soares 2011; Carcinogens 2011; Harrison et al. 2007).

Another mechanism of metal toxicity involves the substitution of essential metals in protein structures with non-essential metals resulting in the destruction of the protein's biological function. Certain proteins, metalloproteins, depend on essential metals such as Fe, Zn and Co for their structures and functions. The replacement of these metals could lead to the inactivation of proteins. For instance, the inactivation of the Fe-dependent enzymes (Fe-S clusters) called the Fe-S enzymes have been reported to occur due to displacement by metals such as Ag, Hg, Cd, Co, Ni and Pb (Harrison et al. 2007; Lemire et al. 2013; Gadd and Griffiths 1977; Gadd 2004; Gadd 2010; Giller et al. 1998). In addition, the displacement of Zn in δ-aminolevulinic acid dehydratase (ALAD) by Pb resulted in the inhibition of the enzyme activity. Another example is Ni disposition of Zn in the glycolytic enzyme, Frutose-6-bisphosphate aldolase, which leads to the inactivation of the enzyme (Lemire et al. 2013). Other mechanisms of metal toxicity include the disruption of electron transport chains and interference with cell membrane function (Lemire et al. 2013; Harrison et al. 2007). Certain metals such as Ag instigate the syphoning of protons from the electron transport chain. As a consequence, the proton motive force (PMF) is disturbed subsequently resulting in the destruction of the cells electron transport chain. Also, metals interfere with the assimilation of nutrients, a primary function of the cell membrane, by interfering with the transportation systems (Lemire et al. 2013; Harrison et al. 2007). Metals competitively occupy the binding sites resulting in an inhibition of uptakes of essential nutrients such as sulphate -and cell starvation (Lemire et al. 2013).

1.16. Mechanism of metal resistance

Metal toxicity to microbes has been widely acknowledged. However, certain microbes develop adaptation mechanisms to overcome toxicity effects on cells thereby resisting metal stress in environments containing heavy metals at toxic levels. Some of these mechanisms include the development of permeability barriers, active transport systems, intracellular sequestration, extracellular sequestration, enzymatic detoxification and metal sensitivity reduction (Bruins et al., 1999).

Microbes develop permeability barrier by altering the cell envelopes to exclude toxic heavy metals thereby protecting metal-sensitive cellular components. For instance, Almárcegui et al. (2014) documented the adjustment of cellular proteins, Porins (channels through which nutrients are diffused into the cells) in *Acidithiobacillus ferroxidans*. This minimised the stress impact of Cu on the bacteria. The reduction in the expression of the gene that encodes Porin production in the presence of Cu, OMP40 was the evidence given that Porin production alteration was responsible for Cu resistance. Some bacteria produce extracellular polysaccharide (EPS) coatings to exclude toxic metals. The metals are usually adsorbed to the EPS layer. Cd adsorption by encapsulated strains of *Klebsiella aerogens*, *Pseudomonas putida* and *Arthrobacter viscosus* have been documented (Bruins et al., 1999). In addition, the EPS-producing strain of *Pseudomonas sp* was observed to adsorb Cd and Pb (Huang and Liu 2013). Another observation was made by (Deb et al. 2013) in encapsulated cd resistant strain of *P. stutzeri* where cd accumulated on the polysaccharide coated cell surface. Also, soil bacteria isolates were observed to accumulate Hg on the EPS coated cell envelope (François et al. 2012). In addition, there has been evidence of metal accumulation within the periplasmic region of the cell envelope. For instance, (Choudhary and Sar 2009) identified a metal resistant strain of a *Pseudomonas* isolated from a uranium mine to exclude Ni, Co, Cu and Cd by accumulating the metals within the periplasmic region.

Apart from cell envelope adjustment, microbes develop active transport systems, which extrude toxic metals that were previously transported into the cytoplasm. These transport systems are efflux pumps. Efflux systems are highly specific for metals, are ATP or proton motive force (PMF)-dependent and are encoded on either plasmids or the chromosome. The microbial resistance of Cd by efflux systems has been studied extensively. A good example of such an efflux system is the ATP- dependent cad-systems in Cd-resistant bacteria (Bruins et al, 1999). The cad-System consists of plasmid encoded proteins cadA- and cadC-proteins. The cadC proteins sort Cd and present the cation to cadA for exportation out of the cell before the toxic metal could interact with the sensitive essential cellular component. The process of extrusion starts when Cd attaches to the cysteine residue of cadC protein, which then facilitates the metals attachment to cadA proteins. The formation of cadA-Cd complex initiates the attachment of ATP to cadA and subsequent phosphorylation. This enables the extrusion of the metal across the membrane. Another efflux system that is capable of

Cd extrusion from cells is the czc –efflux system. This is PMF-dependent. The czc-efflux system was first identified in *Alcaligenes eutrophus* (Nies, 1992; bruins et al., 1999).

Recently, a czc-efflux system was observed in *Acidothiocacillus ferroxidans* (Chen et al. 2014). Other efflux systems responsible for the putative extrusion of other metals include: a P-type ATPase efflux system (NmtA) which excluded Ni and Co in the *Actinobacterium*, *Streptomycetes coelicolor* (Kim et al. 2015); the DmeRF system was responsible for Ni and Co exclusions from cells of *Rhizobium leguminosarum bv. Viciae*; the resistant nodulation cell division (RND) efflux systems in Gram-negative bacteria such as in *E. coli* responsible for resistance to Cu and Ag (Mealman et al. 2012; Su et al. 2011; Delmar et al. 2013); the CopA system that extrudes Cu in *Metellosphaera sedula*, an extreme thermoacidophilic *Archeae* (McCarthy et al. 2014) and so many other efflux systems. Figure 1.6 shows the transport systems by which metals are transported into microbial cell membranes into the cytoplasm and extruded out of the cell.

Furthermore, certain microorganisms develop resistance to toxic metals by sequestration of metals within the cell cytoplasm (intracellular sequestration). When toxic metal are transported into the cell, cytoplasmic proteins (the metallothioneines (MTs) scavenge the metals preventing their binding to essential cell components. MTs have been identified in many prokaryotic organisms including *Cyanobacteria*, *Pseudomonads*, *α-Proteobacteria*, *γ-proteobacteria*, and *Firmicutes* (Blindauer 2011). Some of the binding proteins include: the SmtA proteins that were observed in the metal resistant strains of the cyanobacteria *Synechococcus* (Blindauer 2011; Olafson 1984) which sequester Cd, Cu, Hg and Zn (Blindauer 2011; Nies 1992); the CdBP1 protein from *Pseudomonas putida* that sequestered Cd and Cu; the MymT protein in *Mycobacterium sp* that binds to Cu (Blindauer 2011; Rowland and Niederweis 2012). Also, on exposure to toxic concentrations of Fe, bacteria such as *Mycobacterium tuberculosis* secrete the *Bacterioferrins*, which sequester excess Fe (Rodriguez 2006).

Apart from intracellular sequestration, microorganisms secrete organic compounds and inorganic compounds for extracellular sequestration of toxic metals thereby preventing the metal uptake by cells. The binding of metals to these chelating molecules transforms the size and chemical features of the metals resulting in reduced intake through the non-specific porins (Schalk et al. 2011). For instance, siderophores,

organic compounds that usually bind to Fe, have been identified to be secreted by certain bacteria for the purpose of preventing to uptake of toxic metals (Schalk et al. 2011; Lemire et al. 2013). Pb-resistant strain of *Bacillus sp*, MN34, associated with promoting the hyperaccumulation in *Alnus firma*, produced siderosphores to sequester

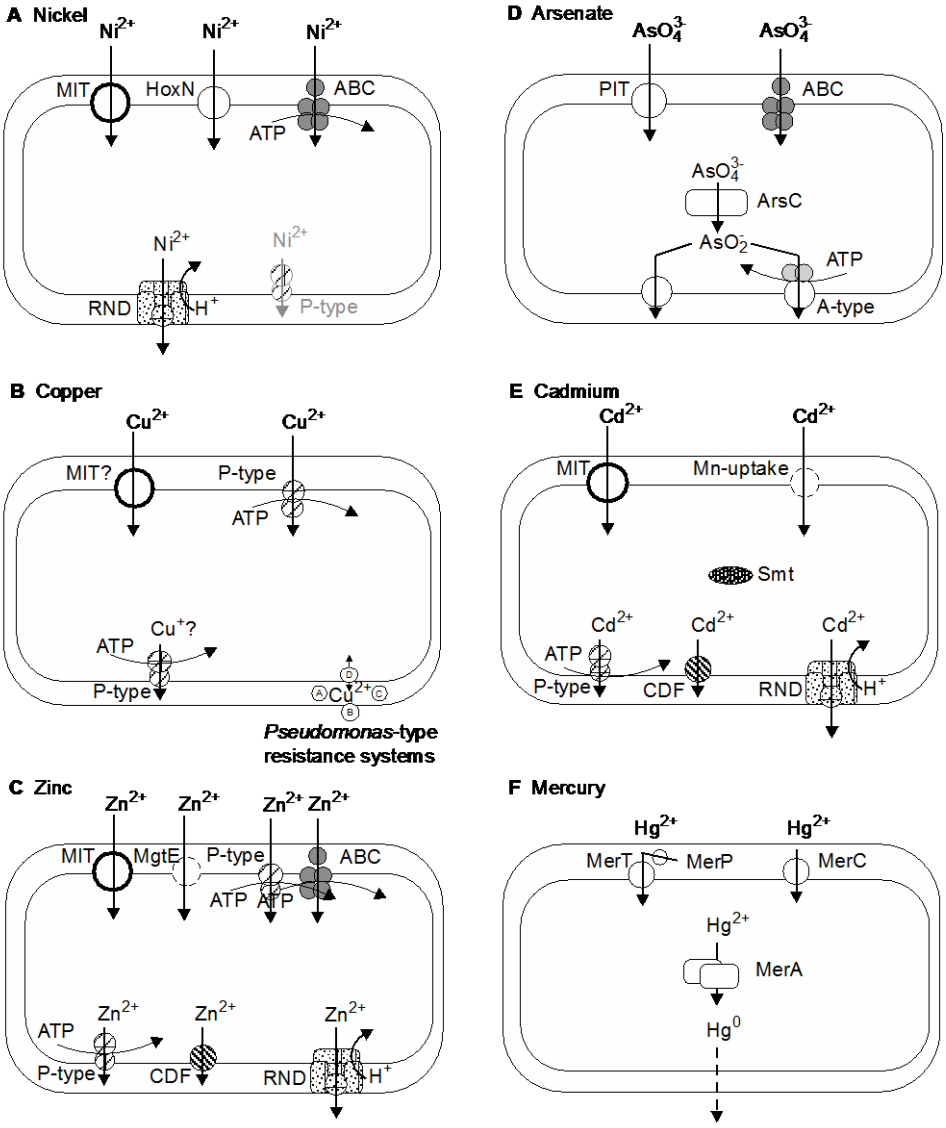


Figure 1.6: Protein families involved in bacterial heavy-metal metabolism. Figures A-F indicate the energy dependent specific transportation systems for Ni, Cu, Zn, As, Cd and Hg respectively. *(source:* (Nies 1999)

Pb (Shin et al. 2012). Also, Pyoverdine (PvdI), a major siderophore secreted by *Pseudomonas aeruginosa* PAOI to bind Fe, was observed to chelate other metals including Ag, Al, Cd, Co, Cu, Hg, Mn, Ni and Zn (Braud et al. 2009). In addition, certain microbes secret thiol-rich (-SH) compounds in response to metal stress. For

example, *Acidithiobacillus ferroxidans* secreted the amino acids with –SH groups, namely, cysteine and glutathione in response to Cd stress (Zheng et al. 2015). Apart from organic compounds, microbes also secret inorganic compounds such as sulphides and phosphates to precipitate metals. For instance, Siripornadulsil and Siripornadulsil (2013) reported the secretion of sulphides and -SH-rich compounds by Cd-resistant soil bacteria in metal contaminated rice field, which formed the insoluble form of Cd and prevented the uptake of the metal by both rice plants and bacteria.

Other mechanisms of resistance include: the transformation of metals to non-toxic forms e.g. the transformation of Mercuric (Hg^{2+}) to metallic Mercury (Hg^{0}) catalysed by the Hg-reductase (MerA) secreted by microorganisms (Simbahan et al. 2005; Møller et al. 2011; Freedman et al. 2012); cell aggregation resulting in the formation of biofilm by microbes (Harrison et al. 2007; Poirier et al. 2013); metabolic bypass to prevent the effect by ROS (Poirier et al. 2013; Nies 1999; Bruins et al. 1999).

Chapter 2

Methods

2.1 Sampling Sites Description

Soil samples were obtained from two different sites in this study: the Nafferton Ecological group farm and the former Lead Works site, St. Anthony's Newcastle upon Tyne. Soil samples for Nafferton farms were used to determine the effect of Ni (see chapter 4), Cd (see chapter 5) and Pb (short-term contamination; see chapter 6) on biodegradation whereas St. Anthony's soils were used for the determination of the effect of long-term Pb contamination on crude oil biodegradation (see chapter 6).

Nafferton farm occupies approximately 294 ha land managed by the Nafferton ecological farming group (Newcastle University). It is located on the north side of the Tyne valley approximately 24 km west of Newcastle upon Tyne (54°59'08.6"N 1°53'56.2"W). The farm practices livestock rearing and crop cultivation practices. The crop cultivation involves organic and conventional farm. The soil used for this project was obtained from a fallow plot within the organic section of the farm. The soil of Nafferton farm has been described as loamy clay soil by the Cranfield Soil and AgriFood Institute; technically classified as the Cambic Stagnogley (Avery, 1980; (Orr et al. 2012; Orr et al. 2011). This means that it is a uniform clay loam formed in slowly permeable Devensian glacial till deposits of the Brickfield series (Avery, 1980). This soil has a pH of 7.13±0.03.

The choice of an agricultural soil is based on the idea that it is valuable agricultural soils, which are subject to contamination in places like the Niger delta, Nigeria

The second sampling site is the former St. Anthony Lead Works located approximately 7 km south-east of the Newcastle City Centre by the Tyne River (54°57'40.0"N 1°33'15.1"W). It is a site already heavily contaminated with lead and other heavy metals. Established in the 1840's, St. Anthony's works activities included processing lead-silver ores from Spain into the white and red lead as well as silver. It was closed down in the 1940s. Newcastle City council landscaped the site in the 1960s such that the ground was covered with topsoil, which was subsequently planted with trees, shrubs and grasses transforming the site to a recreational ground for public use. Presently, it is a part of the Riverside Park, Walker, Newcastle upon Tyne. A study of soils from this site revealed the presence of high concentration of the heavy metals Pb, As, Cd, Ni, Zn, Cu, Cr and

Mo (in order of magnitude) which were significantly above the recommended Soil Guideline values (SGVs) (Okorie et al. 2011; Okorie et al. 2012; Okorie 2010). Further studies on the oral bioavailability of these Potentially Toxic Elements (PTEs) were discovered to be within the range of 21- 96% with the values of Pb and As being considered as a pose to significant possibility of significant Harm (Okorie et al. 2011; Okorie et al. 2012; Okorie 2010). The soils from this site are loamy-clayey soil with pH of 7.1±0.06 at lower Pb contaminated site and 7.83±0.18 at high Pb contaminated site.

2.2 Soil Sample collection and Handling Methods

Sampling at Nafferton farm was conducted twice during the period of the project; on the 27th of July 2012 and on the 23rd of August 2013. Sampling at the Former St. Anthony's lead works was conducted once on the 23rd of August 2013. The period of sampling on these days was between 9am and 12pm. Soil samples were collected based on the guidelines of the international Standard organisation (ISO) and the British Standard Institution (BSI) for collection, handling and storage of soil under aerobic conditions for assessment of microbiological processing biomass and diversity in the laboratory (ISO 10381-6, 2009).

Triplicate samples were collected from both sampling sites by clearing vegetation covering the soil before digging into the ground to about 10cm3 depth using a clean hand garden trowel. The soil was collected in clean plastic sampling bags and safely transported immediately back to the laboratory for processing. In the Laboratory, the spatially obtained samples were pooled into a composite bulk sample and samples were stored away at 4oC and were used within 2 weeks after sampling. A proportion of soil was stored at -20oC for DNA analysis. Prior to use, soil samples were conditioned to room temperature and air-dried by spreading evenly on a plastic tray before being passed through a 2mm sieve. Passing the soils through the sieve facilitated gaseous exchange hence maintaining the desired aerobic condition.

Sieved soils were rewetted (adjusting the soil moisture to 60% of the water holding capacity of soil) and incubated at room temperature for 7days to stabilise the soil environment (Ouyang and Li 2013; Riepert and Felgentreu 2002; Van Gestel et al. 1993; Edition 2006)

Caution was taken to minimise physical contact with the soil; hence, gloves were worn on sampling site and in the laboratory during sampling handling.

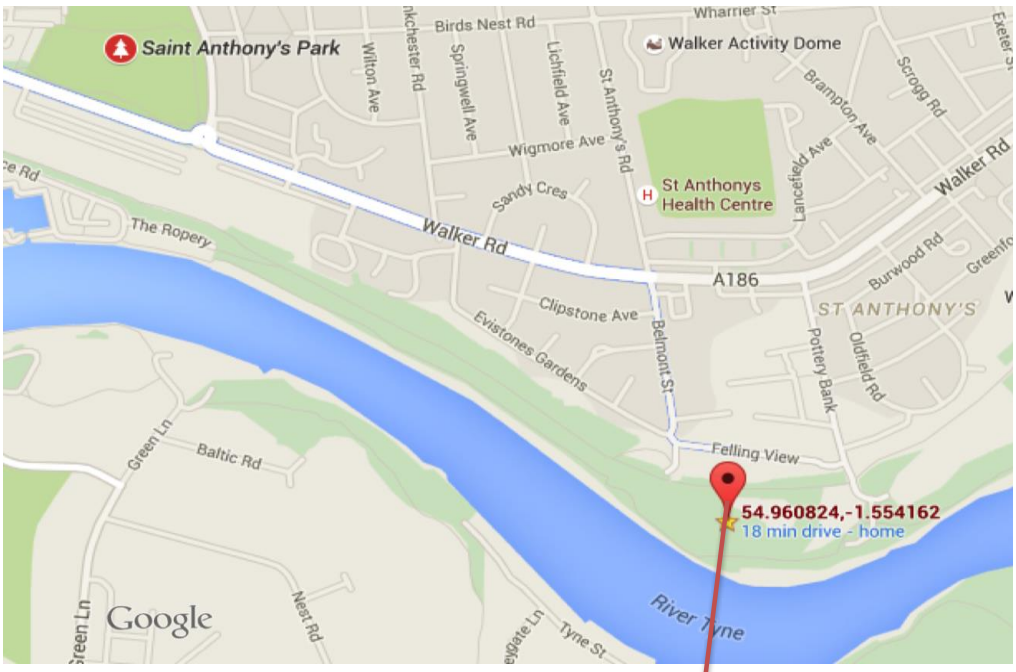
2.3 Soil Preliminary Analysis

Baseline Physicochemical parameters of the soil samples were determined. These parameters included soil moisture content, soil pH, soil water holding capacity, Soil total organic carbon and Soil heavy metal content

2.3.1 Soil pH

Soil pH is the measure of alkalinity and acidity of soil and it is an important parameter that is considered in bioremediation processes. Its importance in petroleum degradation has been documented in the past studies (Dibble and Bartha 1979; Leahy and Colwell 1990; Thapa et al. 2012)

Figure 2.1: location of the first soil sampling site, Nafferton ecological farm (a) a google map showing the location of Nafferton farm; (b) a schematic map of Nafferton ecological farm (curtsey of Nafferton ecological group. Sampling site was located at plot 14 of the organic part of the farm. The plot was fallowed at the time of sampling and is a part of rotation system for the cultivation of wheat.



a

b

Figure 2.2: Google map showing location of the second soil sampling site, the former St. Anthony lead works, walker, Newcastle upon Tyne, the site is contaminated with Pb and other heavy metals. (a) A map showing the location of the sample collection; (b) the map of the site showing the concentrations of Pb in ppm. See appendix II for the enlarged version of st. Anthony work's map.

In this research, the soil pH was determined using the ISO 10390-2005. This is a standard developed for the determination of soil pH using a glass electrode.

Pretreated soils (see section 2.2) were suspended in in deionized water in triplicate samples 1:5 weight by weight. The mixtures were shaken thoroughly for 1hr using a shaker and were left to equilibrate overnight. The pH of the soil suspension was measured using the 3020-pH metre (Jenway Ltd UK) that was calibrated with standard solutions of pH 4 and 7.

2.3.2 Soil moisture content

Soil moisture content was determined using the mass based gravimetric method outlined in ISO 7755-3.1-1994 (ISO, 1994). Replicate of 10g of pretreated soil sample was dried to a constant mass at a temperature of 105oC in an oven (Memmert, Germany). The moisture content was determined by using the equation below

Equation 2.1

$$\textbf{Moisture content} = \frac{M_1 - M_2}{M_2 - M_0} \times 100$$

Where

M_1 =mass of crucible plus soil before drying

M_2 =mass of crucible + soil after drying to constant mass

M_0 =mass of crucible (i. e. the tare)

2.3.3. Soil water holding capacity

In this study, the soil water holding capacity (W_{H2O}) was determined in triplicate using the specifications in ISO 14238-2013 (ISO 2012). A perforated cylinder, with a filter paper (Whatmann filter paper, No1) covered base, was partially filled with pretreated soil (see section 2.2) and partially submerged in water for 2 hours at room temperature. After 2 hours, the cylinder was

completely immersed in water for 1 hr and then drained on a tray of wet fine quartz sand for 2hrs. The soil was then dried to a constant mass at 105^{o}C in an oven (Memmert, Germany). The weights were measured to determine the W_{H2O} using the equation 2.2.

Equation 2.2

$$W_{H2O} = \frac{m_S - m_T - m_D}{m_D} X 100$$

Where

m_S= the mass of water-saturated soil plus the mass of the tube plus the mass of the Filter paper, in grams;

m_T= the mass of tube plus mass of filter paper, in grams;

m_D= dry mass of soil in grams

2.3.4 Soil total organic carbon

The determination of total organic carbon (TOC) of the soils was carried out by Derwentside Environmental Testing Services (DETS) Durham, UK. DETS in-house method DETSC 2084 was used for this analysis. Soil samples were pretreated with 5% phosphoric acid to destroy the inorganic carbon by the consistent addition of 1ml of 5% H_3PO_4 to (by dry weight) of soil sample (homogenised and wetted) until no effervescence was observed. Total carbon was determined by combustion of the pretreated soil and detection of CO_2 by infrared method during combustion.

2.3.5 Soil heavy metal content

The heavy metal content of the soils was carried out by Derwentside Environmental Testing Services (DETS) Durham, UK. Soil samples were analysed for heavy metals including Hg, As, Cd, Cr, Cu, Ni, Pb, Se, V, Zn and B. First, samples were dried and crushed to pass through a 425μm sieve based on BS 1377 ((BS) 1990). Then, to determine the metals, in-house documented

methods based on the Standing Committee of Analysts methods were used (UK Environmental Agency 2006; DEFRA 2009). To test for heavy metals in soils (Apart from Hg and B), Aqua regia digestion was carried out based on specifications in BS EN 13657:2002 (Standard 2002). Aqua regia digestion involves the digestion of sample using aqua regia reagent which is basically a mixture of 65% to 70% Nitric Acid (HNO_3) and 35% to 37% Hydrochloric Acid (HCl) in the ratio of 1:3 volume by volume respectively. Apart from Hg and B, heavy metals were determined using inductively coupled plasma optical emission spectroscopy (ICP-OES). For Hg testing, soil samples are purged with argon, the elemental mercury is collected on a silica-gold vapour trap and the collected elemental mercury analysed by atomic fluorescence spectroscopy. Quantification is performed by comparison to a generated calibration curve. The analysis of B was carried out using colorimetric method (Spencer and Erdmann 1979). First, B was extracted by hot water method. This is followed by the addition of the colorimetric reagent Azomethine-H and detection using the spectrophotometric method.

2.4 Experimental design

In brief, Soil microcosms were set up to simulate heavy metal-petroleum co-contaminated soils and were incubated for approximately 2 weeks. Within the incubation period, the microbial activity, which measures CO_2 production in each microcosm, was determined to ascertain biodegradation of petroleum. Microbial activity is as a result of complete mineralisation of soil organics to CO_2 and H_2O. In aerobic soils, the process involves the oxidation of organic materials coupled to the reduction of molecular oxygen to release carbon for biomass and energy conservation while releasing CO_2. After the incubation period, the geochemical analysis was carried out to determine the extent and pattern of biodegradation solvent-solvent extraction, solid phase extraction and Gas Chromatography.

Also, microbiological analysis to determine the microbial phylogeny and microbial diversity of soils using molecular microbiological methods including Polymerase Chain Reaction (PCR), Agarose Gel Electrophoresis (AGE), Denaturing Gradient Gel Electrophoresis (DGGE) and Ion Torrent-Personal Genomic Machine (Ion Torrent PGM) sequencing. Real time-qPCR was used to enumerate soil microbes by targeting 16S rRNA gene specific for

taxonomic groups. The design of Primer sets, the validation of qPCR assay and the enumeration of target genes are described in the next Chapter.

2. 4. 1 Soil Microcosm

Metal-crude oil co-contaminated soil environment was simulated in soil microcosms. All, soil microcosms were set up in 150ml amber serum bottles such that there was increasing concentrations of different mineral forms heavy metals (see 2.4). Primarily, each microcosm consists of 10g of soil, 100 mg of undegraded medium gravity North Sea crude oil (obtained from British Petroleum) and appropriate concentration and mineral form of heavy metals. Inorganic nutrient in the form of sodium nitrate and potassium dihydrogen phosphate were added such that there were C: N: P ratio of 100: 10: 1 weight by weight. The moisture of the soil was adjusted to the 60% of the soil's water holding capacity. The microcosms were incubated in a dark room at room temperature for 14-16 days.

This project investigates the effects of Ni, Cd and Pb. The Heavy metal concentrations and mineral form varied depending on different heavy metals. In choosing the metal concentration the UK environmental agency's soil guideline values (SGV) in allotment areas, Dutch list recommendation values and the documented metal concentrations in soils associated with the history of Petroleum spill were considered. Presently, the SGV of Pb is presently being reconsidered by UK environmental agency. However, previous SGV was considered in this project. Values below and above the considered guideline for each metal were used in this project. Metals were added in soluble and insoluble mineral forms. The Cd was added as $CdCl_2$ and CdO while Pb was added in the form of $PbCl_2$ and PbS. As for Ni, three mineral forms were used. This included NiO, $NiCl_2$ and Ni-porphyrin, the form in which Ni exist in the soil, was also investigated. Table 2.1 shows the variations of metal concentrations, metal Mineral forms the SGVs and Dutch-Lists intervention

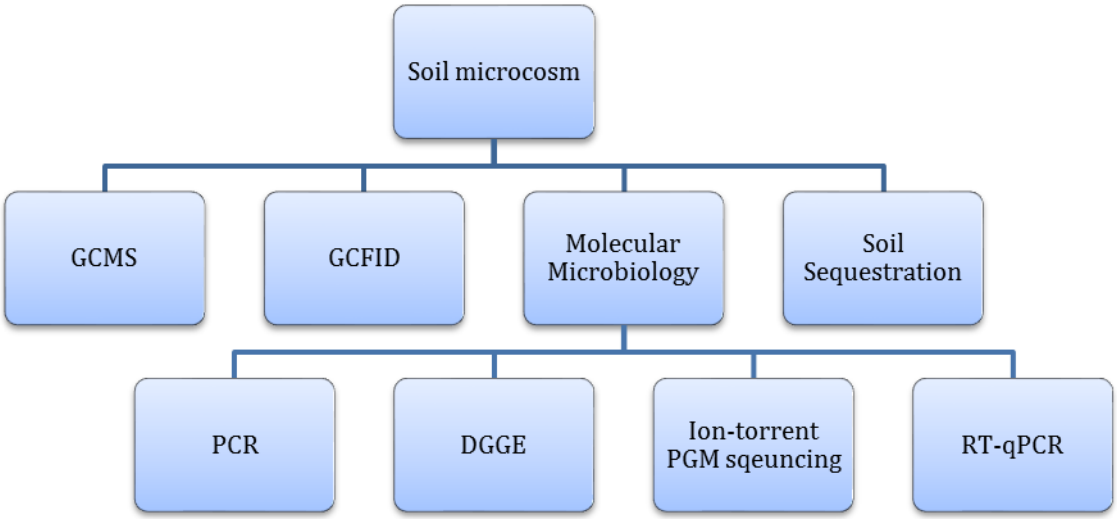


Figure 2.3: diagrammatic representation of the project's experimental design. The methods used in this project include geochemical techniques and molecular microbiological techniques.

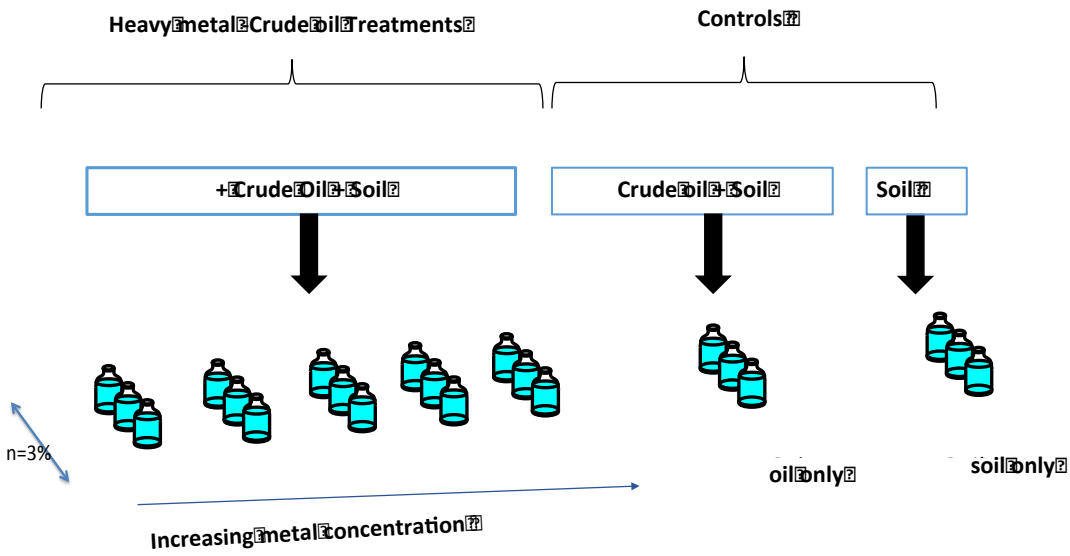


Figure 2.4: A diagrammatic representation of soil microcosm set up. Each heavy metals studied in this project have a triplicate of 5 concentration levels and different mineral forms.

Table 2.1: forms and concentrations of metals studied to determine metal effects on biodegradation of petroleum hydrocarbon in soil and metal UK environmental agency's soil guideline value and Dutch list recommendation values

Metals	Mineral forms	[1]Concentrations (ppm)	[2]SGV (ppm)	[3]Dutchlist value (ppm)
Ni	$NiCl_2$, NiO, Ni-Porph	12.5, 25, 50, 200, 350	230	210
Cd	$CdCl_2$, CdO	0.1, 1.0, 10, 100, 1000	1.8	12
Pb	$PbCl_2$, PbS	150, 1500, 15000	*530	530
[4]historically Pb Contaminated soil	High Pb soil	12866.67		
	Lower Pb soil	580		

1. *Concentration levels pertain to each metal form*
2. *UK environmental agency's Soil guideline values for allotment areas*
3. *Dutch list values representing action values*
4. *Historically contaminated Pb soils from former Anthony works, Newcastle; the concentrations are the average value of triplicate samples*

2.5. Soil Microbial Activities

In this project, the soil microbial activity was measured by determining CO_2 flux in the headspace of experimental soil microcosms. Specifically, CO_2 production was used to assess the extent of hydrocarbon degradation by comparison of CO_2 production rates and total yields in oil-amended and unamended soil controls. Biodegradation of the petroleum was monitored in the soil microcosms by analysing the headspace gas composition using a Gas Chromatography-Mass Spectroscopy (GC-MS) method (a modified version of international standard organisation's guideline for the determination of microbial soil respiration using GC ISO 16072 2002). Headspace CO_2 was analysed using the GC-MS. 100 µl of headspace gases were manually injected into GC-MS system, Fision MD800 (electron voltage 70 eV, filament current 4 A, source current 800 µA, source temperature 200 °C, multiplier voltage 500 V, interface temperature 150 °C), using a gas-tight syringe (SGE Analytical, Australia). The gas sample was separated in the system using the HP-PLOT Q

capillary column (30 m X 0.32 m) with 20-µm Q phase. The carrier gas in this system is the Helium gas (1ml/min, 65kpa, split at 100m/min, 35oC). Xcalibur software was used to determine the data. Headspace gases were determined using parent ion at mass to charge ratio (m/z) of 44. Chromatographic peaks of m/z 44 were integrated and quantified and saved as excel file for further data processing. In addition, calibration curves were determined by using different volumes of mixed gas with 10% CO_2. The slopes of the calibration curves were determined and the R2 values of curves determined were above 0.99. The slope was used to determine the cumulative CO_2 in microcosms. Cumulative CO_2 values were calculated and expressed in MolCO_2*g-1 dry soil, using the ideal gas equation (PV=nRT) modified to resolve into two component terms i.e. mass of CO_2 and molecular weight CO_2. The rate of CO_2 Flux was calculated from the linear accumulation of CO_2 and expressed in milliMol CO_2 g-1 dry soil day-1 as well.

To ensure that oxic conditions were maintained in microcosms, the Oxygen, O_2, content were monitored simultaneously. Integrating and quantifying chromatographic peak of m/z 32 determined the O2 in headspace gases. O2 of the headspace gases was replaced with atmospheric air when oxygen dropped below15%.

The differences in cumulative CO_2 production and rate of microbial activities in microcosms were assessed statistically using the Analysis of Variance (ANOVA) coupled with multiple comparison analyses by Tukey's and Fisher's methods and Regression analysis in MINITAB EXPRESS software and SPSS.

2.6 Compositional changes in petroleum hydrocarbon

The total n-alkanes were determined in the experimental microcosms after 15 days of incubation. First, hydrocarbons were extracted from the soils using solvent-solvent extraction method coupled with the solid phase extraction the method developed by (Bennett et al. 2007). Finally, the total n-alkanes were determined using the Gas Chromatography (GC). The methods used are discussed in this section.

2.6.1 Solvent- Solvent extraction

250 ul of the standard solution of squalane (~200mg / L representing ~0.5% of the initial added crude oil was dispensed into the soil microcosm bottles. Here squalane acts as a surrogate extraction standard. 10ul each of the solvents Methanol, Toluene and Dichloromethane (DCM) were added to the microcosms sequentially to extract soil extractable organic components. The microcosms were vigorously agitated and emptied unto a Buchner filtration system for subsequent extraction by exerting pressure. The Buchner filtration system consists of Buchner perforated filter, Whatman filter paper, grade 1 (Sigma-Aldrich, UK) and Buchner filter flask connected to a laboratory water tap. While still under pressure, 10ml each of methanol, toluene and DCM were used to rinse the soil twice. The filtrate /extract was loaded into 500 ml separating funnels for further extraction. 30 ml each of methanol and DCM was sequentially used to rinse the Buchner flask and the rinse was dispensed into the separating funnel. Also, 50 ml of deionized water was added to the separating funnel. The content of the separating funnel was manually and continuously agitated for 5 minutes. The funnel was set to stand for 1 hr using a tripod stand to separate the liquids. After 1 hr, a mixture of organic solvents and extractable organics, which settles at the lower layer of the separating funnel, was eluted into a round bottom flask. Organics remaining in the upper layer was extracted, 3 times, by adding 30 ml each of DCM and deionized water and agitating for 5 minutes. Separation occurred for 15 minutes and organics were, again, eluted into the round bottom flask. As stated earlier, the liquid collected in the round bottom flask consists of a mixture of organic solvents and extractable organics. To remove the solvents, the liquid in the round bottom flask was rotary evaporated to ~5 ml. Subsequently, the liquid was transferred to a clean 10 ml vial and blown down to dryness under a gentle stream of Nitrogen gas.

2.6.2. Solid Phase extraction

Hydrocarbon and the polar non-hydrocarbon fraction of the Extractable organic matter were isolated independently using the Solid Phase extraction (SPE) Method. Generally, SPE involves the isolation based on the partitioning of analyte into a solid and a liquid medium. Compounds to be isolated adsorb to the solid matrix (Sorbent) base on the intermolecular forces between the

analyte, the liquid phase and the active sites on the sorbent. The isolates are then eluted with the appropriate solvents.

The SPE method developed by Bennett et al (2007) was adapted for this project. The sorbent used in this method was a non-endcapped octadecyl (C_{18}) silicate. This is silica, which is bonded to the hydrophobic methyl group (in this case octadecyl-). This is important in retaining the polar compounds allowing isolation of petroleum fractions. The concept of non-endcapped C_{18} is based on the fact that the non-polar compounds interact with the hydrophobic sorbent by van-der-Waal forces and are easily eluted with a non-polar solvent. In contrast, the polar or moderately compounds are bound to the –OH group on the silica surface and are eluted later with a polar solvent. Hexane was used to elute the non-polar fractions of the Extractable Organic Matter while DCM was used to elute the Polar fraction. To achieve this, SPE column, Isolute C_{18} (Biotage, UK) was prepared by rinsing with DCM. This was left for approximately 24 hrs or until DCM has evaporated. Subsequently, the column was conditioned by rinsing with 10 ml of Hexane. 10 ul of internal standard, Heptadeclycyclohexane (at a concentration of ~1mg/ ml), was added to 50% of EOM. This was transferred to the conditioned SPE column and was eluted with 5 ml hexane. The eluate, which consists of the non-polar hydrocarbon fraction, was collected in a clean 10 ml vial. Subsequently, the polar compounds were eluted with DCM in a clean 10 ml Vial. 0.5ml of the non-polar fraction was transferred to a Gas Chromatography –Flame Ionisation Detector auto-sampler vial for analysis. The GC-FID system used was the Hewlett- Packard 5890 series II with FID, connected to a computer. The GC-FID analysis enables the identification n-Alkanes and determination of the total n-alkanes by the identification and integration of peaks. The quantification of recovered hydrocarbons was calculated by using the equations 2.3, 2.4 and 2.5.

Equation 2.3

Relative response factor (RRF) =

$$\frac{\text{Peak area of Surrogate standard (SS)}}{\text{Peak of internal standard (IS)}} \times \frac{\text{weight of IS}}{\text{weight of SS}}$$

Equation 2.4

Percentage recovery =

$$\frac{\text{Peak area of SS}}{\text{IS peak Area}} \times \frac{\text{Weight of IS} \times 100}{\text{RFF} \times \text{weight of SS} \times \text{fraction}}$$

Equation 2.5

$$\text{Weight of analyte} = \frac{\text{Peak area of Analyte} \times 100}{(\text{Peak Area of IS}) \times (\%\text{Recovery})}$$

2.7 Heavy metal partitioning in petroleum soil

Determination of the fate of added metals in the soil provides an understanding of the mobility and toxicity of these metals and thus an understanding of the various observed effects on microbial activities in the soil microcosm experiments detailed above. To achieve this, the chemical associations of added heavy metals in the soils were determined using the sequential extraction method of Tessier et al. (1979). This method involves a sequential partitioning of metals into five main fractions – exchangeable fractions, fractions bound to carbonates, fractions bound to oxides of Fe and Mn, fractions bound up into organic matter and residuals.

Triplicate soil samples for this experiment were prepared by adding known concentrations of metals into soils. The soils were dried overnight at room temperature. 1g of the dry soil was weighed into 50ml centrifuge tube and sequentially extraction of metals commenced by adding appropriate reagents (see table 2.2), which selectively extract the heavy metals bound to appropriate fractions. The metals investigated were Cd, Ni and Pb in soluble and insoluble forms. The mineral forms of metals were $CdCl_2$, CdO, $NiCl_2$, NiO, $PbCl_2$ and PbS.

The exchangeable are the metal fractions that are usually the free cation in the soil water solution plus those that are loosely bound/adsorbed to soil surfaces at the exchangeable sites. They are the most mobile, in other words, they are the most readily available fraction of soil metals. A change in the ionic composition soil water affects the sorption and desorption cations. Hence,

usually, salt solutions are used to leach the metal fractions from the solid matrix (Zimmerman and Weindorf 2010). In this experiment, exchangeable were extracted using 10 ml of magnesium chloride ($MgCl_2$) for 1 hr. at neutral pH.

Table 2.2: Experimental design for the sequential extraction of heavy metal from soils

Fraction	Reagents	Volume (ml)	Extraction duration
Exchangeable	1M Magnesium Chloride ($MgCl_2$) (pH 7)	8	1hr
Bound to Cabonates	1M sodium acetate solution (NaOAc) (pH 5 adjusted with Acetic Acid (HOAc))	8	6hrs
Bound to Fe and Mn Oxides	0.04M NH2OH.HCl in 25% v/v HOAc	20	6hrs
Bound to organics	i) 0.02M HNO3 + ii) 30% v/v H_2O_2 (pH 2; 85±2°C)	3 5	2hrs
	iii) 30% v/v H_2O_2 (pH 2; 85±2°C)		
	iv) 3.2M NH_4OAc in 20% v/v HNO_3 +	3	3hrs
	v) Deionized water	5	
		Dilute to total volume of 20	30minutes

The metal fractions associated with carbonates are otherwise known as the acid soluble fractions. Usually, the metal fraction is susceptible to pH. The increase in pH enhances the fixation of metals to carbonates through the incorporation of cations in carbonate unit cells (Zimmerman and Weindorf 2010; Plassard et al. 2000). Therefore, to dissociate cations, an acidic condition is essential. In the present experiment, carbonate associates were leached at acidic pH of 5 using an acidic salt solution of sodium acetate. pH was adjusted to 5 using acetic acid. Continuous agitation was maintained for 6hrs.

The oxides of Fe and Mn are known to act as concretions between soil or sediment particles or as simple coatings on particle surfaces and are excellent cations scavengers (Tessier et al. 1979). The metals bound to Fe- and Mn-oxides are affected by redox condition. Under anoxic/reduced conditions, metals bound to these oxides are released but are immediately precipitated as sulfides (Zimmerman and Weindorf 2010). Therefore, extraction of the metal fractions requires solutions capable of dissolving insoluble sulfide salts. In this experiment, Metal fraction was extracted with 20 mL of 0.04 M NH_2OH-HCl in 25% (V/V) HOAc. Extraction was performed at 96± 3 ^oC with occasional agitation for a duration of 6 hrs.

Usually, metals in the soil bind to soil organic forms (Zimmerman and Weindorf 2010; Gadd 2004; Gadd and Griffiths 1977; Mclean and Bledsoe 1992; Ankley et al. 1994) such as living cells/ organisms, detritus, organic acids such as humic and citric acids, small molecular weight organic compounds such amino acids and proteins, soil organic contaminants such as petroleum hydrocarbon and PAHs e. t. c. Metals bound to Organic Matter were extracted using 3 mL of 0.02 M $HN0_3$ and 5 mL of 30% H_2O_2 adjusted to pH 2 with $HN0_3$, and the mixture was heated to 85 ± 2 ^oC for 2 hr. duration with occasional agitation. A second 3-ml aliquot of 30% H_20_2 (pH 2 with H_2SO_4) was then added and the sample was heated again to 65 ± 2 ^oC for 3 hr. duration within which periodic agitation was carried out. After cooling, 5 ml of 3.2 M NH_4OAc in 20% (v/v) $HN0_3$ was added and the sample was diluted to 20 ml and agitated continuously for 30 min. The addition of NH_4OAc is designed to prevent adsorption of extracted metals onto the oxidized sediment.

Residual fractions consist of metals embedded in the crystal structure of the primary and secondary minerals. The metals are not easily dissociated. Hence, strong acids such as HF-$HClO_4$ (Tessier et al. 1979) are usually employed to extract metals. However, in this experiment, residual fractions were not extracted due to safety considerations. Therefore, residual fractions' concentrations were assumed to be the metal concentrations that were not accounted for in the other fractions.

Separation was performed by centrifugation (Sorvall, model RC 5B Plus) at

10000 rpm (12000 g) for 30 min between successive extractions. The supernatant was removed with a pipet and analysed for trace metals by Atomic Absorption spectrometry. The residue was washed with 8 ml of deionized water. After a repeated centrifugation for 30 min, supernatant was discarded.

2.8. Soil Microbiology

2.8.1 DNA extraction

Genomic DNA was extracted from ~0.25 g of soil samples from each microcosms using the PowerSoil ® DNA Isolation Kit (MOBIO Laboratories, USA) in triplicate according to the manufacturer's protocol. Procedural blanks were also carried out. These provided the template for the microbiological studies. The end volume of the extract was 100 µl. The DNA isolation procedure comprises of a combination mechanical and chemical methods. First, bead breaking using the ribolyser, FastPrep-24 instrument(MP biomedicals, USA) for 30 secs combined with chemical cell disruption using sodium dodecyl sulphate (a detergent) were employed to lyse soil microbial cells. This was followed by a series of chemical purification, which involved the precipitation, and removal of non-DNA material (organic and inorganic. Eventually, silica membrane was used to selectively isolate DNA in concentrated salt solution (NaCl). Subsequently, the bound DNA was washed with ethanol to remove residual salts and other impurities. DNA was eluted through the membrane using elution buffer, which is basically sterile salt-free 10mM Tris solution. The credibility of the DNA extractions was determined by checking the DNA extracts and the Procedural blanks using Agarose gel electrophoresis (AGE; see section 2.8.3).

2.8.2. Polymerase chain reaction (PCR)

DNA extracts from the original bulk soils, soil microcosms and procedural blanks were subjected to PCR amplification targeting the highly conserved 16S rRNA gene specific for bacteria and Archaea. For DGGE analysis (discussed in the next session), the V3 region of the 16S rRNA gene was targeted using primer 2 (Vr) and primer 3 (Vf) with a GC-clamp (Muyzer et al. 1993; Sherry et al. 2013). These primers anneal to positions 518-534 and 341-

357 of the 16S rRNA gene based on E. coli 16S ribosomal RNA gene numbering (Brosius et al. 1978) at 53^{o}C. The sequence for Vf with the GC

Clamp (in bracket) was 5'-
(CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGC
ACGGGGG) GCCTACGGGAGGCAGCAG-3'. Also, the sequence of Vr primer was 5'-ATTACCGCGGCTGCTGG-3'. To conduct PCR, 47 µl Mega Mix-Blue (Microzone Limited, UK) reagent, 1ul soil genomic DNA extract and 1ul of each primer (at a concentration of 10 pmol per µl) were dispensed into a clean molecular biology grade 200 µl Eppendorf tubes (StarLab, UK). Amplifications were carried out using an automated thermal cycler Techne TC-5000 (Bibby Scientific, UK). The amplification reaction included 1 min initial denaturation at 95oC followed by 24 cycles of 30 sec denaturation at 95^{o}C, 1 min initial annealing at 65oC but reduced by 1^{o}C after every 2 cycles and 1 min extension 72^{o}C. This is followed by 15 cycles with the annealing temperature of 53^{o}C and 10 min final extension at 72^{o}C. DNA samples were amplified in triplicate. Procedural blanks were carried out as well.

In addition, Amplicons were generated for ion torrent / PGM sequencing and different 16S rRNA primer sets were employed to generate amplicons. Usually, amplicon library is constructed prior to sequencing. Here, fusion primer set that compliment the V4 and V5 region of 16S rRNA genes was used.

This primer set was F515-R926 and have been used in previous studies (Quince et al. 2011). The sequence of the primers included 5'-GTGNCAGCMGCCGCGGTAA-3' for F515 and 5'-CCGYCAATTYMTTTRAGTTT-3' for R926. Usually, for ion torrent amplicon library construction, the forward primers are ligated PGM linker primer/ adapter, a Golay barcode (unique for each sample DNA, hence, distinguishing DNA samples) and a barcode spacer, which corrects sequencing errors (Hamady et al. 2008; Whiteley et al. 2012) at the 5' end of the primer. These are presented in table 2.3 Also, the revers primer was ligated to truncated P1 (TrP1) adapter at 5' end of the primer and the sequence of this attachment was 5'-ATCACCGACTGCCCATAGAGAGG-3'. The amplicon size is 481 bps.

Table 2.3: the PGM linker primer/ adapter, a Unique Golay barcode DNA and barcode spacer used as Tags for ion torrent sequence forward primer F515.

A ADAPTOR (5'-3')	BARCODE (5'-3')	BARCODE SPACER (5'-3')
5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'	5'-AACGCACGCTAG-3'	5'-GAT-3'
5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'	5'-AACTCGTCGATG-3'	5'-GAT-3'
5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'	5'-AACTGTGCGTAC-3'	5'-GAT-3'
5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'	5'-AAGAGATGTCGA3-'	5'-GAT-3'
5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'	5'-AAGCTGCAGTCG-3'	5'-GAT-3'
5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'	5'-AATCAGTCTCGT-3'	5'-GAT-3'
5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'	5'-AATCGTGACTCG-3'	5'-GAT-3'
5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'	5'-ACACACTATGGC-3'	5'-GAT-3'
5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'	5'-ACACATGTCTAC-3'	5'-GAT-3'
5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'	5'-ACACGAGCCACA-3'	5'-GAT-3'
5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'	5'-ACACGGTGTCTA-3'	5'-GAT-3'
5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'	5'-ACACTAGATCCG-3'	5'-GAT-3'
5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'	5'-ACACTGTTCATG-3'	5'-GAT-3'
5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'	5'-ACAGACCACTCA-3'	5'-GAT-3'
5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'	5'-ACAGAGTCGGCT-3'	5'-GAT-3'
5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'	5'-ACAGCAGTGGTC-3'	5'-GAT-3'
5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'	5'-ACAGCTAGCTTG-3'	5'-GAT-3'

PCR procedure was as described above and by Whiteley et al. (2012) but with little modifications. Bioline's TaqMan DNA amplification kit (Bioline UK) was employed in lieu of mega-mix blue reagent as used above. The reaction conditions were 95 °C for 3 mins initial denaturation followed by 30 cycles of 95 °C 1 min denaturation, 55 °C, 45 secs annealing 72 °C 1 mins (extension) and a final extension at 72°C for 10 mins. The experiment was equally carried out in triplicates. Procedural samples were also carried out simultaneously.

2.8.3. Agarose gel electrophoresis

AGE was used to ascertain the credibility of the genomic DNA after DNA extraction from the soils and to determine that the correct amplicon size was produced by PCR reactions. The products of DNA extraction and PCR were analysed using ethidium bromide (EtBr) (1.6 µl EtBr in 100 ml gel solution) stained 1% Agarose gel (Sigma-Aldrich, UK) prepared with 1X TAE buffer (2 M Tris-Acetate, 0.05 M EDTA, pH 8.3). 5 µl of DNA extract, PCR product and reference DNA size marker, Hyperladder II DNA Marker (Bioline, UK) were loaded into the wells of the cast Agarose gel. Electrophoresis was conducted in 1X TAE buffer at 100 volts for 60 minutes. The gels were visualised by UV illumination using a gel visualising and documentation system, Automated bioSpectrum Imaging system (Ultra-Violet Products (UVP), UK).

2.8.4. Denaturing gradient gel electrophoresis (DGGE)

Microbial community diversity in original soils and soil microcosms was studied using Denaturing Gradient Gel Electrophoresis (DGGE). This method separates the constituent sequences based on their differential mobility in a denaturing gradient polyacrylamide gel. The result is a pattern of bands representing the prominent microbial types present in the sample (Muyzer et al. 1993). This method has been used successfully in the past for microbial community studies (Slatko and Albright 2001; Gray et al. 2011; Nakatsu 2007; Muyzer and Smalla 1998; Muyzer et al. 1993; Sherry et al. 2013; Hubert et al. 2012; Hale et al. 2010). DGGE is limited in the resolution and information it can provide but in the context of these studies, DGGE was used to indicate diversity variation between original soil, treatments, experimental replicates and to identify community selection to focus on the ion torrent sequencing strategy.

INGENYPhorU-2x2 system, which consists of the buffer tank, gradient maker and an electrophoresis cassette, was used to conduct DGGE. The 10% acrylamide gels used consisted of a mixture of 40% BisAcrylamide (Sigma-Aldrich Corporation, USA), 50X TAE buffer, Deionized Formamide (Sigma-

Aldrich Corporation, USA), urea (the denaturant; Sigma-Aldrich Corporation, USA) and deionized water. The solidifying agents, APS and TEMED were also added to the mixtures in the ratio of (100:10) just before gel casting. The denaturing gradient was created on the gel by combining denaturing solutions consisting of 40% denaturant and 70% denaturant in the electrophoresis cassette using a gradient maker-peristaltic pump system. Reagent mixture consisting of 0% denaturant was used as the stack. Wells were created in the gel to accommodate DNA samples. The cast gel was placed in the preheated buffer tank consisting of 1X TAE. Each well on the gel was loaded with a combination of 22 µl PCR product / marker and 22 µl loading buffer. The maker used consisted of 12 cloned 16S rRNA gene fragments. They were loaded in multiple regularly spaced lanes in gels. Electrophoresis was carried out at 100 volts for 16 hours. Gels were stained with a fluorescent dye, the SyBr green (sigma Aldrich, USA) for 30 minutes. The stained gel was visualised by UV illumination using gel visualising and documentation system, Automated bioSpectrum Imaging system (Ultra-Violet Products (UVP), UK)

2.8.5 Statistical Analysis DGGE Gels

The electrophoresis gels obtained from DGGE-fingerprinting were analysed using the BioNumerics software (Applied Math, Belgium). The tools of this software make the normalisation of the gel efficient, allowing for compensation of in travel and internal variations in gradient. This was achieved by inclusion of the same marker profile in all gels. Bands representing different components of the microbial communities in different profiles were detected automatically. Band assignments were then manually checked. Comparative quantification of band patterns was automatically computed within and between gels generating Band presence/absence data. Similarities were measured using the Jaccard coefficients. Data generated were exported from Bionumerics for further statistical analysis of community structure. The Multidimensional scale analysis (MDS) in PRIMER 6 software ((Primer-E Ltd., Plymouth, UK) was performed on the data generated to identify profile relatedness between communities (Clarke and Gorley 2006; Clarke and Warwick 2001). MDS uses an algorithm which successively refines the positions of the points until they satisfy, as closely as possible, the

dissimilarity between samples (Foster 1983) providing a 2 dimensional (2D) graphical representation, where points that are close together represent samples that are very similar in composition. This is achieved by using the Bray-Curtis similarity coefficient to generate similarity matrix. Also, the algorithm generates a 2D stress value, which denotes how accurately the dimensional relationships among samples are represented in the 2D output. Low stress values close to 0 are indicative of excellent representation, values of 0.1 showing good representation and stresses of 0.2 demonstrating potentially useful outputs. However, values close to and exceeding 0.3 indicate that placement of points in 2D space is close to random and should be discounted (Clarke and Warwick, 2001). The MDS plot was overlaid with clusters generated from Cluster Analysis, which was based on CLUSTER method in primer 6. This was performed to identify groups using contours that specify 20, 40, 60, and 80 % similarity (Foster 1983).

Furthermore, pairwise analysis, the Analysis of similarities (ANOSIM), was used to assess discriminations between communities. Here, the statistics, Global R-Value (R), was used to determine the degree of dissimilarities between communities. R-values range from 0 to 1. The closer R is to 0, the more similar the communities. On the contrary, the closer R is to 1, the more the discrimination between replicate communities (Foster 1983; Clarke, KR, Gorley 2006). The significance of R was assessed by generalised randomization based on the Monte Carlo simulation test ((Hope 1968; Clarke and Green 1988) generating the statistics, p-values. P-values were reported as percentages with significant values between 0 to 5 %.

2.8.6. Ion torrent -PGM sequencing of tagged 16S rRNA PCR products

In this project, ion torrent/ personal genome machine (PGM) sequencing method (Life technologies, USA) was applied to sequence 16S rRNA gene, which was subsequently analysed to determine a more detailed diversity and phylogeny of communities. This is to complement the DGGE diversity studies which have its limitations including the possibility of a band representing multiple species or multiple bands representing a single species (Samarajeewa et al. 2014). The stepwise procedure for ion torrent sequencing involves DNA

Library construction, template preparation and detection of incorporated nucleotides. Prepared DNA library was submitted to the in-house environmental engineering research laboratory for sequencing.

2.8.6.1. DNA Library Construction

DNA library was generated by carrying out PCR as described in section 2.8.2 tagged primer set was used for the PCR reaction to distinguish sample DNA. The amplicons were purified using Agencourt Ampure XP purification Kit (Beckman Coulter Ltd, UK). This kit utilises Beckman Coulter's solid-phase reversible immobilisation (SPRI) paramagnetic bead technology for high-throughput purification of PCR amplicons. Here, 90 µl of Agencourt Ampure XP reagent (at room temperature) was added to 50ul amplified DNA sample in a 200 µl PCR tube. The tube was placed on a magnet for 2 minutes to allow the clearing of the solution. The supernatant was discarded. To wash the samples, 30 ul freshly prepared 70% ethanol was added to tubes still on the magnetic and incubated at room temperature for 30 seconds. The supernatant was removed. This was repeated twice. The samples were air dried for ~ 5 minutes. 20 ul PCR-grade water was mixed thoroughly with the samples using a vortex and was left to stand for 1 minute. Cleared supernatant containing the purified amplified DNA was carefully transferred to a clean PCR tube. Purified amplicons were quantified using the high sensitive fluorescence dye based Qubit Fluorometer 2.0 combined with Qubit® dsDNA HS Assay Kit (life technologies) to determine the concentration of double stranded DNA of PCR products. The Qubit® dsDNA HS Assay Kit consist of assay reagent, dilution buffer, and pre-diluted DNA standards. To quantify DNA in samples, working solution was prepared by diluting reagent in a buffer in the ratio of 1: 200 v/v in a clean 500 µl tube. Subsequently, samples and standards were prepared for quantification by diluting 10 µl of each in 190 µl of working solution. These were incubated for 2 minutes at room temperature. Qubit fluorometer was calibrated with the standards prior to the quantification of samples' DNA concentration with the instrument. Concentrations were determined in ng/ml using the calculator system of Qubit fluorometer. Subsequently, an equimolar DNA library, consisting of amplified tagged DNA samples, was generated such that the concentration of the pooled library is 25.3 ng/µl. This was achieved by determining the volume of amplicons per

sample that is equivalent to the 1 ul of the sample having the highest concentration of DNA (see equation 2.6)

$$\text{Volume of X (µl)} = \frac{\text{Highest concentration}}{\text{Concentration of X}} \qquad \textbf{(Equation 2.6)}$$

Where X is the amplicons per sample

2.8.6.2. DNA Template preparation for sequencing

Prior to sequencing, sequencing templates were generated by attaching the DNA samples to ion sphere particles using ion PGMTM Template OT2 400 kit (life Technologies, USA) according to manufacturer's instructions. This Kit consisted of on Sphere™ particles, buffers, PCR reagent and enzyme mixes, consumables, and primers. It amplifies DNA based on 400 bp. This process was carried out in OneTouch™ 2 System. The system consists of the Ion OneTouch™ 2 Instrument (an integration of reaction filter, thermal cycler and micro-centrifuge) and the Ion OneTouch™ ES. 1ul of amplicon library diluted in 24 µl of nuclease-free water was mixed with amplification solution to a volume of 900 µl in a 1.5ml Eppendorf tube. Amplification solution comprised of PCR enzymes, Primers and other reagents. The PCR primers are usually oligonucleotides complementary to the Adapter attached to the fusion primers used for amplicon library construction. 100 µl of ion sphere particles, ISP, was added to the Eppendorf tube containing the amplification solution mixture to a final volume of 1000 µl. This was loaded onto the filter assemble in the One Touch instrument. 2000 µl of reaction oil was sequentially loaded to the filter at the initial volume of 1000 µl and subsequent volume of 500 µl. Emulsion PCR was performed with the appropriate program for ~16 hours. ISP was recovered by centrifugation. The recovered ISP was washed with 500 µl wash solutions. This was centrifuged for 2.5 minutes at 15,500 X g. 100 µl of the supernatant was retained and was enriched in the One TouchTM Enrichment System.

2.8.6.3. Personal Genomic Machine (PGM) sequencing

Enriched ion sphere particles were sequenced using PGM sequencing Platform with Ion PGM TM sequencing 400 kits. PGM sequencing involves the real-time measurement of Hydrogen ion produced during DNA hybridization. The incorporation of nucleotide base results to a release of H+. The kit used

comprised of the reagents required for PGM sequencing of 400 bp run on semi-conductor chips. The reagents include the polymerase, nucleotides, sequencing reagents and sequencing control. First, 5 µl of sequencing control was mixed thoroughly with enriched ion sphere particle. To anneal primer to Ion sphere particles, 15 µl of the ISP- sequencing control mixture was added to 12 µl of primer. The primer was annealed at 37oC for 2 minutes in a lid heated (95 oC for 2 minutes) thermal cycler. After annealing of primer to ISP, 3 µl of polymerase was added to the reaction. This was succeeded by incubation of reaction at room temperature for 5 minutes. The reaction mixture (now consisting of ISP ligated to Primers and polymerase) was loaded on sequencing chip for sequencing. For this project, Ion 316 Tm Chip was used. This chip could recover up to 1 Gigabases and a data of up to 3 million sequences reads. Sequencing was run in the PGM sequencer, which was controlled using the ion Torrent Suite software (Life technologies, USA).

Furthermore, Ion torrent suite software was used to retrieve sequence data in FASTA file format after the sequencing had occurred and after filtering individual sequence reads within the software to remove low quality and polyclonal sequences.

The sequence data obtained was analysed using the QIIME (Caporaso et al. 2010; Kuczynski et al. 2012) and the Mothur software (Schloss et al. 2009) to trim sequence data, cluster sequence data into OTUs, assign taxonomic groups to OTUs and analyse community diversity (alpha- and beta- diversity of communities). Furthermore, using the Sequence Match platform on Ribosomal Database Project database Release 11 (Metzker 2010; Cole et al. 2014) (RDP) and Basic Local Alignment Search Tool databases, close relatives of the most predominant OTUs in communities were determined and their sequences were aligned up to MEGA 6 to obtain a phylogenetic tree (using the neighbour joning method and clustering in boostrap tes 50 replicates), which elaborates relatedness. This is essential in designing specific qPCR primers, which are specific taxonomic groups, required in the enumeration of the taxonomic group of interest. The Design of Primers, validation of qPCR assay and enumeration of target genes are described in the next chapter.

2.8.7. Primer validation and Optimisation of Real time-qPCR assay

In this project, validation of the primer sets involved, sequentially, the determination of optimum annealing temperature, melt curve analysis of amplicons and agarose gel analysis to verify the specificity of amplicon size and the determination of standard curve to ensure that a linear response of the assay in response to a standard dilution series of the target taxon is achieved. Samples of Typed 16S rRNA sequences of representative bacteria of target taxonomic group were obtained commercially (DSMZ, Germany). These included the 16S rRNA sequences samples of *Rhodococcus erythropolis* (DSM 43066) representing the *Rhodococcus sp* enriched in Cd and Ni amended microcosms and Scopulibacillus darangshiesis (DSM 19377) representing the *Bacillales* group that enriched in Pb soils. To prepare the standards, typed 16S rRNA DNA obtained commercially (DSMZ, Germany) were quantified by determining the molecular concentration of DNA using a spectrophotometric method (NanoDrop 1000 model, Thermo Fisher Scientific, USA). The standards were serially diluted to attain dilutions 109, 108, 107, 105 and 104 standards.

The optimum annealing temperatures (T_a) for the individual primer pairs were determine using a thermal gradient of annealing temperatures in parallel PCR amplifications and subsequent melt curve analysis (see below) to determine amplification specificity. To determine the Temperature range for the analysis the estimated T_m of primers were considered and temperatures below and above these estimated T_m values were tested.

The qPCR reagent used was the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, UK), which consists of a mixture of polymerase, dNTPs, MgCl2, buffer and SYBR green. Amplification was performed using a CFX96 Real-Time system, which is a PCR thermal cycler that has thermal gradient feature. CFX96 Real-Time system is operated remotely using the BioRad's CFX Manager Software. PCR reactions occurred in 96- well plates (BioRad UK) consisting of 200 µl wells covered with an optically clear cover (Bio-Rad UK). In each well the reaction mix consisted of 5 µl of SsoAdvanced™ Universal SYBR® Green Supermix, 0.5 µl of reverse and forward primer (concentration of 10 picomoles per primer), 1 µl of PCR-grade water and 3 µl

of template. A range of annealing temperatures was tested for each primer set and temperature range varies between primers but temperatures are within 50 to 70 oC. Each PCR cycle consisted of denaturation, annealing and elongation stages. Initially, the thermocycler Lid was heated to 105 ^{o}C for 3 minutes to activate the polymerase enzyme. This step was followed by 40 cycles of 10 seconds denaturation at 95oC and a combination of 30 seconds annealing at the varied temperature within the range specified. Subsequently, a melt curve analysis was carried out between 55 and 90 ^{o}C at increments of 0.5 ^{o}C at every 30 seconds. Specific amplification is attained when only one peak is present on the melt curve. Conversely, non-specific binding of primers is detected when 2 or more peaks are visualised on the melt curve. The optimal Ta is the temperature, which has the lowest quantification value (C_q value) and specific amplification of a single amplicon peak. C_q-values are that cycle when amplification has reached its threshold; at this cycle, the fluorescence exceeds the background fluorescence, hence, quantification ensues. To further verify that correct amplicon were produced during amplification an agarose gel analysis was also carried out to visually view the amplicon size of PCR product.

A standard curve analysis was carried out to determine the efficiency of amplification across a range of concentrations of the target sequence. To this end, a set of standards made of known concentrations of the target gene and prepared by serial dilution was subjected to qPCR to generate a standard curve. The standard curve is basically the plot of the C_q-values plotted against the log of the standard concentration. The resultant linear equation which can be fitted to the plot, is:

$$Cq = K \text{ Log } (No) + Cq1$$

Where

K=slope

No= initial copies of DNA

Cq1 = interception of the curve

PCR efficiency is calculated from the slope of the curve with the equation

$$E = 10^{-1/k} - 1$$

For an acceptable qPCR assay, amplification efficiency is expected to have values between 80 and 115 % (Callbeck et al., 2014). Furthermore, a correlation coefficient (r^2) of the standard curve above 0.95 is desirable (Callbeck et al., 2014).

Chapter 3

Development and optimization of qPCR assays for Hydrocarbon degrading taxa enriched in the oil degrading experiments

3.1. Introduction

Quantitative PCR (qPCR) was used to determine the absolute bacterial 16S rRNA gene abundances in the experimental microcosms detailed in chapters 4, 5 and 6. Critically, theses abundances were determined in both the original soils and the microcosms variably amended with oil and heavy metals to determine the level of enrichment of total bacterial abundances and specific taxa that had occurred during the incubation period. To achieve this, qPCR assays that targeted the 16S rRNA genes of the bacterial domain or specific genera of major hydrocarbon degraders (identified from ion torrent PGM analysis) were designed and or applied to microcosm DNA extracts.

This chapter describes in detail the steps taken to design the primers, which targeted different OTU sequences, enriched during hydrocarbon degradation and validate the resultant qPCR assays.

3.2. qPCR assay Target

The major hydrocarbon degrader enriched based on OTU sequence frequency in the oil and Ni and the oil and Cd amended microcosms, based on BLAST and RDP sequence match, was closely related (between 97 to 100% similarities) to strains of *Rhodococcus sp* (see chapters 4 and 5). In the same vein, the major OTU enriched in Pb contaminated (both long term and short term Pb contamination) oil-amended soil microcosms was closely related to strains of the order *Bacillales* (100% similarity, see chapter 6). At the genus level, the predominant OTU was 98% similar to *Scopulibacillus darangshiesis* (DSM 19377) and *Anaerobacillus arseniciselenatis* (DSM 15340) and 97% related to strains of varied genera including *Bacillus*, *Anaerobacillus*, *Volcanobacillus, Pullulanibacillus, Salinibacillus, Virgibacillus, Salirhabdus Anoxybacillus, Thalossobacillus, Microaerobacter, Listeria* and *Staphylococcus*. Due to these close relatives of various genera of Bacillales,

primers were designed to target the order *Bacillales* in Pb amended petroleum degraded communities instead of a particular genus.

For both 16S rRNA assay targets, phylogenetic affiliations of target OTUs were determined using the Molecular Evolutionary Genetics Analysis software version 6 (MEGA 6) (Tamura et al. 2013). This analysis (see chapters 4, 5 and 6) compared both closely and more distantly related sequences with the target OTUs by the construction of phylogenetic trees (Kumar et al. 1994; Tamura et al. 2011). These trees were then used to select 16S rRNA gene sequences to augment the 16S rRNA sequence library with representative sequences from the target and non-target groups.

3.3. Primer Design

Primers employed in the qPCR assays and developed in this study were designed using the PRIMROSE Software (Ashelford et al. 2002). PRIMROSE is a relatively old computer program designed to identify 16S rRNA probes and PCR primers for use as phylogenetic and ecological tools in the identification and enumeration of bacteria (Ashelford et al., 2002). PRIMROSE was designed to work closely with a relatively small downloadable database from an earlier version of the Ribosomal Database Project specifically RDP release 8 (Ashelford et al., 2002). This database, however, was augmented by desired target and non-target sequences (which were >1200 bp) sequences (see tables 3.1 and 3.2) retrieved from the more up to date RDP release 11. These assembled sequences in conjunction with sequence file(s) from the OTU target identified by sample sequencing was used to create oligonucleotides that potentially target 16S rRNA genes fragments of specific taxonomic groups or individual taxa.

In determining the suitability of oligonucleotides as primers required for PCR optimisation, a set of rules outlined by Innis and Gelfand (1990) were followed. First, the oligonucleotide length should be between 17 and 28 bp. Secondly, the G+C content of the oligonucleotide should be between 50 and 60%. Also, the optimal melting temperature (Tm) should be between 55 and 80 oC. The 3’ end of the oligonucleotide is also a very important feature of the oligonucleotide and a set of guidelines are provided to obtain desired

features thereby preventing mispriming. The Innis and Gelfand's guidelines point out that the base at the 3' end should be either G or C. However, G or C-rich ends consisting of a run of 3 or more Gs or Cs should be avoided. It is also important that the bases at the 3'-end are not complimentary to each other. Complimentary bases at the 3'-end of primers promote non-specific binding that results in primer-dimer amplification thereby reducing the desired yield. Another feature that could result in non-specific pairing is the presence of palindromic sequences within the oligonucleotide and this should be avoided while considering potential primers.

Primers were designed to specifically target *Rhodococcus* and *Bacillales* groups closely related to enriched sequences in microcosms and oligonucleotides were chosen after careful considerations following Innis and Gelfand's guidelines. The primers generated are described in table 3.3 below.

3.4. Primer pair selection and validation

To validate the specificity of different primer pair combinations, primers sequences were analysed in pairs using the RDP release 11 ProbeMatch tool (https://rdp.cme.msu.edu/probematch/search.jsp). ProbeMatch determines the sequences within the RDP database that match the submitted oligonucleotides. The pairs are submitted with one pair in a reverse complemented form (submitted as probe negative strand). Running the ProbeMatch tool determined the specificity of primer pairs in combination and not just the specificity of the individual component oligonucleotides. Potentially useful primer pairs were chosen principally based on their giving the least number of hits within the non-target taxonomic groups. While considering the primer pairs for a qPCR assay, it is important to identify the annealing position of primers on the target gene in order to determination amplicon length. This property was determined using the PRIMROSE software during primer design but was cross-checked manually against downloaded target sequences. Identifying the position of primers is essential For qPCR assay optimisation as efficient primer pair combinations should produce amplicons with fragment sizes between 50 bp and 250bp (Thornton and Basu 2011; Price et al. 2011).

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TABLE 3.1: Rhodococcus strains that are closely related to OTU 1 (most dominant OTU in Cd and Ni crude on degraded soils) used to design primers that targeted OTU 1 sequences in Microcosms

Accession Number	Organism	Source	Similarity to OTU 1	References
DQ125618	unculturered bacterium AKAU3638	Uranium contamianted soil, Tennessee, USA	100	Brodie, et al., 2006
AY512641	*Rhodococcus sp. Amico42*	Oil-refinery site, Northern Bohemia, Czech Republic	100	Hendrickx et al., 2006
AY512639	*Rhodococcus sp. Amico51*	Oil-refinery site, Northern Bohemia, Czech Republic	100	Hendrickx et al., 2006
AJ490526	*Rhodococcus erythropolis*	Newcastleupon Tyne soil, UK	100	O'Reilly, 2002
X89240	*Rhodococcus sp.*	disused site of munitions manufacture, Australia	100	Coleman, et al., 1998
AJ505559	*Rhodococcus sp. 4115*	Cultured Atlantic Salmon	100	Colquhoun, 2002
AJ244660	*Rhodococcus sp. VA.BO.16*	Bacterioplankton from Nice, France	100	Fritz, 1999
AB037105	*Rhodococcus erythropolis*	pesticide contaminated soil, Japan; activated sludge from dye factory, japan	100	Kimura, et al., 2000
AF487704	*Nocardia sp. H17-1*	oil contaminated soil	100	Baek, et al., 2002
AF512838	*Rhodococcus erythropolis*	soil sample, france	100	Lagnel, et al., 2002
AJ457058	*Rhodococcus erythropolis*	soil from car workshop, Germany	100	van Beilen, et al., 2002
AJ237967	*Rhodococcus erythropolis*	industrial waste water	100	Katsivela, et al., 1999
AJ250924	*Rhodococcus erythropolis*	isolated from trichloropropane-polluted soil sample	100	Poelarends, et al., 2000
GQ012727	unculturered bacterium	Human skin	100	Grice, et al., 2009
JF775491	*Rhodococcus sp. S2*	biofilm reactor, Portugal	100	Duque, et al., 2014
JQ769908	unculturered bacterium	biological soil crust of copper mine tailings wastelands in china	100	Liu and Sun, 2014
KC541081	unculturered bacterium RS-B27	River sediment associated with mining	100	Liu and Sun, 2013
KC764993	*Rhodococcus erythropolis*	Tobbaco Rhizosphere	100	you and Zhang, 2013
KC136826	*Rhodococcus erythropolis R8LFS*	Marine sample from Palk Bay, India	100	Bakkiyaraj, et al., 2012
KC136831	*Rhodococcus erythropolis G19*	Marine sample from Palk Bay, India	100	Bakkiyaraj, et al., 2012
KF113581	*Rhodococcus sp. D4*	constructed wetland	100	Dan, 2013
KC881306	*Rhodococcus sp. CH-H63-1*	Hydrocarbon polluted soil in Serbia	100	Beskoski , 2013
KF280392	*Rhodococcus sp.XF-8*	soil	100	Wang, 2013
Z37138	*Rhodococcus wratislaviensis*	soil sample from Newcastley upon Tyne	98	Chun and Goodfellow, 1994
AF124342	*R. koreensis*	industrial waste water, Korea	97.5	Yoon, et al., 2000
AY525785	*R .imtechnesis*	Pesticide contamianted soil in india	97	Ghosh et al., 2006
AB046357	*R. jostii*	Medieval grave	97	Takeuchi, et al., 2002
X79289	*Rhodococcus erythropolis*	crude oil contamianted soil, Kazakhstan	100	Shevtsov et al., 2013
DQ090961	*R. qingshengii*	Cabendazim contaminated soil, China	100	Jing-Liang, et al., 2006
DQ185597	*R. Jialinglae*	sludge of a carbendazim wastewater treatment facility in China	100	Wang, et al., 2012
AB071951	*R. baikonurensis*	isolated from air in the Russian space laboratory Mir	99	Li, et al., 2004
AJ131637	*Rhodococcus erythropolis*	sediment sample from a ditch	99	van der Werf, et al., 1999
EU747697	*Rhodococcus sp. S23*	industrial waste water, Mexico	99	Mauricio-Gutierrez, et al., 2010

TABLE 3.2: *Bacillales* strains that are closely related to OTU 5 the most Dominant OTU in Pb-crude oil degraded soil used to design primers that targeted OTU 5 sequences in Microcosms

Accession Number	Organism	Sources	% Similarity	Reference
NR_115059.1	*Scopulibacillus darangshiensis strain DLS-06*	Isolated from rock	98%	Lee and Lee, 2009
NR_025650.1	*Anaerobacillus macyae strain JMM-4*	Australian gold mine	98%	Santini et al., 2004
NR_109010.1	*Bacillus hemicentroti strain JSM 076093*	Sea urchin, china	98%	Chen et al., 2011
NR_025264.1	*Bacillus hwajinpoensis strain SW-72*	Sea water of the east sea and the yellow sea in Korea	97%	Yoon et al., 2004
NR_024694.1	*Pullulanibacillus naganoensis strain ATCC 53909*	Soil from Japan	97%	Goto et al., 2000
NR_044420.1	*Anaerobacillus alkalidiazotrophicus strain MS 6*	Mongolian soda soil	97%	Sorokin et al., 2008
NR_115854.1	*Anaerobacillus alkalilacustris strain Z-0521*	Soda lake, Russia	97%	Zavarzina et al., 2009
NR_042421.1	*Vulcanibacillus modesticaldus strain BR*	The rainbow vent field, Atlantic mid ridge	97%	Swiderski, 2005
NR_125645.1	*Listeria floridensis strain FSL S10-1187*	Running water	97%	den Bakker et al., 2014
NR_117854.1	*Bacillus zhanjiangensis strain JSM 099021*	Oyster in south china sea	97%	Chen et al., 2014
NR_104553.1	*Thalassobacillus pellis strain 18OM*	Isolated from salted hides	97%	Sanchez-Porro et al., 2011
NR_116709.1	*Bacillus chungangensis strain CAU 348*	Isolated from sea sand, USA	97%	Cho et al., 2010
NR_042538.1	*Salirhabdus euzebyi strain CVS-14*	Sea salt pan pond,the island of sal in the Cape Verde archipelago	97%	Albuquerque et al., 2007
NR_043012.1	*Bacillus taeanensis strain BH030017*	Solar saltern soil in Korea	97%	Lim et al., 2006
NR_042273.1	*Thalassobacillus devorans strain G-19.1*	Saline soil, Spain	97%	Garcia et al., 2005
NR_114912.1	*Bacillus alkalisediminis strain K1-25*	Soda lake sediment from Hungary	97%	Borsodi et al., 2011
NR_024798.1	*Bacillus krulwichiae strain AM31D*	Soil from Japan	97%	Yumoto et al., 2003
NR_029077.1	*Bacillus algicola strain F12*	Brown *algae Fucus evanescens*	97%	Ivanova, et al., 2004
NR_026139.1	*Bacillus pseudofirmus strain DSM 8715*	Lake bank soil in Germany	97%	Nielsen, et al., 1994
NR_117184.1	*Bacillus algicola strain AB423f*	Algae *`fucus vesiculosus*	97%	Wiese, et al., 2004
NR_116915.1	*Thalassobacillus cyri strain HS286*	Hypersaline lake in Iran	97%	Sanchez-Porro, et al., 2009
NR_125494.1	*Pullulanibacillus uraniitolerans strain UG-2*	Acid effluent from a uranium mining site in Portugal	97%	Pereira, et al., 2013
NR_125532.1	*Anoxybacillus calidus strain C161ab*	Soil near a thermal power plant in turkey	97%	Cihan, et al., 2014
NR_109677.1	*Bacillus thermophilus strain SgZ-10*	High-temperature compost	97%	Yang, et al., 2013
NR_109676.1	*Bacillus composti strain SgZ-9*	High-temperature compost	97%	Yang, et al., 2013
NR_125634.1	*Salinibacillus xinjiangensis strain J4*	Hypersaline lake in china	97%	Yang, et al., 2014
NR_109613.1	*Virgibacillus albus strain YIM 93624*	Lop nur salt lake in china	97%	Zhang, et al., 2012
NR_113776.1	*Bacillus cohnii strain NBRC 15565*	Soil from japan	97%	Nakagawa, et al., 2014
NR_028620.1	*Bacillus akibai strain 1139*	Soil from japan	97%	Nogi, et al., 2005
NR_117002.1	*Microaerobacter geothermalis strain Nad S1*	Terrestrial hot spring in Tunisia	97%	Hauschild, et al., 2010
NR_117252.1	*Staphylococcus stepanovicii strain 196*	Skin of small mammals	97%	Hauschild, et al., 2010
NR_108379.1	*Anoxybacillus vitaminiphilus strain 3nP4*	Hot spring in china	97%	Zhang, et al., 2013
NR_116351.1	*Listeria grayi strain H3506*	Feces, animal, Denmark	97%	Graves, et al., 2012
NR_104552.1	*Thalassobacillus hwangdonensis strain AD-1*	Tidal flat sediment of the yellow sea in south Korea	97%	Lee, et al., 2010
NR_043334.1	*Bacillus niabensis strain 4T19*	Cotton waste compost	97%	Kwon, et al., 2007
NR_043025.1	*Virgibacillus koreensis strain BH30097*	Yellow sea in Korea.	97%	Lee, et al., 2006
NR_040985.1	*Bacillus methanolicus strain NCIMB 13113*	Acidic sphagnum peat bogs in Wisconsin	97%	Goto, et al., 2004
NR_041942.1	*Bacillus acidicola strain 105-2*	Acidic sphagnum peat bogs in Wisconsin	97%	Albert, et al., 2005
NR_026349.1	*Listeria grayi strain DSM 20601*	Feces of chinchilla	97%	Vaneechoutte, et al., 1998
NR_026138.1	*Bacillus cohnii strain DSM 6307*	Soil from Germany	97%	Nielsen, et al., 1994
NR_044204.1	*Bacillus alkalinitrilicus strain ANL-iso4*	Soda solonchak soil from Mongolia	97%	Sorokin, et al., 2008

Table 3.3: Prospective primers for *Rhodococcus* and *Bacillales* target group (see table 3.1 and 3.2) designed with PRIMROSE

Oligonucleotides	Position relative to *E. coli* sequence numbering	Primer length (bp)	Estimated t_m °C	G + C %	Target taxonomic group
TTTGTCGCGTCGTTTGTG	544-561	17	53.7	50	*Rhodococcus*
CGCGTCGTTTGTGAAAAC	549-566	17	53.7	50	*Rhodococcus*
TAACTGACGCTGAGGAAC	702-719	17	53.7	50	*Rhodococcus*
GGTTTGTCGCGTCGTTTG	542-559	17	56	55.6	*Rhodococcus*
AGCTCAACTGCTGGCTTG	571-588	17	56	55.6	*Rhodococcus*
TGTGAAAACCAGCAGCTC	558-575	17	53.7	50	*Rhodococcus*
GAAAACCAGCAGCTCAAC	567-578	17	53.7	50	*Rhodococcus*
AACCAGCAGCTCAACTGC	564-581	17	56	55.6	*Rhodococcus*
TCAACTGCTGGCTTGCAG	574-591	17	56	55.6	*Rhodococcus*
CGTAACAAGGTAGCCGTACC	1390 to 1409	19	61.4	60	*Rhodococcus*
CGGTGGCTTAACCCCTTGTG	1454 to 1473	19	57.3	50	*Rhodococcus*
GCATGACTTGGGGTGGAAAG	164 t0 183	19	61.4	60	*Rhodococcus*
GGTTGCATGACTTGGGGTGG	1451 to 1470	19	57.3	50	*Rhodococcus*
GAGGGTCATTGGAAACTG	603-620	17	53.7	50	*Bacillales*
TAGCGGTGAAATGCGTAG	661-678	17	53.7	50	*Bacillales*
TATTGGGCGTAAAGCGCG	537-554	17	56	56	*Bacillales*
GTGTAGCGGTGAAATGCG	658-675	17	56	56	*Bacillales*
CTGGTCTGTAACTGACGC	714-731	17	56	56	*Bacillales*
TGTAACTGACGCTGAGGC	720-737	17	56	56	*Bacillales*
GATACCCTGGTAGTCCAC	766-783	17	56	56	*Bacillales*

When possible primer pairs were matched against the RDP Database release 11 the database comprised 3,224,600 archaeal and bacterial 16S rRNA gene sequences as well as 108,901 fungal 23S rRNA sequences. The bacterial 16S rRNA gene database comprised 3,070,243 sequences. Probe match was carried out in the entire database and the target taxonomic groups were bacterial order, *Bacillales* and bacterial genus, *Rhodococcus*. Primer pairs were analysed for specificity to these target taxonomic groups. Table 3.4 shows the result of the ProbeMatch analysis of primer pairs.

Table 3.4: Specificity of Primer pairs for *Rhodococcus* and *Bacillales* generated from the ProbeMatch platform on Ribosomal Database Project (RDP)

			Total *Rhodococcus* in RDP:	4830		Total *Bacillales* in RDP:	267120	
			Total *Actinobacteria* in RDP:	348028		Total *Firmicutes* in RDP:	811625	
Forward Primer	**Reverse Primer**	**Length (bp)**	**Bacterial hits**	***Actinobacteria* Hits**	**% hits relative to total *Actinobacteria* in RDP**	***Rhodococcus* Hits**	**% hits relative to total *Rhodococcus* in RDP**	***Rhodococcus* Hits/Bacterial Hits (%)**
TAACTGACGCTGAGGAAC	**CACAAACGACGCGACAAA**	**158**	**1328**	**1324**	0.38	**1294**	26.79	**97.44**
GGTTCCTTCCACGGAATC	CAAACGACGCGACAAACC	248	1239	1236	0.36	1223	25.32	98.71
TAACTGACGCTGAGGAAC	GTTTTCACAAACGACGCG	153	1309	1305	0.37	1285	26.60	98.17
GGTTCCTTCCACGGAATC	CAAGCCAGCAGTTGAGCT	219	1159	1158	0.33	1148	23.77	99.05
CGCAACCCCTATCTTATG	CATAAGATAGGGGTTGCG	101	9857	1503	0.43	1136	23.52	11.52
AATACGTAGGGTGCAAGC	CCATCCTGCACCGATAAA	298	1284	1284	0.37	1282	26.54	99.84
GGTACGGCTACCTTGTTACG	**CACAAGGGGTTAAGCCACCG**	**82**	**125**	**122**	0.04	**122**	2.53	**97.60**
		Amplicon Length	**Bacterial Hits**	***Firmicutes* Hits**	**% hits relative to total *Firmicutes* in RDP**	***Bacillales* Hits**	**% hits relative to total *Bacillales* in RDP**	***Bacillales* hits/Bacterial hits %**
TAGCGGTGAAATGCGTAG	CAGTTTCCAATGACCCTC	59	88153	88017	10.84	70130	26.25	79.68
GTGTAGCGGTGAAATGCG	CGCGCTTTACGCCCAATA	122	187700	186481	22.98	172845	64.71	92.69
TGTAACTGACGCTGAGGC	**CGCGCTTTACGCCCAATA**	**184**	**33685**	**33625**	4.14	**33539**	12.56	**99.74**
GATACCCTGGTAGTCCAC	CGCGCTTTACGCCCAATA	230	184085	166974	20.57	163824	61.33	98.11
TGTAACTGACGCTGAGGC	CGCATTTCACCGCTACAC	63	153847	152948	18.84	38328	14.35	25.06
GATACCCTGGTAGTCCAC	CGCATTTCACCGCTACAC	109	759980	463170	57.07	188823	70.69	40.77

**the reverse primers are the reverse complement of the oligonucleotide originally designed with PRIMROSE ;*

+ the primer pairs considered for the Optimization of qPCR assays

***Bold:** the most specific primers for target taxonomic group based on RDP ProbeMatch; these primers were employed in investigations to validate qPCR assay*

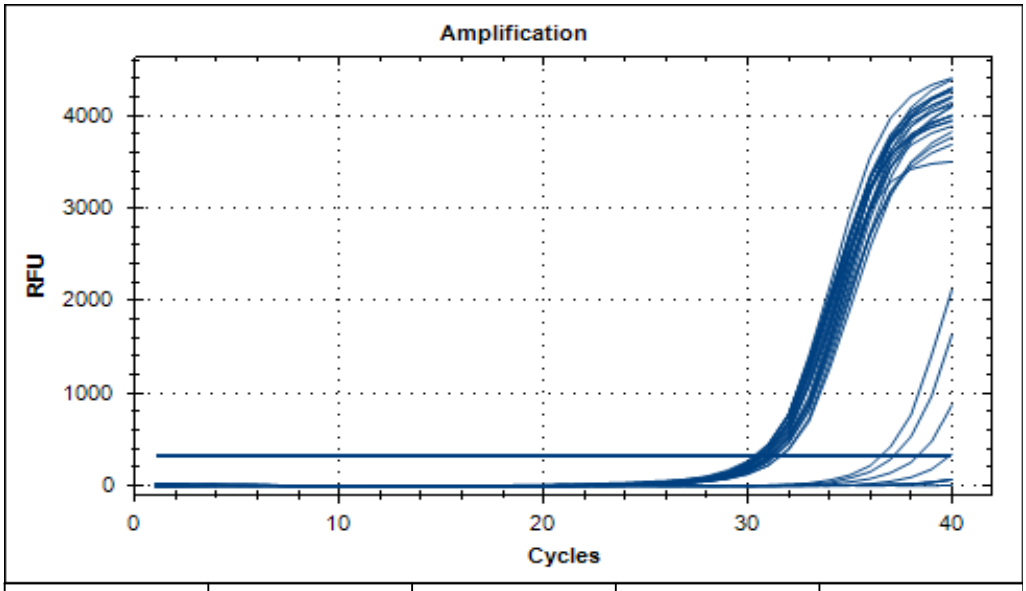
The order *Bacillales* and the genus *Rhodococcus* belong to the bacterial phylogenetic groups *Firmicutes* and *Actinobacteria*, respectively. The RDP ProbeMatch tool presents output-results/ hits in a Hierarchal format; hence, the number of matched sequences at all taxonomic levels are identified, from the domain to genus level. The percentage of hits from target groups (at order or genus level as the case may be) relative to the bacterial hits is a measure of specificity. A high percentage implies a high specificity for the target (see table 3.2). Based on this % specificity and amplicon fragment length, 2 primers sets were considered for use in the enumeration of 16S rRNA genes of target *Rhodococcus sp*. in Ni or Cd and/or oil-amended microcosms detailed in chapters 4 and 5. In the same vein, a single primer set was considered for *Bacillales* enumeration in the Pb and oil-amended microcosms (see chapter 6). The optimisation of these qPCR assays using selected primer set is discussed in the section 2.8.7.

3.3: Selected Primer sets employed in the qPCR assay optimisation for bacterial 16S gene abundance determination

Following the ProbeMatch analysis, primer sets that have high % specificity for target groups, 2 primer sets for *Rhodococcus* (RhO1390F-RhO1454R and RhO53F-RhO189R) and 1 primer set for *Bacillales* (Bac537F-Bac702R), were analysed further towards optimisation of qPCR assays. The comparative analysis of the primer pairs that targeted 16S rRNA gene targets for *Rhodococcus* indicated that the RhO1390F-RhO1454R primer pair was a better primer set than RhO53F-RhO189R. Analysis of the later (Fig 3.1), indicated that the lowest Cq-value was attained at a T_a of 61.4 ^oC. A melt curve analysis (Fig 3.2a) of the curve generated at 61.4 ^oC, however, indicated that incomplete amplification of target sequence within the 40 cycles occurred see figure 3.2 a and b). From figure 3.2a, it could be observed that not all samples attained the maximum peak. Most peaks of samples are slightly above or below the baseline level. Also, standard curve analysis used only two data points of standard samples out of 6 standard samples, to attain an efficiency of 79.2% and R^2 of 0.92. A lower T_a at 59 ^oC resulted in the amplification of non-specific PCR products (see figure 3.2 c and d) and on increasing Ta to 63.3 oC, no amplification of the target sequence was observed (see figure 3.2 e).

For the RhO1390F-RhO1454R primer pair a thermal gradient-melt curve analysis indicated that the optimal T_a was 58.1 ^oC (see figure 3.3) however, amplification of standard dilutions using this temperature as the Ta resulted in non-specific amplification (see figure 3.4 a), which was reflected in a low amplification efficiency of 76% (Figure 3.4b). However, increasing the Ta to 62^oC resulted in a higher amplification efficiency of 81.9% (figures 3.4c and d) and non-specific amplification. Based on these results, RhO1390F-RhO1454 was accepted as the primer set for the enumeration of 16S rRNA gene in samples at T_a of 62 ^oC. Agarose gel electrophoresis visually showed the amplified genes were the correct amplicon length (see figure 3.5). Also, the agarose gel electrophoresis confirmed specific amplification of target gene.

In the same vain, validation analysis was carried out for Bac537F-Bac702R that target species of the order Bacillales. Figures 3.6 and 3.7 present the results of the analysis. The thermal gradient- melt curve analysis indicated that the lowest Cq was attained at 62 ^oC (see figure 3.6). As shown in figure 3.7b, there was no amplification of now- targets sequences at this annealing temperature. The efficiency of amplification was 86% and the R^2 was 0.99 (see figure 3.7a). In addition, RT-PCR analysis at an annealing temperature of 65 ^oC also resulted in non-specific amplification but the amplification efficiency at this temperature was much higher at 106% and the R^2 of the standard curve was 0.95 (see figures 3.7 c and d). This observation suggests that annealing temperature within the range of 62 to 65 ^oC is valid for the amplification 16S rRNA of species belonging to the order Bacillales. In this project, the enumeration of 16S rRNA genes of Bacillales in the aerobic petroleum degrading metal contaminated soils was carried out at Ta of 65 oC. Agarose gel electrophoresis confirmed specific amplification of genes with the appropriate amplicon size (see figure 3.8).



Temperature °C	Cq_a	Cq_b	Cq_c	Mean Cq ± Standard error
65	30.45	31.07	31.53	31.02±0.31
64.5	30.61	31.22	30.74	30.86±0.19
63.3	30.94	31.62	30.58	31.05±0.30
61.4	**30.5**	**30.21**	**30.71**	**30.47±0.14**
59	30.41	30.98	30.39	30.59±0.19
57	30.54	30.5	31.11	30.72±0.20
55.7	31.3	31.12	30.78	31.07±0.15
55	31.27	30.33	31.09	30.90±0.29

Figure 3.1: the determination of optimal Annealing Temperature (T_a) for the optimization of 16S rRNA gene amplification using the primer set Rho53F-Rho189R by thermal gradient qPCR. The blue horizontal line spanning across the graph represents the threshold level of Relative Fluorescent Unit (RFU), above which significant amplification of gene occurs and the Cycle that the RFU is above the threshold Level is the quantification cycle (Cq). The Table attached to the figure shows the Cq of triplicate samples and the mean Cq (±Standard error) at temperatures tested. The row in bold represents the temperature with the lowest Cq value.

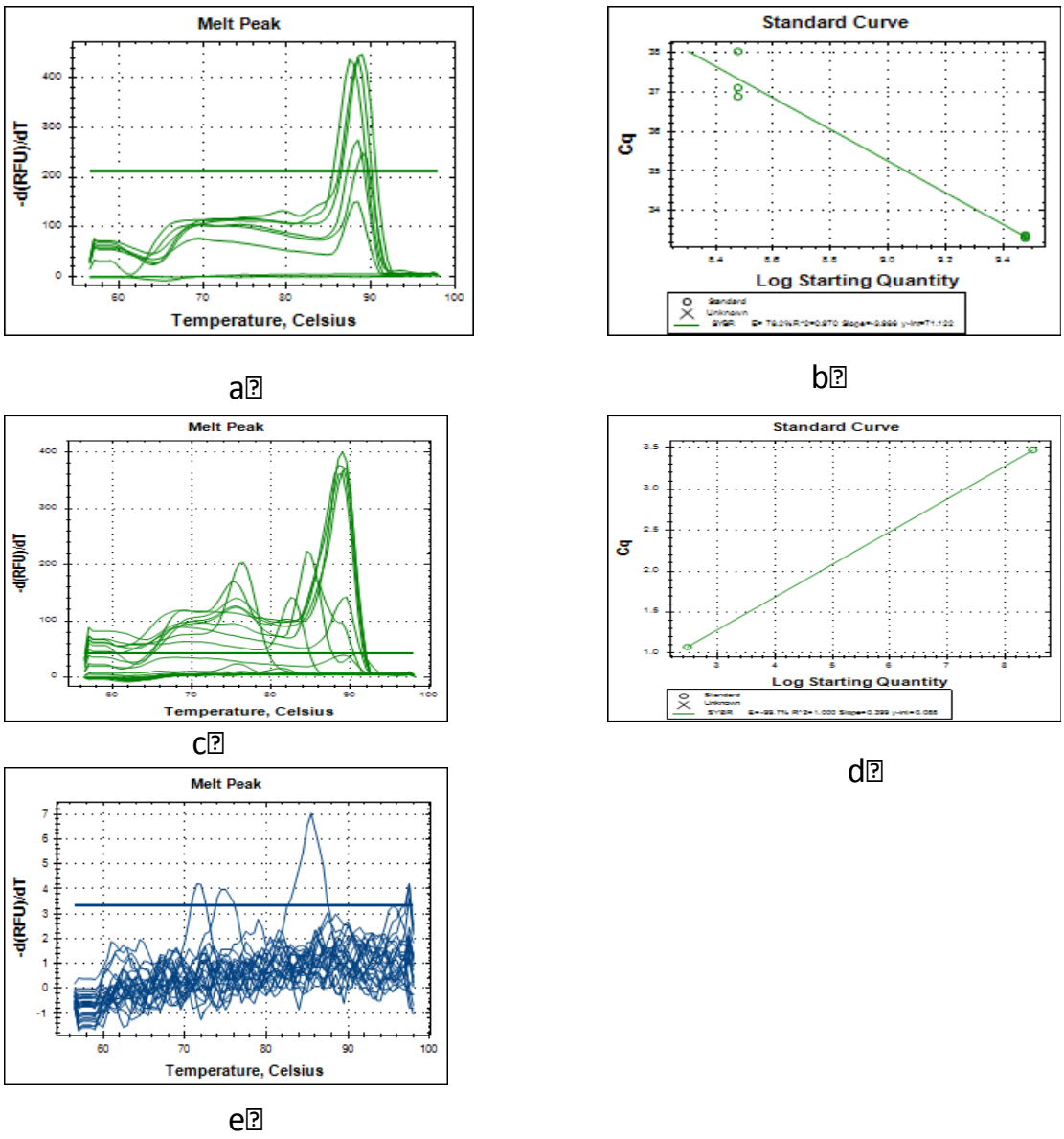
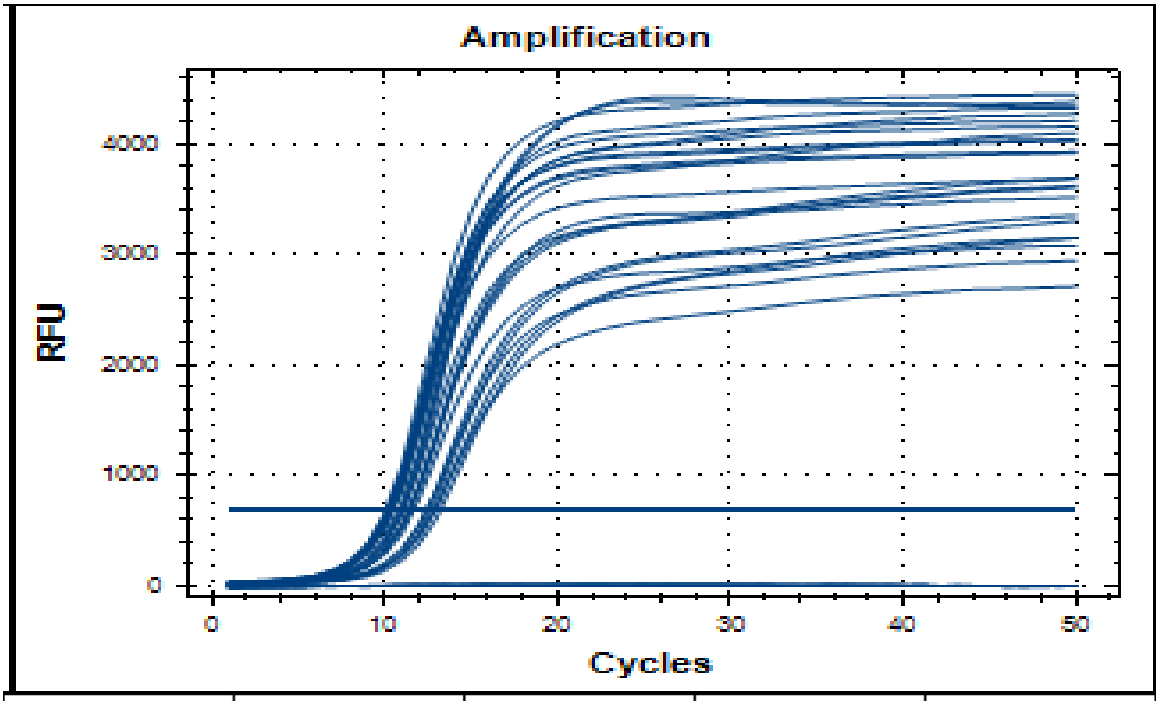
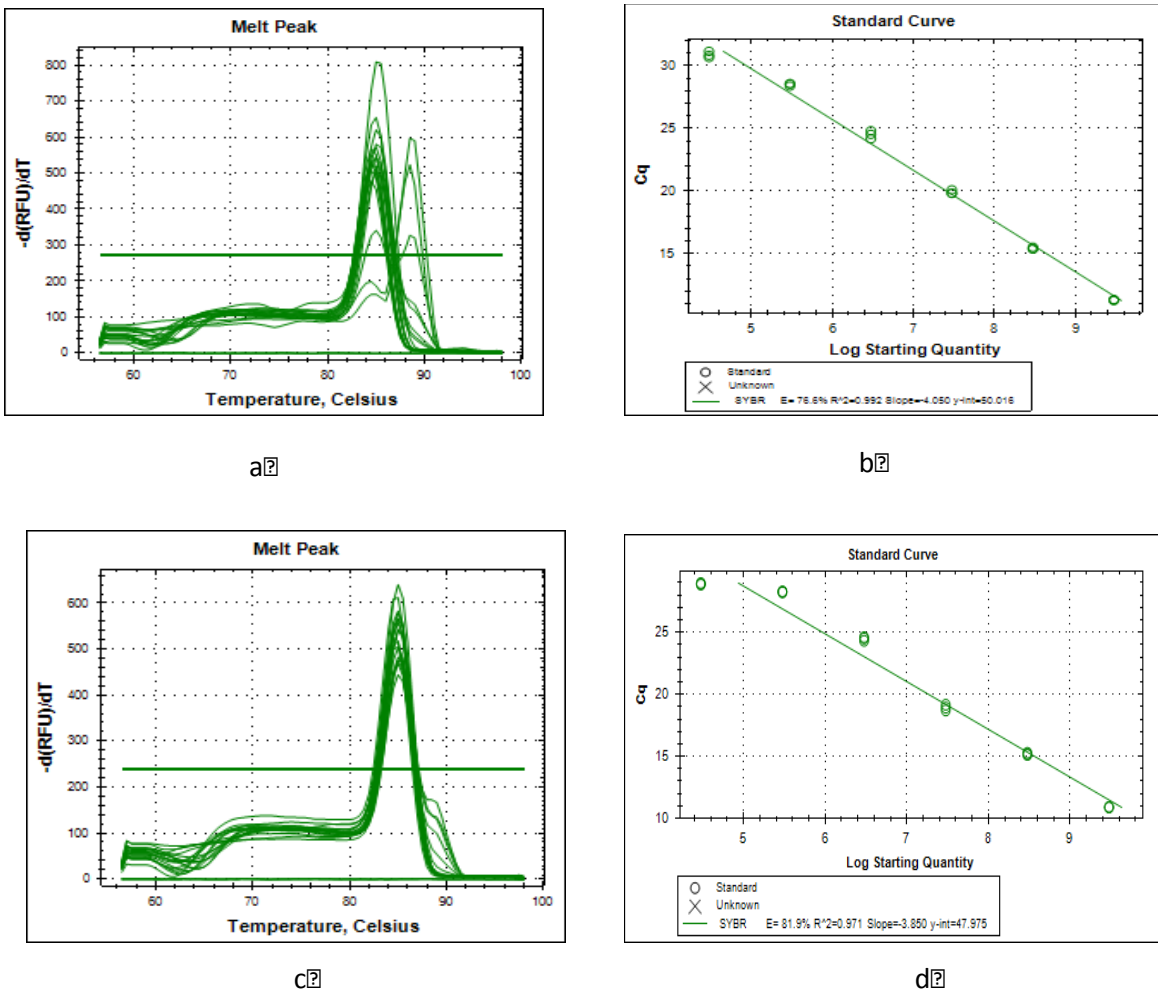


Figure 3.2: amplification of 16S rRNA gene at different using Rho53F-Rho189R primer set at different annealing Temperatures (T_a). a) and b): melt curve and standard curve at T_a =61.4 ^oC ; c) and d): melt curve and standard curve at T_a= 59 ^oC; e) the melt curve at T_a= 63.3 ^oC; no amplification occurred at this temperature. The horizontal line in the melt curve represents the threshold level of Relative fluorescent unit (RFU). Significant amplification occurred above threshold level and quantification begins



Temperature (°C)	Cq_a	Cq_b	Cq_c	Mean Cq ± Standard Error
70.9	12.84	12.91	13.21	12.99±0.11
70	12.41	12.64	12.41	12.49±0.08
68.2	11.67	11.45	11.41	11.51±0.08
65	11.37	11.09	10.78	11.08±0.17
62	10.39	10.49	10.3	10.39±0.05
58.1	**10.19**	**10.27**	**10.06**	**10.17±0.06**
56	10.23	10.17	10.19	10.20±0.02
55	10.44	11.05	10.93	10.81±0.19

Figure 3.3: The determination of optimal Annealing Temperature (T_a) for the optimization of 16S rRNA gene amplification using the primer set Rho1390F-Rho1454R by thermal gradient qPCR. The blue horizontal line spanning across the graph represents the threshold level of Relative Fluorescent Unit (RFU), above which significant amplification of gene occurs and the Cycle that the RFU is above the threshold Level is the quantification cycle (Cq). The Table attached to the figure shows the Cq of triplicate samples and the mean Cq (±Standard error) at temperatures tested. The row in bold represents the temperature with the lowest Cq value.



a b c d

Figure 3.4: the Validation analysis of annealing temperatures (T_a) for primer set Rho1390F-Rho1454R targeting the 16S rRNA gene of *Rhodococcus*. a and b show the melt peak and standard curves at T_a= 58.1 ºC (the optimal temperature determined with thermal gradient-melt curve analysis) while c and d show melt peak and standard curve at T_a= 62 ºC. The horizontal line in the melt curve represents the threshold level of Relative fluorescent unit (RFU). Significant amplification occurred above threshold level and quantification begins.

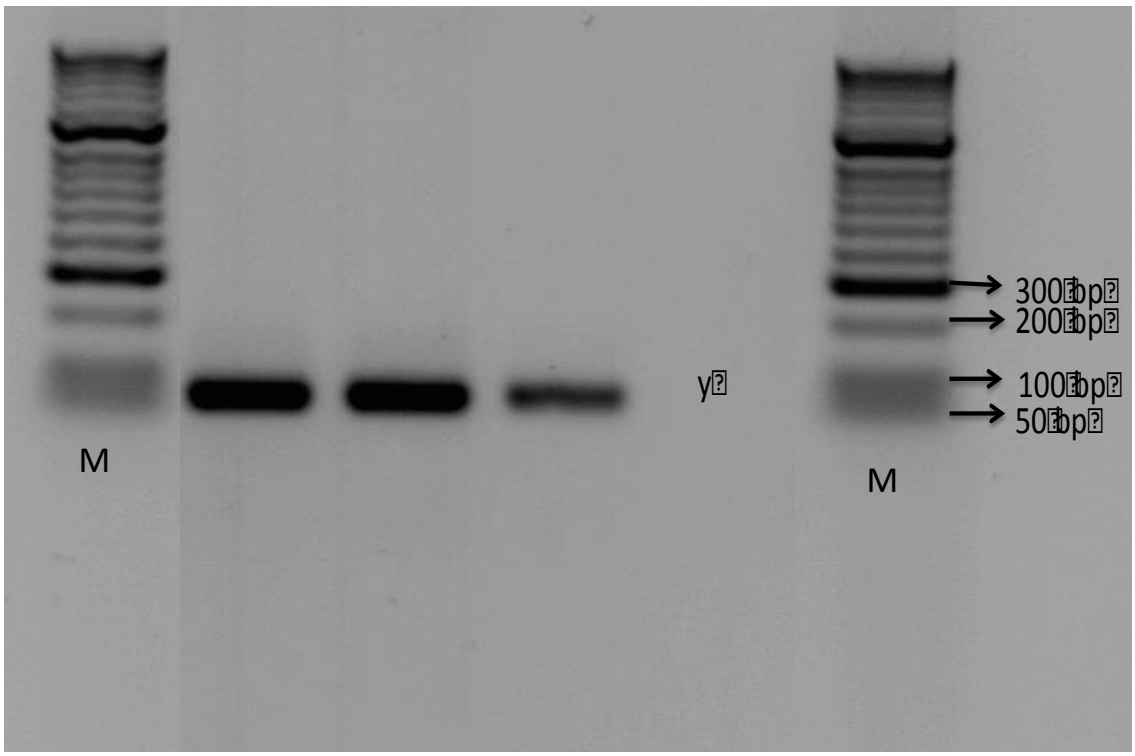
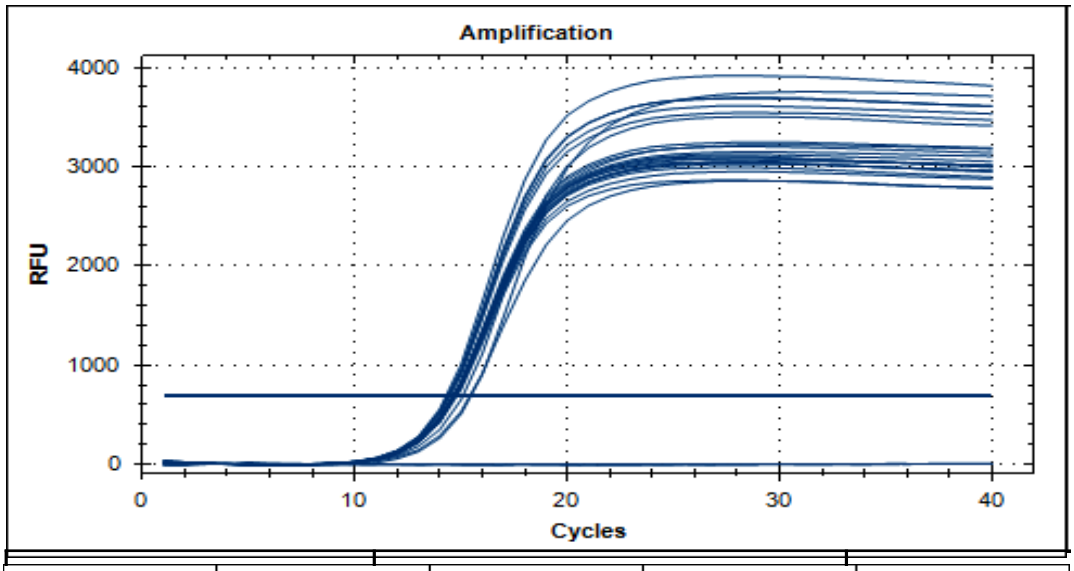


Figure 3.5: specific amplification of 16S rRNA genes of *Rhodococcus* using the primer sets Rho1390F-Rho1454R at annealing temperature of 62 ºC. Bands are represents triplicate amplicons from the pure 16S rRNA DNA of typed *Rhodococcus erythropolis* (DSM 43066) at concentration of 10^9. The amplicon size is 82 bp. M represents the marker lane, which indicates the position of the fragment sizes. Y is the experimental blank (a blank contained no DNA t, only reagents and used to validate the efficiency of experimental reagents).



Temperature (° C)	Cq_a	Cq_b	Cq_c	Mean Cq ± standard error
70	14.76	14.64	14.68	14.69±0.03
69	14.72	14.62	14.49	14.61±0.07
68	14.74	14.66	14.44	14.62±0.09
65	14.73	14.38	14.4	14.50±0.11
62	**14.63**	**14.31**	**14.47**	**14.47±0.13**
58	14.66	14.82	14.74	14.74±0.05
56	14.72	14.66	14.69	14.69±0.02
55	14.72	14.7	14.71	14.71±0.01

Figure 3.6: the determination of optimal Annealing Temperature (T_a) for the optimization of 16S rRNA gene amplification using the primer set Bac537F-Bac720R by thermal gradient qPCR. The blue horizontal line spanning across the graph represents the threshold level of Relative Fluorescent Unit (RFU), above which significant amplification of gene occurs and the Cycle that the RFU is above the threshold Level is the quantification cycle (Cq). The Table attached to the figure shows the Cq of triplicate samples and the mean Cq (±Standard error) at temperatures tested. The row in bold represents the temperature with the lowest Cq value.

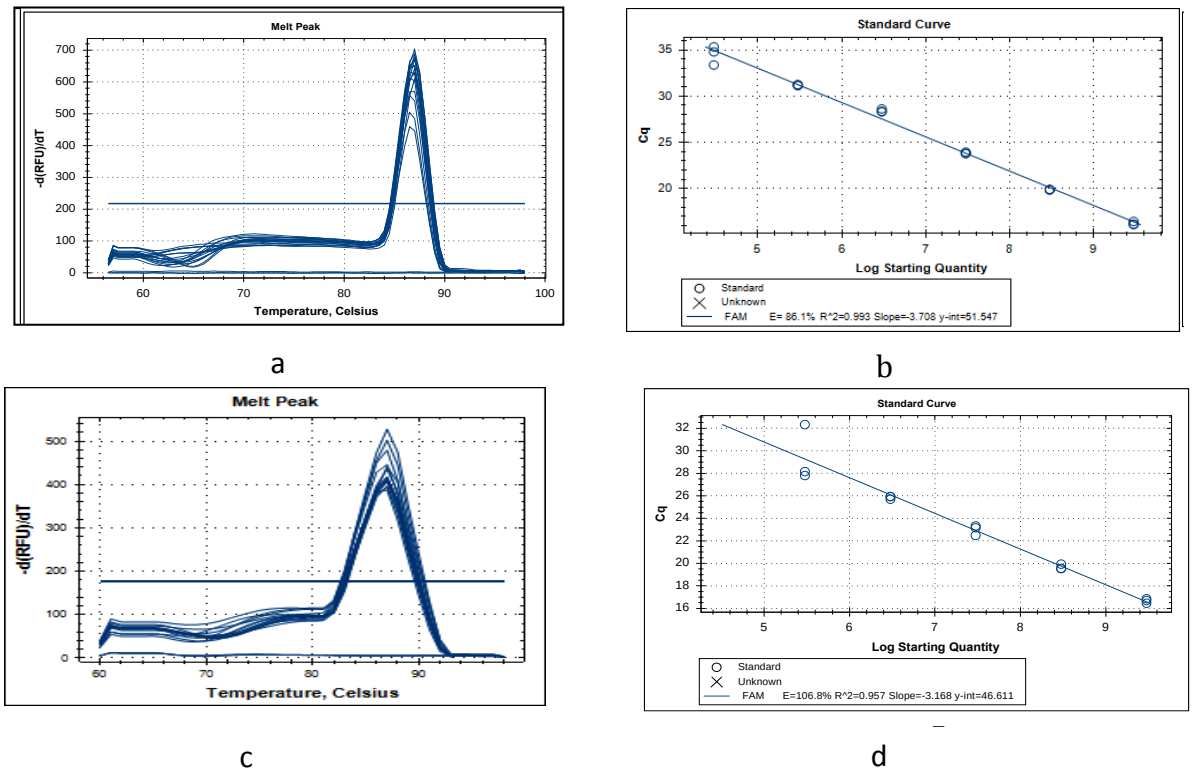


Figure 3.7: the Validation analysis of annealing temperatures (T_a) for primer set Bac537F-Bac720R targeting the 16S rRNA gene of *Bacillales*. a and b show the melt peak and standard curves at T_a= 62 ^oC (the optimal temperature determined with thermal gradient-melt curve analysis) while c and d show melt peak and standard curve at Ta= 65 ^oC. The horizontal line in the melt curve represents the threshold level of Relative fluorescent unit (RFU). Significant amplification occurred above threshold level and quantification begins

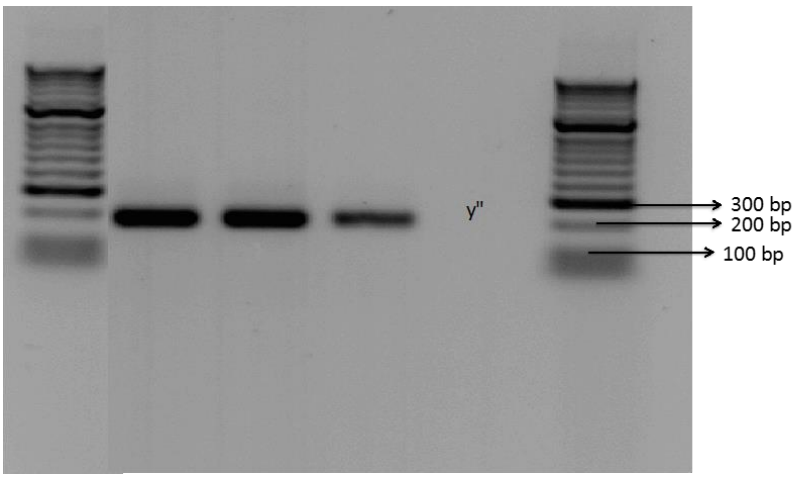


Figure 3.8: specific amplification of 16S rRNA genes of Bacillales using the primer sets Bac537F-Bac720R at annealing temperature of 65 ^{o}C. The amplicon length for primer set is 201 bp. Bands are represents triplicate amplicons from the typed pure 16S rRNA DNA of *Scopulibacillus darangshiensis* (DSM 19377) at concentration of 10^{9}. The amplicon size is 184 bp. M represents the marker lane, which indicates the position of the fragment sizes. Y is the blank (a blank contained no DNA samples, only reagents and used to validate the efficiency of experimental reagents).

3.6. Application of 16Sr RNA gene abundance in petroleum degrading-heavy metal contaminated aerobic soil microcosms

Polymerase Chain Reaction (qPCR) targeting 16S rRNA gene sequences was used to determine the abundances of the total bacterial population and key organisms, which appeared to be enriched in the microcosms from community analysis and thus could be considered to be involved in aerobic hydrocarbon degradation. Based on sequence match analysis using the RDP database, the dominant organisms enriched are 100% similar to strains of *Rhodococcus* and *Bacillales*.

The bacterial primers used were those previously designed and applied in the quantification of 16S rRNA gene in metagenomic oil degrading sediment microcosms (Gray et al. 2011). In addition, specific primers that targeted *Rhodococcus* and *Bacillales* previously validated (see sections 3.1 to 3.) were used to enumerate the 16S rRNA genes of target taxonomic groups.

The enumerations of 16S rRNA genes are possible by calibration with standards, which consist of known quantity of 16S rRNA gene of target taxonomic group. The standard used for bacteria was purified 16S rRNA gene

of *Syntrophic* sp from an environmental sample (Gray et al. 2011). Other standards were as described in the section above. Preparation of standards was also as describe in the section above. Using the PCR protocols developed above, gene abundances were determined against the standards.

Two approaches have been developed by scientists to determine the absolute abundance of target genes in samples. These are the standard curve method (Roberts et al. 2014; Schriewer et al. 2011; Fronhoffs et al. 2002; C. Lee et al. 2006; Larionov et al. 2005; Rutledge and Côté 2003) and one-point calibration method (Brankatschk, Bodenhausen, Zeyer and Bürgmann 2012; Brankatschk, Bodenhausen, Zeyer and Burgmann 2012; Ruijter et al. 2013).

The standard curve method involves the generation of a standard curve from qPCR assay of serially diluted standards with known template concentration, which is the used to calculate the target gene concentration of the sample extracts. With such an approach the standard curve is generated from plotting of the log of template concentration ($LogN_0$) against the C_q- values and the resultant linear regression can be used to convert sample C_q-values into gene abundances. Critically this approach assumes that the PCR Efficiency, E, of the standard is equal to the E of all the samples, which may not be true due to the fact that standards are generated from pure cultures, unlike environmental samples. As a result, it has been stated that efficiencies of qPCR assay are likely affected (Brankatschk, Bodenhausen, Zeyer and Bürgmann 2012) by, for example, the co-extraction of PCR inhibitors. Accordingly, in this project, determination of the absolute abundance of 16S rRNA genes in samples was also carried out using the one-point calibration (OPC) approach (Brankatschk, Bodenhausen, Zeyer and Bürgmann 2012; Brankatschk, Bodenhausen, Zeyer and Burgmann 2012). This method involved the determination of individual amplification efficiency in samples, accounting for template-related variability of amplification efficiency. In this way, differences between amplification efficiency are corrected between samples and standards. To this end raw data from the qPCR, which is non-baseline, corrected is exported into the LinRegPCR program. LinReg basically carries out a baseline correction on each individual sample and then regression analysis is used to determine a

PCR efficiency value for each individual amplification profile from the raw data and calculated from the equation

$$\mathbf{E = (N_A / N_B)^{1/(Cq_A - Cq_B)}}$$

Where:

$\mathbf{N_A}$ and N_B = amount of fluorescence at two arbitrary threshold points (A and B) along the exponential phase of an individual amplification profile and Cq_A and C_{qB}= corresponding Cycles at points A and B.

The gene copies in the sample template at the inception of qPCR, $N_{0\ sample}$, is then calculated using the equation

$$N_{0\ sample} = N_{0\ standard} * (E_{Standard} \wedge Cq_{standard} / E_{sample} \wedge Cq_{sample})$$

Where

$N_{0\ standard}$ =initial copies of template of a defined standard,

E_{sample}= the efficiencies of the sample PCR amplification

$E_{Standard}$ = the average efficiency of standard PCR amplification , values and

Cq_{sample} = the cycle threshold for sample

$Cq_{standard}$ =Cycle threshold for Standard

The 16S rRNA gene abundance in samples was then calculated using the equation below

$$\text{Copies } (ng^{-1} \text{ DNA Extract } g^{-1} \text{ dry soil}) = \frac{N_{0\ sample} * \text{Dilution factor}}{\text{Concentration of DNA } (ng\ ul^{-1} \text{ extract } g^{-1} \text{ dry soil})}$$

Conclusions

The design of qPCR primer pairs that target the most dominant OTU sequences in crude oil-metal (Ni, Cd and Pb) amended degraded soil microcosms and the optimization of qPCR assays that these sequences was described in this chapter. Based on careful considerations of oligonucleotides

designed using PRIMROSE software from the sequences of Target OTUs and sequences of close relatives to Target sequence using Innis and Gelfand's guidelines, temperature gradient analysis, melt curve analysis and standard curve analysis, primers that target were designed and optimized successfully. RhO1390F-RhO1454 at T_a of 62 ^oC was accepted as the primer set for the enumeration most dominant OTU which is closely related to strains of Rhodococcus Sp in Ni and Cd crude oil degraded soil microcosms while Bac537F-Bac702R at T_a of 65 ^oC was accepted primer set for the enumeration of most dominant 16S rRNA gene closely related to strains of Bacillales in Pb crude oil degraded soil microcosm.

For the absolute quantification of 16S rRNA genes in samples, one point caliberation (OPC) method was used to eliminate errors caused by differences between standards DNA samples and DNA samples from Microcosms. Unlike Standard curve method where equal efficiency value is assumed, OPC method determines individual amplification efficiency in samples.

Chapter 4

The Effects of Nickel on the Biodegradation of Petroleum Hydrocarbon in the Soil

4.1. Introduction

Nickel (Ni) is an element of group 10 in the periodic table. The atomic number and mass of the element are 28 and 58.71mg mol-1 respectively. With a density is 8.9 g/cm3 at 20 ^{o}C Ni can be described as a heavy metal. The oxidation states of the metal are predominantly 0 and +2. In certain conditions, Nickel also exhibits +1 and +3 oxidation states (Gonnelli and Giancarlo 2012). The most dominant species of inorganic nickel is Ni2+. Nickel (Ni^{2+}) can substitute alkaline and metallic cations such as Mg^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+} and Mn^{2+} in primary minerals (Kabata-Pendias 2007; Martin, I., Morgan, H., Jones, C., Waterfall, e. and Jeffries 2009).

Ni exists predominantly in the earth crust as Fe-Ni molten core (up to 10% of global Nickel exists in this form) (Gonelli and Rennela, 2012). This is owed to its affinity to Iron. Furthermore, Ni has a high affinity for sulfur, hence, its existence as sulfide minerals such as pentlandite ([(Ni, Fe) 9S8]), Millerite (NiS), Niccolite (NiAS) and Breithauptite (NiSb) (Martin, I., Morgan, H., Jones, C., Waterfall, e. and Jeffries 2009). Ni also exists as carbonate, oxide, phosphate and silicate minerals. A significant amount of Ni has also been observed in petroleum and coal and for years, Ni has been considered one of the primary heavy metal in petroleum. Ni concentration in petroleum varies with the petroleum source rock, ranging from few ppb to tens of ppm. The concentrations of Ni in different petroleum all around the world is shown in table 1.2 of chapter 1. The significant of Ni in petroleum has lead to the use of its quantification (together with vanadium) in the geochemical studies of petroleum (Barwise 1990). Usually, it exists in porphyrin form in the petroleum originating from its substitution of the metallic ions of natural porphyrins such as chlorophyll and bacteriophyll during petroleum formation (Peters et al. 2007a).

Anthropogenic sources of Ni have resulted in increases in the Ni content of soils. The main Ni sources are mining activities such as metal mining,

petroleum and coal mining (Gonnelli and Renella, 2013(Martin, et al., 2009). The accumulations of Ni in soils and sediments due to continuous contamination with petroleum have been reported in the past from across the globe. For instance, Obiajunwa et al (2002) reported an accumulation of up to 76 ppm in chronically contaminated soils in Niger Delta, Nigeria. Also, 122 ppm Ni, and 89.3 ppm Ni were reported to accumulate in soils of Bahrain (Mandany et al 1994), et al. 2014) and Kuwait (Al-Saleh and Akbar 2015) respectively. Other activities that contribute to Ni accumulation in the environment include the addition of fertiliser and heavy metal containing sludge.

Ni has been identified as a potentially toxic element and its concentrations in the terrestrial and aquatic environments are therefore guided. Studies on Ni effects on human health suggest that the metal is a potential toxin, mutagen and carcinogen (Cameron et al. 2011; Kodama and Ishinishi 1980; Schaumlöffel 2012; Barceloux 1999). In the UK, the soil guideline value for Ni is 130 parts per million (ppm) in residential areas, 230 ppm in allotment areas and 1800 ppm in commercial areas (Martin, I., Morgan, H., Jones, C., Waterfall, e. and Jeffries 2009).

Ni has been reported as a co-factor for certain microbial enzymes. Boer et al (2014) described some Ni-dependent enzymes. These include glyoxalase I, acireductone dioxygenase, urease, superoxide dismutase, [NiFe]-hydrogenase, carbon monoxide dehydrogenase, acetyl-coenzyme A synthase/decarbonylase, methyl-coenzyme M reductase, and lactate racemase. As co-factor to these enzymes, Ni becomes essential nutrient for the physiological functions of microbes. This might explain the increased hydrocarbon degraders in soil by the range of 8.4 to 17.2%, resulting in a relative increase in gasoline biodegradation (57.5–62.4%) (Agarry et al. 2013). Although essentiality of Ni to soil plants and microbes is known, its toxicity at high concentrations to plants and microbes has been well documented (Martin, I., Morgan, H., Jones, C., Waterfall, e. and Jeffries 2009; Cempel and Nikel 2006; Gadd 2010; Cameron et al. 2011; Korthals et al. 1996; Weng et al. 2004; Sparks 2005; Kelly et al. 2010) it is worth noting that this contaminant could, therefore, base on this preamble represent both a nutrient and a toxic component of a system and this is reflected in this chapter.

This chapter will discuss the effect of nickel on the biodegradation of petroleum hydrocarbons. The effect on soil respiration during biodegradation, the pattern of change in in the aliphatic component of petroleum during degradation and the effect on the microbial community during biodegradation are highlighted.

4.2. Aim and objective

The aim of the experiments detailed in this chapter is to investigate the effect of Nickel on hydrocarbon degradation in complex natural systems using a microbial ecological approach combined with geochemical methods.

The specific objectives were:

1. To determine the effect of different mineral forms of nickel at different concentrations on the biodegradation of petroleum hydrocarbon to determine likely thresholds of positive and negative impacts on hydrocarbon degrading bacteria

2. To simultaneously in the same experimental system determine the compositional changes of whole petroleum oil relative to imposed Ni contamination.

3. To simultaneously determine the ecological diversity and dynamics of microbial communities relative to variable petroleum hydrocarbon and Ni contamination.

4. To determine the geochemical fate of added Ni in its different added forms and interpret this fate in the context of the observed effect on hydrocarbon degradation and microbial communities.

4.3. Methods

The effects of Ni on the biodegradation of petroleum hydrocarbons was studied in soil microcosms designed to simulate Heavy Metal-Petroleum co-contaminated soils. The general experimental setup and geochemical and microbiological methods used have been described extensively in chapter 2 but are briefly recapped here with the inclusion of specific details of the Ni amended experiments. The soil samples used were obtained from the organically farmed section of the Nafferton ecological group farm described

extensively in section 2.1. Sampling methods, sample handling, pretreatment methods and storage methods were based on ISO guidelines (Standard 2003) as outlined in section 2.2. Initial soil pH, moisture content, water holding capacity, heavy metal content and organic carbon were determined as outlined in section 2.3.

Soil microcosm treatments were set up in triplicate such that there were treatments of increasing Ni concentration in different mineral forms (see Figure 4.1). Ni effects were investigated at 12.5, 25, 50, 200 and 350 ppm. These concentrations were arranged around the environmental agency's soil guideline value (SGV) for Ni as well as Dutch list contaminated land recommendations. In addition, the Ni concentrations in real documented cases of Metal-petroleum contamination were considered as well. The SGV for Ni is 130 ppm for residential areas, 230 ppm for allotment areas and 1800 ppm for commercial areas (UK Environmental Agency 2009). As for the Dutch list, Ni concentration of 35 ppm is considered as the optimum soil value and 210 ppm as the action concentration in soils and sediments (see table 4.1). Furthermore, soluble Ni ($NiCl_2$), insoluble Ni (NiO) and organic Ni (Ni-Porphyrin (Ni-Porph)) forms of the metal were used for this investigation. Control microcosms consisted of either soil contaminated with petroleum hydrocarbon but no added Ni (PHC) or soils without Ni or oil amendments. The third set of controls was set up to ascertain the effect of the porphyrin without Ni on hydrocarbon degradation. In addition, soils were amended with porphyrins as controls for the Ni-porphyrin amendment. Petroleum degradation in these controls was compared with the Ni-Porphyrin amended microcosms. Soil microcosms were incubated in the dark and at ~21 ^{o}C (i. e. room temperature) for 15 days.

Microbial activity in all microcosms was determined by measuring CO_2 accumulation (mmol CO_2 g^{-1} dry soil) in the headspace gas of microcosms determined by GC-MS (see section 2.4) at two-day intervals. Rates of CO_2 production (mmol g^{-1} dry soil day^{-1}) were then calculated from the maximal linear phase of CO_2 accumulation, which occurred after a short lag phase. Changes in n-alkane composition were determined (as outlined in section 2.7) in selected replicated microcosm treatments chosen on the basis of statistically significant differences in CO_2 production profiles. Variations in the means of

total n-alkanes components relative to other treatments and controls were assessed by one way-ANOVA and individual means were compared by Fisher's multiple pairwise comparison analysis. Also, experiments were compared with controls using the Dunnett's pairwise comparison analysis.

Furthermore, the fate of the added heavy metals in soil was determined by a sequential extraction Ni in Ni-Porph and NiO amended soils based on the methods by (Tessier et al. 1979). This method used was described in details in in section 2.5.

The effects of Ni concentrations and mineral forms on the changes in microbial community composition and diversity in the experimental oil degrading microcosms was analysed using microbiological molecular tools targeting the 16S rRNA gene. These methods have been described in section 2.6 and included DGGE analysis of PCR-amplified bacterial 16S rRNA gene fragments to determine the influence of experimental treatment effects relative to within treatment variations in community composition. Following DGGE analysis, studies based on the sequencing of amplified 16S rRNA gene fragments were carried out to obtain a greater understanding of the differences in the microbial community structures in the experimental microcosm treatments. For this purpose, 16Sr RNA genes of communities were sequenced using the ion torrent PGM sequencing methods. Due to the high reproducibility of communities identified from the DGGE analysis, representative samples were used in this analysis. Tagged 16S rRNA samples from 17 soil samples consisting of metal (Ni, Cd and Pb) petroleum degraded soils and controls, which were the petroleum amended, and unamended soils made up the clone library used for the ion torrent sequencing. The sequence data obtained were analysed using the MOTHUR and QIIME software to trim sequence data, cluster sequence data into OTUs, assign taxonomic groups to OTUs and analyse community diversity (alpha- and beta- diversity of communities). Sequence data were trimmed to recover sequences that have a minimum length of 300 bp, a maximum of 500 bp. and homopolymers lower than 7 bp were trimmed. Communities of the Ni-amended soils consisted of 6 representative samples including Ni-Porph amended soils at 12.5, 50 and 350 ppm Ni concentrations and NiO amended soils at 12.5, 50 and 350-ppm Ni concentrations. Sequences of OTUs were, further, matched within the RDP

release 11 and BLAST databases to determine the closest relatives. Based on this match, the phylogenetic tree was generated using the MEGA 6.

Furthermore, qPCR assays that specifically target the 16S rRNA gene of taxonomic groups of interest was carried out to determine the abundance of the target taxonomic group in communities (see chapter 3). The target groups were general bacteria and *Rhodococcus* (closest relatives (100% similar) to the predominant OTU in the oil degraded communities).

For the molecular microbial studies, a subset of samples was selected for analysis based on observed significant differences in microbial activities as determined by CO_2 production rates. Samples from soil microcosms contaminated with NiO and Ni-Porph at Ni-concentrations of 12.5, 50, and 350 ppm were used for Microbiological analysis. The oil only amended and unamended soils were also subjected to this investigation to compare with the test samples.

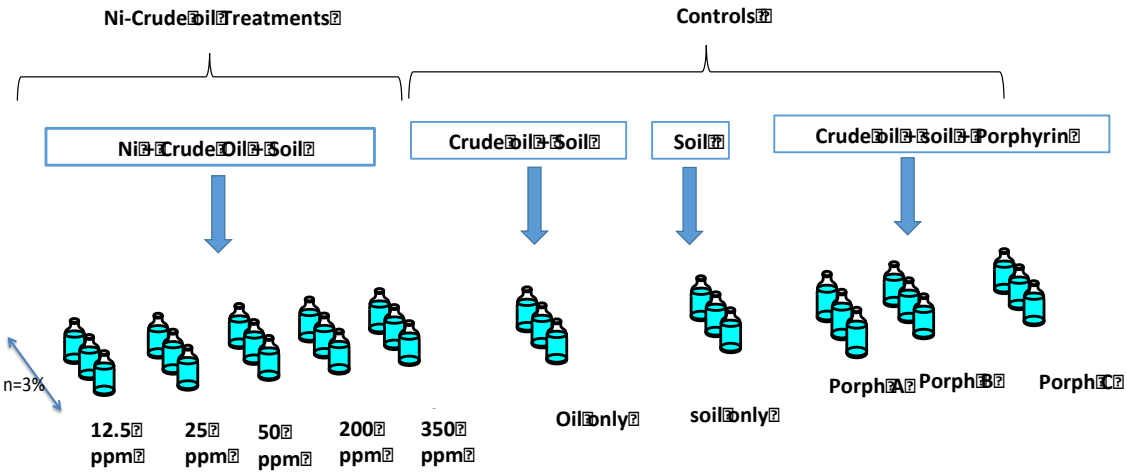


Figure 4.1: the experimental design for the study of crude oil biodegradation in Ni-crude oil amended soil microcosms at increasing Ni-concentrations ranging from 12.5 ppm to 350 ppm relative to oil only amended and no oil amended (soil only) controls in triplicates. Ni amendment was carried out with Ni-Porph, NiO and $NiCl_2$. Porphyrin amended soils were also set up to relate to Ni-Porph amended soils.

4.4. Results

4.4.1. Chemical properties of soil

Physicochemical analysis of the Nafferton soils, as presented in table 4.1, confirmed that these soils samples were not initially contaminated with heavy metals. Specifically with respect to the subject of this chapter the results revealed that the average soil nickel concentration was 9.06 ± 0.64 ppm, which is below the recommendation values by the UK Environmental guideline values and the Dutch list. In addition, the concentration values of other heavy metals were below their respective recommendation values (see table 4.1). With the average value of 7.13 ±0.03, the pH of the soil is approximately neutral.

Table 4.1: Metal Concentration of Nafferton ecological farm soil samples

Metals	Concentration of Heavy metals in soil (ppm)	UK SGV (ppm)	Dutch list (ppm)
Mercury	0.077±0.012	80	10
Arsenic	11.2±4.4	43	55
Cadmium	0.73±0.088	1.8	12
Chromium	17.33±1.45	380	380
Copper	15±1	190	190
Nickel	9.07±0.64	230	210
Lead	125.67±71.19	530*	530
Selenium	<0.5	100	n/a
Vanadium	27±3.06	n/a	n/a
Zinc	81.67±9.91	720	140-720
Hexavalent Chromium	<1.0	n/a	n/a
Boron (water soluble	2.77±0.13	**n/a**	n/a

**Note: ***_The SGV for lead has been withdrawn and is being reconsidered presently. The value used on this table is the value used previously before the withdrawal of the SGV (values ± the standard error)._
n/a means not available

Also, the average total organic matter of the soil was 3.13 ±0.12% by weight. This is within the range of typical soil organic matter content for arable soils (Hobson 1983), therefore, this suggests that the soil was not contaminated with organic contaminants. Also, this soil has no history of contamination in the past. These results, therefore, imply that the soil is a typical pristine soil and is suitable for the experiment simulating an oil spill event onto agricultural land.

4.4.2. Soil microbial activities

4.4.2.1. Lag and linear phase time comparison of oil-amended with unamended controls indicating the presence of HC degrading bacteria

In oil-amended soil microcosms, lag phase period occurred at the inception of degradation, followed by a period of maximum production, which resulted in a steep rise in CO_2 production and a period of reduced CO_2 production (see figure 4.2).

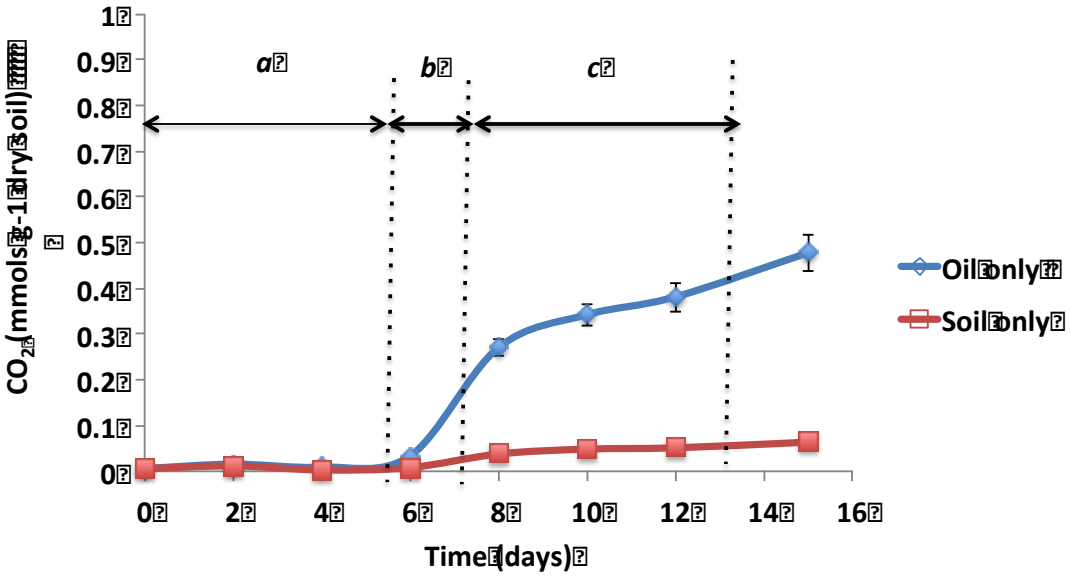


Figure 4.2: CO_2 production profiles in oil amended (oil only) and unamended (soil only) controls. The data points represent the mean cumulative CO_2 of triplicate samples in mmol g^{-1} dry soil. The error bars represent the standard error of triplicate samples. Graph legend indicates oil amended (oil only) and unamended (soil only) controls. a, b and c indicate the lag phase, log phase and decelerating growth phase, respectively.

The Analysis of Variance, ANOVA, and the Dunnett's multiple comparison analysis were used to compare cumulative CO_2 in oil-amended and unamended controls. ANOVA identified that cumulative CO_2 produced in oil-amended controls was significantly higher than the cumulative CO_2 produced in unamended controls ($p=0.0007$). While cumulative CO_2 in oil-amended controls increased from 0.00592 ± 0.000265 mmol g-1 dry soils on day 0 to 0.478 ± 0.04-mmol g-1 dry soils on the day 15, in unamended control, CO_2 increased from 0.000559 ± 0.00018 mmol g-1 dry soils on day 0 to 0.064 ± 0.018 mmol g-1 dry soils day 15. The Dunnet's comparison analysis identified that the difference of means was 0.414 ± 0.0434-mmol g-1 soils.

To statistically determine the changes in cumulative CO_2 during degradation, cumulative CO_2, at 2-day interval, was statistically compared using a combination of ANOVA and multiple pairwise comparisons analysis by Fisher's test (which compared differences of means of cumulative CO_2 at 2-day interval during oil degradation). This way, discrepancies in the cumulative CO_2 produced at each observed period during degradation period were identified. Analyses revealed that no significant changes in cumulative CO_2 occurred between day 0 and day 6 in oil-amended soils ($p=0.36$; differences in means =0.027 mmol g-1 soils). This implies that lag phases in microcosms were approximately 6 days. Successively, cumulative CO_2 increased significantly by 0.236 ± 0.0295 between day 6 and 8 ($p<0.0001$), and by 0.072 ± 0.0295 mmol g-1 soil between day 8 and 10 ($p=0.024$). This was followed by an increase in CO2 by 0.039 ± 0.0295 mmol g-1 soil within days 10 and 12, which was not significant ($p=0.207$) and a slight increase by 0.097 ± 0.0295 mmol g-1 between days 12 and 15 ($p=0.005$). However, in the unamended control, cumulative CO_2 observed on days 0 to day 12 were not significantly different ($p=0.097$). Moreover, means of cumulative CO_2 significantly increased by 0.058 ± 0.0017 mmol g-1 soils on day 15 ($p=0.02$).

These observations coincided with figure 4.2. Also, the observations correspond to standard graphs of microbial population growth phases; lag-phase (period of adaptation to growth condition), log-phase (period of exponential growth) and decelerating growth phase (period of reduced growth) (GUNSALUS 1951; Mason and Egli 1993; Buchanan et al. 1997). The

significant differences in CO_2 production between oil-amended and no oil controls indicate the presence of hydrocarbon-degrading bacteria in oil-amended soils. The increased proliferation of hydrocarbon degraders in the oil-amended soil accounts for the increased CO_2 production in the oil-amended soils.

4.4.2.2. The effect of Ni concentrations and Ni forms on the Lag and Linear phase time during petroleum degradation

The CO_2 production profile in Ni-containing oil-degrading microcosms observed the periods of lag phase, log phase and decelerating growth as was seen in oil only control (see figures 4.3, 4.4 and 4.5). However, differences were observed in microcosms' CO_2 profiles. Cumulative CO_2 at the observed time during the period of degradation in experiment microcosms were compared with the controls using ANOVA and Dunnett's multiple comparison methods to determine the differentiation in CO_2 production profile in Ni containing microcosms. ANOVA revealed that the CO_2 production profile observed in $NiCl_2$ contaminated soils, irrespective of Ni concentration, were not significantly different from the Oil only control ($p=0.545$). Similarly, CO_2 production profile in Ni-Porph containing soils, irrespective of concentration, were not significantly different from the oil only control. In contrast, ANOVA revealed that there were significant differences between the profile of oil only control and NiO containing soils ($p=0.0014$). Pairwise comparison of NiO soils with Oil only control revealed that while the CO_2 production profile of oil only control was not significantly different at 12.5, 25, 50,and 200 ppm Ni, at 350 ppm Ni ($p=0.9255$, 0.9256, 0.9836, 0.5353 respectively), CO_2 production was significantly slower than production in oil only control ($p=0.0006$). Also, Dunnett's pairwise comparison of NiO 350 ppm soil with soil only control revealed that there was no significant difference in the CO_2 production profile ($p=1$).

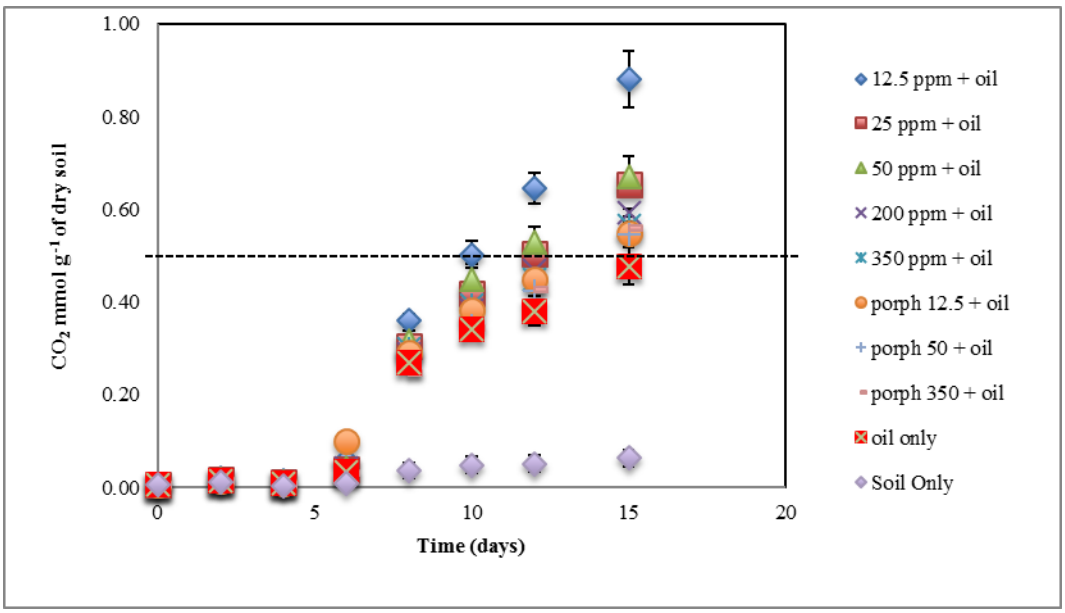


Figure 4.3: CO_2 production profiles of Ni and petroleum hydrocarbon co-contaminated soil microcosms relative to oil amended and unamended controls. Microcosms were amended with Ni-Porphyrin. The data points represent the mean cumulative CO_2 of triplicate samples in mmol g^{-1} dry soil. The error bars represent the standard error of triplicate sample and graph legend indicates the added Ni concentration in ppm and the controls. The dotted line indicates the highest amount CO_2 produced in oil only control.

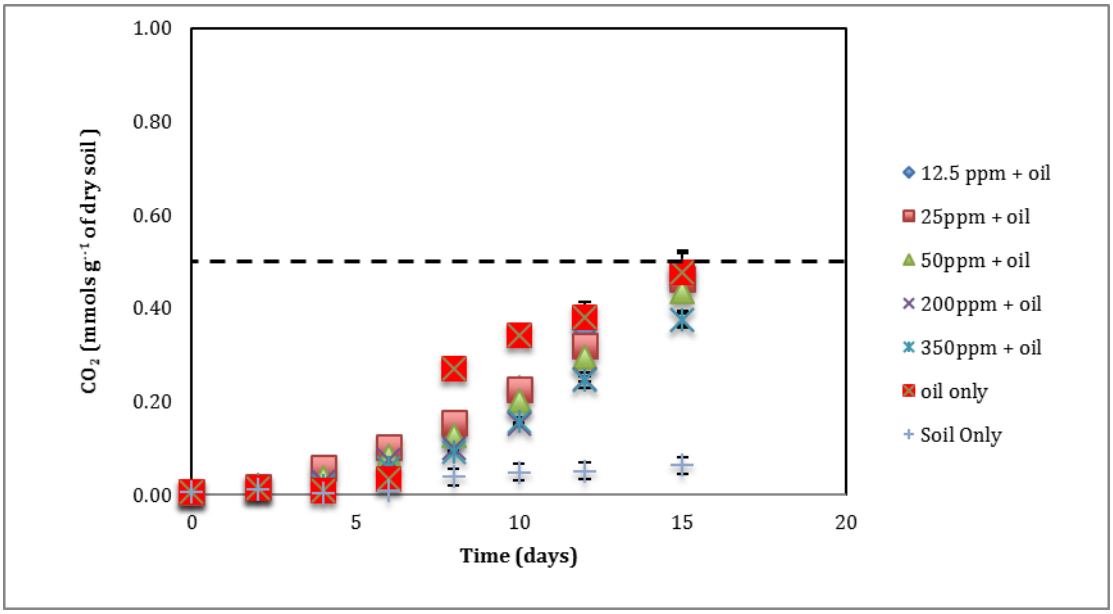


Figure 4.4: CO_2 production profiles of Ni and petroleum hydrocarbon co-contaminated soil microcosms relative to oil amended and unamended controls. Microcosms were amended with $NiCl_2$. The data points represent the mean cumulative CO_2 of triplicate samples in mmol g^{-1} dry soil. The error bars represent the standard error of triplicate sample and graph legend indicates the added Ni concentration in ppm and the controls. The dotted line indicates the highest amount CO_2 produced in oil only control

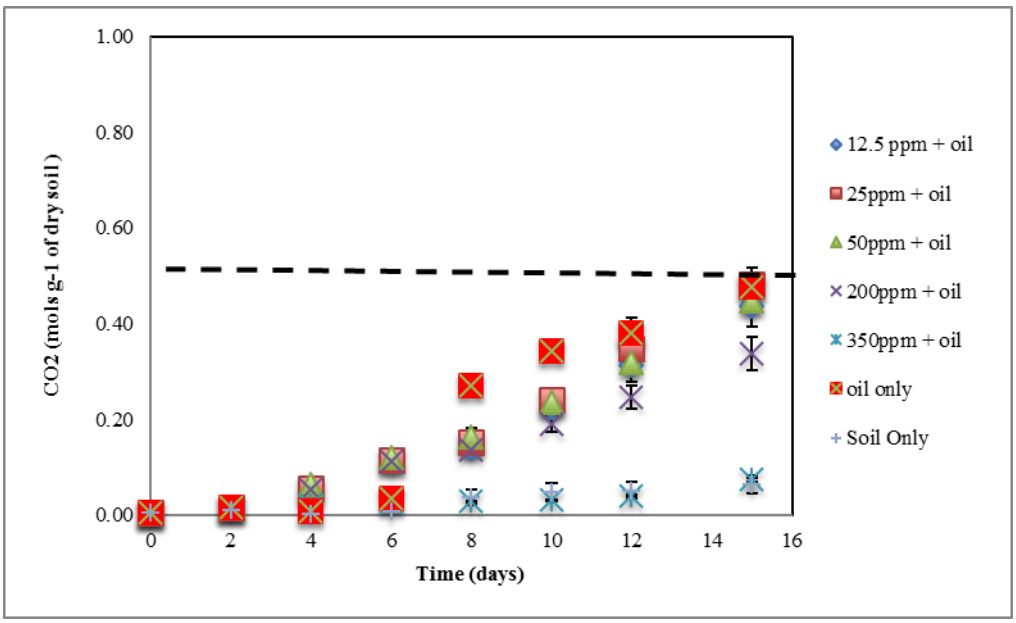


Figure 4.5: CO_2 production profiles of Ni and petroleum hydrocarbon co-contaminated soil microcosms relative to oil amended and unamended controls. Microcosms were amended with NiO. The data points represent the mean cumulative CO_2 of triplicate samples in mmol g^{-1} dry soil. The error bars represent the standard error of triplicate sample and graph legend indicates the added Ni concentration in ppm and the controls. The dotted line indicates the highest amount CO_2 produced in oil only control.

4.4.2.3. Effect of Ni on the maximum cumulative CO_2 during the degradation of petroleum hydrocarbon

The maximum cumulative CO2 (observed on the 15th day of petroleum degradation) in experiment microcosms were compared with oil only amended and soil controls using ANOVA and Dunnett's pairwise comparison methods. ANOVA revealed the chemical form of added Ni ($p<0.0001$) and the concentrations of Ni ($p=0.03$) influenced the maximum cumulative CO_2 in microcosms, significantly. Dunnett's pairwise comparison of maximum cumulative CO_2 in Ni-porph contaminated soils with oil only control revealed that the maximum CO_2 at lower Ni concentrations of 12.5, 25 and 50 were significantly higher than the maximum cumulative CO_2 in the control ($p<0.0001$; $p=0.015$; $p=0.0079$ respectively). However, at higher Ni concentrations of 200 and 350, there was no significant difference in maximum cumulative CO_2 relative to oil only control ($p=0.13$; $p=0.30$ respectively). Also, in $NiCl_2$ soils, ANOVA indicated that maximum cumulative CO_2, irrespective of concentration, were not significantly different

from maximum cumulative CO_2 of the oil only control. In addition, Dunnett's pairwise comparison analysis identified no significant difference between maximum cumulative CO_2 of $NiCl_2$ soils and oil only control. Furthermore, Dunnett's pairwise comparison revealed that while Maximum cumulative CO_2 were significantly lower than oil only control at high concentrations of 200 and 350 ppm Ni in NiO soils ($p=0.022$; $p<0.0001$), there were no significant differences at 12.5, 25 and 50 ppm Ni-concentrations. Also, a pairwise comparison of NiO soils with soil only control revealed that there no significant difference in maximum cumulative CO_2 of the control and soils contaminated at 350 ppm ($p=0.9986$).

4.4.2.4 Effect of Ni on the maximal rate of CO_2 production during the degradation of petroleum hydrocarbon

The maximal rate of CO_2 production during degradation of petroleum was determined and analysed to determine the effect of Ni on the maximal rate of CO_2 production. Figure 4.6 shows a plot of maximal rates per treatment relative to oil-amended and unamended soils. The figure shows that the chemical form of added Ni influenced the effect of Ni amendment. Ni-porph stimulated the maximal rate of CO_2 production relative to oil control, significantly ($p<0.0001$).

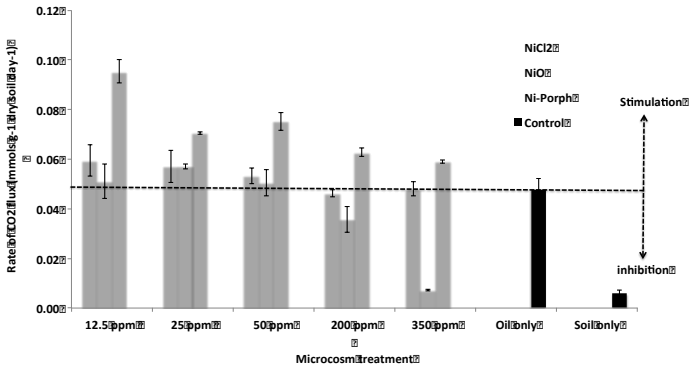


Figure 4.6: The maximal rate of CO_2 production in Ni-oil amended soils relative to oil amended and unamended controls. Each bar represents the mean maximal rate of triplicate sample. The error bars represent the standard error of rate of triplicate samples. The graph legend indicates the chemical form of added Ni and controls. The microcosm treatment on the x-axis indicates the concentration of added Ni and controls. Oil only control is the oil-amended soil while soil only control indicates the unamended soil. Dotted line indicates the rate of oil-amended control; treatments with rates above the dotted lines are stimulatory while treatments with rates below the dotted lines are inhibitory.

This stimulatory effect was highest at the lowest Ni amendment concentration (12.5 ppm) and was progressively less pronounced with increasing Ni concentration. Moreover, Dunnett's pairwise comparison revealed that the while stimulatory effects on maximal rate of CO2 production were significantly higher than the oil control at 12.5 ppm ($p<0.0001$), 25 ppm ($p=0.0179$) and 50 ppm ($p=0.0048$), maximal rates of CO2 production were not significantly different at 200 ppm ($p=0.1462$) and 350 ppm ($p=0.2177$) (see figure 4.7). Furthermore, maximal rates in NiCl2 contaminated soils, at all concentrations, were not significantly different from the maximal rates of the oil only control ($p=0.3297$; see figure 4.8). A different situation was obtained in the NiO contaminated soils. Here, the rates of CO2 production decreased with increased Ni concentration. As a result, inhibitory effects on maximal rates of CO2 production were significantly high at 200 ppm ($p=0.0314$) and 300 ppm ($p<0.0001$) relative to oil only control (see figure 4.9). However, maximal rates at lower concentrations of 12.5, 25 and 50 ppm were not significantly different from the oil only control (See figure 4.9).

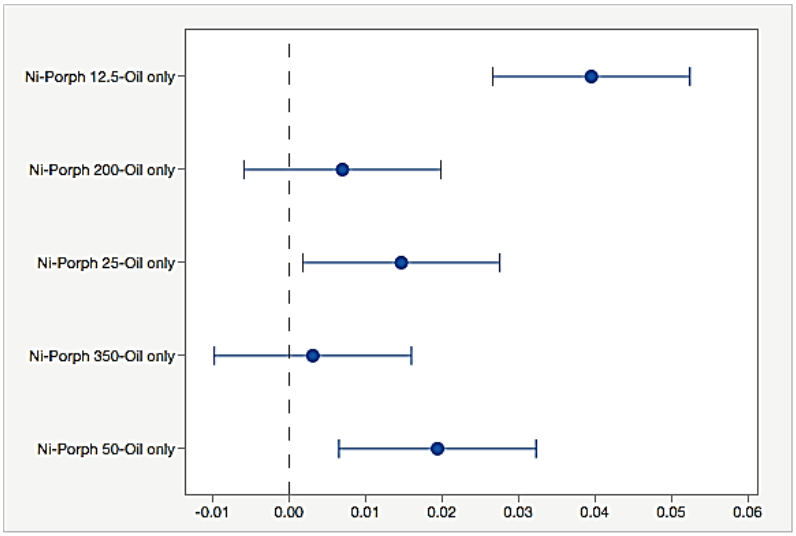


Figure 4.7: comparative analysis of maximal CO_2 production rates in Ni-porph amended oil degraded soil microcosms at Ni concentrations of 12.5, 25, 50, 200 and 350 ppm relative to oil only amended control. Analysis was by Dunnets's multiple comparison analysis at 95% confidence interval. The values include the difference between the treatment mean and the control mean. A mean is not significantly different if its interval contains zero (0).

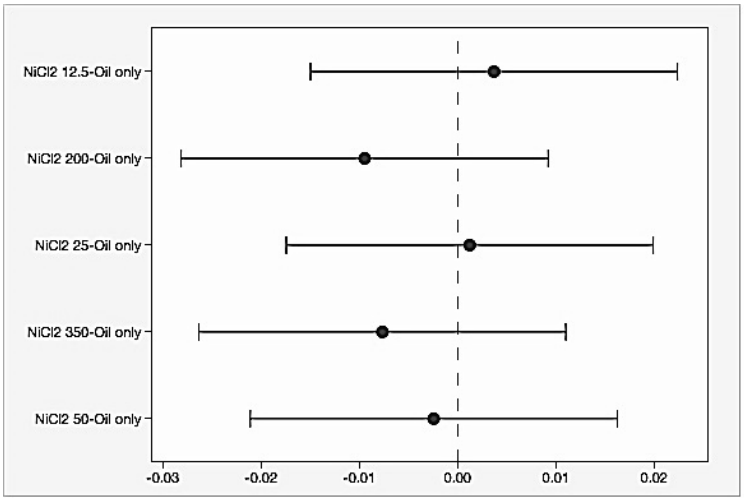


Figure 4.8: Comparative analysis of maximal CO_2 production rates in $NiCl_2$ amended oil degraded soil microcosms at Ni concentrations of 12.5, 25, 50, 200 and 350 ppm relative to oil only amended control. Analysis was by Dunnets's multiple comparison analysis at 95% confidence interval. The values include the difference between the treatment mean and the control mean. A mean is not significantly different if its interval contains zero (0).

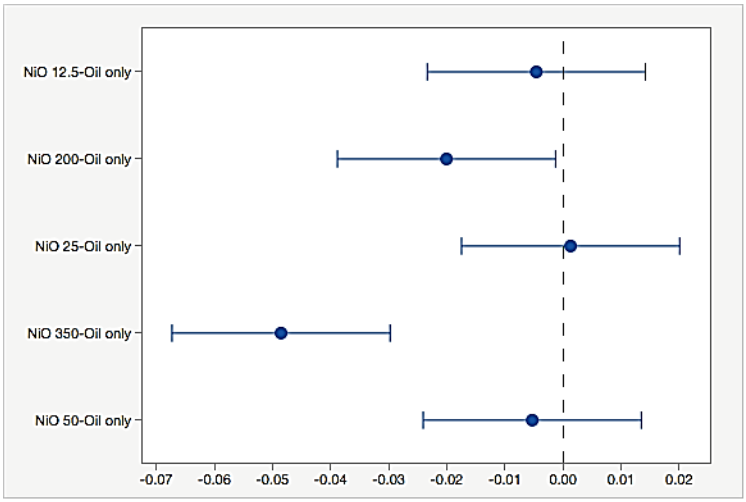


Figure 4.9: Comparative analysis of maximal CO2 production rates in NiO amended oil degraded soil microcosms at Ni concentrations of 12.5, 25, 50, 200 and 350 ppm relative to oil only amended control. Analysis was by Dunnet's multiple comparison analysis at 95% confidence interval. The values include the difference between the treatment mean and the control mean. A mean is not significantly different if its interval contains zero (0).

4.4.2.5 Influence of porphyrin ligand of Ni-porph on the stimulation of petroleum hydrocarbon degradation

Given the nature of the Ni-Porph amendment, i.e. a Ni atom complexed to an organic porphyrin complexing ligand, there are two possible explanations for the observed stimulatory effect on CO_2 production, namely that Ni itself stimulated microbial activity or that degradation of porphyrin ligand might have influenced the stimulation of CO_2 production. To discriminate the influence of the porphyrin component of the Ni-Porphyrin complex from the effect of the metal addition, porphyrin only amended experiments were set up and run concurrently as controls for Ni-Porph experiments. Figure 4.10 shows the maximal rates of CO_2 production in the Ni-Porph amended microcosms compared to their corresponding porphyrin-amended microcosms and oil-amended soil control. ANOVA revealed that maximal rates of CO_2 production in Ni-Porph amended soils were significantly higher than their corresponding porphyrin controls ($p=0.0019$). Furthermore, analysis by Fisher's multiple pairwise comparison showed that maximal rates in the porphyrin controls were not significantly different from the oil only control ($p=0.8858$ for Porph A; $p=0.2933$ for Porph B and $p=0.2414$ for Porph C; where Porph A, B and C are corresponding porphyrins containing controls to Ni-Porph treatments at 12.5, 50 and 350 ppm Ni, respectively).

These observations imply that amendment with porphyrin did not affect the rate of CO_2 production. Hence, CO_2 production was neither stimulated nor inhibited in the porphyrin amended soils. In addition, Fisher's multiple pairwise comparison showed that while the maximal rates of CO_2 in Ni-Porph soils at 12.5 and 50 ppm Ni are significantly higher relative to the corresponding porphyrin control ($p<0.0001$ at 12.5 ppm and $p=0.0316$ at 50 ppm), at 350 ppm Ni, corresponding porphyrin control was not significantly different ($p=0.6676$). These imply that possible porphyrin ligand degradation did not interfere with the stimulation of CO_2 production observed in Ni-porph amended soils at 12.5 and 50 ppm Ni-concentrations.

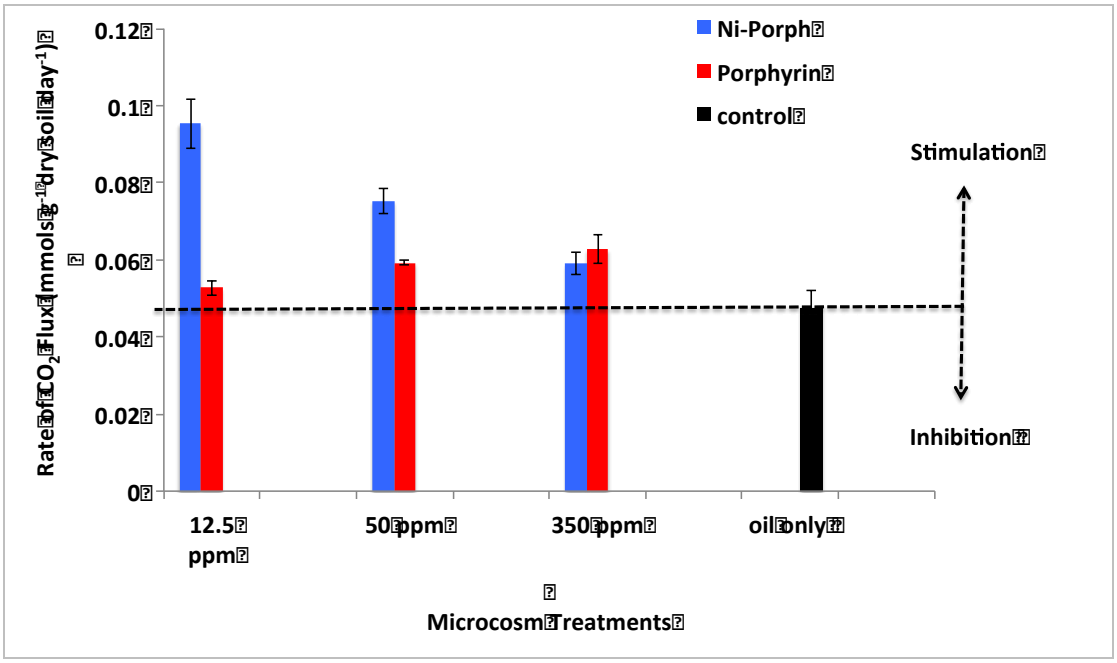


Figure 4.10: the effect of porphyrin on the maximal rate of CO_2 production in soils during the degradation of petroleum hydrocarbon. Each bar represents the mean maximal rate of triplicate sample. The error bars represent the standard error of rate of triplicate samples. The graph legend indicates the Ni-porphyrin, porphyrin and control. The microcosm treatments on the x-axis indicate microcosm treatments' added Ni-concentration and equivalent porphyrin. Dotted line indicates the rate of oil amended control; treatments with rates above the dotted lines are stimulatory while treatments with rates below the dotted lines are inhibitory.

4.4.3. The fate and chemical association of Ni amendments after addition to the soil microcosms

The distribution and chemical associations of Ni added as two amendment forms, NiO and Ni-Porph, was investigated in the soil microcosms by sequential extraction of metals from the soil. The choice of these two Ni forms was because of their observed differences in behaviour relative to stimulation and inhibition of petroleum degradation in soils, which was entirely a different case for $NiCl_2$ contaminated soils where no significant effect on biodegradation was observed.

Triplicate soil samples were contaminated with NiO and Ni-Porph at Ni concentration of 50 ppm and this was incubated at room temperature for 1 week before the sequential extraction of added Ni using the Tessier's sequential extraction method for partitioning developed for the partitioning of metals in soils and sediments (see section 2.7). Ni in the soils was partitioned

into associated soil fractions namely, fractions that were exchangeable, bound to carbonates, bound to Fe- and Mn- oxides, bound to organics and residual fractions of soils (see figure 4.11). The investigation revealed that generally, the majority of the added Ni were associated with the organic and residual fractions. Organic fractions of soils may include living cells/ organisms, detritus, organic acids such as humic and citric acids, small molecular weight organic compounds such amino acids and proteins, soil organic contaminants such as petroleum hydrocarbon and PAHs while the residual fractions include metals embedded in the crystal structure of the primary and secondary minerals.

The individual concentrations of Ni recovered in the five fractions were determined and compared using ANOVA and pairwise comparison by Fisher's multiple comparison tests. In NiO containing soil, ANOVA showed that the concentrations of Ni in the different fractions of the soil partitioning varied significantly ($p<0.0001$). Undetectable concentration levels of Ni (<0.001 ppm) were identified as exchangeable fractions and carbonate fractions. In addition, 6.96 ± 1.05 % of added Ni was associated with Fe- and Mn-oxides relative to the total Ni-concentration added to the soil.

Also, while 26.267 ± 1.053 % of added Ni was bound to soil organics, 48.64 ± 5.82 % of added Ni was recovered from soil residuals. Fisher's pairwise comparison analysis showed that the residual fraction was significantly higher than the organic fractions by 11.189 ± 2.867 ppm Ni-concentration ($p=0.0029$). Similarly, the majority of added Ni were associated with soil organic and residuals in Ni-Porph containing soils, while, 22.56 ± 0.35 % of added Ni were associated soil organic 56.79 ± 2.68 % was recovered as residual. In addition, undetectable Ni concentrations were recovered as exchangeable and carbonate associates. Also, 2.52 ± 0.35% of added Ni was recovered as Fe-

and Mn-oxide associates.

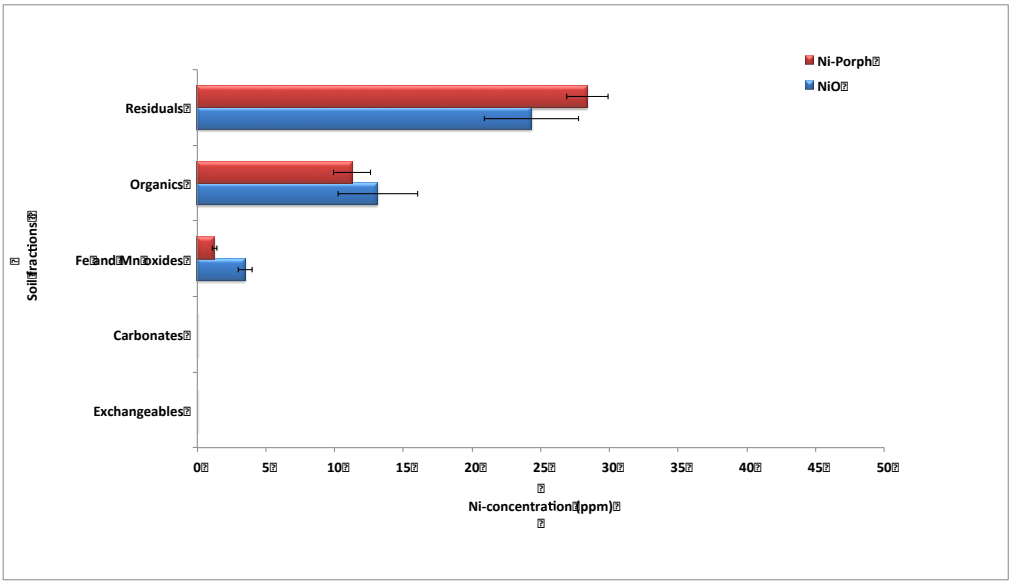


Figure 4.11: Comparative analysis of the fate of added Ni in soil by sequential extraction of Ni fractions associated with exchangeable ions, carbonates, oxides of Fe and Mn, organics and residuals. The graph legend indicates the different chemical forms of Ni used for this study.

4.4.4. Geochemistry of degraded soil microcosms

4.4.4 .1 Degradation of n-Alkanes in microcosms

The residual petroleum hydrocarbons in the soil microcosms contaminated with NiO and Ni-Porph at 12.5, 50 and 350 ppm were recovered after the 15-day incubation period and were analysed by GCFID. Specifically, the n-alkanes were isolated and quantified in microcosms to ascertain the degree of petroleum degradation in microcosms. The n-alkanes identified in the recovered oils were mostly within the range of n-C_{12} to n-C_{32}. The n-alkane profile varied between treatments depending on the degree of petroleum degradation (see figure 4.12). The extraction process loses the light hydrocarbons, i.e. n-C_5 to n-C_{10} or sometimes, nC_{11}), which are known to be present. Typically, n-C_{12} to n-C_{32} makeup ~10% of total petroleum hydrocarbon (Singh et al. 2014). Their quantification, together with pristane and phytane, presents a realistic analysis of the extent of petroleum degradation which will occur in short-term incubations of the type described in this study (Head et al. 2006). In this experiment, 100 mg of petroleum was used to amend microcosms. Hence, it is expected that the n-alkanes consist of

~10 mg of the added petroleum. However, analysis of undegraded oil revealed that n-C_{11} to n-C_{32} consisted of approximately 8.6% of the crude oil used to amend microcosms. The total n-alkanes ranged from 828.1 $\pm$ 618.5 to 5203.6 $\pm$ 1651.8 μ g (see figure 4.13). This wide range indicated variation in petroleum degradation influenced by metal forms and concentrations.

The Total n-Alkanes recovered per treatment microcosm, which was determined by the summation of identified n-alkanes were compared with undegraded crude oil and the oil only controls (see figure 4.13). ANOVA and Dunnett's pairwise comparison was used to statistically identify discrepancies between recovered TPH of treatments and controls.

Interestingly, ANOVA revealed that total n-alkane was significantly influenced by the Ni concentration ($p= 0.0046$) but not Ni form ($p= 0.6279$). ANOVA of the TPH recovered from experiment microcosms and oil only amended control relative to undegraded oil showed that there was significant degradation of whole crude oil during the period of degradation in the soil microcosms ($p=0.0002$). Pairwise comparison using Dunnett's test revealed that total n-alkane of experiment microcosms was significantly lower than the undegraded crude oil ($p<0.005$) except NiO 350 ppm microcosm, which showed no significant difference with undegraded crude oil ($p=0.1398$). This observation implies that degradation of whole petroleum was inhibited in NiO contaminated microcosm at 350 ppm and corresponded with the results from CO2 production experiment.

To identify the influence of Ni amendment on the total n-alkane recovered after degradation, Dunnett's pairwise comparison test was used to compare each of the different Ni-treated microcosms with the oil only control. Analysis revealed that total n-alkanes recovered in Ni-Porph at 12.5 ppm Ni concentration were significantly lower than that obtained at oil only control ($p=0.0484$). However, in contaminated soils, no significant differences in recovered TPH were observed at 50 ppm and 350 ppm relative to oil only control ($p= 0.078$ and 0.973 respectively). Also, Dunnett's pairwise comparison analysis revealed that in NiO containing soils, at 12.5 ppm, total n-alkane was significantly lower than total n-alkane in the oil only control by 2.136 ± 0.5972 ppm. However, there is no significant difference between NiO

amended microcosm at 50 ppm and oil only control. At 350 ppm, total n-alkane was significantly higher than oil only control. This observation suggests that stimulation of crude oil degradation occurred at lower concentration relative to the oil only control and stimulation decreased with increased concentration.

4.4.4.2. Pattern of n-alkane degradation in microcosms

To understand the pattern of n-alkane degradation, n-alkanes identified in microcosm extracts were grouped into 3 categories comprising a low molecular weight group (nC_{12}-nC_{18}), a medium molecular weight group (nC_{19}-nC_{25}) and a high molecular weight (nC_{26}-nC32) group. Quantification revealed that pooled concentration of low molecular weight group was less than medium molecular weight group, which was equally less than High molecular weight group in all microcosms. Hence, the more the molecular weight, the more the concentration of n-alkane. ANOVA was evaluated to distinguish the groups. The analysis revealed significant differences between the groups of n-alkanes observed ($p<0.0001$). In addition to this, Dunnett's comparison tests for differences in means revealed no significant differences between low molecular weight and medium molecular weight groups ($p=0.12$).

However, comparison of high molecular weight group with low and medium molecular weight groups indicated that total concentration of high molecular weight n-alkanes was significantly higher than total concentrations of lower and higher molecular weight n-alkanes. Thus, lower molecular weight and medium molecular weight n-alkanes were preferentially degraded prior to the degradation of the higher molecular weight n-alkanes. Dunnett's comparison revealed that apart from microcosms amended with NiO at 350 ppm Ni, the pattern of degradation was similar across treatments. This corresponds to the results recovered TPH and n-alkane/ isoprenoid ratios, which showed that little if any degradation occurred on amendment of soil with NiO at 350 ppm Ni.

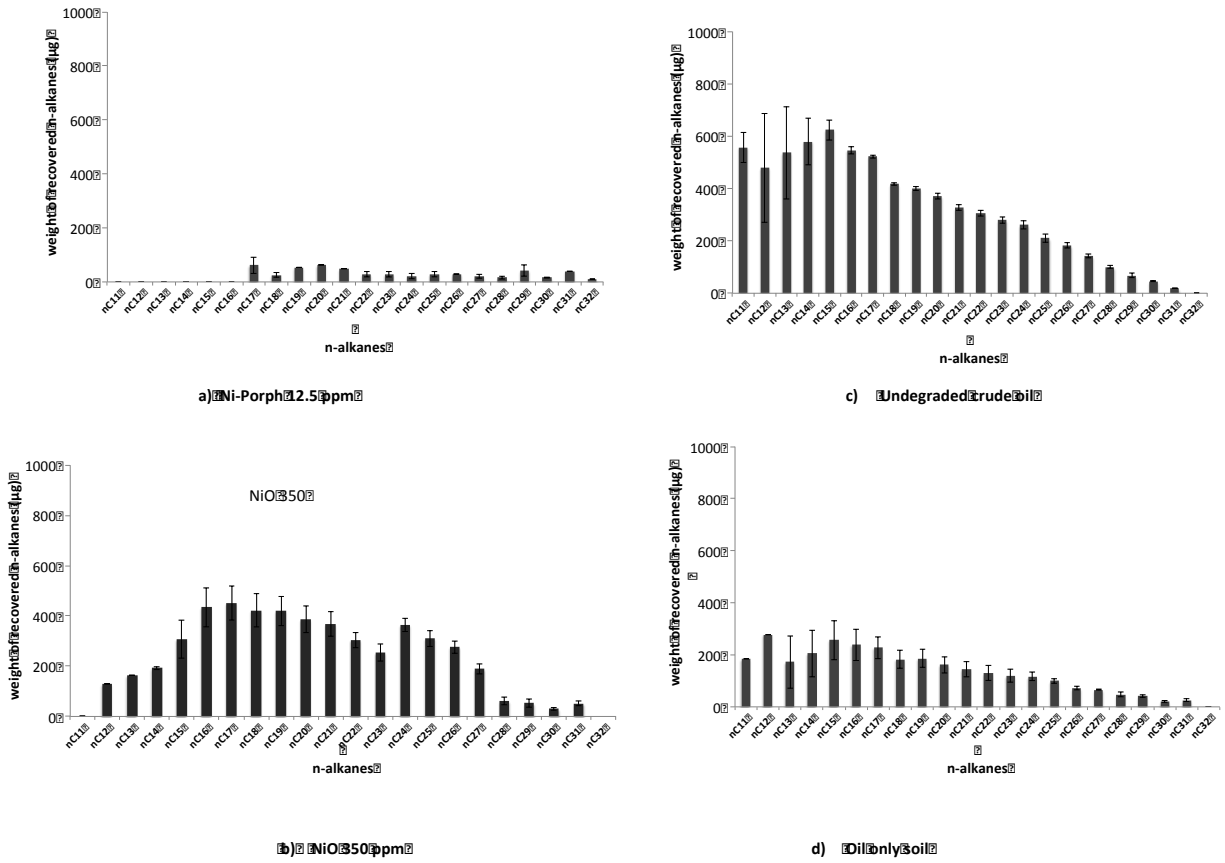


Figure 4.12: the n-alkane profile indicating the degree of degradation of whole petroleum in soil microcosms studied. The individual n-alkanes ranged from nC11 to nC12. Each bar represent the mean sum of recovered individual n-alkane of triplicate samples and the error bars represent the standard error of triplicate samples. a) Represents the n-alkane profile in soil amended with Ni-porph at 12.5 ppm; b) represents the n-alkane profile in soil amended with NiO at 350 ppm; c) represents the n-alkane profile of undegraded crude oil and d) represents the n-alkane profile in soil amended with crude oil only.

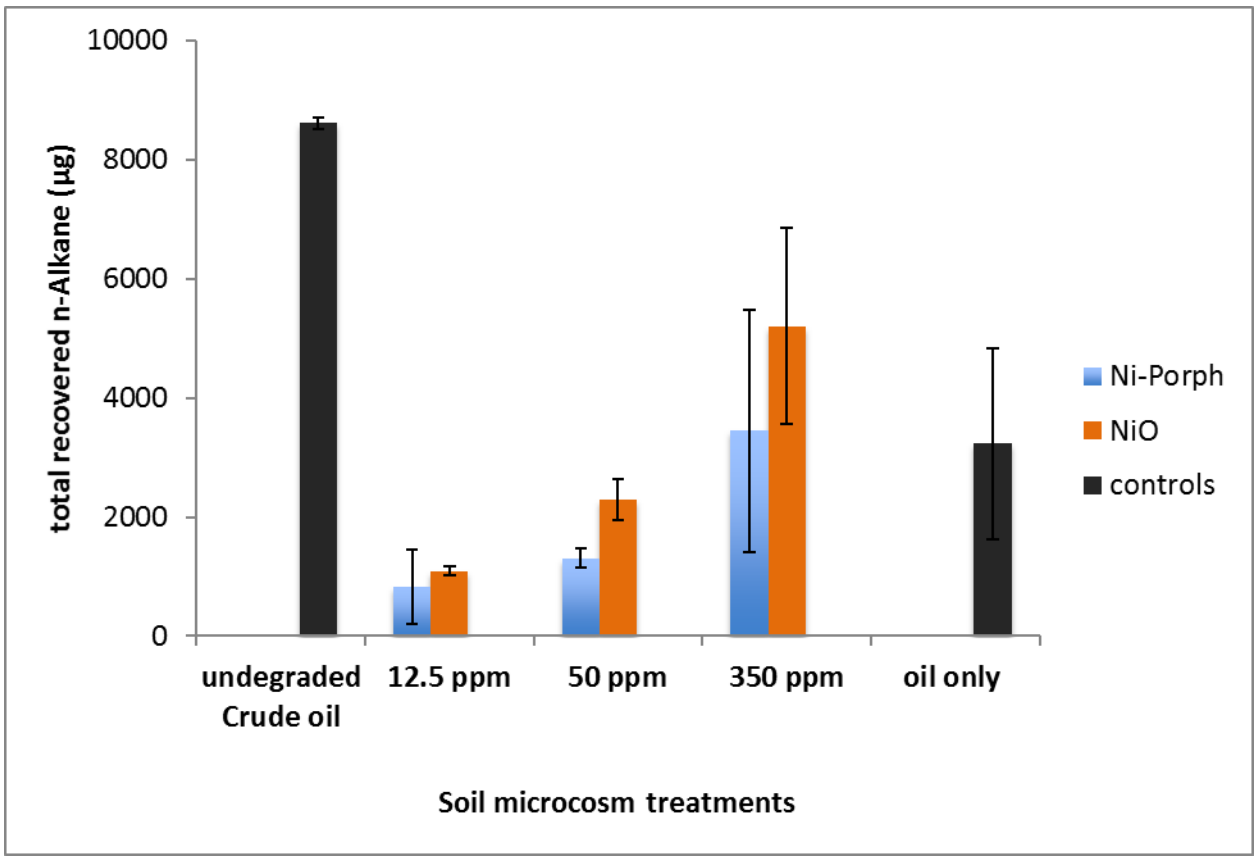


Figure 4.13: recovered Total n-alkanes identified in microcosms contaminated with Ni and/or 100 mg oil after 15 days degradation period. Each bar represents the average of triplicate samples. Error bars represent the standard error of triplicate samples.

4.4.5. Effect of Ni on Microbial community diversity

4.4.5.1. Denaturing gel electrophoresis indicating the microbial communities influenced by Ni-amendment and the reproducibility of communities

A range of molecular techniques was used to study the dynamics of microbial community diversity and phylogeny in response to the different experimental treatments. Initially a PCR-DGGE approach was used to identify variations in patterns of bacterial diversity change within and between the different experimental treatments and correlate these patterns with the measured differences in gas flux and oil chemistry described above. The results from this DGGE analysis were also used to inform selection of samples for subsequent next generation sequencing.

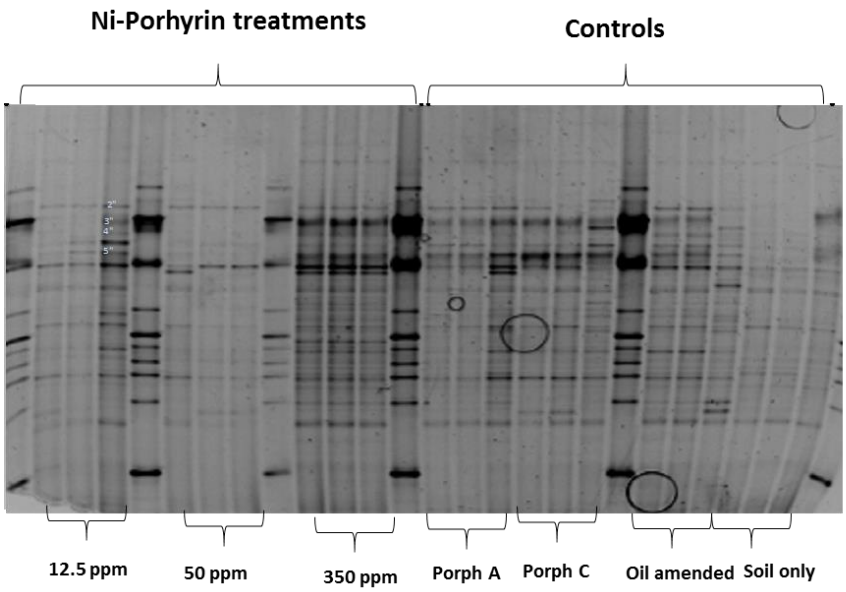
Simple visual inspection of the DGGE fingerprints of amplified bacterial 16s

rRNA gene fragments from soil DNA extracts (figure 4.14) revealed diverse bands amplified from all the soils analysed indicating the presence of a broad range of bacterial species including those with low GC-content and those high GC-content. These profiles also indicate a high degree of reproducibility between experimental replicates allowing clear identification of treatment related effects on community diversity. Comparisons of the unamended soil control with contaminated soils showed selective enrichment of some species of microbes in contaminated soils determined based on the intensity of bands. Although enrichments were observed in most petroleum contaminated soils (see figure 4.14), an exception was obtained in microcosm contaminated with NiO at Ni-concentration of 350 ppm, which appear to have fingerprint comparable with the unamended soil only control. The enrichment of species in soils was influenced by Ni-presence and meal effects depend on chemical form of added Ni and concentration. While a few bands which are reproducibly present in the oil only do not seem so enriched in the Ni treatments there are much more dominant enrichment of a band in Ni treatments which although appearing in the oil only is less dominant. These observations indicate evidence of the impact of metal toxicity on the diversity of the HC degraders enriched and enrichment and evidence of Ni-effect on selected specie growth.

Further elaboration in the community structure differences using multidimensional scaling (MDS) plotted with Bray-Curtis similarity matrix overlaid with similarity contour generated from clustering analysis revealed clustering of microcosms contaminated with same Ni-form at 40% similarity (see figure 4.15). This plot also revealed the entire communities cluster within 20% similarity.

The analysis of the similarities (ANOSIM), carried out using Primer 6 software, revealed significant separation/differences between Ni forms ($R=0.907$; $p=0.002$) and moderate separation among Ni-concentrations ($R=0.451$; $p=0.001$). This implies that the microbial community structures of soils vary significantly in relation to Ni-form and concentration.

a)



b)

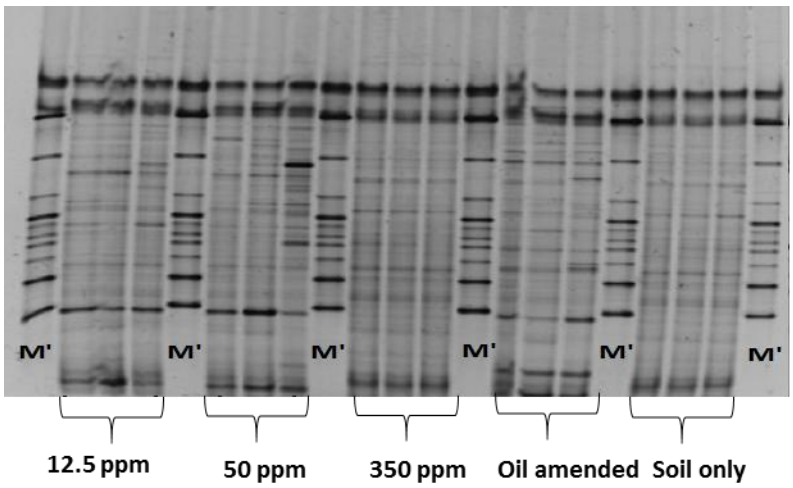


Figure 4.14: PCR amplified 16S rRNA gene fragment DGGE profiles indicating the diversity of bacterial communities and reproducibility of experimental triplicates of soil microcosms contaminated with Petroleum hydrocarbon and Ni of different forms (NiO and Ni-Porph) and at Ni concentrations of 12.5, 50, and 350 ppm. a) shows the DGGE fingerprint of Ni-Porph amended soils at Ni concentrations of 12.5, 50 and 350 ppm relative to porphyrin controls, Porph A and C (bearing the same porphyrin concentration as Ni-Porph at Ni concentrations of 12.5 and 350, respectively) crude oil amended soils and pristine soil (unamended soils) controls; b) shows the DGGE fingerprint of NiO amended soils at Ni concentrations of 12.5, 50 and 350 ppm relative to crude oil amended soils and pristine soil (unamended soils). Each band represents the presence of amplified species in the community and the intensity of band indicate the dominance of specie. M indicate the markers which consisted of cloned 16S rRNA gene fragments. The arrows indicate the direction of denaturant gradient from 40 to 70 % denaturant.

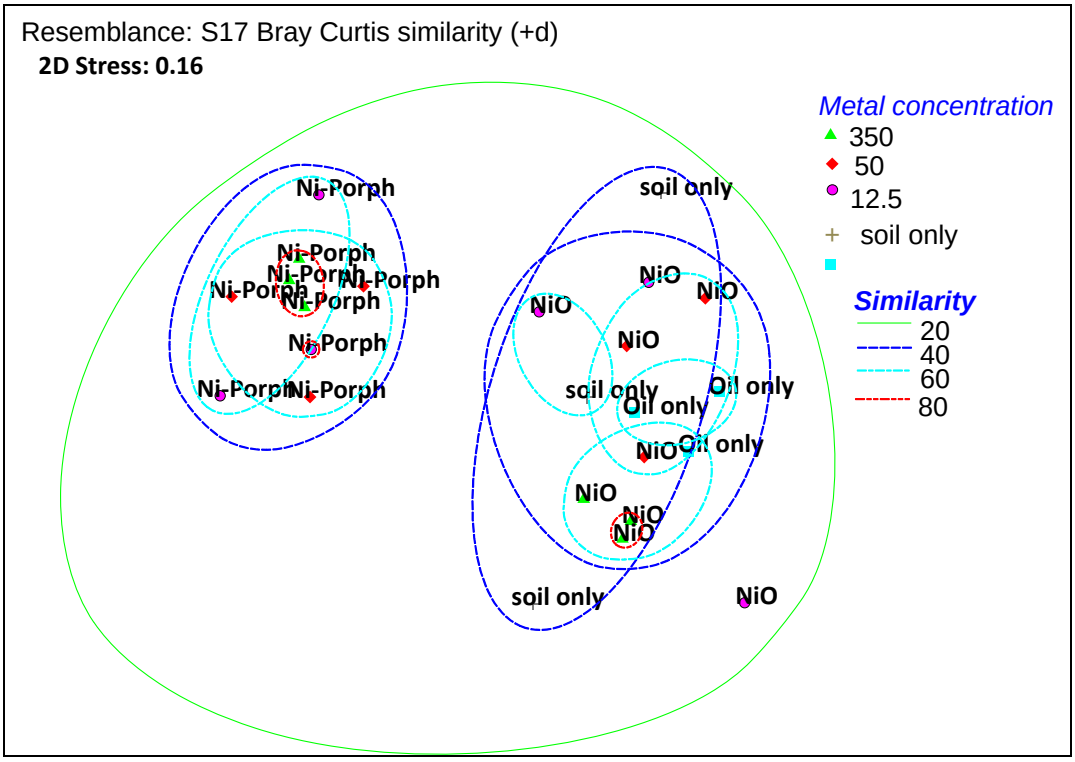


Figure 4.15: Non-metric MDS analysis of the bacterial communities present in Ni-crude oil amended soil microcosms, oil only amended microcosms and unamended soils (soil only) after 15 days of incubation period. MDS plots are representations of how different the communities are from each other based on clustering of like samples. Similarity contour lines from cluster analyses are superimposed on to the MDS plots. Ni-porph indicate Ni-Porphyrin amended soils while NiO indicate NiO amended soils. Oil only indicate crude oil amended soils (no metal amendment) while Soil only indicate unamended soils. The legend indicate the concentrations of added Ni and the controls (i. e. oil only and soil only) as well as similarity contour lines

4.4.5.2. Qualitative analysis of sequencing data set used for the biodiversity study of microbial communities of Ni amended crude oil degraded soil

The ion torrent sequencing recovered high throughput sequence data set of 492574 sequences. Sequence data from Ni-amended communities accounted for 211284 sequences that are approximately 42.89% of the sequence data from the clone library (consisting of tagged 16S rRNA sequences from 17 samples) used for sequencing with the average number of 35214 ± 8980 sequences. The trimming of sequence data on Mothur resulted to the omission of ~33% of the raw sequence data, reducing the sequence recovery to a total 141404 sequences and an average sequence count of 23567 ± 5963 sequences

(see table 4.2).

The number of sequences recovered from the oil amended control was 25,278 sequences before trimming. After trimming, the number of sequences recovered was 21,315 sequences. However, 19,937 sequences was recovered from unamended soil control before trimming and 17,343 sequnces after trimming.

For further analysis on mothur software, sequences were normalised to 8000 sequences per sample (the highest number of sequences mothur could analyse without crashing). Also, for QIIME analysis, sequences were normalised to 13500 sequences per sample; reasonably the least sequence count obtained considering all 17 samples analysed.

Table 4.2: Number of sequences recovered from microbial communities of crude oil –Ni amended soils before and after trimming of sequence data

Sample	Sequence count (raw)*	Sequence count (trimmed)**
Ni-Porph 12.5	23849	16332
Ni-Porph 50	36985	26374
Ni-Porph 350	29129	20062
NiO 12.5	65672	43287
NiO 50	26529	16551
NiO 350	29120	18798
Total	**211284**	**141404**
Mean sequence ± standard error	**35214 ± 8980**	**23567 ± 5963**

note

- *Sequence count from the raw sequence data*

** *Sequence count after the trimming of raw sequence data to eliminate homopolymers and sequences lower than 300 bp and higher than 500 bp*

Table 4.3: qualitative estimation of the sampling of 16 S rRNA sequences used for microbial community and phylogenetic studies on QIIME and Mothur platforms to determine the influence of Ni on the microbial community after oil degradation soil microcosms for 15 days

Samples	Chao estimators (13500 sequences per sample)	Number of observed species (13500 sequences per sample)	Chao estimator (8000 sequences per sample)	Number of observed species (8000 sequences per sample)
Ni-Porph 12.5	7732.227273	3312	5076.636364	1506
Ni-Porph 50	8099.165644	3251	4635.463855	1412
Ni-Porph 350	8353.775551	3391	5447.259887	1610
NiO 12.5	9906.175585	4019	6805.875	2025
NiO 50	8098.297909	3580	5181.456522	1782
NiO 350	9295.013717	4378	7027.780669	2308
oil only	8754.92543	3567	6104.180723	1676
soil only	9637.722008	4562	7331.279605	2457

Note

13500 sequences were used for microbial community studies on QIIME

8000 sequences were used for microbial community studies on Mothur platform

4.4.5.3. OTU-base studies on the influence of Ni on the community diversity of oil degraded soil microcosms

Operational Taxonomic Units (OTUs) generated using the average Neighbor clustering method based on 97% similarity cutoff of 8000 16S rRNA sequences were analysed to determine the alpha diversity; species richness, Shannon Evenness ($E_{H'}$), and Simpson's diversity (λ) were evaluated using the mothur (Schloss et al. 2009) platform (table 4.3). These estimators of diversity evaluated established microbial species diversity in communities and the degree of dominance of communities by species in crude oil containing microcosms with or without Ni.

The species richness was established by determining the number of observed OTUs in communities. Generally, 16382 OTUs were observed in the whole library analysed. Of these, 1676 and 2457 OTUs were observed oil only

amended control and soil only control were, respectively. This implies that dominance of microbial community by hydrocarbon degraders in the oil-amended soil was 46.70% relative to the unamended soil.

Furthermore, the number of observed OTUs in Ni-Porph amended soils at 12.5, 50 and 350 ppm Ni were 1506, 1412 and 1610 OTUs respectively implying that the dominance of communities by hydrocarbon degraders were 11.29 %, 18.70 %, 4.10 % more at 12.5, 25 and 350 ppm respectively, relative to oil only control. More so, the number of OTUs in NiO amended soils at 12.5, 50 and 350 ppm Ni were 2025, 1782 and 2308, respectively implying that relative to oil only control, dominance of communities by hydrocarbon degraders were 17.24 %, 5.95 % and 27.38 % lower in NiO amended soils at 12.5, 50 and 350 ppm Ni, respectively. These observations suggest that microbial diversity in communities are influenced by the chemical form of added Ni. While Ni-Porph amendment improved hydrocarbon dominance in communities, NiO amendment decreased hydrocarbon dominance of degraders in communities.

The evenness of community species was determined using EH'. The $E_{H'}$ values in are usually between 0 and 1 with 0 indicating perfect community species unevenness (i. e. a perfect dominance by a species) and 1 a perfect community species evenness (a perfect heterogeneous community). The $E_{H'}$ of oil-amended soil and unamended soil controls are 0.710628 and 0.874792, respectively. This implies that the dominance of species (hydrocarbon degraders) in oil-amended soils were 23.10 % higher relative to unamended soil. To clearly establish the effect of Ni on community species evenness, $E_{H'}$ in Ni amended soils were compared with the oil only amended soil control. The $E_{H'}$ of Ni-Porph amended soils were 0.62582, 0.564626, and 0.6365 at 12.5, 50 and 350 ppm Ni, respectively. This implies that relative to oil only control, dominance by hydrocarbon degraders increased by 13.55 %, 25.86 % and 11.65 % at 12.5, 50 and 350 respectively. On the other hand, $E_{H'}$ of NiO contaminated soils, which were 0.764972, 0.737043 and 0.873217 at 12.5, 50 and 350 ppm Ni respectively, were lower by 7.10 %, 3.58% and 18.62% respectively. These observations imply that the chemical form of added Ni affects selective enrichment of species in communities with Ni-Porph effecting increase in selective enrichment and NiO effecting decrease in selective enrichment by community species.

Furthermore, λ determined in communities clearly identified the extent of selective enrichment of species in communities by considering species richness and evenness. λ in oil only amended soil and soil only controls were 0.051148 and 0.003842 respectively. These values of λ imply that community of the oil-amended control was 92.25 % more homogenous than the soil only control due to the selective enrichment of hydrocarbon degraders. Similarly, H' of in oil only amended soil and soil only control were 0.00699 and 0.0075 respectively implying that the homogeneity of communities was 7.29 % more in oil-amended soils. Indicating slight change in community diversity. Comparison of λ in oil Ni-Porph amended soil communities relative to oil only amended control indicated that with the λ values of 0.126888, 0.192441 and 0.128645, homogeneity in communities were 59.69 %, 73.43 % and 60.24 % more at 12.5, 50 and 350 ppm Ni respectively. However, relative to in NiO amended soil communities, with λ values of 0.033501, 0.033929 and 0.004012, homogeneity in soils decreased by 52.68 %, 50.25 % and over 100% at 12.5, 50 and 350 ppm Ni, respectively.

In addition, the distinction between communities and controls was also tested using Yue and Clayton's dissimilarity coefficient, θ (see table 4.4). The value of θ ranges from 0 to 1 with 0 denoting a perfect similarity between communities and 1 a perfect dissimilarity between communities. Comparing communities of the oil-amended and unamended controls identified that there are dissimilarities in diversity between the communities of oil-amended soil control and soil-only control (θ=0.946). In Ni-amended soils, the most similar community was observed to be Ni-porph at 12.5 ppm Ni (θ=0.15). While most of the communities of Ni-amended soils show similarity with oil only control, NiO 350 ppm Ni community was highly dissimilar to the oil only amended control (θ=0.946) (see table 4.4 for other values of θ). More so, θ value obtained in NiO 350 ppm soil relative to soil-only control identified the high similarity between the two communities (θ=0.097158), while other communities show dissimilarity to soil only control (see table 4.4 for θ values).

Table 4.3: Community species richness and diversity indices evaluated with 8000 16S rRNA sequences of Ni-oil contaminated soil microcosms

Sample	Number Of Observed OTUs (Specie Richness)	Shannon Evenness ($E_{H'}$)	Simpson's Diverisity Indices
Ni-porph 12.5	1506	0.62582	0.12688
Ni-porph 50	1412	0.564626	0.192441
Ni-porph 350	1610	0.6365	0.128645
Nio 12.5	2025	0.764972	0.033501
Nio 50	1782	0.737043	0.033929
Nio 350	2308	0.873217	0.004012
Oil Only	1676	0.710628	0.051148
Soil Only	2457	0.874792	0.003842

Notes:

1. $0 \leq \lambda \leq 1$ where 0= perfect heterogeneous community and 1= perfect homogenous community

2. $0 \leq EH' \leq 1$ where 0= complete unevenness of community and 1= complete evenness of community specie

3. OTUs are discriminated at 97% similarity cut off

TABLE 4.4: Comparison of microbial community structure in soil microcosms based on 8000 16S rRNA gene sequences

	Oil only amended soil (θ)	Soil only (1-θ)
NiO 12.5	0.22	0.98
NiO 50	0.36	0.99
NiO 350	0.24	0.98
Ni-Porph 12.5	0.15	0.93
Ni-Porph 50	0.22	0.94
Ni-Porph 350	0.95	0.097
Oil only amended soil	0	0.95
soil only	0.95	0

Note: $0 \leq \theta \leq 1$ where 0= perfect similarity and 1= perfect dissimilarity; PHC indicates oil

only control

4.4.5.4. Community diversity study; Ni effects on the diversity of hydrocarbon degraders in oil degraded soil microcosms based on phylogenetic analysis

A Phylogenetic study of the 16S rRNA gene sequences was used to determine the key OTUs, which were present in the soil without and without Ni and oil amendment. To further understand the details of species dominance pattern in the communities, the minimum number of OTUs that made up 50% of the relative abundance of communities was calculated per sample and interestingly a discriminatory pattern of dominance influenced by Ni-form and concentration was observed. For instance, three (3), eight (8) and eleven (11) OTUs made were found to make up approximately 50% of the relative abundance of the communities in the Ni-Porph contaminated systems at 12.5, 50 and 350 ppm Ni-concentrations respectively. This clear evidence of the selective enrichment of a few OTUs greatly contrasted with the number of OTUs that made up 50% of the relative abundance of OTUs in the NiO contaminated systems where Fifty-eight (58), thirty-one 31 and one hundred and sixty-five (165) OTUs made up 50% relative abundances at 12.5, 50 and 350 ppm Ni-concentration respectively. A comparison of these numbers with the number of OTUs (one hundred and seventy-three (173) calculated for the Soil only control revealed that no increase in species dominance had occurred in the NiO contaminated soil at 350ppm. In the same way, a comparison with the oil only control, which had a minimum of 28 OTUs making up 50% abundance of microbial species, indicates that nickel amendment influences petroleum degradation and that this influence depends on the chemical form or the Ni amendment.

4.4.5.5. Comparative phylogenetic analysis of microbial communities of Ni-Crude oil amended soils relative to oil only amended soils and unamended soils

Classification of OTUs carried out using the Bayesian method by (Wang, George M. Garrity, et al. 2007) matches sequences against the Silva, RDP and greengenes reference files on the mothur and QIIME platform revealed that the most dominant organisms in most oil amended systems irrespective of Ni

contamination with the exception of NiO amended community at 350ppm Ni and representing approximately 50% of the relative abundance of the community population per sample, included OTUs that are closely related to bacterial and archaeal strains . The bacterial species that predominated belonged to members of 9 phyla. These were *Actinobacteria*, *Firmicutes*, α-, β-, γ-*Proteobacteria*, *Verrucomicrobia*, *Bacteriodetes*, *Chloroflexi* and *Acidobacteria* (see figure 4.16). The archaeal species were members of the *Crenarcheota*. Most importantly, an observable enrichment of *Actinobacteria* occurred in Ni-oil amended soils and oil only contaminated soils. An exception was observed in the soil contaminated with NiO at Ni-concentration of 350 ppm, which was comparable to soil only control. A careful consideration of the relative abundances of the *Actinobacteria* group in the petroleum contaminated soils revealed an observable increase in relative abundances, which were approximately or more than twice of the relative abundance in the soil only control. Further classification to genus level identified the most dominant OTU (OTU1) in crude oil contaminated soil systems (with or without Ni with the exception of 350 ppm Ni, NiO soil) is closely related to the species of the genus Rhodococcus with 100% similarity (see figure 4.17). Analysing 8000 sequences per sample, OTU1 consisted of approximately 36%, 43% and 35% in Ni-Porph contaminated microcosms at 12.5, 50 and 350 ppm respectively. This result is comparable to that obtained in the soil control amended with only oil, which has OTU1 comprising approximately 21% of the total sequences for that individual library. In the same vain, OTU1 represented approximately 17% and 15% in NiO microcosms at 12.5 and 50 ppm Ni-concentration respectively. Most interestingly, OTU1 consisted of less than 1% in microcosm contaminated with NiO at 350 ppm Ni-Concentration. This representation is similar to the result obtained for the soil only control library. These observations indicated that OTU 1 generally predominance in oil degraded soil is stimulated on amendment with Ni-porph. However, amendment with NiO did not effect the stimulation of OTU1 dominance in microcosms. Infact, amendment with NiO at 350 ppm Ni hindered the dominance of OTU1.

To identify the closest related strains of *Rhodococcus* to OTU1, sequences were further analysed using the basic local alignment search tool (BLAST) and RDP sequence Match. This analysis revealed that OTU1 was very closely

related to strains isolated from hydrocarbon-contaminated and metal contaminated environments (see table 4.5). Some these close relatives that showed 100% similarity to OTU1 were *R. erythropolis* (AJ576250) from Antarctica soil (Shivaji et al. 2004) , *R. erythropolis DN1* (X79289) from Kazakhstan soil polluted with crude oil (Shevtsov et al. 2013), *Rhodococcus sp. YE1 (*EU293153) crude oil contaminated soil (Cao 2007), *Rhodococcus sp. Pi71* (AM110074) from benzene-contaminated groundwater (Fahy et al. 2006), *Rhodococcus sp. SQ8* (DQ366088) from oil polluted soil (Nohit et al. 2006), *R. qingshengii djl-6* (DQ90961) from carbendazim contaminated soil (Jing-Liang et al. 2006; Xu et al. 2007) and *R. jialingiae strain djl-6-2* (DQ185597) from carbendazim contaminated sludge. Others included Rhodococcus sp. Q15 (99.7 %) psychrophilic bacteria from Bay of Quinte, Ontario (Whyte et al. 1998), uncultured bacterium (DQ125599) (99%) from uranium-contaminated soil (Brodie et al. 2006) bromate-reducing bacterium B8 (AF442524) (99%) from drinking water (Davidson et al. 2011) and R. koreensis (AM497794) (97.5%) from a German forest soil (Makdessi et al. 2007; Becker et al. 1997).

Phylogenetic and molecular evolutionary analyses were conducted using the Molecular Evolutionary Genetic Analysis (MEGA 6) software. The sequences of closely and more distantly related strains identified in the BLAST analysis including Rhodococcus type sequences were aligned with the OTU1 sequence identified in this study and an evolutionary tree using the Neighbor-joining method was constructed (see figure 4.18).

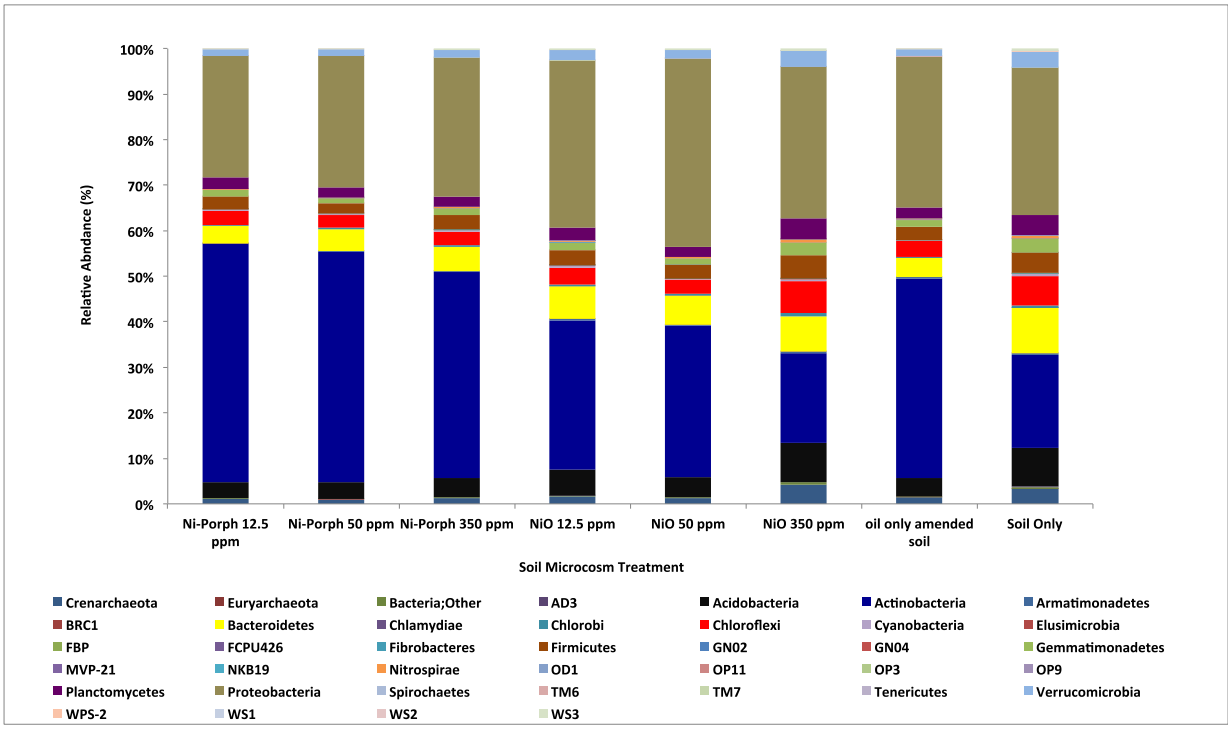


Figure 4.16: Comparative analysis of relative abundances of phylogenic group of 16S rRNA sequences recovered from representative Ni- crude oil amended soil microcosms relative to oil only amended soil and unamended soil. The relative abundance was determined using the QIIME and each treatment was normalized to 13500 16S rRNA sequences. The Legend indicate the different phyla represented in the microcosms

4.4.5.6. Evidence of Ni-influence on selective enrichment of hydrocarbon degraders in soil

Apart from the unequal enrichment of *Rhodococcus* across all treatments, other evidences of selective enrichment, which are influenced by soil treatments, were observed (see figure 4.17). Enrichment with unknown *Comamonadaceae*, a β-proteobacteria, was observed in oil-amended control. However, enrichment of this bacterium is more pronounced in NiO amended soils at 12.5 and 50 ppm Ni (~2x enrichment in oil only control) but not in Ni-Porph amended soil at 12.5 ppm Ni. Enrichment of unknown *Comamonadaceae* in Ni-Porph amended soils at 50 and 350 ppm Ni is comparable to enrichment of the bacterium in oil only amended soil. Also, *Mycobacterium* was observed to be clearly enriched in oil only amended soil. Enrichment of *Mycobacterium* is ~2X lower than enrichment in Ni-Porph amended soils relative to oil only control. However, in NiO soils, *Mycobactrium* was rarely enriched.

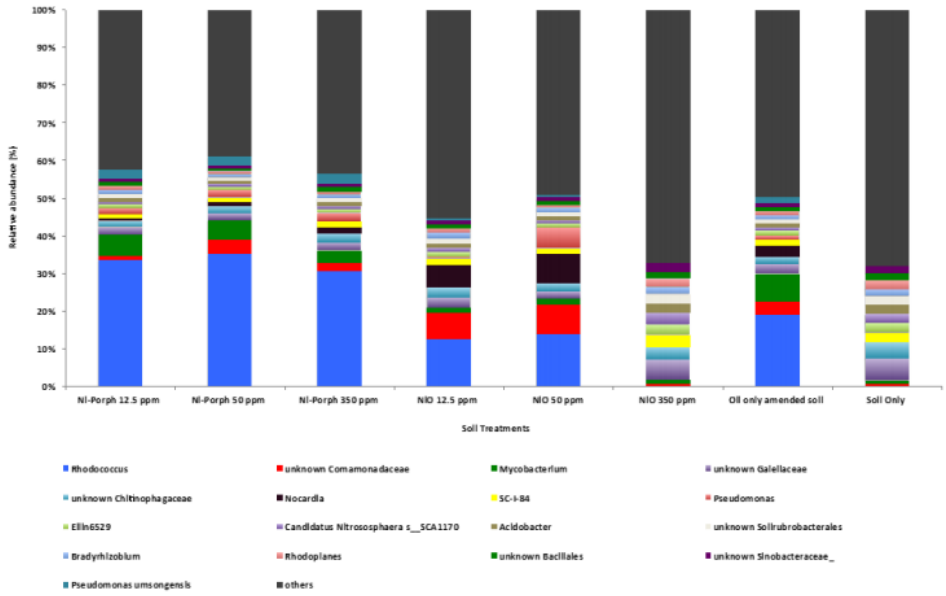


Figure 4.17: Comparative analysis of relative abundances of genera of 16S rRNA sequences recovered from representative Ni- crude oil amended soil microcosms relative to oil only amended soil and unamended soil. The relative abundance was determined using the QIIME and each treatment was normalized to 13500 16S rRNA sequences. 50 most dominant genera were used in this analysis. The percentage sequences represented relative to the number of sequences per treatment is indicated in brackets. The Legend indicates the different genera represented in the microcosm. Others in the legend indicate the collective relative abundance of genera with individual relative abundance less than 1%.

Contrary to this is the selective enrichment of *Nocardia*, which was very prominent in NiO soils and oil only amended soils but not in Ni-Porph soils. Further observation showing selective enrichment, which was influenced by both Ni forms and concentrations, was the selective enrichment of *Pseudomonas* in NiO amended soil at 50 ppm Ni. *Pseudomonas* was 78.6 % more enriched in NiO amended soil at 50 ppm Ni than enrichment in oil only amended soil. Also, enrichment in NiO at 12.5 ppm is 92.3% less than enrichment at 50 ppm. Compared to the Ni-Porph soils, enrichment of *Pseudomonas* is 71%, 66% and 60% less relative to NiO 50 ppm soil. Interestingly, *Pseudomonas umsongensis* was clearly enriched in oil only amended and Ni-Porph amended soils, but was rarely enriched in NiO soils.

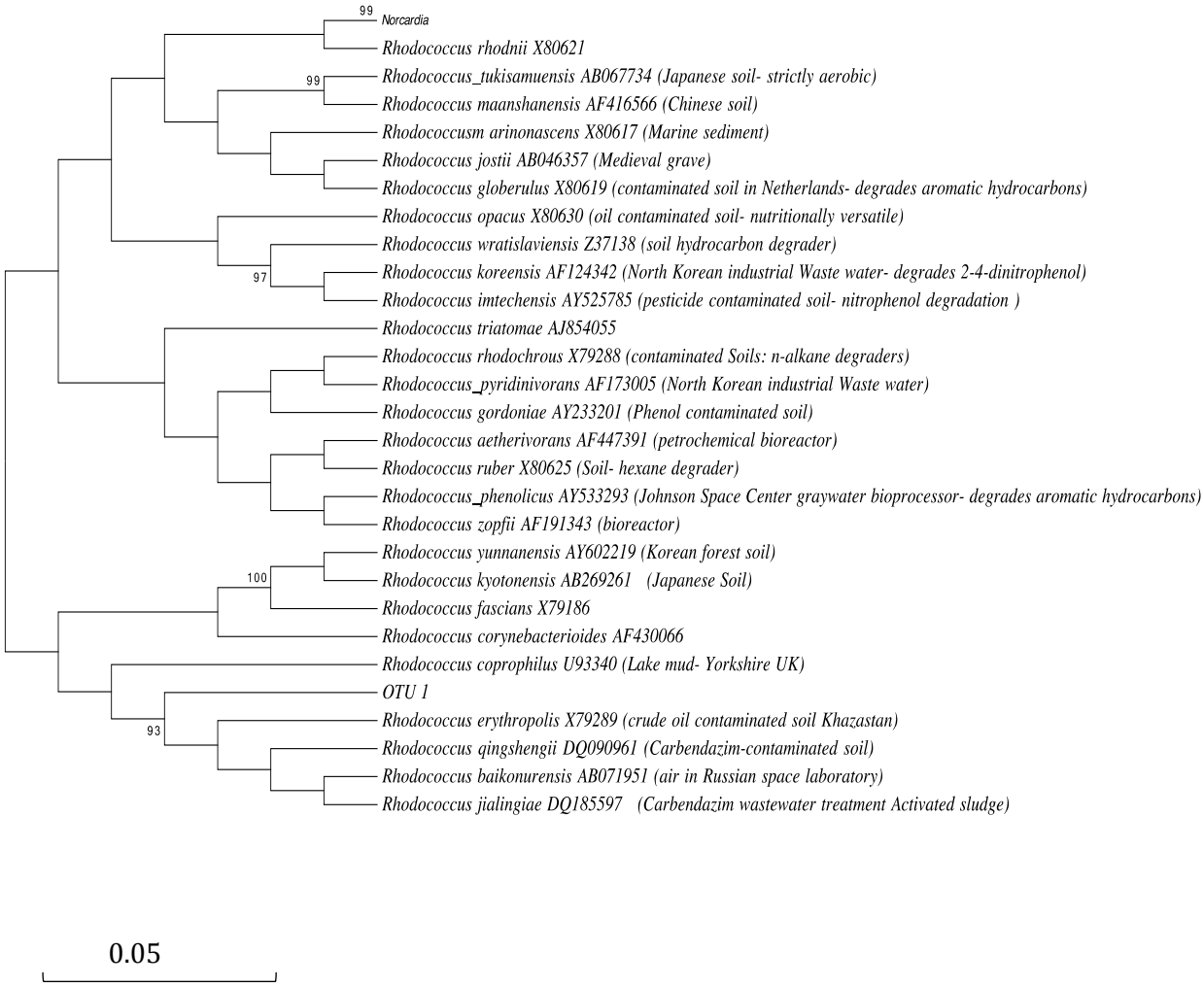


Figure 4.18: Comparison of the dominant sequence recovered from Ni-crude oil amended and oil only amended soil microcosms with closely related bacteria (mostly bacteria belonging to the phylum *Corynebacterium)* on phylogenetic distance tree based on the Neighbour-joining method. All sequences matched to the test sequence are typed 16S rRNA sequences. GenBank accession number for all data sequences are provided and a brief description of each strain of bacteria source are provided in brackets. The scale bar denotes 0.005 divergence and the values at the nodes indicate the percentage bootstrap trees that contained the cluster to the right of the node. Bootstrap values lower than 70 are not shown.

Table 4.5: Close relatives of the most abundant sequence recovered from oil degraded microcosms with or without Ni

Bacteria strain	Accession number	Similarity (%)	Source	Reference
Rhodococcus erythropolis	AJ576250	100	soil sample from Antarctica	Shivaji et al 2004
Rhodococcus erythropolis DN1	X79289	100	crude oil contaminated soil, Kazakhstan	Shevtson et al., 2013
Rhodococcus spp YIE	EU293153	100	Crude oil contaminated soil	Cao, 2007*
Rhodococcus sp. Pi71	AM110074	100	benzene contaminated sandstone aquifer	Fahy et al 2006
uncultured Rhodococcus sp. SQ8	DQ366088	100	oil polluted soil	Nohit et al 2006
Rhodococcus quinshengii djl-6	DQ90961	100	Carbendazim containing soil	Jing-Liang et al 2006
Rhodococcus jialingiae	DQ185597	100	Carbendazim containing sludge	Wang et al 2010
Rhodococcus sp. Q15	AF046885	99.7	Bay of Quinte, Ontario	Whyte et al 1998
uncultured bacterium clone AKAU3602	DQ125599	99	uranium contaminated site	Brodie et al 2006
Bromate reducing bacterium 88	AF442524	99	Drinking water	Davidson et al 2011
Rhodococcus koreensis	AM497794	97.5	German forest soil	Makdessi 2007

Note:

1. *Table provides the genebank accession number of the bacteria strains*
2. *The % similarity with the most dominant OTU*
3. *Source of bacterial strain isolation*

* *Unpublished work*

4.4.6. Absolute 16S rRNA gene abundances of general bacteria and *Rhodococcus* in the microbial communities of degraded soil microcosms

Oil contamination of soil environment has been found to affect the soil microbial populations by the enrichment of specific individual OTUs. The abundance of the 16Sr RNA gene of bacteria in communities of Ni-oil contaminated soils and oil only amended and unamended controls were determined to assess the change in the bacterial population. This was carried out by qPCR assay, which targeted the 16S rRNA of bacteria in communities using the universal primer set, U1048f-U1371r (Gray et al., 2011). In addition, the relative abundance indicated that strains of *Rhodococcus* sp dominated in the Ni-oil contaminated soil systems in the previous section. Based on this knowledge, a specific qPCR assay was developed to target the 16S rRNA gene of *Rhodococcus* group (i. e. OTU1 sequences). Here, the des primer designed for *Rhodococcus* quantification was RhO1390F-RhO1454R (see chapter 3, section 3.3). Quantification was carried out to clearly determine the Ni effect on soil bacterial populations during the biodegradation of crude oil in the soil. 16S rRNA gene abundances were determined in each replicate of the microcosm treatments. The 16S rRNA gene abundances of treatments relative

to oil only amended and unamended control are presented in figure 4.19. It could be observed from the figure that the general 16S rRNA gene abundance of bacteria in most microcosms is comparable. On the contrary, the figure shows discrepancies in the *Rhodococcal* 16S rRNA gene abundances between treatments. Mean abundances were analysed using ANOVA followed by pairwise comparisons of means using the Dunnett's comparison analysis to statistically identify the significance of the influence of amendments on the bacterial and *Rhodococcal* abundances in soils.

4.4.6.1 Influence of oil amendment on Bacterial abundance in oil degraded soil

Analysis of the bacterial 16S rRNA gene in oil-amended and unamended soils revealed that soil amendment with oil only increased the mean abundance of bacterial 16S rRNA gene by 9.10×10^7 sequences relative to soil only control. This increased bacterial abundance could be attributed to the proliferation of hydrocarbon degraders in the oil-amended soils. This increment in bacterial abundance was not significant ($p=0.97$).

4.4.6.2. Influence of Ni on the bacterial abundance in oil-amended soil systems

Ni influence on bacterial abundance due to oil amendment was investigated by comparing the mean abundance of 16S rRNA gene abundance in Ni amended soils relative to the oil only amended. Generally, ANOVA revealed that there were no significant differences in mean abundance across treatments ($p=0.192$). In Ni-Porph amended soil at 12.5 ppm comparison analysis by Dennett's method revealed that the mean abundance of bacterial 16S rRNA gene in Ni-Porph amended soils at 12.5 ppm, which was 3.91×10^8 sequences, increased by 2.77×10^8 sequences relative to the oil only amended soil. However, at the higher Ni concentrations of 50 and 350, bacterial 16S rRNA gene abundance decreased by 2.25×10^6 and 6.76×10^7, respectively. Generally, Dunnett's comparison analyses revealed that the bacterial 16S rRNA gene abundances in Ni-Porph soils were not significantly different relative oil only control (see figure 4.19).

In NiO amended soils, the 16Sr RNA gene abundances at 12, 50 and 350 ppm were decreased by 5.34 X 10^7, 4.86 X 10^7 and 8.46 X 10^7 relative to oil only amended soil, respectively. These differences in bacterial abundance of Ni-amended systems are not significantly different with the oil only control (see figure 4.23).

4.4.6.3. The influence of oil amendment on the absolute abundance of Rhodococcus in soils

The amendment of soil with crude oil stimulated the proliferation of Rhodococcus in soils (see figure 4.17). Analysis of the 16S rRNA gene abundance of Rhodococcus in oil only amended soil control relative to the unamended soil revealed that there was a significant increase in the abundance of Rhodococcus in oil-amended soils (p=0.043). While the mean abundance in oil-amended soil was 2.84 X 10^7, in soil only control, the mean abundance of 16S rRNA gene of Rhodococcus was 3.06 X 10^6 the difference in soil only control. Hence, mean Rhodococcus 16S rRNA gene abundance in oil only amended soil and soil only controls 4.46 X 10^7 + 1.52 X 10^7 sequences.

4.4.6.4. The influence of Ni on absolute abundance of Rhodococcus in oil degraded soil microcosms

To understand the effect of Ni amendment on *Rhodococcus* abundance in oil degraded soils, Ni-oil amended soils were analysed statistically relative to oil only control by using the ANOVA and Dunnett's pairwise comparison analysis. ANOVA revealed the abundance of 16S rRNA gene of *Rhodococcus* differed significantly across treatments (p=0.0009). The analysis revealed that in soils amended with Ni-Porph at 12.5 ppm, the mean *Rhodococcal* 16S rRNA gene abundance was 8.82 X 107 ±2.33 X 10 7 sequences, which was higher than the mean abundance obtained in the oil only amended control by 4.21 X 10 7 ± 2.10 X 10 7 sequences. Also, the mean 16S rRNA gene of *Rhodococcus* in Ni-Porph amended soils at 50 and 350 ppm Ni were 4.49 X 10 7 ±1.03 X 10 7 and 1.54 X 107 ±2.06 X 107, respectively. These were lower relative to the oil only amended soils by 1.12 X 106 ±2.10 X 107 and 3.06 X 107 ± 2.10 X 107 sequences, respectively. Dunnett's comparison of Ni-porph amended soils revealed that the differences observed per treatment

were not statistically significant relative to oil only control (p=0.183 at 12.5 ppm; p= 0.9999 at 50 ppm; p=0.3839 at 350 ppm) (see figure 4.20). Also, the abundances of *Rhodococcal* 16Sr RNA significantly decreased with increased concentration of Ni (0.05)

In NiO amended soils, the mean *Rhodococcal* 16S rRNA genes abundance at 12.5 and 50 ppm were 5.97 X 10^{6} ±3.84 X 10^{5} and 9.72 X 10^{6} ±7.55 X 10^{6} sequences, which were not significantly lower than the relative abundance of oil only control by 4.01 X 10^{7} ± 1.72 X 10^{7} and 3.63 X 10^{7} ±1.54 X 10^{7}, respectively. In addition, at 350 ppm of NiO amended soil, the mean *Rhodococcal* 16S rRNA genes abundance which was 8.4 X 10^{5} ±5.24 X 10^{4}, was significantly lower by and 4.52 X 10 + 1.54 X 10^{7} relative to oil only amended control (p=0.0485) (see figure 4.21). Also, the abundances of *Rhodococcal* 16Sr RNA significantly decreased with increased concentration of Ni (0.05).

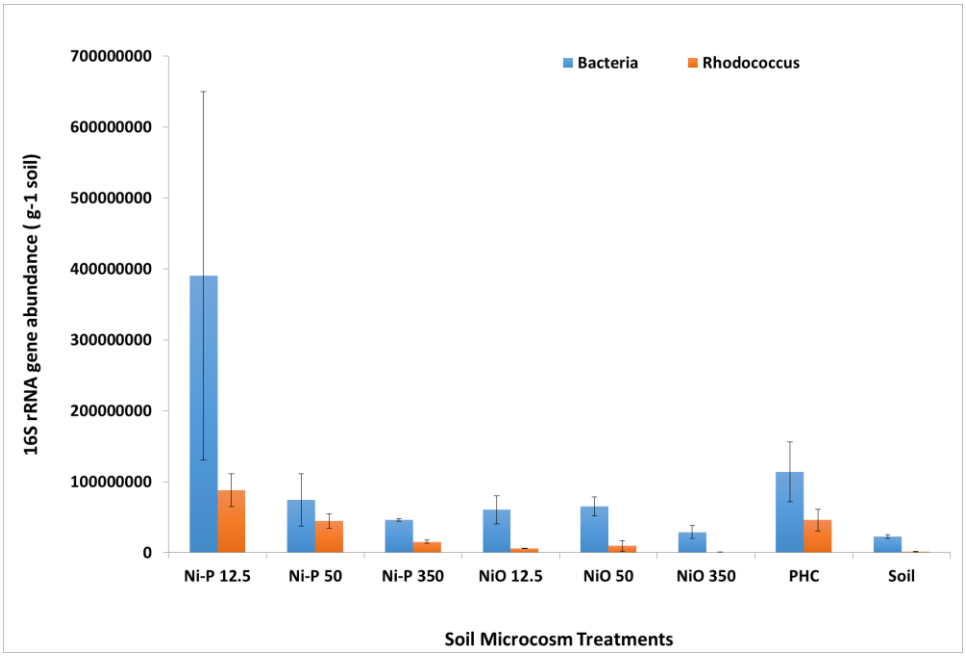


Figure 4.19: the 16S rRNA gene abundance of bacteria and *Rhodococcus* in Ni-crude oil amended soil microcosms after degradation for 15 days. The 16S rRNA gene were quantified by specific qPCR assays that target general bacteria and *Rhodococcus*. Each bar represents the mean 16S rRNA gene abundance of bacteria or *Rhodococcus* of triplicate samples and the error bars are the standard deviation of means of triplicate samples. The graph legend indicate the target taxonomic group quantified.

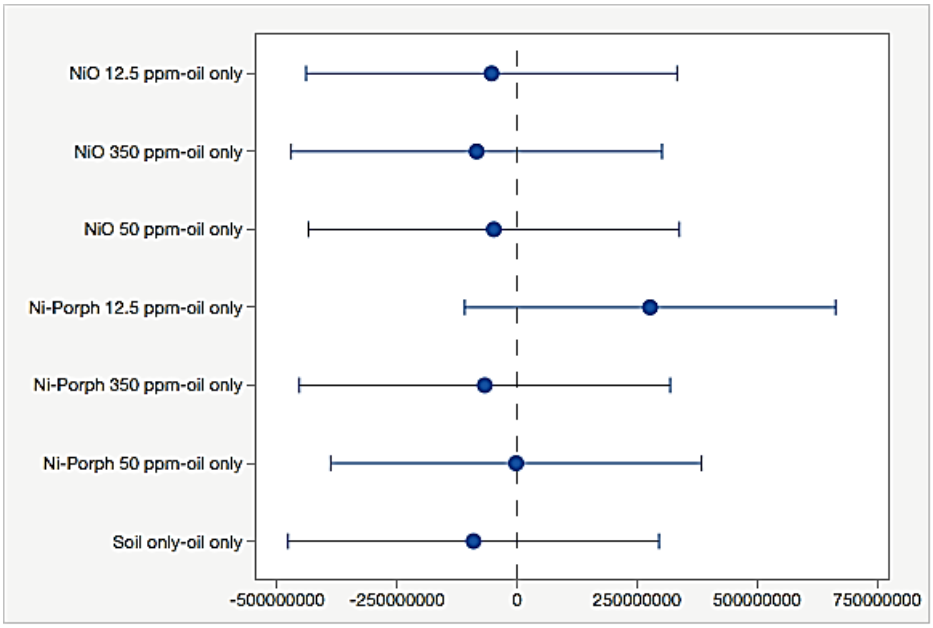


Figure 4.20: comparative analysis of the mean abundance of the bacterial 16S rRNA gene in soils amended with Ni and Crude oil at Ni-concentrations of 12.5, 50 and 350 ppm relative to oil only amended soils and unamended soils. Ni amendment was as Ni-Porph and NiO. Analysis was by Dunnet's multiple comparison analysis at 95% confidence interval. The values include the difference between the treatment mean and the control mean. A mean is not significantly different if its interval contains zero (0).

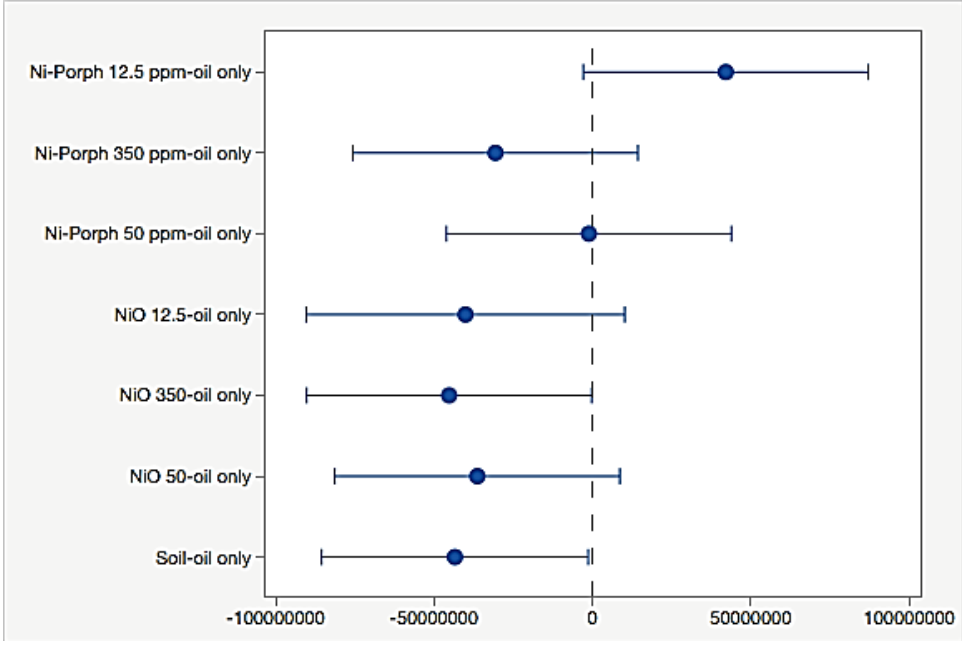


Figure 4.21: comparative analysis of the mean abundance of the *Rhodococcal* 16S rRNA gene in soils amended with Ni and Crude oil at Ni-concentrations of 12.5, 50 and 350 ppm relative to oil only amended soils and unamended soils. Ni amendment was as Ni-Porph and NiO. Analysis was by Dunnet's multiple comparison analysis at 95% confidence interval. The values include the difference between the treatment mean and the control mean. A mean is not significantly different if its interval contains zero (0).

4.4.7. Numerical relationships between cumulative CO2, maximal CO2 production rates, Total petroleum hydrocarbons recovered after degradation period, Bacterial 16S rRNA Gene abundances and *Rhodococcus* 16S rRNA Gene abundances

The objective of this research work was to understand the influence of nickel on the biodegradation of petroleum hydrocarbon using a combined geochemical and microbiological approach. It was expected that by doing this, a detailed understanding of the biological process in soil microcosms will be obtained. So far, the results have been presented separately based on individual analysis techniques. An analysis of the correlation between variables determined in experiments was carried out to identify trends in the relationships between microbiological data and geochemical data. To achieve this, a matrix correlation analyses was carried out to determine the relationships between cumulative CO_2, maximal CO_2 production rate, and Total n-alkane recovered after degradation period, Bacteria 16S rRNA gene abundance and 16S rRNA gene abundance of *Rhodococcus* (see tables 4.6 and 4.7).

4.4.7.1. Correlation of 16S rRNA gene abundance (of bacteria and *Rhodococcus*) relative to maximal CO2 produced during the degradation of petroleum hydrocarbon in soil microcosms

Figure 4.21 is a scattered plot representing the relationship between maximum cumulative CO_2 and Bacterial 16S rRNA abundance of soil microcosms. There is a very weak positive linear relationship between the two variables ($r=0.59$; $p=0.04$; $r^2=0.33$). Based on this, spearman correlation rs was determined to explore the possibility of non-linearity the between the two variables. r_s indicated that indeed there is a week relationship between bacterial abundance and cumulative CO_2 in microcosms ($rs=0.21$; $p=0.65$). However, the there is a strong positive linear between 16S rRNA gene abundance of *Rhodococcus* and maximum cumulative CO_2 In soil microcosms, which is significant ($r=0.82$; $p=0.02$; $R^2=0.68$) (see table 4. 6). Hence, increase in the abundance of *Rhodococcus* results to an increase in the mineralisation of crude oil to CO_2. These observations imply that although there was a general increase in proliferation of bacteria due to increasing

abundance of hydrocarbon degraders in the soil microcosms, the dominance of the bacterial community by *Rhodococcus* contributed significantly to the mineralisation of crude oil to
CO_2.

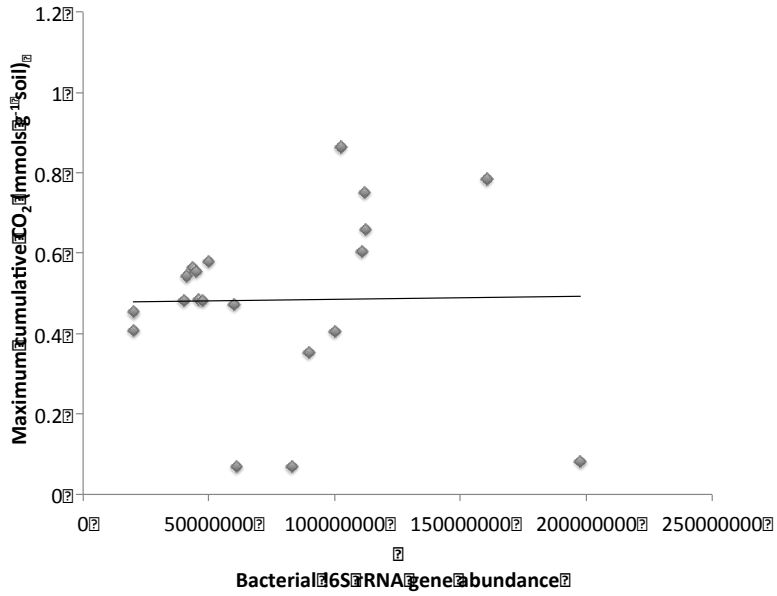


Figure 4.22: correlation analysis between maximum cumulative CO and the bacterial 16S rRNA gene abundance in Ni-crude oil amended soil microcosms. The coefficient of determination (R^2) is indicated.

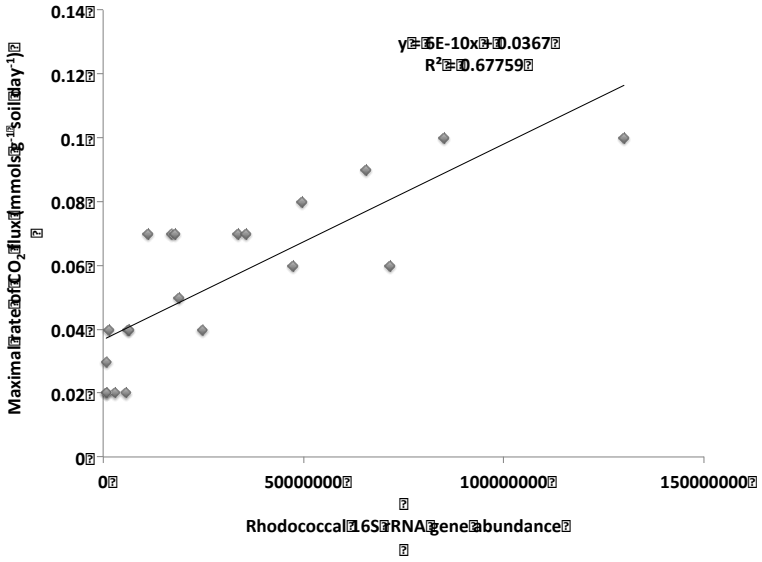


Figure 4.23: correlation analysis between maximum cumulative CO and the *Rhodococcal* 16S rRNA gene abundance in Ni-crude oil amended soil microcosms. The coefficient of determination (R^2) is indicated.

4.4.7.2. Correlation between 16S rRNA gene (bacterial and *Rhodococcal*) and maximal rate of CO_2 production during the degradation of petroleum hydrocarbon in soil microcosms

The Correlation analysis indicated that there is a very weak linear positive correlation between 16S rRNA gene abundance of bacteria and the maximal rate of CO_2 production in microcosms ($r=0.63$; $p=0.052$; $r^2= 0.40$). In addition, r_s was determined to explore the possibility of the non-linear relationship between the 2 variables and the value of r_s indeed indicated that the relationship between 16S rRNA gene abundance of bacteria and maximal rate of CO_2 production in microcosms was very weak and not significant ($r_s=0.27$; $p=0.56$). However, based on the correlation coefficient determined (see table 4.6), strong linear positive relationship, which is significant, exist between the *Rhodococcal* 16S rRNA gene abundance and the maximal rate of CO_2 production in microcosms ($r=0.86$; $p=0.013$; $r^2=0.65$). This implies that increase in the abundance of *Rhodococcus* in the system resulted in an increase in the rate of crude oil mineralisation in microcosms. General, the dominance of microbial community in oil degrading systems by *Rhodococcus*, amongst other hydrocarbon degraders, played a significant role in the acceleration of biodegradation process in petroleum degradation in soil microcosms.

4.4.7.3. Correlation of 16S rRNA gene (bacterial and *Rhodococcal*) relative to Total n-alkanes recovered after the degradation of petroleum hydrocarbon in soil microcosms

The Pearson's correlation analysis, as indicated in table 4.6, between total n-alkanes recovered from microcosms after degradation and bacterial gene abundance suggests that there was a negative relationship between the variables ($r= -0.53$; $p= 0.22$). The coefficient of determination, r^2, indicated that very weak linear correlation existed between the variables ($r^2= 0.0013$) (see figure 4.24). To explore the possibility non-linearity between total n-alkanes and bacterial abundance and this revealed that indeed, there is a very little correlation is between the variables ($r_s= -0.39$; $p=0.38$) (see table 4.7). Likewise, a similar relationship occurred between total n-alkane and Rhodococcal abundance. The Pearson's correlation coefficient indicated that

there is a low negative linear correlation between the variables (see table 4.7 and figure 4.25), which was not significant (r= 0.58; p= 0.17; r^2= 0.33). In addition, r_s between the variables indicated that the relation is indeed weak (r_s= -0.5; p=0.25). The negative correlation coefficients indicate that as total n-alkane decreases, the general bacterial abundance and Rhodococcal abundance increased slightly. The weak relationship observed may be attributed to the fact that not all carbons of crude oil are converted to biomass. These carbons that are not converted to biomass may have been completely mineralized to CO2 or converted to other by-products as a result of degradation.

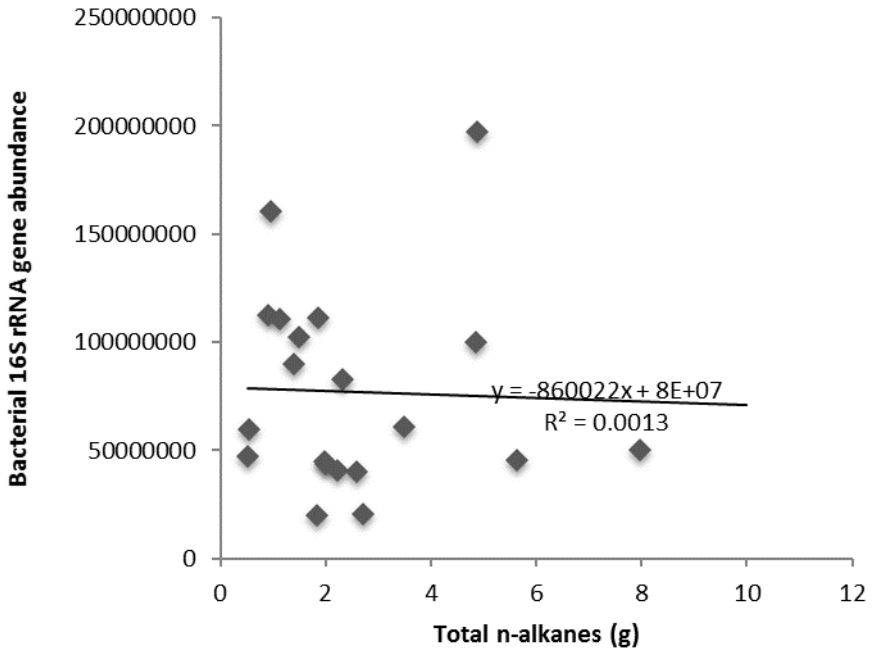


Figure 4.24: correlation analysis between recovered total n-alkanes and the bacterial 16S rRNA gene abundance in Ni-crude oil amended soil microcosms. The coefficient of determination (R^2) is indicated**.**

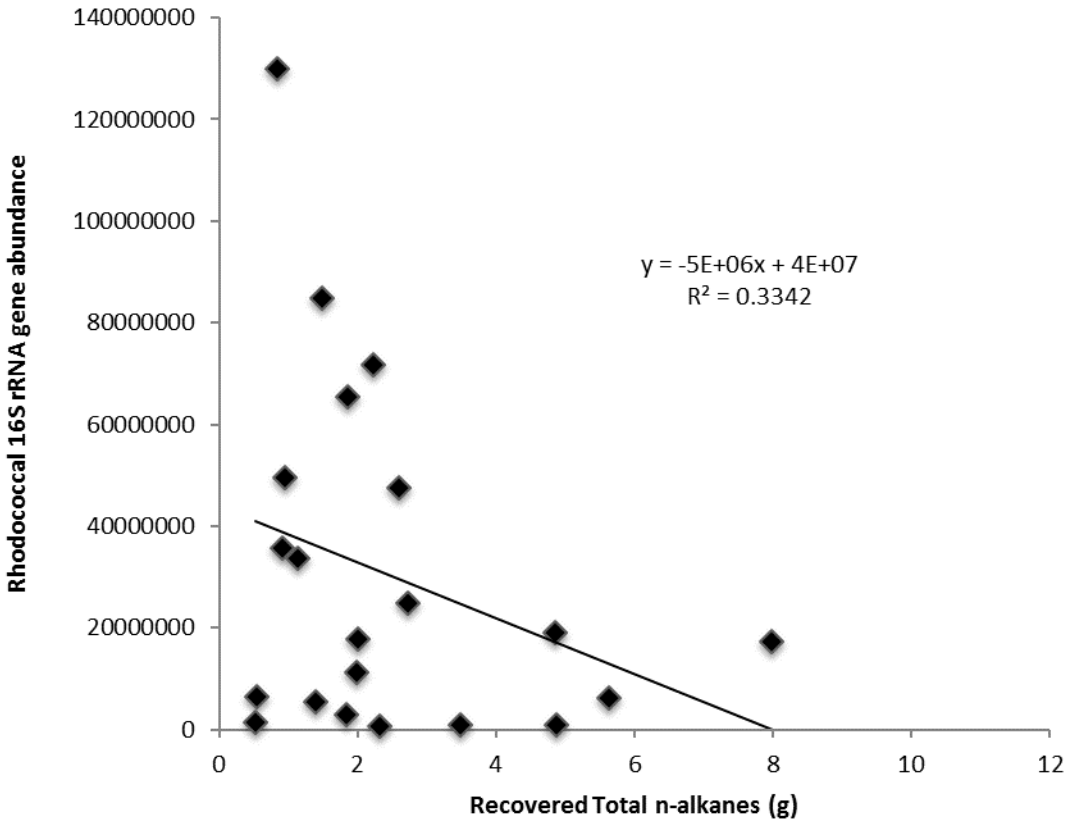


Figure 4.25: correlation analysis between recovered total petroleum hydrocarbon and the *Rhodococcal* 16S rRNA gene abundance in Ni-crude oil amended soil microcosms. The coefficient of determination (R^2) is indicated. The slope and the description of correlation (positive or negative correlation) and the slop of curve are indicated in the equation.

Table 4.6: the Pearson's correlation analysis describing the interaction between microbiological variables and geochemical variables determined from petroleum-nickel contaminated soil microcosms

		CO_2	Rate	Bacteria	*Rhodococcus*	n-Alkanes
CO_2	Correlation Coefficient	1	0.907**	0.588	0.823*	-0.627
	Sig. (2-tailed)		0.0048	0.165	0.023	0.131
	N	21	21	21	21	21
Rate	Correlation Coefficient	0.907**	1	0.630	0.861*	-0.406
	Sig. (2-tailed)	0.0048		0.130	0.013	0.367
	N	21	21	21	21	21
Bacteria	Correlation Coefficient	0.588	0.630	1	0.811*	-0.534
	Sig. (2-tailed)	0.165	0.130		0.027	0.217
	N	21	21	21	21	21
Rhodococcus	Correlation Coefficient	0.823*	0.861*	0.811*	1	-0.578
	Sig. (2-tailed)	0.023	0.013	0.027		0.174
	N	21	21	21	21	21
n-Alkanes	Correlation Coefficient	-0.627	-0.406	-0.534	-0.578	1
	Sig. (2-tailed)	0.131	0.367	0.217	0.174	
	N	21	21	21	21	21

Note:
**Correlation is significant at the 0.05 level (2-tailed).*
*** Correlation is significant at the 0.01 level (2-tailed).*

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Table 4.7: the spearman's correlation analysis describing the interaction between microbiological variables and geochemical variables determined from petroleum-nickel contaminated soil microcosms

		Bacteria	*Rhodococcus*
Rate	Correlation Coefficient	0.270	
	Sig. (2-tailed)	0.558	ND
	N	21	
CO_2	Correlation Coefficient	0.214	
	Sig. (2-tailed)	0.645	ND
	N	21	
n-alkanes	Correlation Coefficient	-0.393	-0.5
	Sig. (2-tailed)	0.383	0.253
	N	21	21

Note:

1. *Spearman's correlation analysis was carried out to identify non-linear relationship in variables with weak linear relations*
2. ND denotes not determined

4.5. Discussions

The geochemistry and microbiological studies of Ni-oil contaminated soil has shown the patterns of Ni effects on the process of petroleum hydrocarbon biodegradation and the associated pattern of change in the community diversity and phylogeny. From the studies, Ni at lower concentrations clearly stimulated the biodegradation process but the stimulatory effect declines with increasing metal concentration. Also, discrepancies were recorded between Ni-forms used with Ni as a Porphyrin complex having the most stimulatory effect on biodegradation over the widest concentration range. In addition, the inorganic Ni forms, though not statistically different, manifested apparent differences between the $NiCl_2$ and NiO. These differences are manifest at high concentrations (200 and 350 ppm) of added Ni with NiO exhibiting stronger inhibitory effect at this concentration. In this section, the finding from these investigations is related to previous works in an effort to fully understand the unique trend of Ni-effects on the biological process.

4.5.1. Microbial metabolic activities in oil-amended soil during a short-term degradation period

In this project, the trend in CO_2 production over time has provided rigorous evidence that biodegradation of petroleum hydrocarbons occurs rapidly (within days) in microcosms comprising pristine soils amended with oil. Similar trends were observed in previous studies e.g. Obire and Nwaubeta (2001) observed progressive emission of CO_2 in soils amended with 5% diesel, gasoline and kerosene, which declined after 4 weeks of incubation. In the same vain, Baptista et al (2005) observed a significant progressive trend in CO_2 production within 29 days of incubation during a 45 day crude oil degradation experiment and again, (Franco et al. 2014) made a similar observation within the first 30 days of a 90 day degradation of diesel in soil amended with 5% diesel.

4.5.2. The influence of Ni on the microbial metabolic activities during oil degradation in soils

Previous studies carried out to determine Ni effect on petroleum hydrocarbon and other hydrocarbon degradation in soil has identified that at low concentrations, Ni has no effect on degradation at low concentrations of Ni but

inhibition by Ni increased with increased NI effect. For instance, Al-Saleh and Obuekwe (2009) studied the effect of Ni (added as $NiSO_4$) on the mineralisation of Crude oil, Hexadecane and Naphthalene in Kuwait soil with the aim of identifying the inhibitory potential of Ni on hydrocarbon degradation in Kuwait soils. In their studies, a comparison of Ni –crude oil-amended soils, crude oil only amended and unamended soils identified no significant Ni effect on the soil metabolic activities at the low concentration of 20 ppm and at higher concentrations of 100 and 200 ppm in the crude oil and Hexadecane contaminated soils. However, in Naphthalene containing soil, metabolic activity was reduced significantly. In all the microcosms, an increase in inhibitory effect was observed with increasing concentration of Ni. This is comparable with the observations made in NiO contaminated soils where Ni at low concentrations of 12.5 25 and 50 ppm has no effect on maximum CO_2 produced but inhibitory effects at 200 and 350 ppm Ni. Also in this study, $NiCl_2$ amendment show slight difference with the studies by Al-Saleh and Obuekwe in that no change in CO_2 production was observed in $NiCl_2$ amended soils. However, an entire contrast was obtained on the amendment with Ni-Porph in the current study. Here, amendment with Ni-Porph stimulated CO_2 production in microcosms and stimulation decreased with increased Ni-concentration.

The observations made in this study may suggest that Ni is performing as an essential nutrient in the soil systems. Majorly, nutrients for oil bioremediation usually consider N and P (Atlas 1995; Bragg et al. 1994; Singh et al. 2014; Walworth and Reynolds 1995; Walworth et al. 2001; Bell et al. 2011; Walworth et al. 2007; Coulon et al. 2005; Sutton et al. 2014) but rarely consider trace element limits. Moreover, there are documentations identifying Ni as an essential nutrient required for microbial growth that could become toxic at high concentrations (Gadd 2010; Gadd 2004; Gadd and Griffiths 1977). Usually, in microorganisms, Ni acts as co-factor to certain enzymes, which are Ni-dependent (Boer et al. 2014; Thauer et al. 1980; Glass and Orphan 2012; Pfaltz et al. 1987; Diekert et al. 1980; Diekert et al. 1981; Jeoung et al. 2014; Desguin et al. 2014). Boer et al (2014) documented 9 important Ni-dependent enzymes. These included glyoxalase 1 responsible for the detoxification of methylglyoxal that occur spontaneously during glycolysis, urease which catalysis the conversion of urea to ammonia and

carbonic acid, and lactase racemase, which catalyses the racemization of L- and D-lactic acids. Others include Superoxide dismutase responsible for the detoxification of reactive oxygen species, ROS, such as H_2O_2 that are produced during respiration due to excess oxygen; Ni-Fe hydrogenase (involves in the reverse activation of molecular hydrogen in anaerobic systems) and methyl-coenzyme M reductase (which are involved in anaerobic methane oxidation and methanogenesis). Also are the enzyme systems involved in the CO_2 cycle i.e. carbon-monoxide hydrogenase that catalyses the anaerobic oxidation of CO to CO_2 and Acetyl-CoA synthase/decarboxylase that is responsible for the anaerobic synthesis of acetyl-CoA from CO_2.

One way Ni may have stimulated biodegradation is to instigate the secretion of some of these Ni- dependent enzymes for the maintenance and growth of microbial cells. One important Ni-dependent enzyme that may have been activated in the soils investigated is the superoxide dismutase. Usually, the uptakes of metals by microbes instigate the production of reactive oxygen species (ROS) in cells, which usually impose oxidative stress in microbial cells. The secretion of superoxide dismutase will effect the detoxification of reactive oxygen species (ROS) generated due to the process of autoxidation, a process usually experienced under oxic conditions (Nies 2003; Harrison et al. 2007; Lemire et al. 2013; Gadd 2004). ROS production is usually activated on uptake by metals due to the presence of electrons from electron donors (Lemire et al. 2013). However, when the rate of ROS production supercedes the elimination rate, oxidative stress occurs. This may explain the inhibitory effects observed at a high concentration of Ni added as NiO. Therefore, the possibility that the rate of ROS production is minimal at lower Ni concentration and higher at high Ni concentration seems to be the case in the soil systems investigated.

Presently, no Ni-dependent enzymes are directly involved in petroleum biodegradation under aerobic conditions that are. In bacteria, most of the enzyme systems that have been documented to be involved in hydrocarbon degradation are involved in the catabolism of alkanes, which involves the initial enzymatic oxidation of terminal or sub-terminal methyl group/ groups (depending on the microbe) to generate a primary alcohol. This is followed by enzymatic oxidation of the primary alcohol to fatty acid, which is

subsequently mineralisation by the process of beta-oxidation, which yields Acetyl CoA. The product is channelled to the Kreb cycle for energy generation and CO_2 generation. These reactions have been studied extensively (KESTER and FOSTER 1963; Wang et al. 2010; Funhoff et al. 2007; Van Beilen et al. 2006; Das and Chandran 2011a; Nelson 2009; Denisov et al. 2005; Van Bogaert et al. 2011; Urlacher and Girhard 2012). One group of enzymes are the Cytochrome P450 alkane hydroxylases which constitute family of monooxygenases which play an important role in the microbial degradation of oil, chlorinated hydrocarbons, fuel additives, and many other compounds (Oldenhuis et al. 1989; De Mot and Parret 2002; Das and Chandran 2011b; S. W. Lee et al. 2006). Cyt P450s are more generally enzyme systems, which catalyse diverse metabolic reactions in living organisms (Urlacher and Girhard 2012; Van Bogaert et al. 2011) and the diversity of Cyt P450s is so great that there are 1105 reported bacterial systems and 27 archaeal systems (Nelson 2009). Although, Cyt P450s do not contain Ni, this metal may have indirectly affected Cytochrome P450 enzyme systems in the microcosms because interestingly, peroxides are known to induce these enzymes (Zu et al. 1999; D Hamdane, H. M. Zhang, et al. 2008; Hamdane et al. 2009; Djemel Hamdane et al. 2008; Hannemann et al. 2007; D Hamdane, H. Zhang, et al. 2008) and thus Ni may have indirectly stimulated the rates of oil biodegradation through the production of peroxides facilitating the secretion of Cyt P450 necessary for the activation of the seemingly inert petroleum hydrocarbons (Das and Chandran 2011a).

Furthermore, in addition to the biological catabolic process of hydrocarbon degradation, certain Cyt p450 enzyme systems have been documented to catalyse the production of biosurfactants by bacteria and fungi (Van Bogaert et al. 2011; Urlacher and Girhard 2012). Hydrocarbon degraders produce biosurfactants increase the surface area of hydrophobic water-insoluble substrates and increase their bioavailability to degraders (Lawniczak et al. 2013; Ron and Rosenberg 2002; Sen et al. 2012). The hydrophobic tail of biosurfactants comprise common Fatty Acids (FAs) and Hydroxylated FAs originating from beta-Oxidation (Van Bogaert et al. 2011) and the study of the microbial communities of the microcosms revealed that known biosurfactant producers such as *Rhodococcus* (Bicca et al. 1999) (Pacheco et al. 2010) and

Pseudomonas (Ben Belgacem et al. 2015; Aparna et al. 2012; Onwosi and Odibo 2012) are enriched/ selected.

More so, some of FAs and Hydroxylated FAs generated during the terminal or sub-terminal oxidation of n-alkanes by microbes are used as building blocks of cell components e.g the cell membranes (Van Bogaert et al. 2011; Sutcliffe 1998; Sutcliffe et al. 2010). Hence, in addition to hydrocarbon degradation, Ni may have indirectly affected the microbial anabolic processes, i. e. biomass build-up in microcosms.

4.5.3. Bioavailability of Ni and its effect on the biodegradation of petroleum hydrocarbons

The observations made in this research identified different stimulatory and inhibitory effects influenced by Ni chemical forms. In the Ni-porphyrin containing microcosms, stimulatory effects on degradation were observed even at high concentrations whereas inhibitory effects were observed in the inorganic forms at high concentrations. One explanation for these observations could be the bioavailability of Ni in the degrading systems. Possibly, Ni in the organic form is more accessible than in inorganic forms in these systems. The transport of organometallic complexes such as metalloporphyrins is a usual strategy by microbes to facilitate uptake of specific metals and to mitigate potential toxic effects of metal ions (Harrison et al. 2007; Lemire et al. 2013; Vraspir and Butler 2009; Nies 2003). Matlakowska and Sklodowska (2010) documented in details the highly efficient assimilation of metalloporphyrins by indigenous microorganisms of the Kuperferschiefer black Shale from Poland, which was subsequently followed by intracellular degradation of the porphyrin, freeing the metal ions within the cells. However, excessive intracellular cations may result in oxidative stress resulting to protein malfunction. This complements the decline in stimulatory effect with increasing concentration observed in Ni-Porph containing soil systems.

There are apparent differences between the two inorganic forms of Ni investigated. Of the two forms, $NiCl_2$ seemed to be less inhibitory than NiO, an effect that was especially clear at high Ni concentrations. This is surprising when it is considered that chlorides of metal salts are readily soluble in water

while oxides are insoluble (Yoder 2006) and are therefore easily dissociated into ionic forms thereby contributing to the cationic pool in soil solutions, which are the bioavailable/mobile forms of metals (Gadd 2004; Gadd and Griffiths 1977; Gadd 2010; Bruins et al. 2000; van Gestel and van Diepen 1997; Olaniran et al. 2013; Roane 2015). An explanation for such an observation may be that Ni added in as $NiCl_2$ may have dissociated readily in the soil solution, chelating the readily available organic compounds, which either facilitates its uptake by soil microbes or reduce its bioavailability at high concentrations of Ni. However, Ni added as NiO dissociated slower and due to depletion of organics, due to crude oil degradation, was not readily bound to organic, hence was readily bioavailable at high concentration, as a consequence, inhibitory effect occurred.

More so, the evidence that certain metal oxides including Ni-oxides can mediate the formation of superoxides during the decomposition of peroxides (Kitajima et al. 1978; Anpo et al. 1999). Also, NiO nanoparticles are reported to induce the production of superoxides in microbes (Horie et al. 2011; Baek and An 2011). Therefore, in NiO containing systems, peroxidases may have been produced at rates that supercede its detoxification resulting to increased metal stress.

4.5.4. Mechanism of Ni Uptake

In this study, metals may have been transported into the microbial cell through microbial transport systems. Generally, the uptake of Ni by microbial cells is typically through transport systems such as the ABC-transporter-like system, which is an ATPase-binding cassette, Nik-ABCDE (Dosanjh and Michel 2006; Eitinger and Mandrand-Berthelot 2000; Macomber and Hausinger 2011; Mulrooney and Hausinger 2003; Nies 1999). Nik-ABCDE comprised of 5 proteins; NikA which a is a periplasmic nickel-binding protein, NikB and NikC which are integral inner membrane proteins that form transmembrane pore for the passage of Ni; and NikD and NikE are membrane-associated proteins with intrinsic ATPase activity i.e the coupling of energy from ATP hydrolyzation (Mulrooney and Hausinger 2003; Dosanjh and Michel 2006; Eitinger and Mandrand-Berthelot 2000; Nies 1999). Apart from NikABCDE transport system, Ni is transported into a prokaryotic cell through Ni-specific permeases such as HoxN transporter initially observed in Ralstonia eutropha,

driven by chemiosmotic gradient (Mulrooney and Hausinger 2003). Others include HoxN homologues proteins such as NhiF transporter which is specific transporter for both Co and Ni initially observed in Rhodococcus rhodochrous J1, NixA observed in Helicobacter pyroli (important factor in the organism's virulence), NicT in Mycobacterium tuberculosis and HupN transporter in Bradyrhizobium japonicum (Mulrooney and Hausinger 2003; Eitinger and Mandrand-Berthelot 2000).

Within the microbial cells, intracellular proteins, which shields and transport Ni to the specific active sites required enzyme, have been reported (O'Halloran and Culotta 2000)(Mulrooney and Hausinger 2003). These are called metallochaperones and they mitigate the toxic effect of metals within microbial cell.

4.5.5. Total petroleum hydrocarbon and degradation pattern in degraded Ni-Petroleum hydrocarbon contaminated soil

In this project, n-Alkanes were determined and quantified and the pattern of degradation was observed (see section 4.4.4.3). The observation made in microcosms was that the lower molecular weight n-Alkanes (n-C_{12} - n-C_{18}) were degraded prior to the medium (n-C_{19} - n-C_{25}) molecular weight hydrocarbon, which was degraded preferentially to the high molecular weight n-Alkanes (n-C_{26} - n-C_{33}). This observation is consistent with the general knowledge of aerobic biodegradation of hydrocarbons where lower molecular weight hydrocarbons are preferentially degraded prior to the degradation high molecular weight hydrocarbons. Increased biodegradability is inhibited with increased complexity of hydrocarbons due to the fact that accessibility by microbes/attack by enzymes are hindered (Head et al. 2006). In fact, Peters et al. (2007) acknowledged that molecular weight is inversely proportional to the degradation of petroleum hydrocarbons in an aerobic environment. Contrary to this is the observation made by (Fayad and Overton 1995) who observed that polycyclic aromatic hydrocarbons (PAHs) were preferentially degraded prior to saturates in the Arabian Gulf spills during the gulf war. This, they observed could be due to the significant presence of indigenous microbial population, which have the capability to degrade PAHs. Also, Atlas and

Hazen (2011) reported that due to the high composition of saturates and less complex compounds in the crude oil spilt during the 2010 BP Deepwater horizon blowout, biodegradation was a much faster than the 1989 Exxon Valdez spill in the united states.

Previous studies have indicated that the pattern of crude oil degradation in aerobic condition differs from anaerobic systems. Under anaerobic conditions, more complex hydrocarbons are degraded prior to the less complex hydrocarbons (Sherry et al. 2013; Aitken et al. 2013; Jones et al. 2008; Hasinger et al. 2012; Cheng et al. 2014; Gray et al. 2011; Atlas 1981; and references therein). For instance, Hasinger et al reported a preferential initial degradation of branched- alkanes over straight-chained n-alkanes in anaerobic system. Also, Hasinger et al observed the preferential degradation of medium to higher molecular weight n-alkane over lower molecular weight n-alkane in systems composed of nitrates as terminal electron acceptors. They also observed that degradation of nC_{31} and nC_{32} were significant in sulphate reducing system. These observations made are contrary to the pattern of n-alkane degradation in this project.

Comparison pattern of degradation across treatments (see section 4.4.4.3.) indicated that apart from soil microcosms amended with NiO at 350 ppm, the pattern of degradation was similar across all treatment. It should be noted that results from recovered TPH, nC_{17}/Pristane ratio and nC_{18}/ phytane ration indicated that little or no degradation occurred on the amendment with NiO at 350 ppm Ni. Therefore, preferential degradation pattern observed in degraded microcosms may be evidence that Ni was indirectly stimulating the secretion of hydrocarbon degrading enzymes. As has been discussed in the previous section, the increased assimilation of Ni by microbes could result in the production of peroxides and it has been established in the past that peroxides induce the Cyt P450 oxygenase enzyme system. This enzyme system has been known to catalyse the metabolism of short carbon chain n-alkanes (nC_5 –nC_{16}) and cycloalkanes in Bacteria (Das and Chandran 2011b).

4.5.6. Community diversity and phylogeny

Atlas (1981) documented that the capability to degrade petroleum is not restricted to a few but extends to diverse phylogenetic groups. This wide

capability across different organisms seem to be the case with the communities studied in this project. As presented earlier, the DGGE analysis revealed that the microbial communities were highly diverse in all soils with phylogenetic groups representing both low and high GC content containing groups. This evidence of high diversity was buttressed with the alpha-diversity assessment from the sequence libraries (i.e the estimation of specie richness, Shannon and Simpsons indices), which indicated slight enrichment in petroleum-contaminated soils (with or without Ni, except for NiO containing soils at Ni concentration of 350 ppm) differing the soils from uncontaminated control. High diversity observed in this study is similar to previous studies on aerobic biodegradation of petroleum in soils within a short period of degradation or the early stage of long-term degradation (LI et al. 2007; Labbé et al. 2007; Muangchinda et al. 2014; Abed et al. 2015; dos Santos et al. 2012; Joynt et al. 2006a). For instance, a slight decrease in the diversity of the community structure within 15 days of laboratory incubation of soil contaminated with diesel fuel has been reported in the past (LI et al. 2007). Abed et al. (2015) reported automated rRNA intergenic spacer (ARISA) analysis profiles which indicated highly diverse communities in 5 dessert soils contaminated with crude oil with the soil containing a relatively higher petroleum concentration harbouring a higher specie /OTU richness. More so, interestingly, high diversity encountered in hydrocarbon-contaminated soils is not impaired by the presence of metals. Similar to the observations in this project, soil contaminated with Cr, Pb and petroleum hydrocarbon indicated phylogenically diverse microbial communities in soil from Indiana, USA and Phylogenetic groups included high and low GC gram-positives, *α-, β-, γ-Proteobacteria*, green non-sulfur bacteria and others reflecting metal and petroleum effects (Joynt et al. 2006a). In addition to this, dos Santos et al. (2012) recorded high diversity in petroleum degrading Brazilian soil contaminated with chromium and copper.

Other than the soil environment, high microbial diversity has also been recorded in marine, freshwater and estuarine sediments contaminated with petroleum under both aerobic and anaerobic systems. Dell'Anno et al. (2012) investigated the bioremediation of habour sediments under oxic and anoxic conditions and identified diverse communities under both conditions. However, diversity was greater under an oxic than an anoxic condition. In

their studies of petroleum degradation, Gray et al 2011 and Sherry et al., 2013 identified a diverse community of bacteria and Archaea in the methanogenic and sulfate-reducing system. These studies imply that communities encountered in petroleum degrading systems, not withstanding the condition are highly diverse.

The high efficiency of hydrocarbon degradation was attributed to the high diversity of communities as opposed to degradation by a consortium comprising of selected few microbial group as typically created in the laboratory (Dell'Anno et al. 2012). Typically an ecological network is created in degrading systems whereby members of the community interact, directly or indirectly, with each other and with the environment (Head et al 2006). Such interactions, which include cooperative relationships, competition for nutrient and space, predation and lysis by phage, contribute to biodegradation efficiency in the natural environment. Of great importance are the mutualistic interactions between members of the microbial community. A good example is the production of biosurfactants by bacteria (Head et al 2006, and reference therein). In their 27 weeks study, Kaplan and Kitts (2004) identified successional changes in community diversity during the petroleum biodegradation in soils of Guadalupe oil field, central Californian coast, California USA. Their findings indicated that *Pseudomonas* and *Flavobacterium* were identified as the dominant organisms during the first 3 weeks of rapid degradation. These phylogenetic groups were succeeded by other members of the community as their abundances decreased. Also, a detailed study of the degradation of Deepwater horizon petroleum in the Gulf of Mexico also identified *Pseudomonas* within the early stage of degradation (Dubinsky et al. 2013). The production of biosurfactants by strains of *Pseudomonas sp* and the improvement on the efficiency of petroleum degradation has been well documented in previous studies. (Zhang et al. 2012; Thavasi et al. 2011; Saikia et al. 2012). Also, strains of *Flavobacterium* identified at an early stage by Kaplan and Kit are known degraders of high molecular hydrocarbon in marine (Dubinsky et al. 2013), degraders of polyaromatics in the marine environment (Gertler et al 2009) and bacterial predators (Banning et al., 2010).

The importance of *Rhodococcus sp*. in the degrading communities is indicated by correlation analysis with strong positive relationships between the *Rhodococcus* abundance and both CO_2 production and rate of CO_2 production. *Rhodococcus* is known to secret extracellular polysaccharides, which reduce the surface tension of hydrophobic media such as oils. In other words, some strains of *Rhodococcus* produce biosurfactant that act to emulsify oils. The production of biosurfactant by *Rhodococcus sp* and their role in hydrocarbon degradation has been well documented (White et al. 2013; Liu and Liu 2011; Kumari et al. 2012; Zheng et al. 2012; Sorkhoh et al. 1990; Iwabuchi et al. 2000; Iwabuchi et al 2002). An example of such role, just like the ones stated in the previous paragraph, is the enhanced degradation of aromatics by the *Rhodococcus* biosurfactant, which promoted community dominance by hydrocarbon degrader, *Cycloclasticus* in seawater (Iwabuchi et al 2002).

Other OTUs identified in this study, as being involved in hydrocarbon degradation are close relatives of, *Bacillales*, unknown *Comamonadaceae*, *Mycobacterium*, *Norcadia*, *Pseudomonas*, *Methylibium*, *Rhodoplanes*, *Arthrobacter* and unclassified *Rhodobium*. These organisms have been isolated in petroleum-contaminated environments and during petroleum degradation in heavy metal-petroleum co-contaminated in soils in previous studies (Máthé et al. 2012; Zhang et al. 2010; Dubinsky et al. 2013; Atlas and Hazen 2011b; LI et al. 2007; Mittal and Singh 2009; Labbé et al. 2007).

The dominance of communities by the *Rhodococcus* OTU in this study may be attributed to the metabolic versatility of the organism. Previous studies have identified *Rhodococcus* species in petroleum hydrocarbon and metal co-contaminated systems (Ivshina and Kuyukina 2014; Ivshina et al. 2013; Máthé et al. 2012). In recent research by Ivshina and Kuyukina (2014) genes encoding enzymes that catalyse catabolism of alkanes, biosurfactant production and Heavy metal resistance were identified in a strain of *Rhodococcus sp, Rhodococcus ruber* IEGM 231. Some of the genes identified included Monooxygenases, Cyt P450, Dioxygenases, peroxidases and dehydrogenases involved in the metabolism of hydrocarbons. Others included genes the encode Acyl-CoA synthase and Fatty Acid synthase that catalyses biosurfactant production and heavy metal resistant genes including genes for

Heavy metal transporters, cation efflux enzymes, metal binding proteins, mercuric reductase and alkyl mercury lyase.

The Real time-qPCR data generated in this study indicated that abundance of the 16S rRNA gene of the *Rhodococcus* OTU across communities differed with increasing Ni concentration resulting in decreasing abundance of the *Rhodococcus*. This corresponds to maximum CO_2 production, maximal rate of C2 production and recovered TPH data. Previous studies have documented Ni toxicity to *Actinobacteria* (Ivshina et al. 2013). In their study, Ivshina et al identified Ni as the third most toxic metal amongst 8 metals tested, which included Cd, Zn, Ni, Cu, Mo, Pb, Cr and V in order of decreasing toxicity. Also, high concentrations of Ni ranging from 130 to 260 mg/Kg decreased microbial degradation of petroleum by communities dominated by *Actinobacteria* and *α-Proteobacteria* (Taketani et al. 2015). However, Ni influence in the enhancement of petroleum degradation have been identified in a previous study where increased degradation of petroleum due to increased hydrocarbon degraders as a result of increased Ni concentration (ranging from 2.5 to 12.5 ppm) was observed during the bioremediation of Gasoline-Nickel co-contaminated soil by phytoremediation (Agarry et al. 2014).

Conclusions

The aim of this project was to understand the effect of Ni, in different chemical forms and at Ni- concentrations on the biodegradation of petroleum hydrocarbon in pristine soils. Using geochemical and microbiological approaches, the Ni effects on the Microbial activities, the compositional change of petroleum and the microbial community diversity and phylogeny were determined successfully on the same experimental systems.

In general, the findings suggest that Ni at a lower concentration, have no or stimulatory effects on biodegradation of petroleum hydrocarbons in soils depending on the chemical form of added Ni. The stimulatory effect was observed in Ni-porph soils and declined with increasing Ni concentration. However, in NiO soils, no effects occurred at low concentrations and increased concentration of Ni resulted in increased inhibition of biodegradation. This is unlike $NiCl_2$ amended soils where Ni effects on biodegradation were neutral irrespective of Ni concentration. These results are

explicable when it is considered that Ni can be linked indirectly to the release of metabolic enzymes, which are important during biodegradation in aerobic environment but also may require the Ni to be in complexed (chaperoned) form to reduce toxic effects. Therefore, observations made in this study suggest that Ni could be a limiting factor during the biodegradation of petroleum hydrocarbon in the soil.

Furthermore, the phylogenetic study detected phylogenetic groups that comprised of important petroleum hydrocarbon degraders including *Rhodococcus, Bacillales,* unknown *Comamonadaceae, Mycobacterium, Norcadia, Pseudomonas, Methylibium, Rhodoplanes, Arthrobacter* and unclassified *Rhodobium*. The most abundant microbe detected was a strain of *Rhodococcus* spp. Their proliferation in Ni-crude oil co-contaminated soil was linked to their metabolic versatility, which enables them to degrade while utilising Ni as a co-factor at low concentration as well as resisting Ni at high concentration. Some of the enzymes systems detected in the genome of *Rhodococcus* strains included oxygenases, Cyt P450, peroxidases and dehydrogenases, all these enzymes important during petroleum biodegradation in an aerobic environment. Others included enzyme system for metal resistant and enzyme systems for biosurfactant production.

Chapter 5

The effect of Cadmium on the biodegradation of petroleum hydrocarbon in the soil environment

5.1. Introduction

Cadmium, with the elemental symbol Cd, is a member of group 12 in the periodic table along with zinc and mercury. Its atomic number and mass are 48 and 112.41 g respectively. The Cd has a density of 8.64 g/cm3 and thus is classified as a heavy metal. Cd mainly exists in an oxidation state of +2 and the predominant species is Cd^{2+} (Greenwood and Earnshaw 1997).

In nature, Cd rarely exists in its elemental form and is found in the earth crust as a co-existing metal in ores of Zn, Pb and Cu. Principally, the mineral forms of the metal are greenockite (CdS), Octavite (CdSe) and Monteponite (CdO). The Cd has low relative abundance in the earth crust with a concentration ranging from 0.08 to 0.1 ppm. Sources of the element in the environment include natural activities such as rock weathering and volcano emission as well as anthropogenic activities such as mining, agricultural and industrial activities. The deposition of Cd in the environment due to these activities has been widely reported (Hartwig 2013). In fact, the biogeochemistry of the metal indicates that the major source of Cd in the environment is through human actives (Hartwig, 2013). The metal has also been found to be a potentially toxic substance to human health and the environment. Specifically, Cd has been reported to be a potential carcinogen and mutagen based on in-vitro and in-vivo studies (Huff et al. 2007; Il'yasova and Schwartz 2005; Waalkes 2003). In addition, Cd has been reported to negatively affect important microbial activities, terrestrial and aquatic animals as well as plants ((Sanità Di Toppi and Gabbrielli 1999; Burger 2008; Benavides et al. 2005; Barrow 2000; Martelli et al. 2006; Satarug et al. 2003). Due to its toxicity to health and the environment, Cd has been prioritised as heavy metal of concern along with Hg and As by the United Nations economic commission for Europe (UNECE).

In the UK, the soil guideline value (Environment Agency 2009) for Cd is 10 mg/ Kg DW of soil for residential, 1.8 mg/ Kg DW for allotment areas and 230 mg/ Kg DW for industrial areas. In addition, the Dutch list (ESDAT 2000)

recommended intervention values of Cd as 12 mg/ Kg DW in soils and sediments.

The Cd is also one of the metals commonly found in petroleum, however, its concentration depends on the petroleum source, formation and origin. This occurrence was discussed in detail in section Chapter 1. Section 1.6. The mining of fossil fuels such as petroleum and coal has contributed to Cd environmental contamination. There has been documented pollution of the environment due to activities of the petroleum industries all over the world. For instance, in the Niger Delta, Nigeria, Cd and other heavy metals have been reported to have increased in the environment by several folds after petroleum mining and exploration activities (Osuji and Onojake 2004; Ohimain et al. 2008; Kadafa 2012; Olawoyin et al. 2012; Osuji and Adesiyan 2005; Adekola and Mitchell 2011; UNEP 2011; Benson and Essien 2009; Arias et al. 2010; Ite et al. 2013). Also, reports on increases of Cd and other heavy metals have been reported in areas where there are chronic exploration and mining of petroleum activities such as Kuwait (Hussain and Gondal 2008; Fields and Massoud 1997; Bou-Olayan et al. 1995). Other reports include the bioaccumulation of Cd in aquatic animals after the blowout at the Gulf of Mexico, United States (Mendelssohn et al. 2012).

Due to its toxicity, the occurrence of Cd above the recommended intervention values may impair important biological activities such as the biodegradation of organic pollutants e. g petroleum. One mechanism of Cd toxicity includes the displacement of divalent essential elements in microbial cells resulting in cell malfunction. Based on this premise, this research will investigate the effect of Cd on the biodegradation of petroleum hydrocarbon in the soil environment.

5.2. Aims and objective

The aim of this research is to determine the effect of Cd on hydrocarbon degradation in complex natural systems such as a soil using a batch microcosm biodegradation study combined with microbial ecological and geochemical analysis approaches.

The objectives were:

1. To determine the effect of different mineral forms of Cd at increasing concentrations on the biodegradation of petroleum hydrocarbon by determining the rates microbial metabolic activities of the soils (CO2 evolution),

2. To determine compositional changes of a whole petroleum during biodegradation in the soil contaminated with different mineral forms of Cd at different concentrations using geochemical techniques,

3. To determine changes the ecological diversity of microorganisms in soils contaminated with petroleum hydrocarbon and different forms of Cd at increasing concentrations during the biodegradation of petroleum hydrocarbon,

4. To determine the geochemical associations and fate of the added Cd in a hydrocarbon contaminated system.

5.3. Methods

Most of the methods used in this research have been described in detail in chapter 2 and include the general details of the molecular microbiological and geochemical techniques used. Provided here are brief summaries and specific details relevant to the Cd experiments.

The soil sample used for this project was the loamy-clayey soil from Nafferton ecological farm. The sampling site and soil properties have been described in details in sections 2.1.

Soil Microcosms consisting of Cd in different mineral forms at different concentration levels, crude oil and soil were set up in triplicates as described in section 2.4. The mineral forms of Cd used were $CdCl_2$ and CdO. The soil amended Cd concentrations were 0.1 ppm, 1.0 ppm, 10 ppm, 100 ppm and 1000 ppm. The controls employed were soil microcosms amended with either crude oil only and or no amendments. The soil microcosms were incubated at room temperature for a period of 16 days. Soil microbial activities were monitored per microcosms by measuring the CO_2 production and O_2 consumption in each microcosm using GC-MS analysis of the headspace at 2-day intervals over the incubation period as described in section 2.5.

At the end of the incubation period, soil samples from each microcosm were subjected to microbiological studies to ascertain the metal effects on soil microbial community profiles. Culture-independent molecular microbiological techniques were used for this purpose (see section 2.8.) Briefly, soil 16S rRNA gene was amplified from extracted DNA using the PCR. For microbial diversity studies, 16S rRNA gene sequences were analysed using PCR-DGGE and for a more detailed community diversity study, Ion torrent sequencing–PGM was used for the 16S rRNA sequence analysis. Finally, Real time-qPCR optimised for the specific quantification of bacteria and the most dominant sequence (closely related to strains of Rhodococcus spp.) was used (see chapter 3).

In addition, geochemical studies were carried out to determine the extent and pattern of petroleum degradation in microcosms. To this end, petroleum hydrocarbon, specifically n-alkanes were determined in soil samples. The methods used in this investigation have been described in details in section 2.6. Briefly, undegraded petroleum hydrocarbon residues were recovered from soil samples using a solvent-solvent extraction method (see section 2. for details). Subsequently, separation of polar and non-polar fractions of extracts was carried out using a solid phase extraction technique, which basically involves a chromatographic separation of oil components in a chromatographic tube comprising non-endcapped octadecyl (C_{18}) sorbent (Bennett et al. 2007). GCFID analysis was used to determine the petroleum hydrocarbons. Peaks of n-alkanes were identified, integrated and quantified.

To understand the chemistry and fate of Cd in the soil, sequestration of Cd in soil was carried out using the sequential extraction method as described in section 2.7.

5.4. Results

5.4.1. Background concentrations of Cd in Nafferton soil

The physicochemical analysis of the soil sample used in this experiment revealed that the Cd concentration in the soil, which was 0.73 ± 0.09 ppm which does not exceed the UK soil guideline or Dutch list value. This signifies that the sample was not ‘contaminated’ with Cd. The other heavy metals tested

were below the SGV and Dutch list values of the heavy metals (see table 4.1). Also, the soils total organic compound value was 3.1 ± 0.12 %.

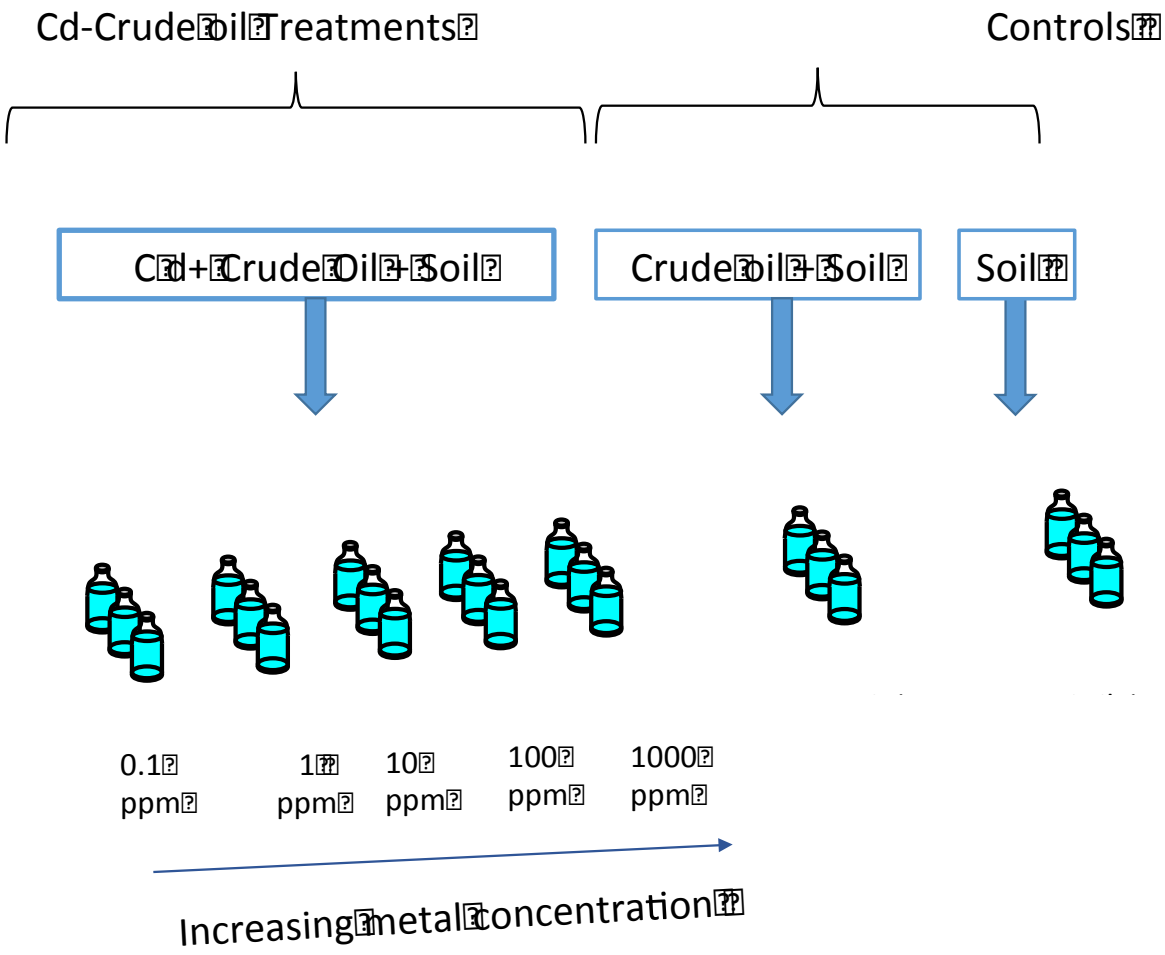

Figure 5.1: the experimental design for the study of crude oil biodegradation in Cd-crude oil amended soil microcosms at Cd-concentrations of 0.1, 1, 10, 100 and 1000 ppm relative to oil only amended (PHC) and no oil amended (soil only) controls in triplicates.

5.4.2.1 Effect of Cd on soil oil biodegradation microbial activities

The CO_2 production profiles, the maximum cumulative CO_2 and maximum CO_2 production rates in oil only amended soils and unamended soils (i. e soil only control) were compared to understand the effect of oil amendment on CO_2 production. Also, Cd-crude oil-amended soils were compared relative to

the oil only amended soil control to ascertain the effect of Cd on microbial activities in the soil. These profiles were statistically compared using analysis of variance (ANOVA) and individual treatments were compared with oil only control using the Dunnett's pairwise comparison analysis. In addition, comparisons were made between treatments using the Fisher's multiple pairwise comparison method.

5.4.2.2. The effect of Cd concentrations and chemical forms on CO_2 production profiles during petroleum degradation

Cumulative CO_2 in Cd and oil-amended microcosms were determined and compared with the oil-amended control to understand the effect of Cd on CO_2 production during the period of crude oil degradation. Figures 5.3 and 5.4 show the changes in cumulative CO_2 during the period of degradation in $CdCl_2$ and CdO amended soils, respectively. From the figures, it was observed that at Cd concentrations of 0.1 and 1 ppm irrespective of Cd form, a similar CO_2 production profile was observed relative to oil only amended soil. However, CO_2 production profiles at 10, 100 and 1000 ppm irrespective of Cd form tended towards that observed in the soil only control. Comparison of Cd forms using ANOVA indicated that while there were no significant differences in CO_2 production between Cd forms ($p=0.47$). But comparisons of concentrations with ANOVA Indicated that there were differences between microcosms at different Cd concentrations ($p<0.0001$). Dunnett's pairwise comparison analysis indicated that irrespective of form, change in cumulative CO_2 during degradation was not significantly different at 0.1 and 1 ppm Cd relative to oil only amended soils. However, change in cumulative CO_2 during degradation was significantly lower at 10 ppm Cd ($p<0.0001$), 100 ppm Cd ($p<0.0001$) and 1000 ($p<0.0001$) ppm Cd relative to oil only amended soils. At these higher concentrations Cd, changes in cumulative CO2 were not significantly different relative to soil only control.

5.4.2.3 Effect of Cd on the maximum cumulative CO_2 during degradation

Differences in the maximum cumulative CO_2 for treatments (as measured after 16 days) were compared relative to oil only amended soil control. ANOVA revealed that there were significant differences in cumulative CO_2 across

treatments ($p<0.0001$). Figure 5.5 shows the Dunnett's comparison analysis of mean maximum cumulative CO_2 of treatments relative to the oil only control.

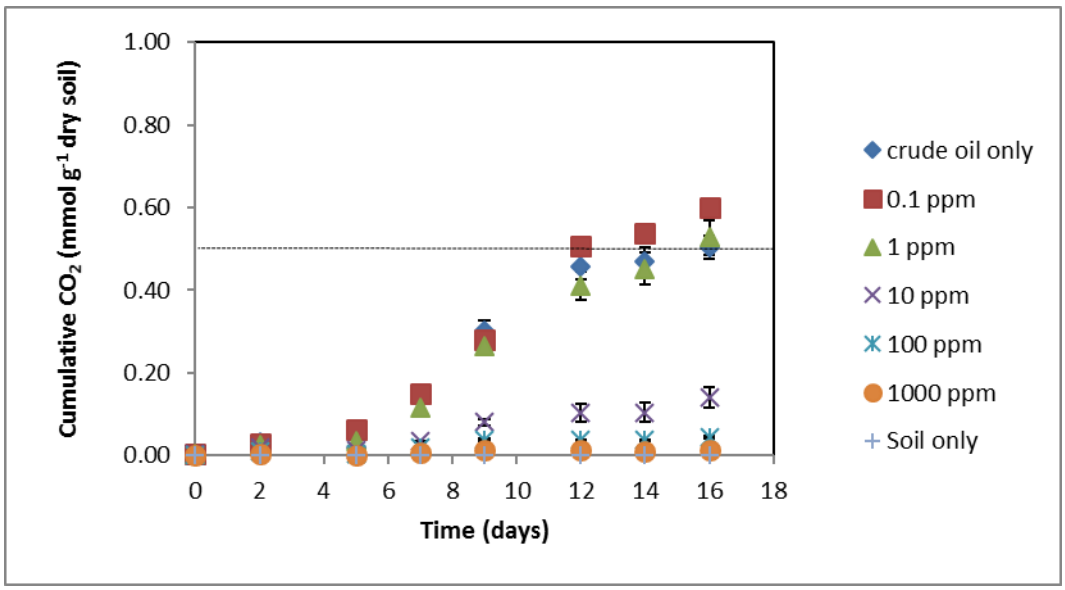

Figure 5.2: CO_2 production profiles of Cd and petroleum hydrocarbon co-contaminated soil microcosms relative to oil amended and unamended controls. Microcosms were amended with $CdCl_2$ (as indicated by ppm). The data points represent the mean cumulative CO_2 of triplicate samples in mmols g^{-1} dry soil. The error bars represent the standard error of triplicate sample and graph legend indicates the added Ni concentration in ppm and the controls. The dotted line indicates the maximum CO_2 produced in control microcosm amended with crude oil, only.

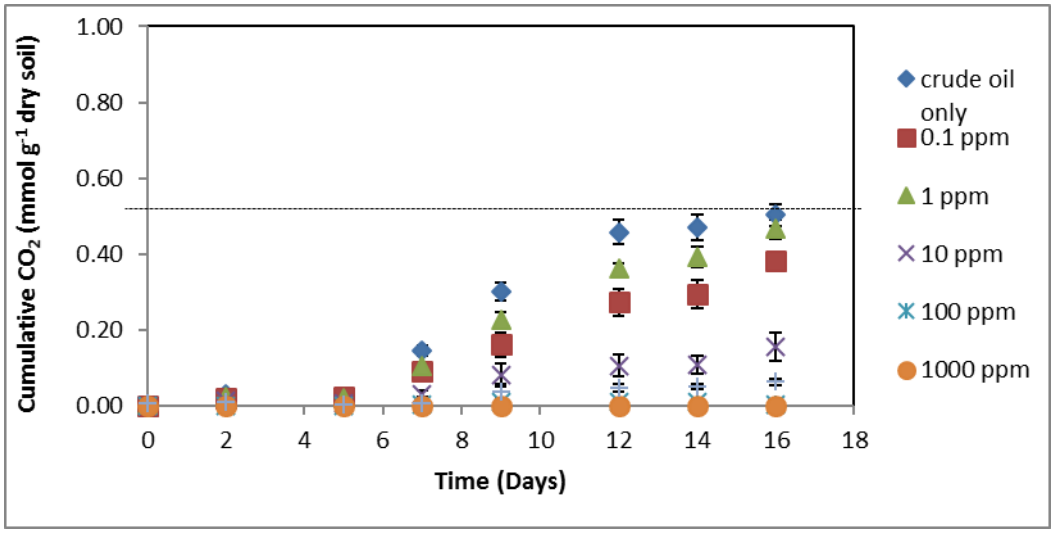

Figure 5.3: CO_2 production profiles of Cd and petroleum hydrocarbon co-contaminated soil microcosms relative to oil amended and unamended controls. Microcosms were amended with CdO (as indicated by ppm). The data points represent the mean cumulative CO_2 of triplicate samples in mmols g^{-1} dry soil. The error bars represent the standard error of triplicate sample and graph legend indicates the added Ni concentration in ppm and the controls. The dotted arrow indicates the maximum CO_2 produced in control microcosm amended with crude oil, only.

The analysis revealed that relative to oil only control, no significant differences in mean cumulative CO_2 at Cd concentrations of 0.1 and 1 ppm occurred irrespective of Cd form. In the $CdCl_2$ amended soil, the differences in means relative to oil only control were 0.06 ± 0.031 mmol g-1 dry soil at 0.1 ppm Cd ($p=0.33$) and 0.03 ± 0.031 mmol g-1 dry soil at 1 ppm Cd ($p=0.97$). Similarly, in CdO amended soils, the differences in means of maximum cumulative CO_2 relative to the oil only control were 0.08 ± 0.031 mmol g-1 dry soil at 0.1 ppm Cd ($p=0.13$) and 0.09 ± 0.031 mmol g-1 dry soil at 1 ppm Cd ($p=0.07$) However, the mean maximum cumulative CO_2 at 10, 100 and 1000 ppm Cd irrespective of Cd form were significantly lower than at of oil only amended soils. For $CdCl_2$ amended soils, maximum cumulative CO_2 was lower by 0.41 ± 0.031 mmol g-1 dry soil at 10 ppm Cd ($p<0.0001$), 0.51 ± 0.031 mmol g-1 dry soil at 100 ppm Cd ($p<0.0001$) and 0.54 ± 0.031 mmol g-1 dry soil at 1000 ppm Cd ($p<0.0001$). Similarly, in CdO amended soils, maximum cumulative CO_2 was lower by 0.39 ± 0.031 mmol g-1 dry soil at 10 ppm Cd ($p<0.0001$), 0.55 ± 0.031 mmol g-1 dry soil at 100 ppm Cd ($p<0.0001$) and 0.55 ± 0.031 mmol g-1 dry soil at 1000 ppm Cd ($p<0.0001$). Looking at the profiles there is definitely a progressive impact of these metals as concentration increases.

5.4.2.4. Effect of Cd on the maximal rate of CO_2 production during the degradation of petroleum hydrocarbon

The maximal rate of CO_2 production in microcosms during the degradation period was achieved between day 5 and day 12. The maximal rate determined in microcosms was compared with the oil only amended soil control. Figure 5.6 compares the maximal rate of CO_2 production in treatment microcosms relative to oil only amended soil control and soil only control. From the figure, it can be seen that amendment with Cd reduced the rate of CO_2 production resulting in the inhibition of petroleum mineralisation. ANOVA indicated that this reduction in maximal rates was significant across all treatments ($p<0.0001$) and influenced significantly Cd concentrations ($p<0.0001$) irrespective of the form of Cd.

The maximal rates of treatments were compared with the oil only control using the Dunnett's pairwise analysis to determine the effect the Cd on the rate

of petroleum mineralization in soils (see figures 5.7 and 5.8). Analysis revealed that in irrespective of Cd-form, the maximal rates at 0.1 and 1 ppm were not significantly different from the oil only amended soil. The maximal rate of CO_2 production in oil only amended soil was 0.07 ± 0.006 mmols g-1 dry soil day. In $CdCl_2$ amended soils, maximal rate was lower by 0.011 ± 0.005 mmols g-1 dry soil day at 0.1ppm cd (p=0.127) and 0.014 ± 0.005 mmols g-1 dry soil day at 1 ppm Cd (p=0.056) relative to oil only control. Similarly, in CdO amended soils, maximum rate is lower by 0.02 ± 0.008 mmols g-1 dry soil day at 0.1 ppm Cd (p=0.08) and 0.018 ± 0.008 mmols g-1 dry soil day at 1.0 ppm (p=0.11).

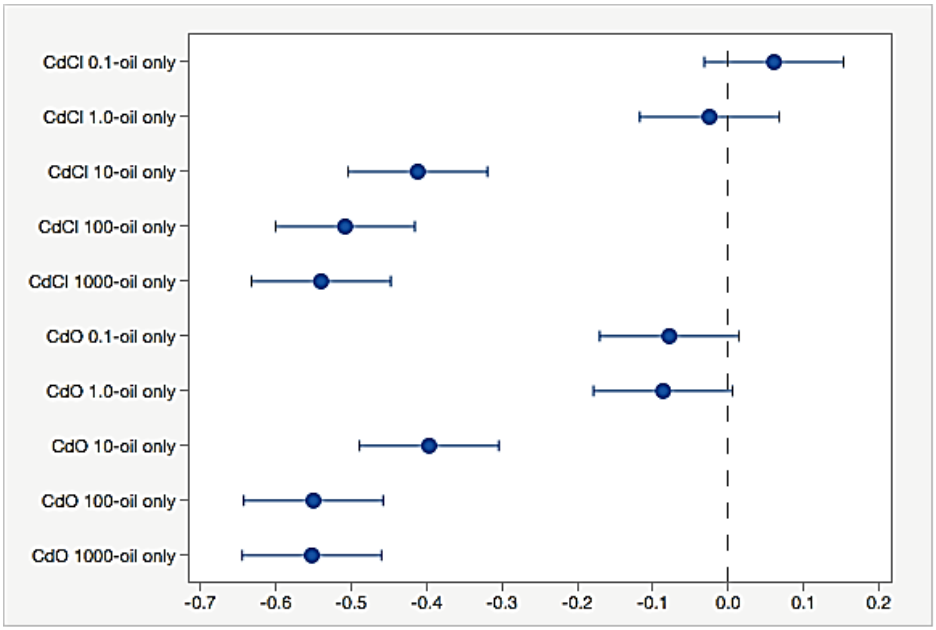

Figure 5.4: Comparative analysis (Dunnets's multiple comparison) of cumulative CO_2 values measured after 16 days in $CdCl_2$ and CdO amended oil degraded soil microcosms at Cd concentrations of 0.1, 1, 10, 100 1nd 1000 ppm relative to oil only amended control. The plot indicates confidence intervals for the range differences (95% confidence) between the individual treatment means and the control mean. A mean is not considered significantly different if the interval spans zero (0)

In contrast, maximal rates at higher concentrations of 10, 100 and 1000 ppm Cd, irrespective of Cd form, were significantly lower relative to the maximal rate of oil only control. In $CdCl_2$ amended soils, maximal rates were lower by 0.055 ± 0.005 mmol g-1 dry soil day, 0.061 ± 0.005 mmol g-1 dry soil day and

0.068 ± 0.005 mmol g-1 dry soil day at 10, 100 and 1000 ppm Cd, respectively, relative to oil only amended control ($p<0.0001$ at all concentrations). Similarly, in CdO amended soil, maximal rates were lower by 0.052 ± 0.008 mmol g-1 dry soil day, 0.068 ± 0.008 mmol g-1 dry soil day and 0.070 ± 0.008 mmol g-1 dry soil day at 10, 100 and 1000 ppm Cd, respectively, relative to oil only amended control ($p<0.0001$ at all concentrations).

These observations indicate that at low concentrations of Cd, Cd did not significantly affect the rate of petroleum mineralisation although it should be noted that the oil only soils gave the highest mineralisation rates and the differences between this rate and the 0.1 and 1 ppm Cd addition experiments were borderline significant ($P \leq 0.1$). This is contrary to high Cd concentrations, which were clearly inhibitory to CO_2 production.

To understand the degree of inhibition by Cd, treatment microcosms at high concentrations were also compared with soil only amended soils. The mean maximal rates of CO_2 production in soil only control is 0.027 mmol g-1 dry soil day. Dunnett's pairwise comparison (see figure 5.9) indicated that rates were not significantly different at 10 ppm irrespective of Cd form, relative to soil only control. However, at higher concentrations of 100 and 1000 ppm, the maximal rates were significantly lower. In $CdCl_2$ amended soil, maximal rates were lower by 0.018 ± 0.005 mmol g-1 dry soil day at 10 ppm ($p=0.01$) and 0.025 ± 0.005 mmol g-1 dry soil day at 1000 ppm ($p=0.001$). Similarly, in CdO amended soils, the maximal rate was lower by 0.025 ± 0.005 mmol g-1 dry soil day at 100 ppm Cd ($p=0.0009$) and 0.027 ± 0.005 mmol g-1 dry soil day at 1000 ppm Cd ($p=0.0004$). These differences observed in the maximal rates at 100 and 1000 ppm relative to soil only control suggests that Cd inhibited the normal soil microbial activities implying that general soil microbes were negatively affected as a result of Cd amendment at 100 and 1000 ppm.

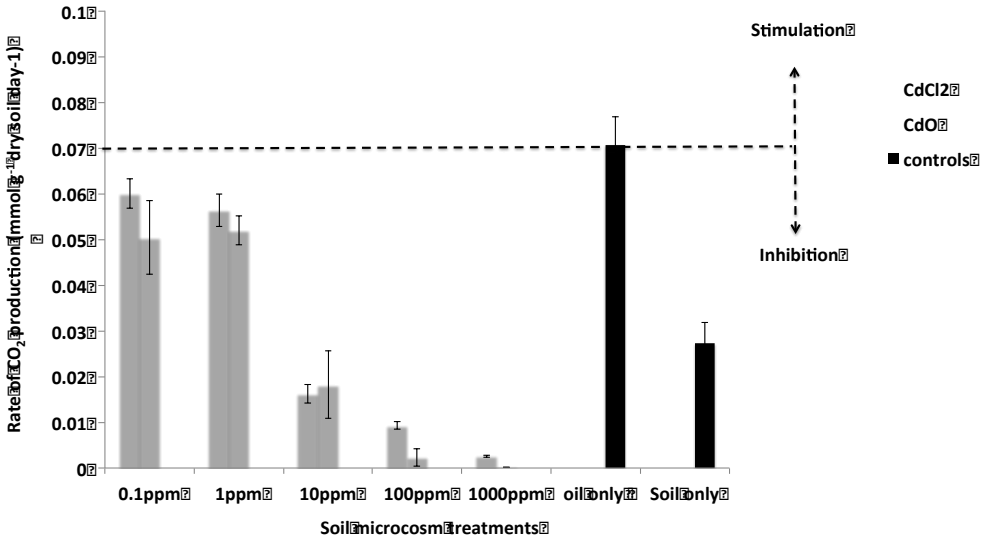

Figure 5.5: The maximal rate of CO_2 production in Cd-oil amended soils relative to oil amended and unamended controls. Each bar represents the mean maximal rate of triplicate samples. The error bars represent the standard error of the rate of triplicate samples. The graph legend indicates the chemical form of added Ni and controls. The microcosm treatment on the x-axis indicates the concentration of added Cd and controls. The oil only control is the oil-amended soil while soil only control indicates the unamended soil. The dotted line indicates the rate of oil-amended control; treatments with rates above the dotted lines considered stimulatory while treatments with rates below the dotted lines are inhibitory.

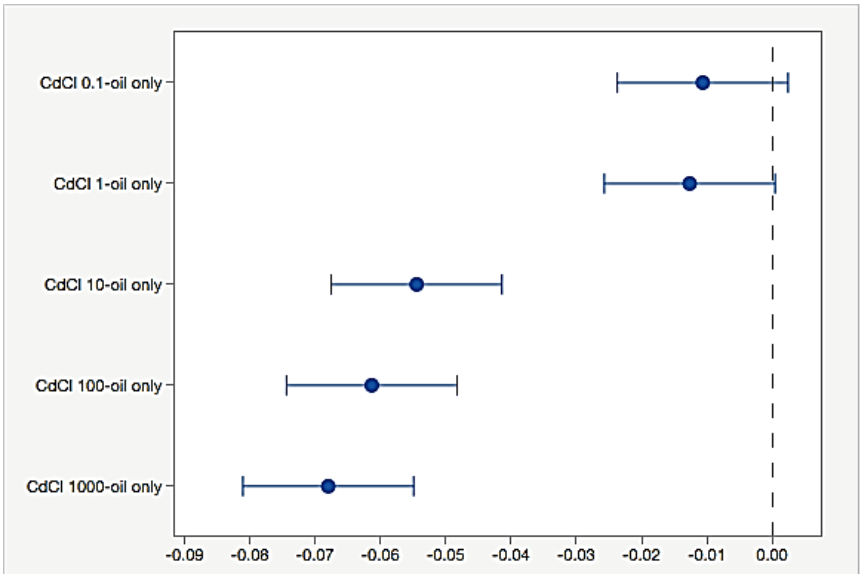

Figure 5.6: comparative analysis of maximal CO_2 production rates in $CdCl_2$ amended oil degraded soil microcosms at Cd concentrations of 0.1, 1, 10, 100 1nd 1000 ppm relative to oil only amended control. Analysis was by Dunnett's multiple comparison analysis at 95% confidence interval. The values include the differences between the treatment mean and the control mean. A mean is not significantly different if its interval encompasses zero (0)

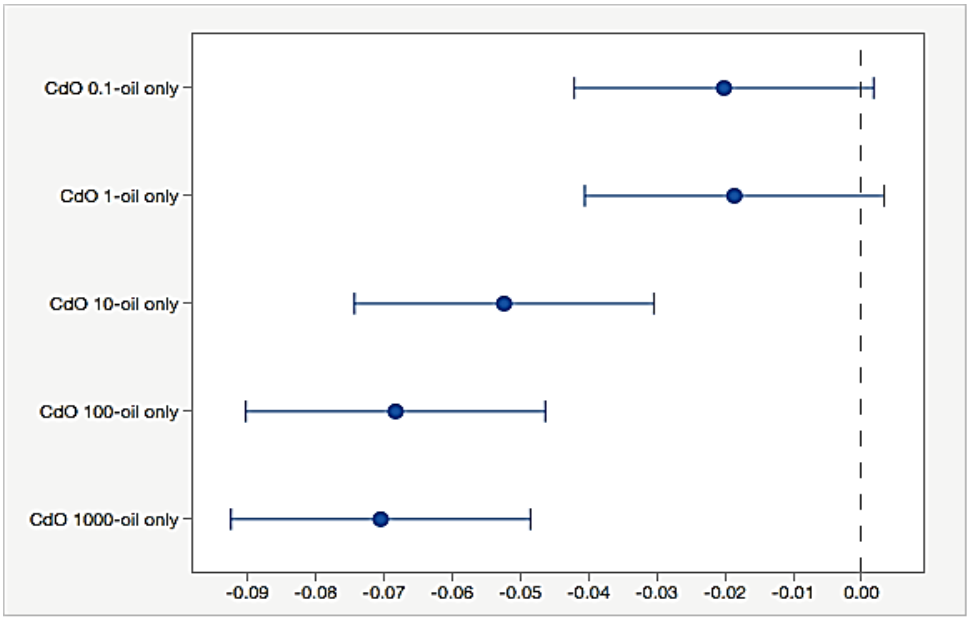

Figure 5.7: comparative analysis of maximal CO_2 production rates in CdO amended oil degraded soil microcosms at Cd concentrations of 0.1, 1, 10, 100 1nd 1000 ppm relative to oil only amended control. Analysis was by Dunnett's multiple comparison analysis at 95% confidence interval. The values include the differences between the treatment mean and the control mean. A mean is not significantly different if its interval encompasses zero (0).

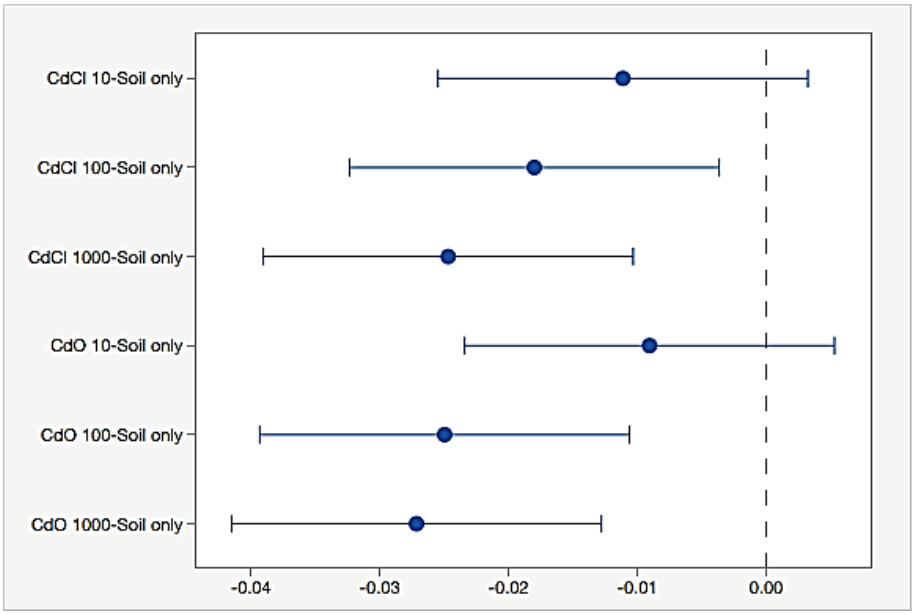

Figure 5.8: comparative analysis of maximal CO_2 production rates in $CdCl_2$ and CdO amended oil degraded soil microcosms at Cd concentrations of 10, 100 1nd 1000 ppm relative to soil only control. Analysis was by Dunnett's multiple comparison analysis at 95% confidence interval. The values include the differences between the treatment mean and the control mean. A mean is not significantly different if its interval encompasses zero (0)

5.4.3. The fate and chemical association of Cd amendments after addition to the soil microcosms

Fractions of added Cd were sequentially partitioned (see section 2.7) to understand the chemical associations of the metal in soils. The aim was to provide details of the fate of added Cd in soils. Here, Triplicate soil samples were amended with CdO and $CdCl_2$ at 10 ppm Cd concentration and were incubated at room temperature (21 ^{o}C) for 1 week. At the end of the incubation period, added Cd was sequentially extracted from the soils using the sequential extraction method for the extraction of metals in soils and sediments developed by Tessier et al (1979). The Cd was partitioned based on their association with soil fractions, which included the exchangeable, the carbonates, oxides of Fe and Me, Organics and residual fractions.

Figure 5.10 shows the results of Cd sequential sequestration in the soil. From the figure, there are apparent differences in the partitioning between the different forms of Cd investigated. In CdCl2 amended soil 2.64 ppm Cd (26% of added Cd), 0.36 ppm Cd (3.6% of added Cd), 1.75 ppm Cd (17.5% of added Cd), 1.22 ppm Cd (12.2% of added Cd) and 4.02 ppm Cd (40.2% of added Cd) were associated with the soil exchangeable fraction, the carbonates, oxides of Fe and Me, Organics and residual fractions, respectively. For CdO, 1.17 ppm Cd (11.7% of added Cd), 0.71 ppm Cd (7.1% of added Cd), 2.61 ppm Cd (26.1% of added Cd), 2.92 ppm Cd (29.2 % of added Cd) and 2.59 ppm Cd (25.9% of added Cd) were associated with the soil exchangeable fraction, the carbonates, oxides of Fe and Me, Organics and residual fraction, respectively. This indicates that exchangeable fractions of the soil (Cd ions easily released into soil solution) and residuals were larger fractions in CdCl2 amended soil than in CdO soils. Whereas Cd associated with carbonates, Fe and Mn and organics were larger fractions in the CdO amended soils. Despite these apparent differences, ANOVA indicated that there were no significant differences in Cd partitioning between $CdCl_2$ and CdO amended soils ($p=0.19$). Also, ANOVA indicated that the concentrations of Cd partitioned across soil fractions were not significantly different in $CdCl_2$ ($p=0.31$) and CdO ($p=0.50$) amended soils.

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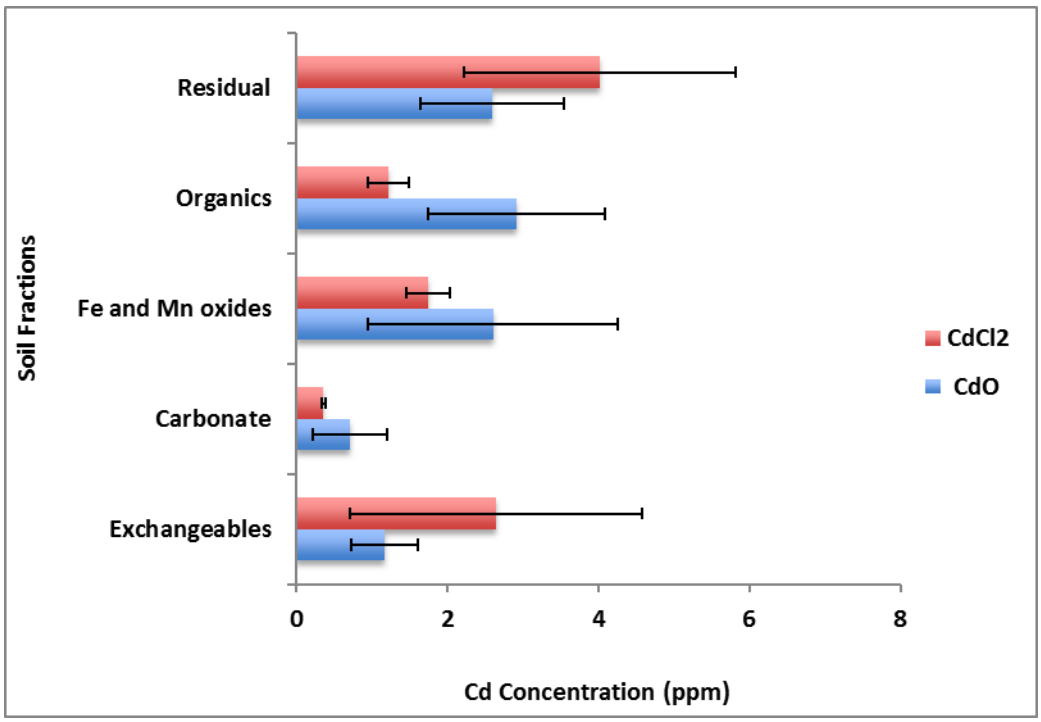


Figure 5.9: comparative analysis of the fate of added Cd in soil by sequential extraction of Cd fractions associated with exchangeable ions, carbonates, oxides of Fe and Mn, organics and residuals. The graph legend indicates the different chemical forms of Cd used for this study. Each bar represent the mean of concentration of Cd associated with the soil fractions in triplicate. The error bars represent the standard error of triplicate microcosms.

5.4.4. Degradation of n-alkanes in soil microcosms; total n-alknanes recovered from Cd-crude oil degraded soil

The residual petroleum hydrocarbons in the soil microcosms contaminated with $CdCl_2$ and CdO at 0.1, 10 and 1000 ppm Cd were extracted after the degradation period and were analysed by GCFID. Total n-alkane (nC_{12}-nC_{32}) determined in the extracts from microcosms were compared with the n-alkanes (nC_{12}-nC_{32}) contents of the undegraded crude oil (see figure 5.10). ANOVA indicated that there was a significant difference across the treatment microcosms ($p=0.0004$). The mean total n-alkanes of the undegraded crude oil was 9626.84 ± 451.59 µg. A pairwise comparison of treatment microcosms relative to undegraded crude oil using the Dunnett's method indicated that n-alkanes recovered from oil only amended soil was significantly lower relative

to the undegraded crude oil by 1398.36 ± 459.17 µg (p=0.0007), signifying that degradation of crude oil occurred in soil microcosms amended with oil. However, in Cd-oil amended microcosms, the mean total n-alkanes were not significantly different relative to undegraded crude oil, irrespective of Cd form or concentration (p>0.05). This implies that amendment with Cd may have deterred the degradation of petroleum hydrocarbons. This is also manifested in the n-alkane profile as seen in the figure 5.12 were n-alkane profiles of Cd-amended soils appear similar to the n-alkane profile undegraded crude oil.

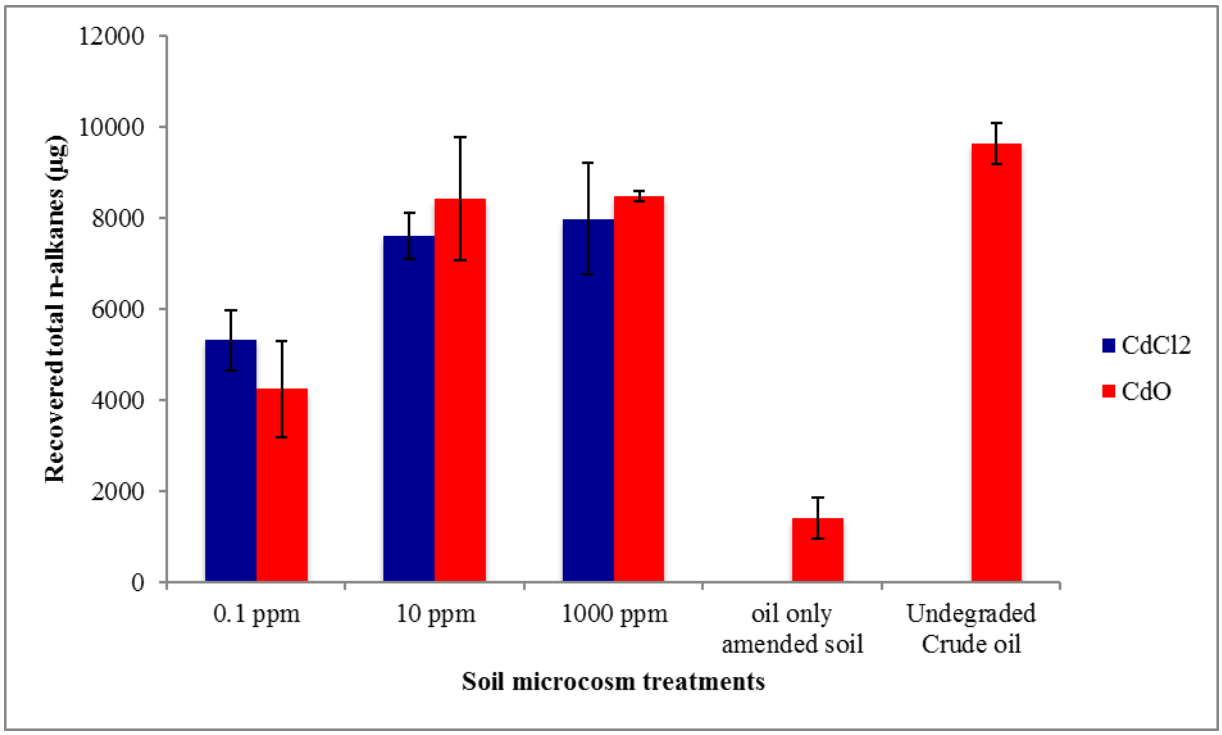


Figure 5.10: comparison total n-alkanes (nC_{12}-nC_{32}) recovered frommicrocosms contaminated with Cd and/or 100 mg oil after 15 days incubation. Each bar represents the average of triplicate samples. Error bars represent the standard error of triplicate samples.

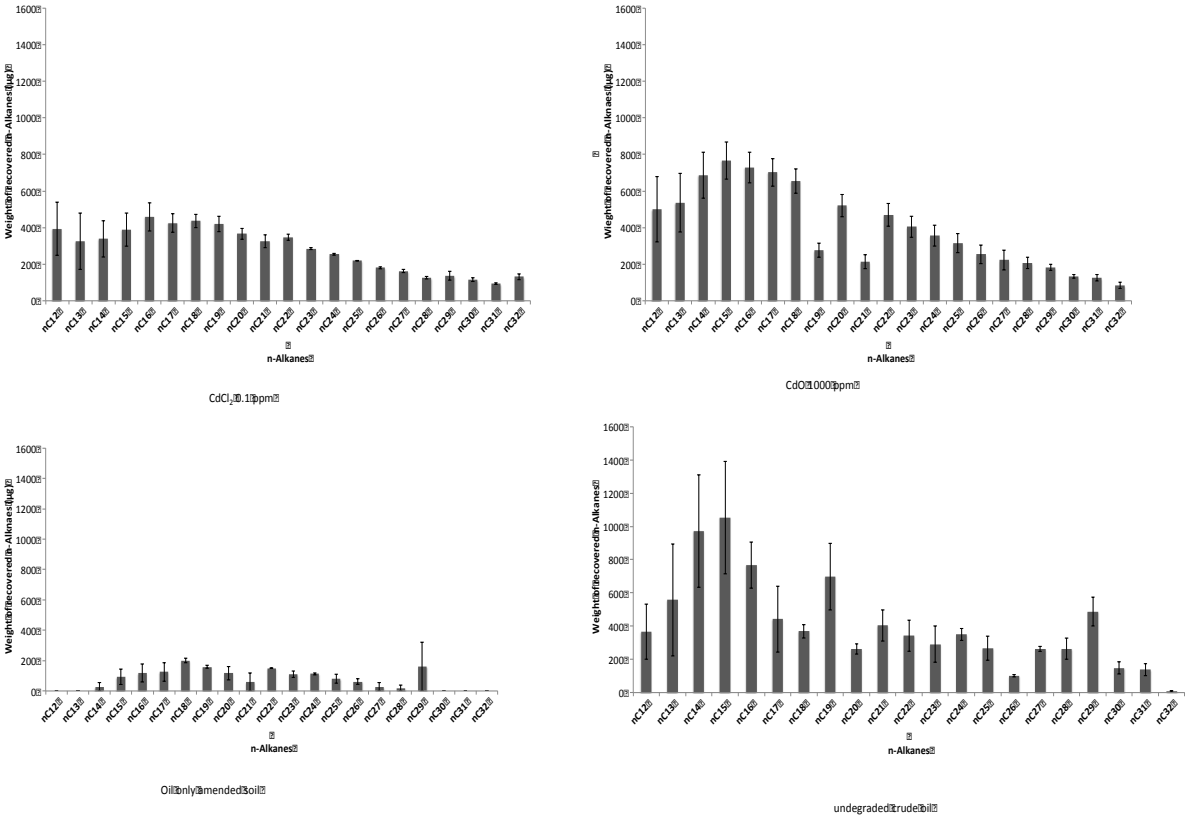


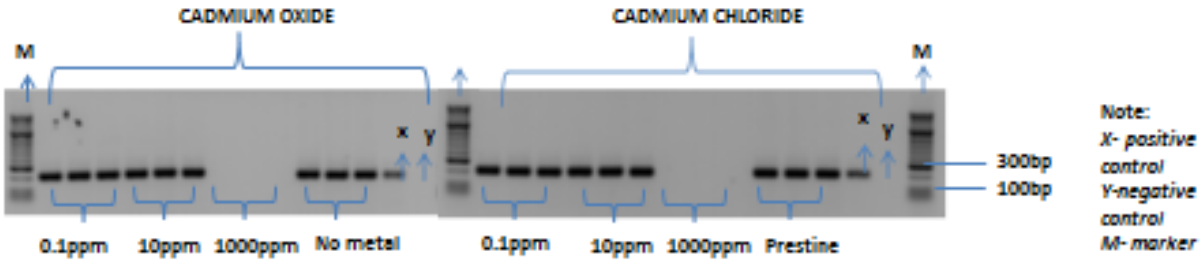
Figure 5.11: comparison of n-alkane profile of petroleum crude oil recovered from Cd-oil amended soil microcosms at low ($CdCl_2$ 0.1 ppm) and high (CdO 1000 ppm) Cd concentrations relative to oil only amended soil and undegraded oil. n-Alkanes were obtained by GCFID analysis of recovered oil from degraded soil microcosms. Each bar represents the mean of weight of recovered n-alkanes. The error bars indicate the standard error of triplicate samples.

5.4.5. Microbiological diversity and phylogeny

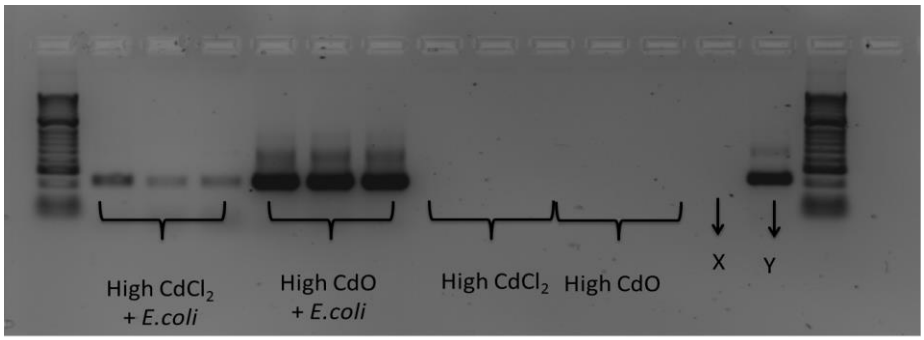
Molecular methods were used to study the microbial community diversity and phylogeny in microcosms to understand the effect of Cd on degraded soil communities. These included DGGE and ion torrent sequencing analysis. These methods were described in chapter 2 and results are presented in this section. Based on the results of the degradation experiment, soil microcosms amended with Cd at 0.1, 10 and 1000 ppm were used for these microbiological studies.

5.4.5.1. The effect of Cd concentration on microcosm bacterial communities identified by endpoint PCR of 16S rRNA genes.

Polymerase Chain reaction, PCR, was used to amplify 16S rRNA genes from DNA extracts of soil microcosms and the resultant amplicons were visualised by agarose gel electrophoresis (AGE) (see figure 5.12a). AGE indicated amplification of 16S rRNA gene in soils amended with Cd at 0.1 and 10 ppm Cd irrespective of Cd forms. However, at 1000 ppm Cd, amplification of 16S rRNA was not visualised on the gel. To eliminate the possibility of non-amplification of 16S rRNA gene at 1000 ppm Cd being due to other factors such as inhibition of amplification as a function of high metal concentration, a control consisting of degraded soil samples amended with Cd at 1000 ppm and inoculated with E. coli cells were amplified alongside the treatment samples for 1000 ppm Cd. AGE indicated the amplification of *E. coli* 16S rRNA genes at 1000 ppm, but no amplification of soil bacterial 16S rRNA in the treatment microcosms (see figure 5.12 b). This indicated that there was likely an inhibition of bacterial growth in soils amended with Cd at 1000 ppm Cd, irrespective of Cd-form. This may have explained the significant reduction in maximum cumulative CO_2 and the rate of CO_2 production in microcosms, relative to soil only microcosms.



(a)



(b)

Figure 5.12: Agarose Gel electrophoresis of amplified 16S rRNA genes from Cd-petroleum co-contaminated soil microcosms. a) Amplification of genes did not occur at 1000 ppm Cd-concentration presumably due to the toxicity of Cd. b) verification that insignificant amplification at 1000 ppm was likely due to inhibition by soil materials.

5.4.5.2. Denaturing gel electrophoresis indicating the microbial communities enrichment influenced by Cd-amendment and the reproducibility of communities

Based on the PCR-AGE analysis, Denaturing gel electrophoresis (DGGE) was carried out using amplified 16S rRNA genes from $CdCl_2$ and CdO treated soil microcosms at 0.1 and 10 ppm Cd. A visual analysis of the DGGE fingerprints of amplified bacterial 16s rRNA gene fragments from soil DNA extracts (figure 5.13) revealed diverse bands amplified from all the soils analysed indicating the presence of a broad range of bacterial species including those with low GC-content and those with high GC-content. A high degree of reproducibility between experimental replicates was also indicated in the DGGE fingerprint allowing clear identification of treatment-related effects on community diversity. Comparisons of the unamended soil control with contaminated soils showed selective enrichment of some species of microbes in contaminated soils determined based on the intensity of bands. Cd-presence and metal effects dependent on the chemical form of added Cd and concentration influenced the enrichment of species in soils. While some bands, which were reproducibly present in the oil only microcosm do not seem so enriched in the Cd treatments there was also some enrichments of bands in Cd treatments, which were less dominant in the oil only. These observations provide evidence of the impact of metal toxicity on the diversity of the HC degraders enriched and evidence that Cd-effects the enrichment of selected species growth.

In addition to the visual evidence of treatment reproducibility and distinct treatment effects on enriched organisms a numerical analysis of these banding profiles identified differences in community structure using multidimensional scaling (MDS) plotted with Bray-Curtis similarity matrix overlaid with similarity contours generated from a clustering analysis. This analysis revealed that all the communities analysed (including the un-amended soil control) clustered within 40% similarity (see figure 5.14). Clustering of communities was based on Cd-form where essentially, $CdCl_2$ amended communities, irrespective of concentration, showed 80% similarity. However, CdO soils were essentially clustered at 60% similarity based on the Cd concentration. Also, $CdCl_2$ treated soils clustered with soils amended oil only

(oil only) and soil only control within 60% similarity. But essentially, reproducibility of communities was indicated at 80% similarity for $CdCl_2$ treated soil and controls and 60% similarity for CdO treated soils.

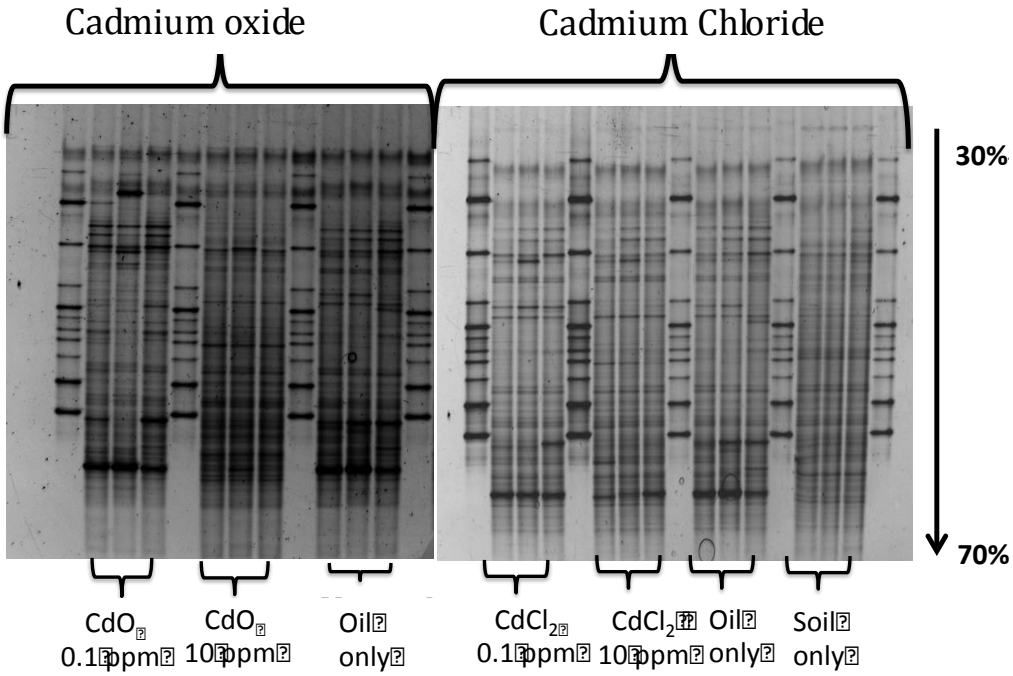


Figure 5.13: the 16S rRNA gene profile on a Denaturing gradient gel indicating the diversity of microbial communities and reproducibility of communities in triplicates of soil Microcosms contaminated with petroleum hydrocarbons and Cd in different forms ($CdCl_2$ and CdO) and at different Cd concentrations (0.1, 10, and 1000 ppm).

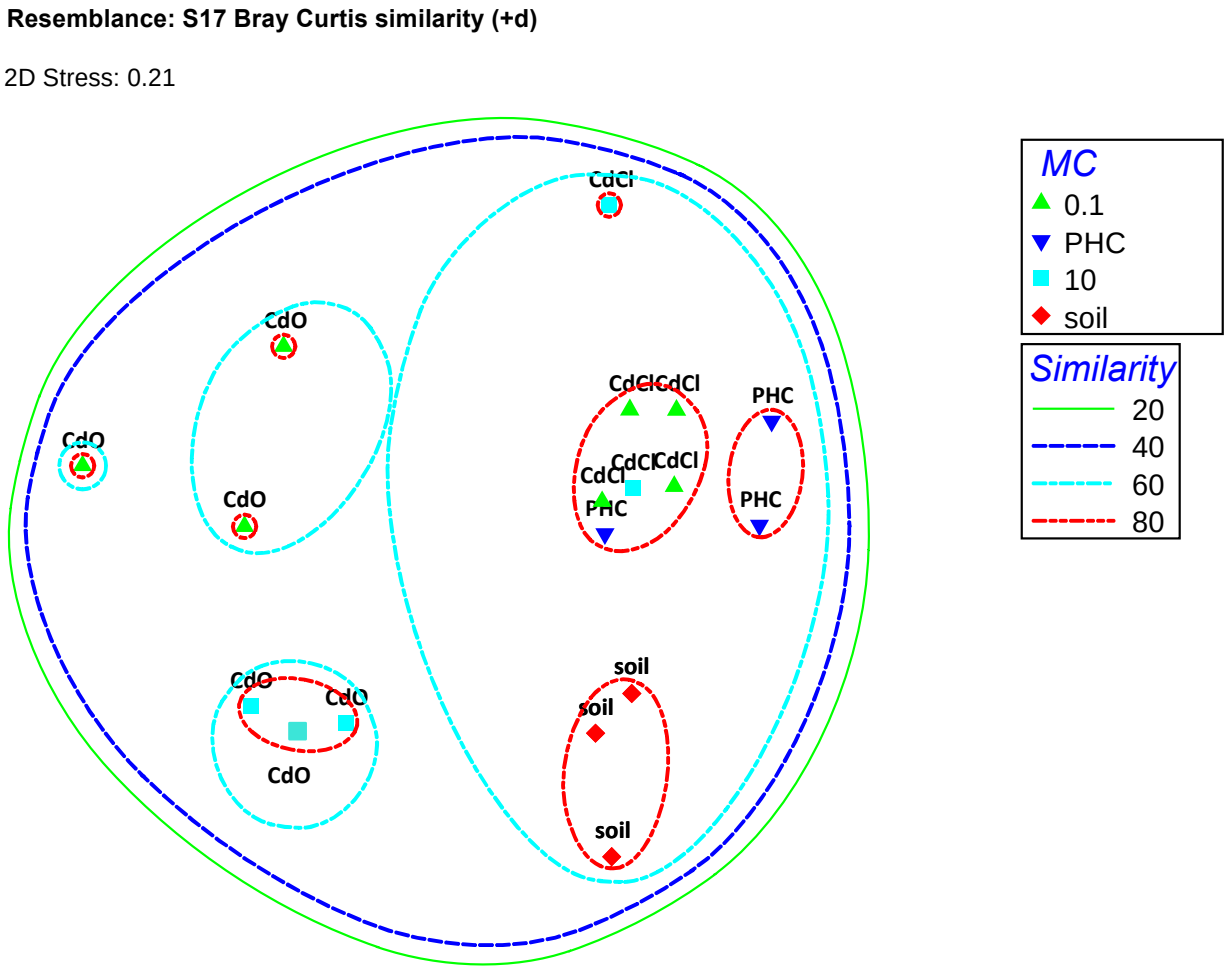


Figure 5.14: Non-metric MDS analysis of the bacterial communities present in Cd-crude oil amended soil microcosms, crude oil only amended microcosms and unamended soils (soil only) after 16 days of degradation period. MDS plots are representations of how different the communities are from each other based on clustering of like samples. Similarity contour lines from cluster analyses are superimposed on to the MDS plots. The legend indicates the concentrations of added Cd and the controls (i. e. oil only and soil only) as well as similarity contour lines.

To statistically determine the influence of Cd form and concentration on community fingerprinting, Analysis of similarity (ANOSIM) was carried out. ANOSIM revealed that with a global r of 0.53 the influence of Cd forms on community variation was moderate but highly significantly (p=0.001). A pairwise comparison of treatment forms relative to oil only amended soils indicated that there was a very little disparity between $CdCl_2$ containing communities and oil only amended control, which was not significant (Global R=0.083; p=0.345). However, significant dissimilarity was identified on the

comparison of oil only control with CdO (Global R=0.75; p=0. 012). Likewise, comparison of treatment concentrations analysed implicated that there was very little separation between concentrations of Cd (Global R=0.228; p=0.033).

5.4.5.3. Qualitative analysis of sequencing data set used for the biodiversity study of microbial communities of Cd amended crude oil degraded soil

For a more detailed understanding of soil microbial community diversity of experimental microcosm treatments soils, studies based on the sequencing of amplified 16S rRNA gene fragments were carried out. For this purpose, 16Sr RNA genes of communities were sequenced using an ion torrent PGM sequencing approach. The sequence data obtained were analysed using the mothur software to trim sequence data, cluster sequence data into OTUs, assign taxonomic groups to OTUs and analyse community diversity (alpha- and beta- diversity of communities) to identify the differences in the microbial community structures. Due to the high reproducibility of communities identified from the DGGE analysis, single representative samples for each treatment were used in this analysis. Tagged 16S rRNA samples from 17 soil samples consisting of metal (Ni, Cd and Pb) petroleum degraded soils and controls, which were the petroleum amended, and unamended soils made up the clone library used for the ion torrent sequencing. Communities of the Cd amended soils consisted of representative samples including $CdCl_2$ amended soils at 0.1 ppm Cd concentrations and CdO amended soils at 0.1 ppm Cd concentrations.

The ion torrent sequencing recovered a high throughput sequence data set of 492574 sequences. Sequence data from Cd-amended communities accounted for 45789 sequences, i. e. approximately 9.30% of the sequence data from the clone library (consisting of tagged 16S rRNA sequences from 17 samples). Sequence data were trimmed to recover sequences that have a minimum length of 300 bp, a maximum of 500 bp. and homopolymers lower than 7 bp were trimmed. This resulted in the omission of ~8.16% of the raw sequence data from the Cd amended communities, reducing the sequence recovery to a total of 42054 Sequences. Before the trimming, the number of sequences recovered in $CdCl_2$ amended soil was 16646 sequences and 29143 sequences

in CdO soils. After trimming, the number of sequences recovered amended soils was 14627 sequences from CdCl2 and 27427 sequences from CdO amended soils.

For further analysis on mothur software, sequences were normalised to 8000 sequences per sample (the highest number of sequences mothur could analyse without crashing). Also, for QIIME analysis, sequences were normalised to 13500 sequences per sample; reasonably the least sequence count obtained considering all 17 samples analysed. Table 5.1 shows the Chao estimator on the number of observed OTUs per sample after sequence sampling of 13500 sequences per treatment on QIIME and 8000 sequences per treatment on mothur platforms.

Table 5.1: the qualitative estimation of the 16S rRNA sequence sampling for the diversity and phylogenetic study of communities of Cd-oil amended soils.

Samples	Chao estimator (13500 sequences)	Number of Observed species (13500 sequences)	Chao estimator (8000 sequences)	Number of Observed species (8000 sequences)
CdCl2	8270.23	4056	6163.18	2103
CdO	9155.84	3838	7533.8	1910
Oil only	8754.93	3567	6104.18	1676
Soil only	9637.72	4562	7331.28	2457

Table 5.2: Community specie richness and diversity indices evaluated with 8000 16S rRNA sequences of Cd-oil amended soil microcosms

Samples	Number of Observed OTUs	Shannon's Evenness (E_H)	Simpson's diversity index (λ)
$CdCl_2$	2103	0.840788	0.00814
CdO	1910	0.701226	0.071885
Oil only	1676	0.710628	0.051145
Soil only	2457	0.874792	0.00384

1. *$0 \leq \lambda \leq 1$ where 0= perfect heterogeneous community and 1= perfect homogenous community*

2. *$0 \leq E_H \leq 1$ where 0= perfect heterogeneous community and 1= perfect homogenous community*

3. *OTUs are discriminated at 97% similarity cut off.*

5.4.5.4. OTU-base studies on the influence of Cd on the community diversity of oil degraded soil microcosms

Operational Taxonomic Units (OTUs) generated using the average Neighbor clustering method based on 97% similarity cutoff of 8000 16S rRNA sequences were analysed to determine the alpha diversity of communities. Species richness, Shannon Evenness ($E_{H'}$), and Simpson's diversity (λ) were estimated using the Mothur (Schloss et al. 2009) platform (table 5.2). These estimators of diversity evaluated established microbial species diversity in communities and the degree of dominance of communities by species in crude oil containing microcosms with or without Cd.

The species richness was established by determining the number of observed OTUs in communities. As has been pointed earlier in chapter 4, 16382 OTUs were observed in the whole library analysed. The observered species in oil only amended and unamended soil communities were 1676 and 2457 OTUs, respectively. Hence, relative to the unamended soil, 31.79% dominance of microbial community by hydrocarbon degraders occurred in the oil-amended soil. Interestingly, 2103 and 1910 OTUs were observed in $CdCl_2$ and CdO amended soils respectively. This implies that relative to oil only control, while CdCl2 amendment resulted in a decrease in community dominance by

17.38%, the dominance by community species increased by 9.52% as a result of amendment with CdO and oil.

Furthermore, the evenness of community species was determined by estimating the EH' of communities. The EH' values of communities are usually between 0 to 1, with 0 indicating perfect community species unevenness (i. e. a perfect dominance by a single species) and 1 a perfect community species evenness (a perfect heterogeneous community). The EH' of oil-amended soil and unamended soil controls are 0.710628 and 0.874792, respectively. To clearly establish the effect of Cd on community species evenness, EH' in Cd amended soils were compared with the oil only amended soil control. The EH' of CdCl2 and CdO amended soils were 0.840788 and 0.701226, respectively. Furthermore, λ determined in communities clearly identified the extent of dominance in communities by considering species richness and evenness. The values of λ are between 0 and 1 with 0 representing the perfect heterogeneous community and 1 representing the perfect homogenous community. λ in oil only amended soil and soil only controls were 0.051148 and 0.003842 respectively. These values of diversity indices indicate that the amendment of soil with oil resulted in the most homogenous community. This could be attributed to the selective enrichment of hydrocarbon degraders in the community. Comparisons of λ in $CdCl_2$ amended soil communities relative to oil only amended control indicated that with the λ values of 0.008142, homogeneity of the $CdCl_2$ community decreased by 84.01% on the amendment with $CdCl_2$ relative to the oil only control. On the contrary, homogeneity of CdO amended oil degraded soil community increased slightly by 9.52% relative to oil only control. A possible explanation for these observations may be that Cd inhibited specific enrichment of hydrocarbon degraders in the Cd amended communities and promoted highly specific enrichment of Cd-resistant species, especially in CdO soils.

In other to distinguish between diversities of control and treatment communities, Yue and Clayton's dissimilarity coefficient, θ (see table 5.3) was evaluated for all treatments relative to oil only amended and unamended controls. The value of θ ranges from 0 to 1 with 0 denoting a perfect similarity between communities and 1 a perfect dissimilarity between communities. The

values of θ indicated that community diversity of CdO (θ=0.6326) and CdCl2 (θ=0.1.218) amended soils were dissimilar to oil only amended soil community with CdO having a greater dissimilar diversity. Similarly, relative to soil only control, with θ values of 0.9650 and 0.5782 in $CdCl_2$ and CdO amended soils respectively, there are dissimilarities between community diversity of Cd amended soils and unamended soils.

Table 5.3: Clayton's dissimilarity coefficient comparison of microbial community structure in soil microcosms based on 8000 16S rRNA gene sequences

	Crude oil only	**Soil only**
Samples	**θ**	**θ**
CdO	0.6326	0.5782
CdCl2	0.1218	0.965
Crude oil only	0	0.946011
Soil only	0.946	0

Note: $0 \leq \theta \leq 1$ where 0= perfect similarity and 1= perfect dissimilarity

5.4.5.5. Community diversity study; Cd effects on the diversity of hydrocarbon degraders in oil degraded soil microcosms based on phylogenetic analysis

The number of OTUs that consisted of ~50% relative abundance of microbes in communities was determined to understand the pattern of dominance in communities. The evaluation identified discrepancies between Cd forms. From the evaluation, 27 OTUs made up 50% relative abundance in CdCl2 amended community. This is comparable to oil only amended control, which has 22 OTUs consisting of 50% relative abundance. On the contrary, 111 OTUs made up 50% relative abundance in CdO contaminated communities indicating inhibition of dominance relative to oil only amended control. In

addition, 171 OTUs consist of 50% of the relative abundance of bacteria in soil only control.

5.4.5.6. Comparative phylogenetic analysis of microbial communities of Cd-Crude oil-amended soils relative to oil only amended soils and unamended soils

Classification of OTUs carried out using a Bayesian method (Wang, George M Garrity, et al. 2007) and by matching sequences against the Silva, RDP and green genes reference files on MOTHUR and QIIME platform revealed that the most abundant organisms growing in all petroleum contaminated communities (with or without Cd) included species of the bacteria and archaea. The bacterial and archaeal species that predominated (representing ~50% of microbial abundance per community) belong to members of 10 phyla *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, *Acidobacteria*, *Chloroflexi*, *Firmicutes*, *Planctomycetes*, *Gemmatimonadetes*, *Verrucomicrobia* and *Crenarchaeota* in the order od decreasing abundance (see figure 5.15). Further classification to genus level identified the most enriched OTUs in all petroleum contaminated soil systems (with or without Cd) were closely related to the species of *Rhodococcus*, *Mycobacterium*, unclassified *Comamonadaceae*, *Nocardia*, *Pseudomonas*, *Achromobacter*, *Sphingobium*, *Bacillales*, *Acidobacteria*, *Parvibaculum* and *Tsukamurella* in order of decreasing dominance (see figure 5.16).

Considering the most abundant OTU (OTU1), which was closely related to *Rhodococcus* sp., analysis on QIIME platform revealed with the relative abundance of 19.90%, *Rhodococcus* in oil only amended soil control was enriched up to 158 times more than unamended soil control in which it was present at 0.13% relative abundance. However, the relative abundances of it in the CdO soil was 8.46%, which was 11.44 %, lower relative to the oil only control. On the contrary, the relative abundance of *Rhodococcus* in the $CdCl_2$ amended soil community was 23.32% and this was 3.43% higher relative to oil only control. This explains the differences in diversities from the previous section where diversity in CdO amended soil were significantly different

relative to oil only control at 0.1 ppm cd whereas $CdCl_2$ amended soils showed similarity in diversity relative to oil only control at 0.1 ppm Cd.

The OTU1 sequence identified in Cd amended soils was the same as that obtained from the soil amended with Ni and oil (see chapter 4). As has been pointed out in chapter 4, the closest related strains of *Rhodococcus* to OTU1 were identified using the basic local alignment search tool (BLAST) and RDP sequence Match. This analysis revealed that OTU1 was very closely related to strains isolated from hydrocarbon-contaminated and metal contaminated environments (see table 4.6). Some these close relatives that showed 100% similarity to OTU1 were *R. erythropolis* (AJ576250) from Antarctica soil (Shivaji et al. 2004) , *R. erythropolis* DN1 (X79289) from Khazastan soil polluted with crude oil (Shevtsov et al. 2013), and *Rhodococcus sp.* YE1 (EU293153) crude oil contaminated soil (Cao 2007). Others include *Rhodococcus sp.* Pi71 (AM110074) from benzene-contaminated groundwater (Fahy et al. 2006), *Rhodococcus sp. SQ8* (DQ366088) from oil polluted soil (Nohit et al. 2006), *and R. qingshengii* djl-6 (DQ90961) from carbendazim contaminated soil (Jing-Liang et al. 2006; Xu et al. 2007) *R. jialingiae* strain djl-6-2 (DQ185597) from carbendazim contaminated sludge. *Rhodococcus sp.* Q15 (99.7 %) psychrophilic bacteria from Bay of Quinte, Ontario (Whyte et al. 1998) and an uncultured bacterium (DQ125599) (99%) from uranium-contaminated soil (Brodie et al. 2006). Others included a bromate-reducing bacterium B8 (AF442524) (99%) from drinking water (Davidson et al. 2011) and R. koreensis (AM497794) (97.5%) from a German forest soil (Makdessi et al. 2007; Becker et al. 1997).

Furthermore, phylogenetic and molecular evolutionary analyses were conducted using the Molecular Evolutionary Genetic Analysis (MEGA 6) software. The sequences of closely and more distantly related strains identified in the BLAST analysis including *Rhodococcus* type sequences were aligned with the OTU1 sequence identified in this study and an evolutionary tree using the Neighbor-joining method was constructed (see figure 4.19).

5.4.5.7. Evidence of Cd-influence on selective enrichment of hydrocarbon degraders in soil

The phylogenetic analysis of communities indicated that species of hydrocarbon degraders being enriched in communities are influenced by Cd-mineral form and presence. First of all, as has been indicated earlier in a previous section, although *Rhodococcus* was the primary hydrocarbon degrader enriched in communities, the relative abundances of *Rhodococcus* in communities varied. In addition to this, evidence of Cd influence on the enrichment of other organisms in the communities was clear. For instance, the relative abundances of *Mycobacterium* (OTU 4) in the oil only amended soil community was 8.47% whereas the relative abundance of *Mycobacterium sp.* in CdO and $CdCl_2$ amended soil communities was 1.56% and 1.41%, respectively. Similarly, enrichment of *Nocardia sp*. (OTU 7) was observed to be more in oil only amended soil community than the Cd-contaminated soil communities. The relative abundance of *Nocardia sp.* in oil only amended soil community (3.50%) was ~ 3X more than that of CdO-amended soil (1.12%) and ~ 1.4X more in $CdCl_2$ amended soil communities (2.48%).

Furthermore, differences in Cd influence on enrichment could be observed from relative abundances of *Pseudomonas sp*. (OTU 3) in microcosms. As was mentioned earlier, *Pseudomonas* was on of the enriched hydrocarbons in soils. Analysis revealed that *Pseudomonas* abundance in soil only control was 0.1%. This increased on an amendment with crude oil to 2.86%. In Cd-crude oil-amended soils, *Pseudomonas* was observed to more enrich in $CdCl_2$-amended soil (relative abundance = 5.9%) than in CdO amended soil (relative abundance = 2.27%). These observations made on the enrichment of *Rhodococcus*, *Mycobacterium*, *Nocardia* and *Pseudomonas* suggested that selective enrichment of hydrocarbon degraders in communities was dependent on Cd presence. Also, Cd form may have influenced species enrichment in communities.

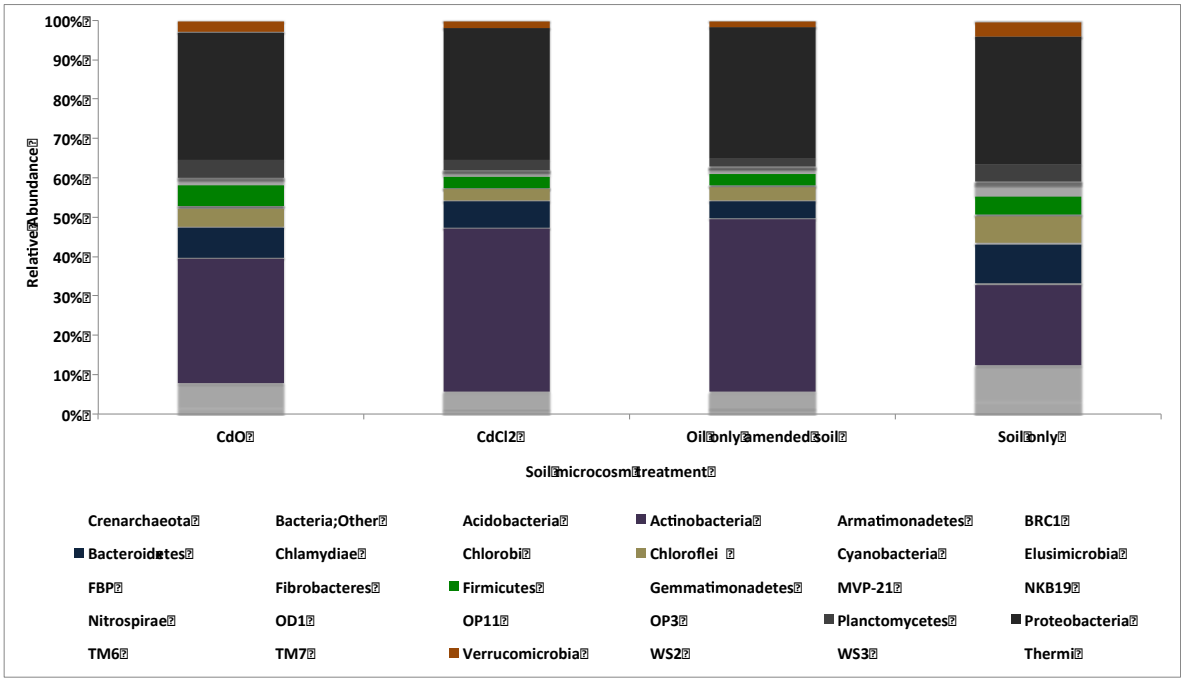


Figure 5.15: Comparative analysis of relative abundances of phylogenic group of 16S rRNA sequences recovered from representative Cd-crude oil amended soil microcosms relative to oil only amended soil and unamended soil. The relative abundance was determined using the QIIME and each treatment was normalized to 13500 16S rRNA sequences. The Legend indicate the different phyla represented in the microcosms

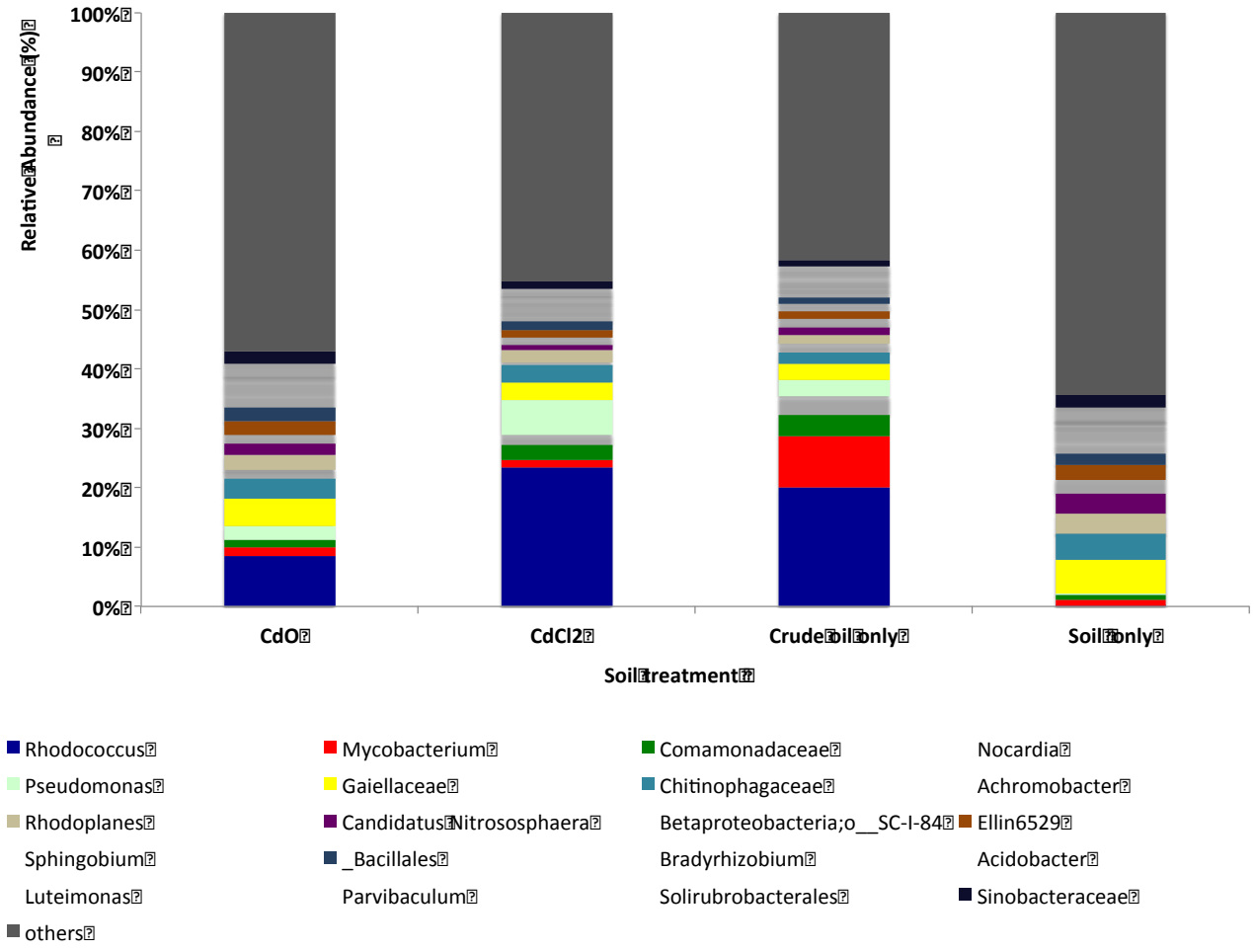


Figure 5.16: Comparative analysis of relative abundances of genera of 16S rRNA sequences recovered from representative Cd- crude oil amended soil microcosms relative to oil only amended soil and unamended soil. The relative abundance was determined using the QIIME and each treatment was normalized to 13500 16S rRNA sequences. The Legend indicates the different genera represented in the microcosms and others included the cumulative relative abundance of microbial genera with individual relative abundance of less than 1%.

5.4.6. Absolute 16S rRNA gene abundances of general bacteria and Rhodococcus in the microbial communities of Cd-crude oil-amended degraded soil microcosms

So far, the most dominant taxonomic group in oil-contaminated microcosms are the strains of the genus Rhodococcus. In addition, the relative abundances in representative microcosms were presented in previous section. The phylogenetic analysis identified the influence of Cd amendment on species richness. To provide a clear understanding of the Cd influence on species abundances in communities, qPCR assays specifically carried out to target 16S rRNA genes of Bacteria and *Rhodococcus* determined the absolute abundance of targeted 16S rRNA genes in the communities. To understand the effect of crude oil amendment on 16S rRNA gene abundance, the community of soil amended with oil only was compared to soil only control. Furthermore, the abundances of the 16S rRNA genes of treatment soils were compared to the controls to determine the absolute influence of Cd on 16S rRNA gene abundances in communities.

5.4.6.1. Influence of crude oil amendment of soil on the abundance of bacteria and *Rhodococcus*

qPCR analysis (see figure 5.17) identified that while the mean 16S rRNA gene copies in unamended soil communities were $5.30 \times 10^7 \pm 9.86 \times 10^6$ sequences, the mean the mean number of 16S rRNA gene copies in the oil only amended soil communities was $4.10 \times 10^7 \pm 1.39 \times 10^6$ gene copies. Comparison of mean 16S rRNA gene copies in the communities identified that there was no significant difference between the sizes of the bacterial gene abundances ($p=0.518$).

However, the amendment of soil with crude oil increased the abundance of *Rhodococcus* in soil communities. Analysis of the 16S rRNA gene abundance of *Rhodococcus* (see figure 5.17) in oil only amended soil control ($1.25 \times 10^7 \pm 4.13 \times 10^6$) relative to the unamended soil ($6.57 \times 10^5 \pm 2.37 \times 10^4$) revealed that there was a significant increase in the abundance of *Rhodococcus* in the oil-amended soils ($p=0.008$). Hence, mean *Rhodococcus* 16S rRNA gene abundance in the oil only amended soil was significantly higher than the

mean *Rhodococcus* 16S rRNA gene abundance in soil only controls by 1.18 X 10^{7} ± 2.39 X 10^{6} sequences.

5.4.6.2. Influence of Cd amendment on the abundance of bacteria and Rhodococcus in crude oil degrading soil

The determination of bacterial 16S rRNA gene abundance by qPCR assay revealed that the amendment of Cd affected the abundance of bacteria in the microbial communities (see figure 5.17). ANOVA indicated that there is a significant difference across treatments ($p=0.013$). To determine the influence of Cd and oil combinations on soil bacterial communities, pairwise comparison of treatments relative to oil only amended soil communities were carried out and this analysis indicated that the mean bacterial 16S rRNA gene abundance at 0.1 and 10 ppm Cd were not significantly different relative to oil only control, irrespective the form of Cd. However, mean bacterial 16S rRNA was significantly lower relative to oil only amended control in communities contaminated with $CdCl_2$ ($p=0.043$) and CdO ($p=0.05$) at 1000 ppm by 4.09 X 10^{7} ± 1.39 X 10^{7} and 3.77 X 10^{7} ± 1.39 X 10^{7}, respectively.

Furthermore, the influence of Cd-oil amendment on the abundance of Rhodococcus was determined by comparing the *Rhodococcus* 16S rRNA gene abundances of communities (see figure 5.17). ANOVA indicated that the Rhodococcus 16S rRNA gene abundance was significantly different across communities ($p<0.0001$). Comparative analysis by Dunnett's pairwise tests (see figure 5.19) revealed that relative to the oil only amended soil community, the mean 16S rRNA gene abundance of *Rhodococcus* was not significantly different to the CdO 0.1 ppm amended soil community. The mean 16S rRNA gene abundances were 1.47 X 10^{7} ± 2.09 X 10^{6} and this was slightly higher relative to oil only control by 2.19 X 10^{6} ($p= 0.93$). However, the mean 16S rRNA gene abundance was significantly lower in the other Cd treatments relative to the oil only control. In the $CdCl_2$ amended soil communities, the mean *Rhodococcal* 16S rRNA gene abundances were 5.22 X 10^{6} ± 2.4 X 10^{6}, 2.91 X 10^{5} ± 3.64 X 10^{4} and 1.61 X 10^{3} ± 5.43 X 10^{2} sequences at 0.1, 10 and 1000 ppm, respectively. These were significantly lower by 7.26 X 10^{6} ± 1.99 X 10^{6}, 1.22 X 10^{7} ± 1.99 X 10^{6} and 1.25 X 10^{7} ± 1.99 X 10^{6} sequences at 0.1 ($p= 0.01$), 10 ($p<0.0001$) and 1000 ($p<0.0001$) ppm Cd respectively, relative to oil only amended soils. Also, in CdO

amended soils, the mean *Rhodococcal* 16S rRNA gene abundances were 1.43 X 10^5 ± 6.04 X 10^3 and 1.84 X 10^2 ± 1.60 X 10^1 sequences at 10 and 1000 ppm Cd, respectively. Hence relative to the microbial community of the soil amended with oil, only, the 16S rRNA gene abundance of *Rhodococcus* were significantly lower by 1.25 X 10^7 ± 1.99 X 10^6 and 1.25 X 10^7 ± 1.99 X 10^6 at 10 and 1000 ppm Cd, respectively.

In general, there is a systematic logarithmic relationship between the concentration of added cadmium and Rhodococcal abundance (see figure 5.21). Regression analysis indicated there was a significant negative relationship between Cd concentration and the abundance of Rhodococcus, which is logarithmic in both CdCl2 (R2= 0.79 p=0.036) and CdO CdCl2 (R2= 0.75 p=0.037) amended soils Hence, increase in Cd concentration resulted in an exponential decrease in *Rhodococcus* abundance.

Furthermore, comparison of treatments with soil only control determined the Cd impact of the Cd-oil amendment on the general soil bacteria. In CdCl2 amended soils, the mean bacterial 16S rRNA gene abundance was significantly lower at 1000 ppm Cd (p=0.006) relative to the soil only control and the difference in the means relative to soil only control were 5.30 X 107 ± 9.84 X 106. However, mean bacterial 16S rRNA gene abundance was not significantly different at 0.1 (p=0.673) and 10 ppm (p=0.281) relative to soil only control. In addition, relative to the soil only control, the bacterial 16S rRNA gene abundances in the CdO contaminated soil community were significantly lower by 4.92 X 10^7 ± 9.86 X 10^6 and 4.97 X 10^7 ± 9.86 X 10^6 at 10 ppm Cd (p=0.013) and 1000 ppm Cd (p=0.007), respectively. However, at 0.1 ppm Cd, the mean bacterial 16S rRNA gene abundance was not significant, relative to soil only control (p=0.93).

The *Rhodococcal* abundance in the unamended soil community (soil only control) was compared with treatments using Dunnett's pairwise comparison (see figure 5.21) to determine the impact of Cd on the abundance of Rhodococcus in communities. Analysis indicated that relative to soil only control, the mean 16S rRNA gene abundance of the microbial community of soil amended with CdO at 0.1 ppm Cd was significantly higher ($p<0.0001$). However, the mean 16S rRNA gene abundance of other Cd amended

microbial community of soil, irrespective of form or concentration was not significantly different relative to the soil only control ($p>0.1$).

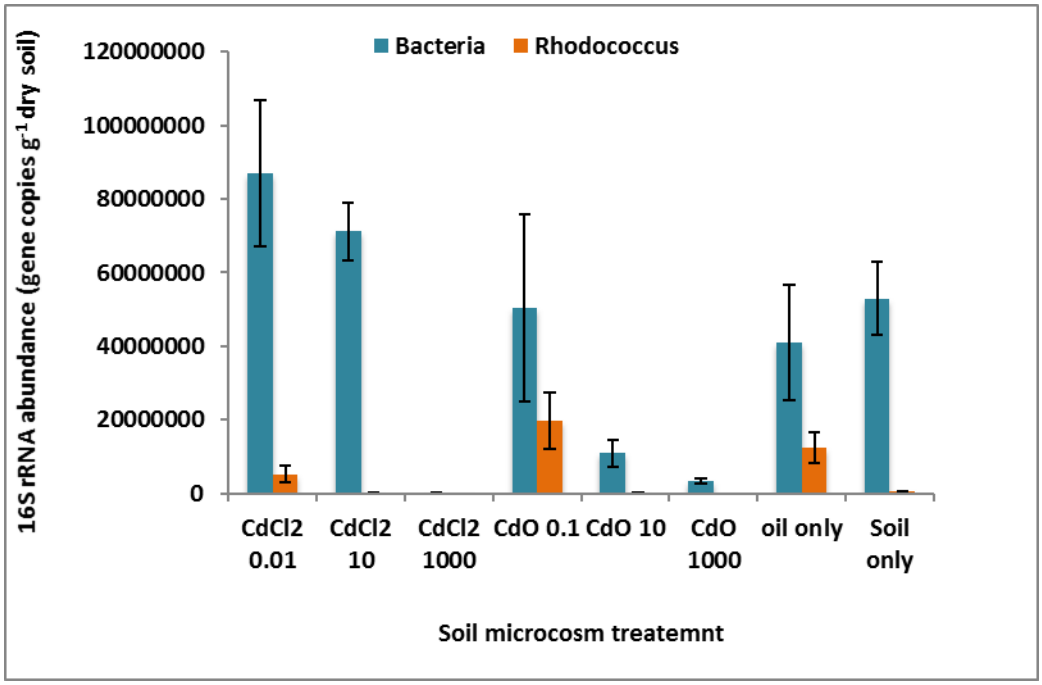


Figure 5.17: the 16S rRNA gene abundance of bacteria and *Rhodococcus* in Cd-crude oil amended soil microcosms after degradation for 15 days. The 16S rRNA genes were quantified by specific qPCR assays that target 16S rRNA genes of general bacteria and *Rhodococcus*. The X-axis indicates the treatment microcosms. Each bar represents the mean 16S rRNA gene abundance of bacteria or *Rhodococcus* of triplicate samples. The error bars are the standard error of means of triplicate samples. The graph legend indicate the target taxonomic group quantified

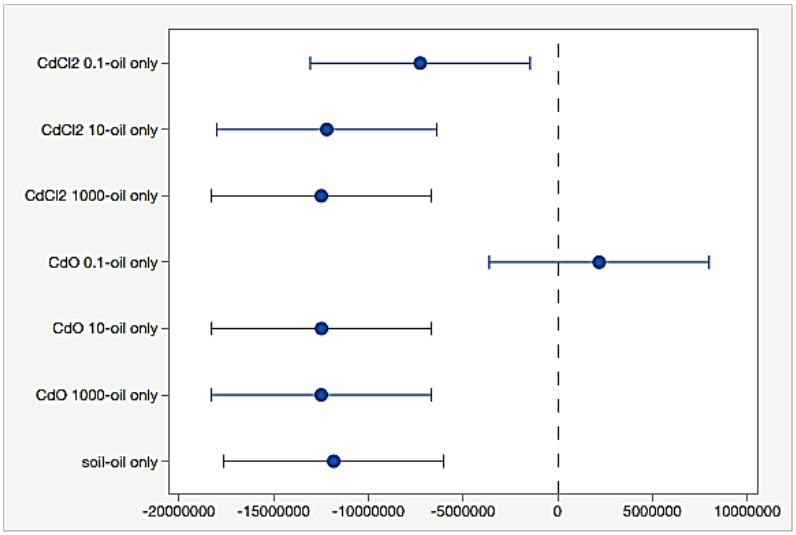


Figure 5.18: comparative analysis of the mean abundance of the *Rhodococcal* 16S rRNA gene copies in soils amended with Cd (CdO and $CdCl_2$) and Crude oil at Cd-concentrations of 0.1, 10 and 1000 ppm relative to oil only amended soils. Analysis was by Dunnett's multiple comparison analysis at 95% confidence interval. The values include the difference between the treatment mean and the control mean. A mean is not significantly different if its interval contains a zero (0).

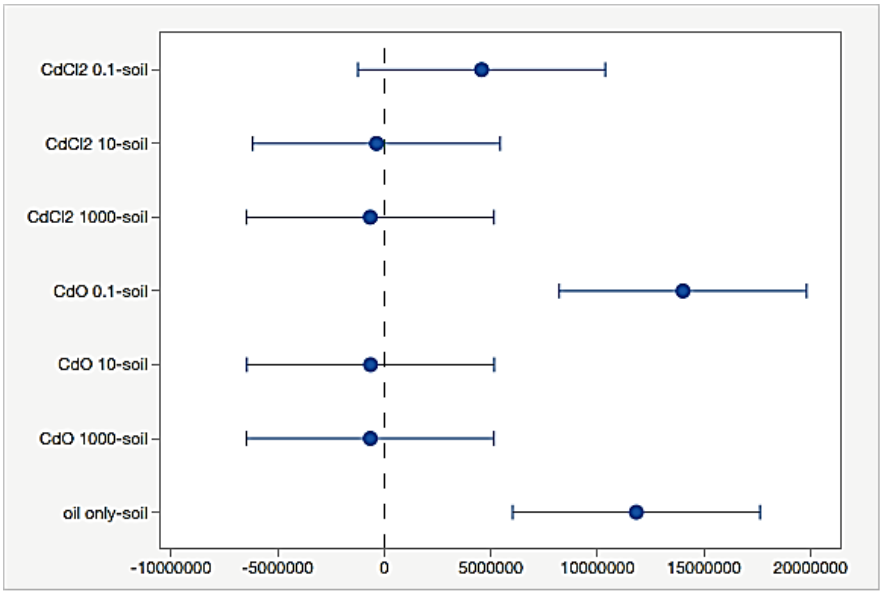


Figure 5.19: comparative analysis of the mean abundance of the *Rhodococcal* 16S rRNA gene copies of soils amended with Cd (CdO and $CdCl_2$) and Crude oil at Cd-concentrations of 0.1, 10 and 1000 ppm relative to unamended soils. Analysis was by Dunnett's multiple comparison analysis at 95% confidence interval. The values include the difference between the treatment mean and the control mean. A mean is not significantly different if its interval contains zero (0).

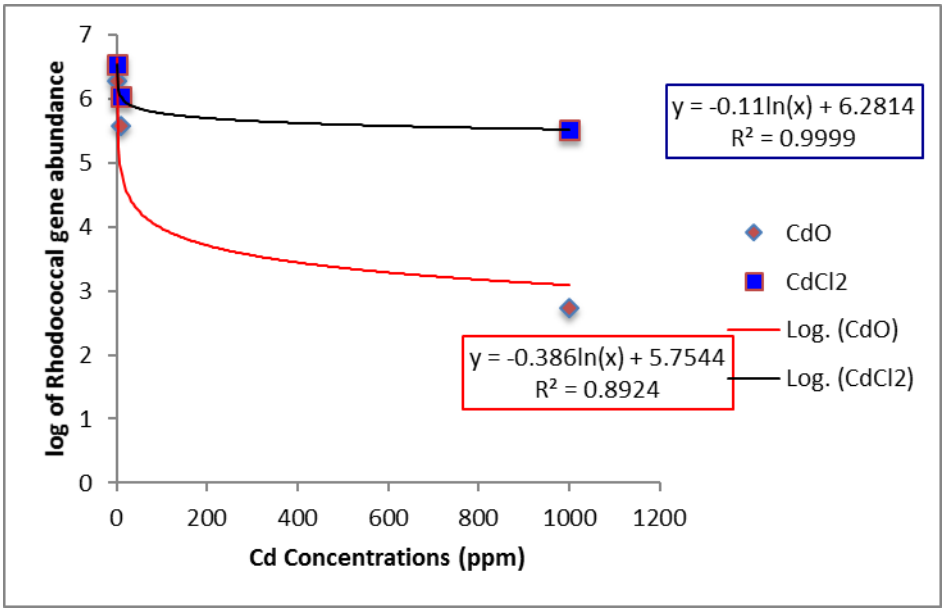


Figure 5.20: the logarithmic relationship between concentration and abundance of *Rhodococcal* 16S rRNA genes copies in degrading soil amended with a combination of Cd and crude oil.

5.4.7. Numerical relationships between cumulative CO_2, maximal CO_2 production rates, recovered petroleum hydrocarbons after degradation period, Bacterial 16S rRNA Gene abundances and *Rhodococcus* 16S rRNA Gene abundances

In this project, geochemical and microbiological approaches were employed to study the biodegradation of petroleum hydrocarbon in Cd-petroleum contaminated soils. The results obtained from the geochemical and microbiological experiments have been presented separately in previous sections. Here, a correlation analysis between these different variables was conducted to identify the relationship between these. Specifically, a matrix based correlation analyses of cumulative CO_2, maximal CO_2 production rate, total n-alkanes recovered after degradation period, Bacterial 16S rRNA gene abundance and 16S rRNA gene abundance of *Rhodococcus* was carried out to achieve this. Pearson's correlations were used to determine the strength of relationships between variables (see table 5.4).

5.4.7.1. Correlation of 16S rRNA gene abundance of bacteria relative Rhodococcal 16S rRNA gene abundance in soil communities

The Pearson's correlation analysis identified a moderate positive linear relationship between 16S rRNA gene abundances of *Rhodococcus* and bacteria (a proxy measure of the absolute abundance of *Rhodococcus* and bacteria cells in soil communities) (see figure 5.22), which was significant (r2= 0.54, r=0.74, P=0.0001). Hence, bacterial enrichment in the Cd contaminated petroleum degrading soils was due to the enrichment of *Rhodococcus* in these systems to a reasonable extent. This identifies the importance of *Rhodococcus* in the degrading soil systems.

5.4.7.2 Correlation of 16S rRNA gene abundances (Bacteria and *Rhodococcus*) relative to maximal cumulative CO2 during the degradation of petroleum hydrocarbons in soil microcosms

The relationship between 16S rRNA gene abundances of bacteria and maximum cumulative CO_2 in communities is a significant positive linear relationship (p=0.001) (see figure 5.23). With r-value of 0.65 (i.e. R^2=0.43), there is an indication that not all CO_2 produced is in microcosms is linked to bacterial abundance. This may suggest that not all species in the microbial communities are involved in petroleum mineralisation on soil amendment with the metal and petroleum. Hence, not all bacteria species in the community are hydrocarbon degraders. On the other hand, the relationship between 16S rRNA gene abundance of *Rhodococcus* in communities is a significant strong positive linear relationship (r= 0.89 r^2=0.80 p<0.0001) relative to maximal CO_2 produced by microbial communities (see figure 5.24 and table 5.4). Hence, an increase in *Rhodococcus* indicates an increase in CO_2 production. This observation may suggest that *Rhodococcus* dominance in communities resulted to increased microbial activities in the soils, which imply an increase in crude oil mineralisation to CO_2.

5.4.7.3 Correlation between 16S rRNA gene abundance (of bacterial and *Rhodococcus*) and maximal rate of CO_2 production during the degradation of petroleum hydrocarbon in soil microcosms

The relationship between 16S rRNA gene abundance of bacteria and Rhodococcus with the maximal rate of CO_2 production is similar to that with maximum CO_2 produced as described above.

Table 5.4: Pearson's correlation analysis describing the interaction between microbiological variables and geochemical variables determined from petroleum-Cd contaminated soil microcosms.

		Bacteria	*Rhodococcus*	Maximum cumulative CO_2	Maximal rates	Total n-alkanes
Bacteria	**Correlation Coefficient**	1	0.737	0.656	0.642	-0.6528
	Sig. (2-tailed)		<0.0001	0.0013	0.0017	0.0013
	N	21	21	21	21	21
Rhodococcus	**Correlation Coefficient**	0.737	1	0.891	0.882	-0.749
	Sig. (2-tailed)	<0.0001		<0.0001	<0.0001	<0.0001
	N	21	21	21	21	21
Maximum cumulative CO2	**Correlation Coefficient**	0.656*	0.891	1	0.962	-0.813
	Sig. (2-tailed)	0.0013	<0.0001		<0.0001	<0.0001
	N	21	21	21	21	21
Maximal rates	**Correlation Coefficient**	0.642	0.882	0.962	1	-0.731
	Sig. (2-tailed)	0.0017	<0.0001	<0.0001		0.0002
	N	21	21	21	21	21
Total n-alkanes	**Correlation Coefficient**	-0.6528	-0.749	-0.813	-0.731	1
	Sig. (2-tailed)	0.0013	<0.0001	<0.0001	0.0002	
	N	21	21	21	21	21

The Correlation analysis indicated that the relationship between 16S rRNA gene of bacteria and maximal rate of CO_2 production was significantly weak positive relationship (r=0.64, r^2=0.39, p=0.002) (see table 5.4 and figure 5.25). However, the correlation between *Rhodococcal* 16S rRNA gene abundance and maximal rate of CO_2 production indicate a strong positive linear relationship between the variables (r=0.88, r^2= 0.78 p<0.0001) (see table 5.4 and figure 5.26). These observations imply that the increased abundance of *Rhodococcus* stimulated the biodegradation of crude oil in soils.

5.4.7.4 Correlation of 16S rRNA gene (bacterial and Rhodococcal) relative to Total petroleum hydrocarbon recovered after the degradation of petroleum hydrocarbon in soil microcosms

The correlation analyses revealed that there is a significant weak negative linear relationship between bacterial abundance and the total n-alkanes recovered from soil microcosms after degradation of petroleum crude oil (r=-0.65; r^2=0.43, p=0.0013) (see table 5.4 and figure 5.28). However, spearman's correlation analysis identified a significant moderately strong non-linear relationship between bacterial abundance and total n-alkanes recovered (r=-0.72; r^2=0.51, p=0.0003). In addition, the linear relationship between *Rhodococcus* and total n-alkanes is moderately strong and significant (r=-0.75; r^2=0.56, p<0.0001) (see table 5.4 and figure 5.28), but spearman's correlation indicated a strong significant negative non-linear relationship between *Rhodococcal* abundance and total n-alkanes (r=-0.83; r^2=0.70, p<0.001). The negative relationships imply that increase in abundance of bacteria and *Rhodococcus* results to decrease in the recovered total n-alkanes. Hence, this indicates that increased the abundance of bacteria and *Rhodococcus* results to increased petroleum hydrocarbon degradation.

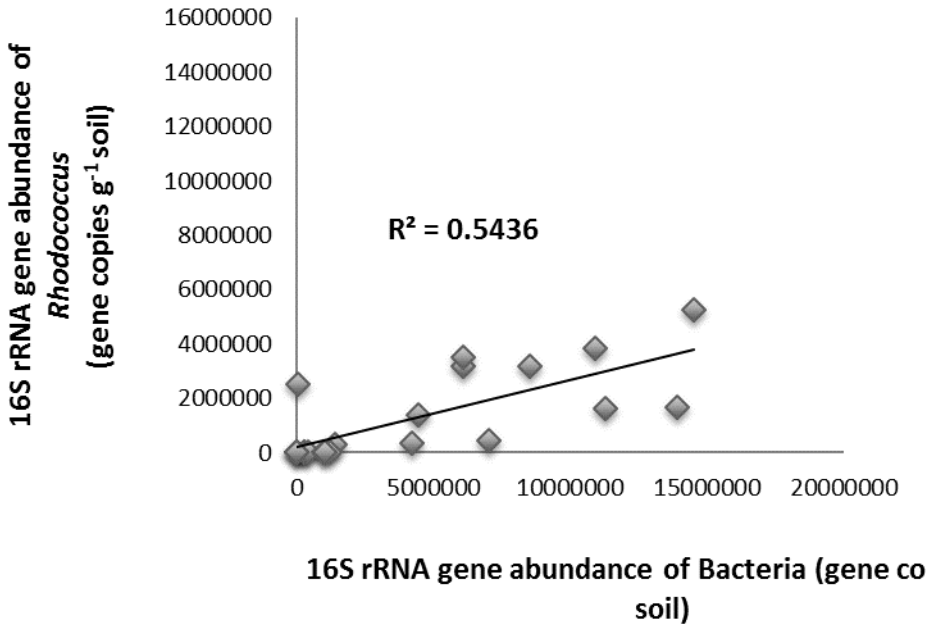


Figure 5.21: correlation analysis between maximum cumulative the bacterial and *Rhodococcal* 16S rRNA gene abundance in Cd-crude oil amended soil microcosms. The graph indicated the coefficient of determinant (R^2).

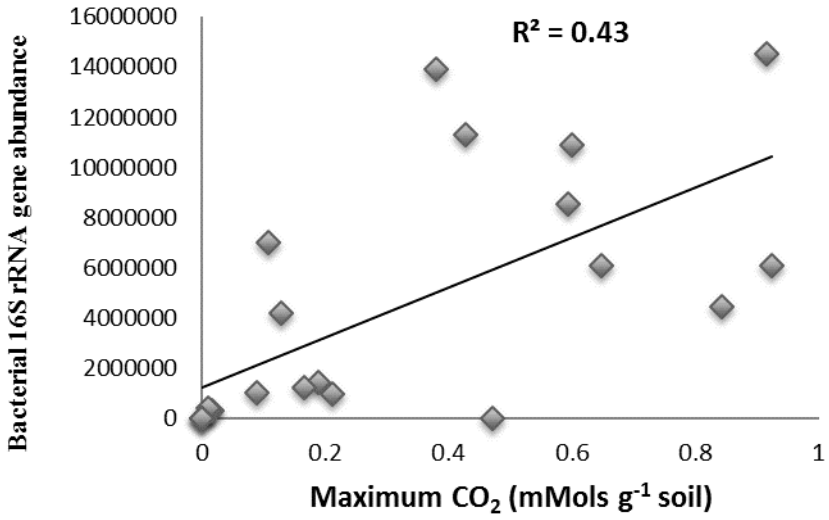


Figure 5.22: correlation analysis between maximum cumulative CO_2 and the bacterial 16S rRNA gene abundance in Cd-crude oil amended soil microcosms. The graph indicated the coefficient of determinant (R^2).

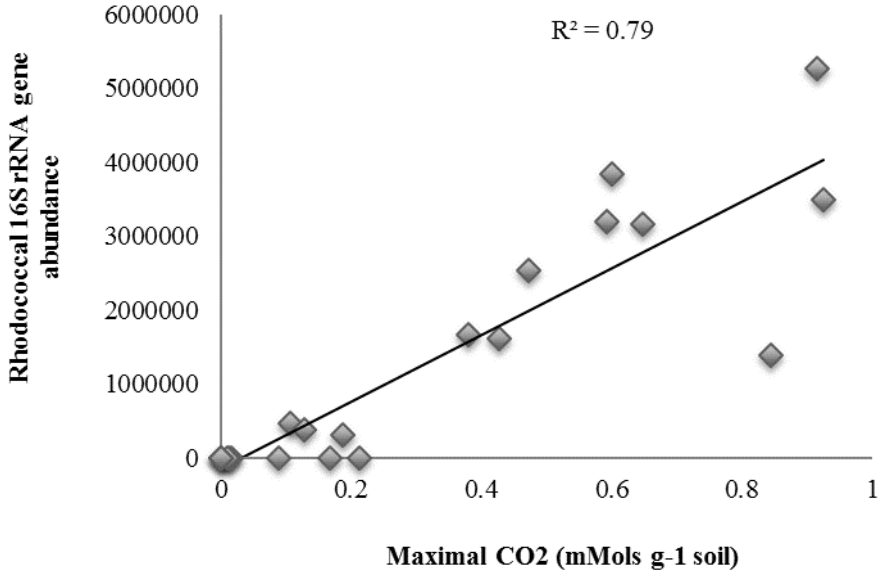


Figure 5.23: correlation analysis between maximum cumulative CO_2 and the *Rhodococcal* 16S rRNA gene abundance in Cd-crude oil amended soil microcosms. The graph indicated the coefficient of determinant (R^2).

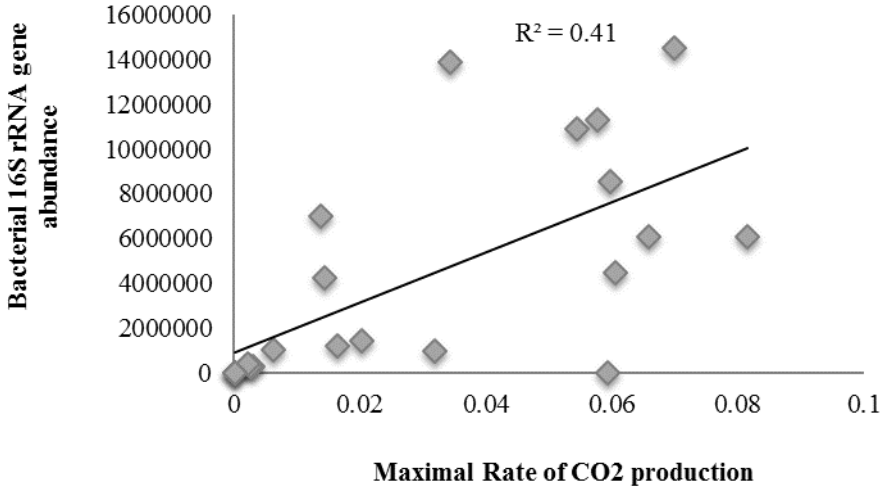


Figure 5.24: correlation analysis between maximal rate of CO_2 production and the bacterial 16S rRNA gene abundance in Cd-crude oil amended soil microcosms. The graph indicated the coefficient of determinant (R^2).

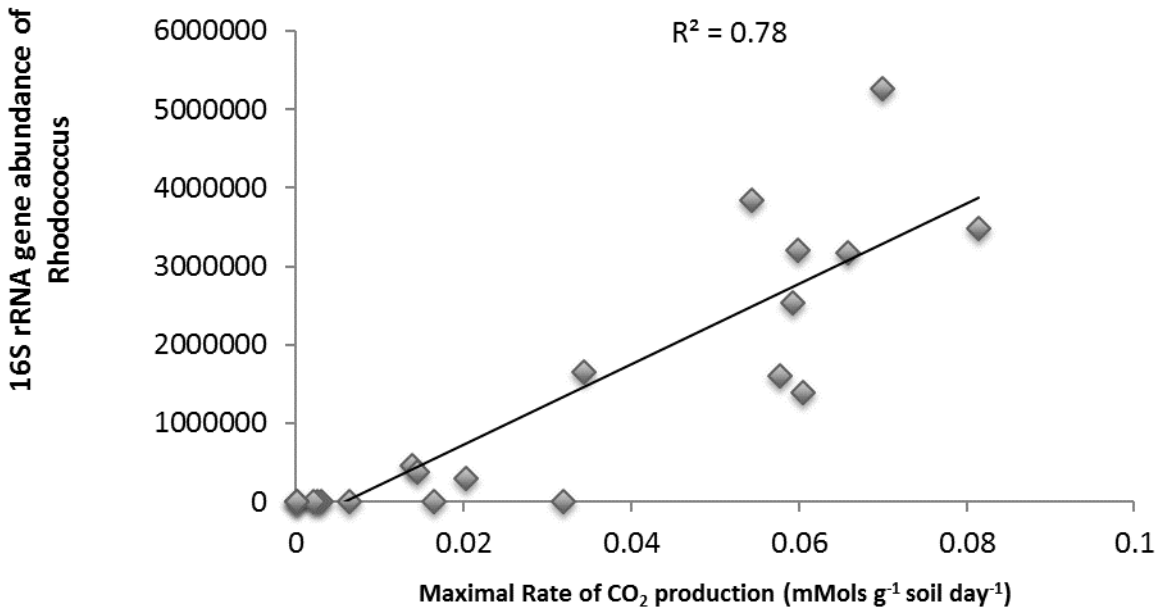


Figure 5.25: correlation analysis between maximal rate of CO_2 production and the *Rhodococcal* 16S rRNA gene abundance in Cd-crude oil amended soil microcosms. The graph indicated the coefficient of determinant (R^2).

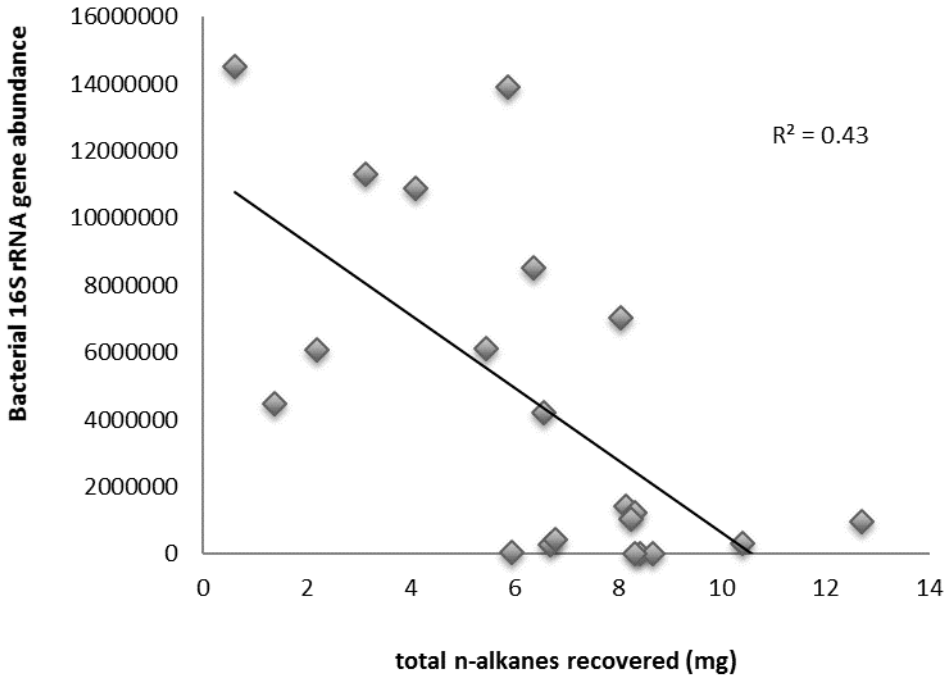


Figure 5.26: correlation analysis between total n-alkanes recovered from degrading soil microcosms and the Bacterial 16S rRNA gene abundance in Cd-crude oil amended soil microcosms. The graph indicated the coefficient of determinant (R^2).

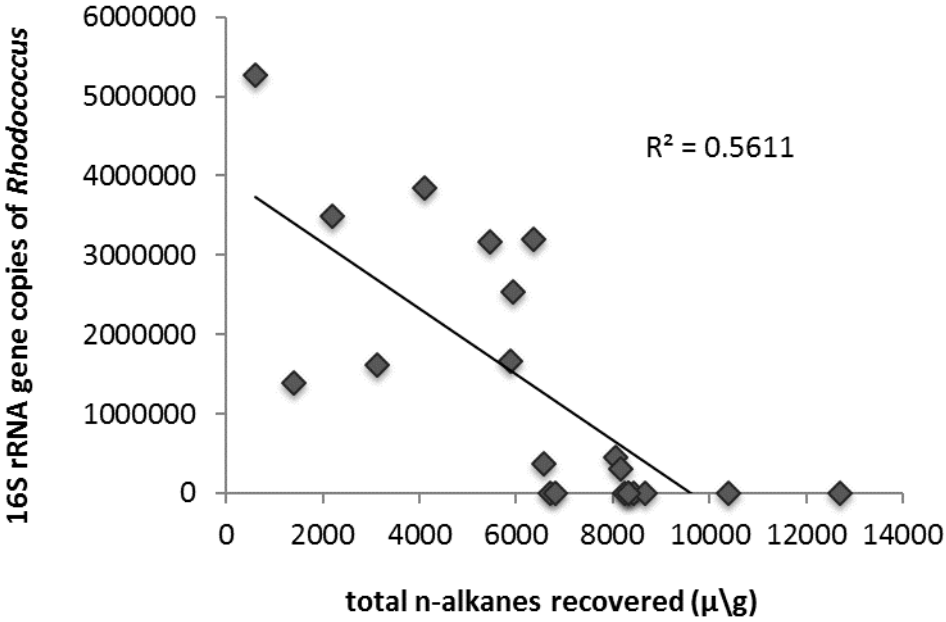


Figure 5.27: correlation analysis between total n-alkanes recovered from the degrading soil microcosms and the *Rhodococcal* 16S rRNA gene abundances in oil amended and Cd-crude oil amended soil microcosms. The graph also indicated the coefficient of determination (R^2).

5.5. Discussions

In this section, the results of microbiological and geological analysis of Cd-oil amended systems are discussed in details from different points of views to derive a more holistic understanding of the complex systems and this approach is lacking in previous studies.

5.5.1. Inhibitory effect of Cd on microbial activities and biodegradation of petroleum in the soils

The results obtained from current study indicated that Cd inhibited the biodegradation of petroleum hydrocarbon in soil microcosms during the period of degradation. This may be attributed to inhibition of the proliferation of petroleum hydrocarbon degrading microorganisms due to inhibition of enzyme activities in soils.

The determination of the absolute abundance of the 16S rRNA gene in soils indicated that bacterial abundance increased during the biodegradation of petroleum hydrocarbon in the soils. However, amendment with Cd in higher

concentrations resulted in decreased bacterial abundance. Increasing Cd concentration decreased in bacterial abundance even more. A decline in *Rhodococcus* abundance with increasing concentration of Cd was also observed. The inhibition of microbial proliferation observed in this study agrees with the studies by Zibilske, L.M. and Wagner (1982) who observed Cd inhibition of soil bacteria at Cd concentrations of 0.1, 1 and 111 micrograms per gramme Cd after a period of two weeks. Apart from soil environment, investigation on the influence of Cd, Pb and Cu on petroleum degradation in estuarine sediments from River Lima indicated that the amendment of the sandy sediment with Cd significantly decreased the total cell count of petroleum degraders (Almeida et al. 2013). In addition, Hoffman et al. (2005), in their study on effects of Cd toxicity studies on naphthalene degradation, documented decline in the growth of a *Comamonas testosteroni* strain (isolated from sediment of river Oshkosh, USA and which has the capability to degrade Naphthalene) at Cd concentration of 100 and 500 μM despite differences in the pattern and degree of inhibition, which was dependent on the mineral salt medium used.

Both bacteria and *Rhodococcus* abundances significantly correlated positively with maximal degradation rates and maximum CO2 produced in microcosms due to petroleum mineralisation, but negatively with the total alkane recovered from microcosms. Also, results indicated that while maximal rates of degradation and maximum CO_2 produced in microcosms decreased with increased Cd concentration, recovered total n-alkanes increased with increased Cd-concentrations. These observations were consistent with previous researches. For instance, (Dar and Mishra 1994) documented Cd inhibition of Carbon and Nitrogen mineralisation in sludge amended and unamended soils. Also, results from the present study suggested that a decrease in the rate of microbial metabolic activity reflected inhibition of important microbial metabolic pathways catalysed by enzymes, hence signifying inhibition of the enzymes in the systems. Although research on enzyme activities in Cd-petroleum co-contaminated soils are lacking, past research on the effect of Cd on enzyme activities is well documented. For instance, Cd has been documented to inhibit dehydrogenase activities in the soil (Dar 1996; Pan and Yu 2011; Alrumman et al. 2015). Dar (1996) identified inhibition of dehydrogenase activity in sludge amended and unamended soils at 10 and 50

microgram Cd per gramme soil. Also, increased inhibition with increased concentration of Cd was observed. In addition to this finding, Pan and Yu (2011) observed significant decreases in soil dehydrogenase activity at 10, 50 and 100 ppm Cd. Dehydrogenase activity is usually used as an indicator for evaluating microbial oxidative activity in soils (Vig et al. 2003). Other soil enzymes important in nutrient cycles that were inhibited by Cd included Urease, nitrogenase and phosphatase (Khan et al. 2010; Pan and Yu 2011; Dar 1996; Baldrian et al. 2000). Cd inhibits enzyme activities through different mechanisms including binding to the active site of an enzyme to reduce enzyme sensitivity, interacting with the enzyme-substrate complex, affecting the synthesis of enzyme within cells and inducing the production of reactive oxygen species (ROS) such as hydrogen oxide (H_2O_2) and superoxide radicals (O_2-) thereby imposing oxidative stress and consequently leading to the damage to DNA, Lipids and proteins (Nies 1992; Nies 1999; Sandrin and Maier 2003; Harrison et al. 2007; Abha and Singh 2012; Lemire et al. 2013). These mechanisms were discussed in chapter 1.

5.5.2. Bioavailability of Cd and uptake by microbes

The deleterious effect of Cd on petroleum degrading soils' metabolic activities, soil microbes, hydrocarbon degrader proliferation and consequent negative effect on petroleum degradation may be attributed to the susceptibility of soil microbes to Cd. However, the degree of susceptibility of microbes to the toxicity of Cd largely depends on the bioavailability of Cd. Results from Cd partitioning in the soil indicated that a good percentage of Cd, 26% in $CdCl_2$ and 12% in CdO contaminated soils, were in form of free Cd cation in the soil solution (exchangeable cations), which are the most mobile form of the metal in soils (Tessier et al. 1979; Vig et al. 2003; Gadd 2010; Tashakor et al. 2015). This result implies that this percentage are may be readily available to soil biota including microbes. Metal toxicity in soils has been shown to significantly correlate with the free metal ion in the soils (Prokop et al. 2003 and references therein).

In this study, significant differences were observed in the percentage of free Cd ions between the Cd forms. This difference may be attributed to the solubility of the forms where $CdCl_2$ is more soluble. It is not surprising that

more Cd ions were observed in the soil solution compared to the insoluble CdO.

Furthermore, good percentages of the Cd in soils were associated with oxides of Fe and Mn and Organic components of the soils. This observation explains the mode of Cd transportation into the microbial cells. The organics soil fraction may include the soil organisms, soil microbes, enzymes and proteins, humic and fulvic acids etc. (Tessier et al., 1979). This may suggest that a good amount of Cd may have been immobilised intracellular in soil's microorganisms and may confirm the susceptibility of Cd to soil microbes.

The Cd is transported into the microbial cells through specific and non-specific transporters, which are essential for the uptake of physiologically important trace metals and nutrients by the cells (Nies 1992; Nies 1999; Lemire et al. 2013; Harrison et al. 2007). For instance, the P-type transporter which transports Mn into cells also transported Cd in cells of Bacillus subtilis (Laddaga et al. 1985), *Staphylococcus aureus* (Weiss et al. 1978; Silver et al. 1989; Gómez-Sanz et al. 2013; Nucifora et al. 1989) and *Lactobacillus plantarum* (Hao, Chen, et al. 1999; Hao, Reiske, et al. 1999). Other examples include the transportation of Cd through Zn-specific transports ZipB and ZupT in *Bordetella bronchiseptica* (Lin et al. 2010) and E. coli (Grass et al. 2005; Taudte and Grass 2010) respectively. In addition, essential trace metals such as Fe are transported bound to low molecular weight ligands such as phosphates, peptides, amino acids and organics. Non-essential trace metals such as Cd may substitute the essential trace metals and hence are transported instead. For instance, the Fe-siderophore transport system is also employed in the transportation of Cd into microbial cells (Lemire et al., 2013). The association of Cd to oxides of Fe and Mn indicates the reducible state of a percentage of Cd in the soil. This is required for easy mobility of Cd, including uptake by living cells.

In this project, *Rhodococcus* was the key hydrocarbon degrader in the system. Although the mechanism of Cd uptake was not studied in this project, in a previous research, genes encoding heavy metal transport systems were identified in the genome of Butane-Oxidizing *Actinobacterium*, *Rhodococcus ruber* IEGM 231.

5.5.3. Microbial community diversity and phylogeny

In this project, DGGE analysis indicated that the microbial communities of soils are highly diverse. However, a slight decrease in community diversity was encountered in petroleum-contaminated microcosms, with or without Cd. This is due to enrichment of a few OTUs, which lead to the lower number of overall species being detected. This is similar to observations made in the previous study by Li et al. (2007) whereby a slight lowering in the diversity of soil microbial communities was observed within 15 days of the onset of petroleum degradation. Furthermore, alpha diversity studies using sequence data obtained by ion-torrent PGM sequencing of amplified 16S rRNA gene identified more diverse communities in Cd-contaminated petroleum degrading soils compared to the soil control amended with oil, only. This indicated that apart from petroleum hydrocarbon degraders, Cd-resistant microbes might have proliferated on the amendment with Cd consequently increasing the diversity of the community. In their study, Li et al. (2006) observed increased diversity in soils co-contaminated with Cd, Zn, and Cu due to the tolerance of metals of the members of the microbial community. Also, diverse community, which included low and high G +C bacteria, was observed in soils co-contaminated with Pb, Cr and Hydrocarbons (Joynt et al. 2006a).

Phylogenetic studies carried out indicated that the most enriched taxonomic group that made up 50% of the microbial relative abundance in petroleum degrading systems with or without Cd were members of the phylogenetic groups *Actinobacteria, α-Proteobacteria, ß-Proteobacteria, γ-Proteobacteria, Bacteriodetes, Chloroflexi* and *Crenarchaeota*. *Rhodococcus sp*. belonging to the Actinobacterial phylum was the most abundant in all degraded microcosms. Moreover, additional phylogenetic groups were identified in Cd amended systems. These included the *Firmicutes, Planctomycetes, Acidobacteria* and *Verucomicrobia*. Members of the observed taxonomic groups have been enriched in hydrocarbon degrading/ degraded systems in previous studies. For instance, the taxonomic group associated with members of the *Actinobacterial* groups including *Corynebacterium, Dietzia, Rhodococcus erythropolis,* and *Nocardioides* were isolated from a petroleum-contaminated soil (Chikere et al. 2012). Also, strains closely relating to *Rhodococcus* and *Gordonia* (both *Actinobacteria*) were isolated from

hydrocarbon contaminated Mediterranean shoreline (Quatrini et al. 2008). Apart from *Actinobacteria*, other phyla identified in this study have been previously acknowledged as hydrocarbon degraders. For instance, both *Actinobacteria* and *α-, ß-* and *γ-Proteobacteria* were isolated in an Austrian petroleum-contaminated alpine soil (Labbé et al. 2007). The importance of *Proteobacteria* in petroleum degradation is also well acknowledged. In their review, Head et al. (2006) represented major hydrocarbon degraders on a phylogenetic tree; including *Firmicutes*, *α-Proteobacteria*, *ß-Proteobacteria* and *γ-Proteobacteria*. Apart from the *Actinobacteria* and *Proteobacteria*, other bacterial groups have been isolated from hydrocarbon contaminated soils. For instance, 16Sr RNA genes closely related to strains of Proteobacteria, *Actinobacteria, Verrucomicrobia, Bacteriodetes, Firmicutes, Chloroflexi, Cyanobacteria* and *Acidobacteria* were isolated from aromatic hydrocarbon degrading Antartica soils and sediments (Muangchinda et al. 2014). Apart from soils, these organisms were also enriched in other hydrocarbon environments such as marine sediments (Hazen et al. 2010; Cai et al. 2014), petroleum contaminated groundwater (Hao et al. 2009), river/estuarine sediments (Leahy and Colwell 1990; LI et al. 2007; Hale et al. 2010). Also, similar taxonomic groups were observed in petroleum-heavy metal co-contaminated systems (Amor et al. 2001; Máthé et al. 2012; Joynt et al. 2006a; Almeida et al. 2013) and Cd contaminated environments (Ivshina et al. 2013).

Certain synergistic interaction exists amongst the members of microbial communities petroleum contaminated environments. Head et al (2006) pointed out the synergetic interaction between members of communities in the petroleum-contaminated environment as a strategy of survival by the microbes. This might be the case in this community. For instance, the enrichment of *Rhodococcus* and *Pseudomonas* in the systems is evidence of the community interaction. The role of *Rhodococcus* in petroleum degradation is well documented. Primarily, the strains of *Rhodococcus sp*. have enzyme systems that enable the production of biosurfactants. Their abundance in the communities observed might suggest their key role of petroleum emulsification to simpler molecules, thereby making the petroleum hydrocarbon easily accessible to other members of the communities. Also, biosurfactant produced by *Rhodococcus* may have immobilised Cd to some extent (especially at a low concentration of Cd) thereby promoting the thriving

of other members of the microbial community such as *Pseudomonas*, *Norcadia*, *Mycobacterium*, *Achromobacter*, *Rhodoplanes*, e. t. c. metal resistance mechanism will be discussed in details the next section. In the same way, well acknowledged the capability of *Pseudomonas* strains to degrade petroleum hydrocarbon (Sharma et al. 2015; Obayori et al. 2013; Barathi and Vasudevan 2001; Kumar et al. 2006) may portray the role of *Pseudomonas* in the system. There is a possibility that *Pseudomonas* were enriched after emulsification of petroleum hydrocarbon by biosurfactant produced by *Rhodococcus*. The previous research reported improved efficiency of petroleum degradation by *Pseudomonas sp.* in the presence of biosurfactant producing bacteria (Kumar et al. 2006). Apart from hydrocarbon degradation, strains of *Pseudomonas* were documented to depend on biosurfactant as a source of energy (Colores et al. 2000) in other to control biosurfactant secretion in the environment.

5.5.4 Cd-resistant mechanism in Microcosms

Phylogenetic results revealed that while the relative abundance of hydrocarbon degraders e.g. *Norcardia* and *Mycobacterium* decreased in Cd-contaminated soils, the relative abundance of other hydrocarbon degraders such as *Rhodococcus* and *Pseudomonas* were not affected in the systems (see figure 4.12). It has been documented that several microbes demonstrate resistance to Cd (Bruins et al. 2000). Microbes adapt to metal presence by developing the mechanism of metal resistance. Theses mechanisms may include extracellular and intracellular sequestration, efflux system development, cell repair mechanism, metabolic by-pass and contaminant detoxification by chemical modification (Nies 1999; Nies 2003; Harrison et al. 2007; Lemire et al. 2013).

As has been pointed out in the previous section, the primary microbes enriched in petroleum degraded system is closely related to strains of *Rhodococcus sp*. *Rhodococcus* is a highly metabolically and physiologically versatile organism and has been associated with both hydrocarbon degradation and metal resistance (Murai 1981; Desomer et al. 1988; Finnerty 1992; Larkin et al. 2005; Larkin et al. 2006; Martínková et al. 2009; Binazadeh et al. 2009; White et al. 2013). However, relatively little is known about the mechanism of metal resistance in *Rhodococcus*. The Ivshina and Kuyukina (2014) identified genes that encode enzymes that catalyse versatile metabolic activities in a

strain of *Rhodococcus* including genes for hydrocarbon degradation, biosurfactant production, and active transport systems. Active transport might give an insight into the mechanism of metal resistance by *Rhodococcus sp*., which might include metal resistance by sequestration and active efflux systems.

In addition to this, plasmids encoded Cd-resistance gene has been reported in strains of *Rhodococcus fasciens* (Desomer et al. 1988; Murai 1981). Murai reported 99 KB plasmid in *R. fasciens* strains MW2, which bears Cd-resisting gene. The loss of the plasmid corresponded to the loss of Cd-resistance and hydrocarbon utilisation by the strain. In addition, the 138 KB Cd-resistance gene bearing Plasmid pD188 was reported by Desomer et al 1988 in strains of *R. fasciens* strain D188. The plasmid correlated strictly with Cd-resistance. The presence of pD188 in mutants, which were previously sensitive to Cd, resulted in Cd resistance in the mutant strains.

Primarily, microbes resist Cd by developing energy dependent efflux systems, which facilitate the export of Cd from the cell. For instance, P-type ATPase, CadA pump, which was responsible for the transportation of Cd out of Cd-resistant *Staphylococcus aureus* documented in previous studies (Nies 1992; Nies 1999; Nies 2003; Nucifora et al. 1989). Also, a csz system (comprising of cszA, cszB and cszC proteins, with cszD as determinant) was identified in Cd-resistant strains of *Alcaligenes eutrophus* (Nies 1999), Ralstonia sp. (Nies 2000; Anton et al. 1999) and Acidithiobacillus ferroxidans (Chen et al. 2014).

Apart from resistance based on efflux systems, microbes have been documented to confer resistance to Cd by metal sequestration intracellularly. For instance, (Hrynkiewicz et al. 2014) attributed the Cd-resistance observed in Cd tolerating *Pseudomonas sp.* IV-111-16 and Bacillus sp. ML1-2 isolated from rhizosphere, *Ectomycorrhizae*, and fruit bodies of *Ectomycorrhizal f*ungi associated with willows (*Salix viminalis L*.) growing at anthropogenic degraded sites to intracellular detoxification of Cd by the formation of Cd-phosphates. In addition, Silicate binding by *Bacillus sp*. ML1-2 was identified. Also, intracellular sequestration of Cd by cysteine and glutathione in Acidithiobacillus ferroxidans has been identified (Zheng et al. 2015).

Furthermore, Cd resistance by extracellular sequestration has been well documented. This strategy involves the exclusion of Cd from the cell by immobilisation of the metal thereby preventing uptake by cells. For example, the sequestration of Cd by extracellular polysaccharides (EPS) of Cd-resistant *Sinorhizobum*, *Pseudomonas putida* and other bacterial strains has been identified (Slaveykova et al. 2010; Ueshima et al. 2008; Wei et al. 2011; Guibaud et al. 2005) and Biosurfactants are usually a good example of EPS.

Conclusions

The aim of this project was to understand the effect of Cd, in different forms and at concentrations on the biodegradation of petroleum hydrocarbon in soils. Using geochemical and microbiological approaches, the Cd effects on the Microbial activities, the compositional change of petroleum, changes in microbial community diversity and phylogeny were established holistically.

Results from investigations revealed that Cd inhibited microbial activities in soils, irrespective of Cd form or concentrations even at concentrations below the recommended soil guideline values of UK environmental agency and below the intervention value of Dutch list. The inhibitory effect increased with increasing concentration. This is likely due to the toxicity of Cd binding to the active site of enzymes to reduce enzyme sensitivity, interacting with an enzyme-substrate complex, affecting the synthesis of the enzyme within cells and inducing the production of ROS thereby imposing oxidative stress and consequently leading to the damages of DNA, Lipids and proteins. In addition to this, the total n-alkanes recovered from microcosms after degradation period indicated that very little percentage of petroleum was degraded in Cd amended microcosms.

Furthermore, phylogenetic analysis results indicated that Cd inhibited the proliferations of hydrocarbon degraders in soils. However, the hydrocarbon degrader, *Rhodococcus*, which dominated the microbial community, was less affected than *Mycobacterium*, *Nocardia* and *Pseudomonas*. These results suggest that there is resilience in these soils whereby even though some hydrocarbon degraders are inhibited at low Cd others including the *Rhodococcus* are able to carry on degrading. This resilience /redundancy is

further illustrated when you consider the effect of Pb in the next chapter where the *Rhodococcus* was not dominant.

Rhodococcus is known for its metabolic versatility, which includes hydrocarbon degradation as well as Cd resistance. The role of *Rhodococcus* in the soil may be to increase the availability of petroleum hydrocarbons to other members of the microbial community by producing biosurfactants while resisting Cd. Despite the Cd-resistance capability, the proliferations of *Rhodococcus* in microcosms were inhibited with increasing Cd concentration.

Although this study established the toxicity of Cd on the biodegradation of petroleum hydrocarbon in soil, the resistance of Cd by the dominant hydrocarbon degraders in the degrading system was not studied. For future studies, understanding the resistance mechanisms of Cd employed by hydrocarbon degraders through genetic studies will contribute to engineering strategies required for the bioremediation of Cd-petroleum co-contaminated soils.

Chapter 6

Effect of Lead on the Bioremediation of Petroleum Hydrocarbons

6.1. Introduction

Lead (Pb) is a member of group 14 in the period table with an atomic number of 82 and an atomic mass of 207.2. Pb exists majorly as the Plumbous ion (Pb II) in nature (Canadian Council of Ministers of the Environment 1999 and references therein) and alloys readily with Tin (Sn), Zinc (Zn), Copper (Cu) and Antimony (Sb). Pb minerals include galena (PbS), cerussite ($PbCO_3$) and anglesite ($PbSO_4$) (Anthony et al. 1990). Also, It is a non-essential element (i.e. element not required for any physiological or biochemical processes) for living organisms and ubiquitous in the soil. Its average concentration in the Earth's crust is 16 mg kg^{-1} and the background concentration natural uncontaminated soils varies with soil (Domy C. Adriano 2001). For instance, In England, the background value of Pb in urban regions varied from 36 ppm in Corby to 224 ppm in Swansea (Rothwell and Cooke 2015). Also, whereas in Canada, the average background value of Pb is 20 ppm in Poland, the average background value of Pb is 10.3 ppm (Domy C. Adriano 2001). Pb contamination, together with Cd and As, is of great global concern (UNEP 2010). In contaminated soils, the value of Pb varies from 10s of mg Pb Kg-1 soil to thousands of mg Pb Kg-1 soil depending on the source of contamination. Pb could be released into the environment as a result of natural events (such as weathering of rock and volcanoes) as well as anthropogenic activities; Metals are released into the environment from mining of metal ores and fossil fuels, petrol additives, agricultural activities and manufacturing activities (UNEP 2010; Canadian Council of Ministers of the Environment, 1999). More important to this study is the contamination of Pb due to oil spills. Pb in petroleum could be up to thousands of µg Pb Kg-1 soil (see table 1.2 in chapter 1). Continuous release of petroleum (accidental or deliberate) due to exploration, mining and transportation activities deposits the accompanying Pb leading to a build up of Pb to levels above the soil guideline values may result (see table 1.1 in chapter 1). The UK environmental agency's soil guideline value (SGV) for Pb is currently being evaluated but the previous UK

environmental agency's SGV for Pb is 530 ppm (i.e. mg Pb Kg-1 soil) (Mccann 2012; Okorie 2010; Okorie et al. 2011; Okorie et al. 2012). In Canada, the SGV for agricultural soils is 70 ppm (Canadian Council of Ministers of the Environment 1999) and the Dutch list value for soil Pb intervention level is 530 ppm (ESDAT 2000).

6.2. Aims and objective

The aim of this research is to determine the effect of Pb on hydrocarbon degradation in complex natural soil systems using microbial ecological approach combined with the geochemical approach.

1. The objectives include
2. To determine the effect of different mineral forms of Pb at increasing concentrations on the biodegradation of petroleum hydrocarbon by determining the rate of microbial metabolic activities in oil and Pb contaminated soils,
3. To determine the compositional changes of whole petroleum in the soil contaminated with different mineral forms of Pb at different concentrations using geochemical analytical techniques,
4. To determine the ecological diversity of microorganisms in soils contaminated with petroleum hydrocarbon and different forms of Pb at increasing concentrations during the biodegradation of petroleum hydrocarbon,
5. To determine the effect of long-term metal contamination on petroleum hydrocarbon degradation
6. To determine the fate of Pb in a hydrocarbon-contaminated system.

6.3. Methods

The basic experimental microcosm setup, the geochemical and molecular microbiological techniques used to investigate the effect of Pb on petroleum degradation are described in detail in chapter 2. Briefly, this Pb orientated study employed two different soil systems to evaluate hydrocarbon degradation with the freshly added metal contaminant (analogous to the Ni and Cd experiments) and; in a soil system where the metal contamination was historic and thus aged. The Nafferton Ecological Soils were used to understand the effect of freshly added Pb contamination on petroleum

degradation and a soil with a history of long-term Pb contamination obtained from former lead ore smelting works at St. Anthony's, Newcastle upon Tyne (see chapter 2, section 2.1).

The soil microcosms used to investigate the effects of Pb addition (in different mineral forms) consisted of added Pb, petroleum crude oil and Nafferton soil (see figure 6.1). The effect of two forms of added Pb (PbCl2 and PbS) was investigated at increasing concentrations 150, 1500 and 15000 ppm.

Soil microcosms used to investigate the biodegradation of crude oil in historically contaminated Pb soil involved the amendment of soils containing lower Pb concentrations (~500 ppm) and high concentrations (~12000 ppm) with crude oil. The soil sample site was described previously in Chapter 2, section 2.1. These soil microcosms were set up in triplicate for all treatments and controls to allow for statistical analysis.

For experiments investigating the effect of short-term Pb contamination on petroleum crude oil degradation, two sets of control soil microcosms were set up. The controls were crude oil only amended soil microcosms and soil only microcosms. For experiments investigating the effect of historical Pb contamination, High-Pb soils and Low-Pb soils (with no crude oil amendment) served as controls.

All microcosms were incubated at room temperature for a period of 14 days. Soil microbial activities were monitored in each microcosm replicate by periodically measuring CO_2 production of headspace gases at 2-day intervals by GC-MS analysis (see Chapter 2, section 2. 5). After incubation, microcosms were subjected to geochemical and microbiological analysis as described in the previous chapters and outlined in Chapter 2, sections 2.6 and 2.8).

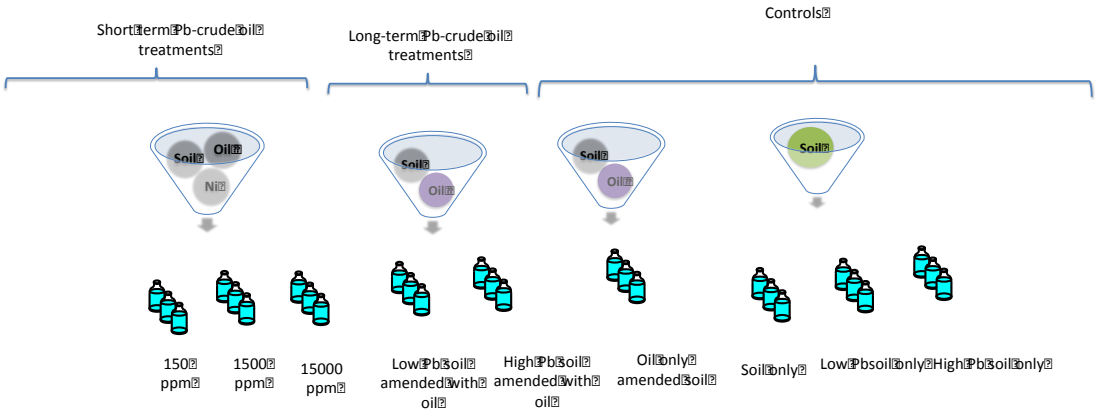


Figure 6.1: Experimental design for the study of crude oil biodegradation in freshly Pb-contaminated soil microcosms and for long-term Pb contaminated soils relative to oil only amended and no oil amended (soil only) controls. Freshly Pb contaminated soils were studied at concentrations of 150, 1500 and 15000 ppm. Long-term Pb-contaminated soils include lower Pb soils (lower Pb concentration of ~500 ppm) and High Pb Soils (high Pb concentration of ~13000 ppm).

6.4. Results

This section presents the results of the microbial respiratory activity, geochemistry and microbial community studies of the petroleum degrading soil microcosms contaminated with Pb.

6.4.1. Physicochemical properties of soils

The soils used for this investigation were analysed to determine background levels of metal contamination (Table 6.1). Comparisons of values with SGV provided an evidence of no metal contamination in Nafferton farm soil. In contrast, in the St Anthony soil samples, contamination by metals, primarily As, Zn, Pb and Cd, occurred. This observation coincided with previous reports on a physicochemical analysis of Anthony works (Okorie et al. 2012; Mccann 2012; Okorie et al. 2011).

In addition to metal concentrations, the pH and total organic carbon (TOC) of the soils were determined. The pH of the soils was approximately neutral. Nafferton soil had a pH of 7.1±0.03, lower Pb soil had pH of 7.1±0.06 and high Pb soil had a pH of 7.83±0.18. TOC values were 3.13±0.12%, 6.26±0.47% and 6.47±1.17% for the Nafferton, lower Pb Anthony and high Pb Anthony soils respectively.

Table 6.1: Concentrations of Metal in Nafferton Ecological Farm and St. Anthony works soils

Metals (ppm)	Nafferton Farm	Anthony Low	Anthony High	Soil Guideline Values	Dutch List values (action values)
Mercury	0.0767±0.01	0.143±0.02	2.133±0.43	80	1
Arsenic	11.2±4.4	33.333±8.57	1323.333±290.77	43	55
Cadmium	0.733±0.088	1.4±0.15	19.667±5.70	1.8	12
Chromium	17.33±1.45	29±2.08	27.333±3.38	389	380
Copper	15±1	47±5.77	210±51.32	190	190
Nickel	9.067±0.64	21.333±1.20	42±10.01	230	210
Lead	125.667±72.2	580±151.43	12866.667±2598.29	530	530
Selenium			15.133±5.95	100	n/a
Vanadium	27±3.06	48.667±0.33	68±12.53	n/a	n/a
Zinc	81.667±9.91	446.667±103.33	3400±1253	720	720
Boron (water soluble)	2.767±0.13	5.1±0.52	3.633±0.23	n/a	n/a

6.4.2. Effect of Pb on soil Microbial activities

CO_2 production profiles, maximum cumulative CO_2 values and maximal CO_2 production rates in oil only amended soils and unamended soil (i. e. soil only control) were compared to understand the impact of oil amendment on CO_2 production. Metal and oil treated soils were then compared to these controls to ascertain the effect of Pb addition on oil degradation.

6.4.2.1. Effect of short-term Pb contamination on oil degradation in the Nafferton farm soils

The CO_2 production profile observed in oil-amended and PbS or $PbCl_2$ amended soils at Pb concentrations of 150, 1500 and 15000 ppm were compared with the oil only amended soils and with the soil only controls to understand the effect of short-term Pb contamination on the degradation petroleum degradation in the soils (see figures 6.2 and 6.3). Generally, CO_2 production profile indicated that CO_2 production in all the Pb amended soils, irrespective of Pb-form or concentration, was lower relative to the oil only-amended soil. More specifically, the CO_2 production in the Pb amended soils was broadly similar to that of soil only control. To statistically differentiate the CO_2 production profile of soils, cumulative CO_2 of treatment soils at different time points during the period of degradation were compared relative to oil only-amended soil and unamended soil using ANOVA and Dunnett's pairwise

comparison analysis. While CO_2 production increased significantly with time in oil only amended soil after a period of lag phase ($p<0.0001$), there were no significant increase in CO_2 production in Pb contaminated soils and unamended control over the period of degradation ($p<0.05$). Dunnett's pairwise comparison analysis revealed that while the CO_2 production profile of all Pb amended soils, irrespective of soil form or concentration, were significantly different relative to oil only-amended soil ($p<0.0001$), there was no significant different relative to unamended soil control ($p=0.078$).

The maximum cumulative CO_2 yield (obtained on the 15th day) of all treatments were compared relative to the oil only amended soil control. The mean maximum cumulative CO2 in oil only amended soil was 0.45 ± 0.0033 mmol g^{-1} dry soil. ANOVA revealed that there were significant differences in the cumulative CO_2 across treatments ($p<0.0001$) relative to the oil only amended soil control. Figure 6.4 shows the Dunnett's pairwise comparison of mean maximum cumulative CO_2 of treatments relative to oil only control in $PbCl_2$ and PbS amended soils, respectively. The analysis revealed that there were significant differences in mean cumulative CO_2 at all concentrations of Pb irrespective of Pb form, relative to the oil only control. In $PbCl_2$ amended soil, the differences in means were significantly lower by 0.375 ± 0.045 mmol g-1 dry soil at 150 ppm Pb ($p=0.0001$), 0.413 ± 0.045 mmol g-1 dry soil at 1500 ppm Pb ($p<0.0001$) and 0.488 ± 0.045 mmol g^{-1} dry soil at 15000 ppm Pb, relative to oil only control. Similarly, in PbS amended soils, the differences in means were significantly lower by 0.448 ± 0.045 mols g-1 dry soil at 150 ppm Pb ($p<0.0001$), 0.468 ± 0.045 mmol g-1 dry soil at 1500 ppm Pb ($p<0.0001$) and 0.469 ± 0.045 mmol g^{-1} dry soil at 15000 ppm Pb, relative to oil only control.

Furthermore, the final cumulative CO_2 yield of treatment microcosms was compared with the unamended soil control, which had a mean maximum cumulative CO_2 yield of to 0.067 ± 0.0044 mmol g^{-1} dry soil. ANOVA and Dunnett's pairwise comparison analysis (see figure 6.5) indicated that the mean maximum cumulative CO_2 from $PbCl_2$ amended soils at 150 ppm and 1500 ppm Pb were significantly higher relative to soil only control by 0.088 ± 0.01 mmol g^{-1} dry soil ($p<0.0001$) and 0.049 ± 0.01 mmol g-1 dry soil ($p=0.002$), respectively. However, at 15000 ppm Pb, mean maximum

cumulative CO_2 was lower by 0.026 ± 0.01 mmol g^{-1} dry soil and this was not significantly different relative to soil only control (p=0.108). In the PbS amended soil the mean maximum cumulative CO_2 at 150 ppm was higher by 0.014± 0.01 mmol g^{-1} dry soil but this was not significantly different relative to soil only control (p=0.6). On amendment with PbS at higher concentrations of 1500 and 15000 ppm, the mean maximum cumulative CO_2 observed was lower by 0.0056 ± 0.01 mmol g^{-1} dry soil and 0.006 ± 0.01 mmol g^{-1} dry soil, respectively, relative to soil only control. Equally, these differences were not significant.

The maximal rate of CO_2 production was calculated from the period of maximal linear accumulation of CO_2 production in microcosms, which was general between days 6 and 10. The rates were presented in figure 6.6. From the figure, the maximal rate of CO_2 production in Pb and crude oil-amended soils are lower than in the oil only amended soils indicating an inhibitory effect of Pb addition. To statistically determine the effect of Pb on the maximal rate of degradation, treatment microcosms were compared with oil only amended control by ANOVA and Dunnett's pairwise comparison (see figures 6.7 and 6.8). ANOVA indicated that the mean maximal rate of CO_2 production differed significantly across treatments relative to oil only control (p<0.0001). The mean maximal rate of degradation in crude oil only amended soil was 0.048 ± 0.005 mmols g^{-1} dry soil day-^{1}. Dunnett's pairwise comparison of Pb and crude oil amended soils relative to oil only control indicated that all Pb-crude oil amended soils were significantly lower. The mean maximal CO_2 production in $PbCl_2$-crude oil amended soils was significantly lower by 0.038 ± 0.0042 mmols g^{-1} dry soil day^{-1} (p<0.0001), 0.044 ± 0.0042 mmols g^{-1} dry soil day-^{1} (p<0.0001), 0.046 ± 0.0042 mmols g-1 dry soil day^{-1} (p<0.0001) at 150, 1500 and 15000 ppm, respectively, relative to the oil only control (see figure 6.7). Similarly, in the PbS-crude oil amended soils, the mean maximal CO2 production was significantly lower by 0.045 ± 0.003 mmols g^{-1} dry soil day^{-1} (p<0.0001), 0.046 ± 0.003 mmols g^{-1} dry soil day^{-1} (p<0.0001), 0.047 ± 0.003 mmols g^{-1} dry soil day-1 (p<0.0001) at 150, 1500 and 15000 ppm, respectively, relative to oil only control (see figure 6.8). ANOVA indicated that there were no significant differences in between forms (p=0.773) and concentrations (p=0.08). These observations suggest that Pb

significantly inhibited the maximal rate of crude oil degradation in soils. These inhibitory effects were not influenced by Pb form or concentration.

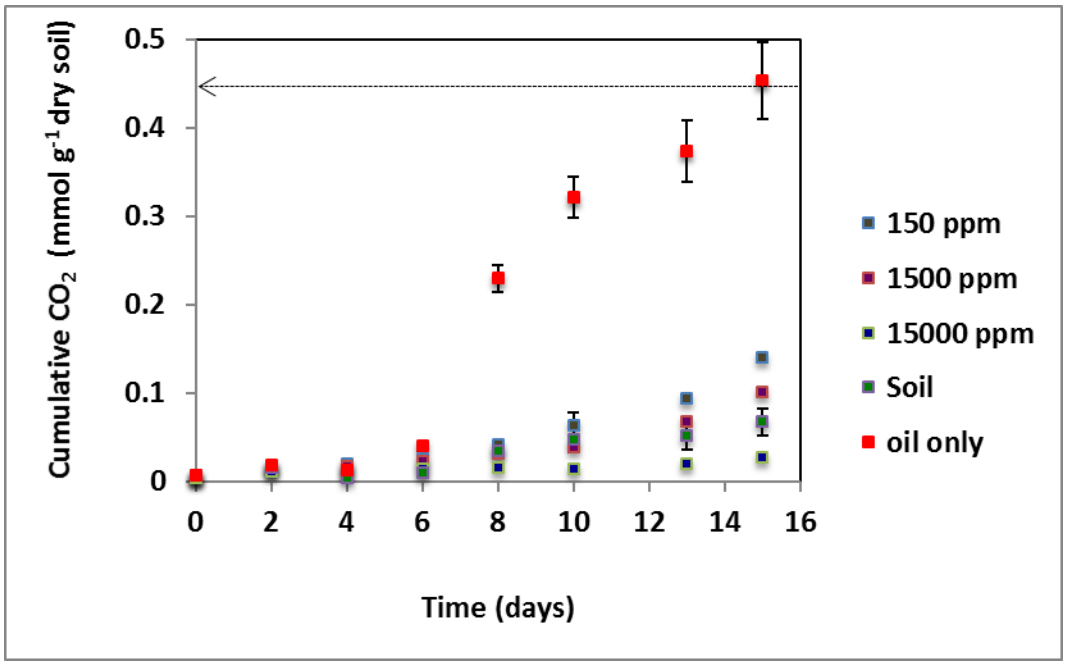


Figure 6.2: CO_2 production (mmols CO_2 g^{-1} dry soil) of Nafferton Farm soil microcosms amended with $PbCl_2$ (150-1500 ppm additions) and oil relative to oil only amended and unamended controls. The data points represent the mean cumulative CO_2 of triplicate samples in mmols g^{-1} dry soil. The error bars represent the standard error of triplicate sample and graph legend indicates the treatment microcosms and the controls (unamended soil is indicated as soil while oil only amended control is indicated as oil only). The dotted arrow indicates the maximum CO_2 produced in crude oil only amended Nafferton farm soil.

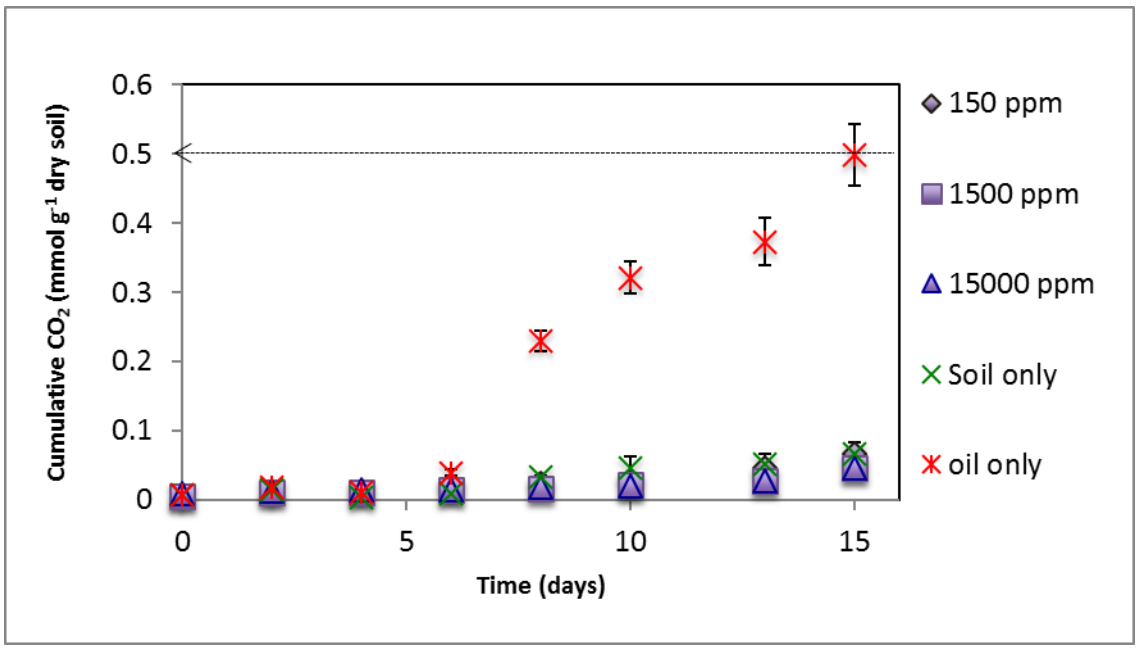


Figure 6.3: CO_2 production profiles of Pb and petroleum hydrocarbon co-contaminated soil microcosms (soil from Nafferton Farm) relative to oil amended and unamended controls. Microcosms were amended with PbS. The data points represent the mean cumulative CO_2 of triplicate samples in mmols g^{-1} dry soil. The error bars represent the standard error of triplicate sample and graph legend indicates the treatment microcosms and the controls (unamended soil is indicated as soil while oil only amended control is indicated as oil only). The dotted arrow indicates the maximum CO_2 produced in crude oil only amended Nafferton farm soil.

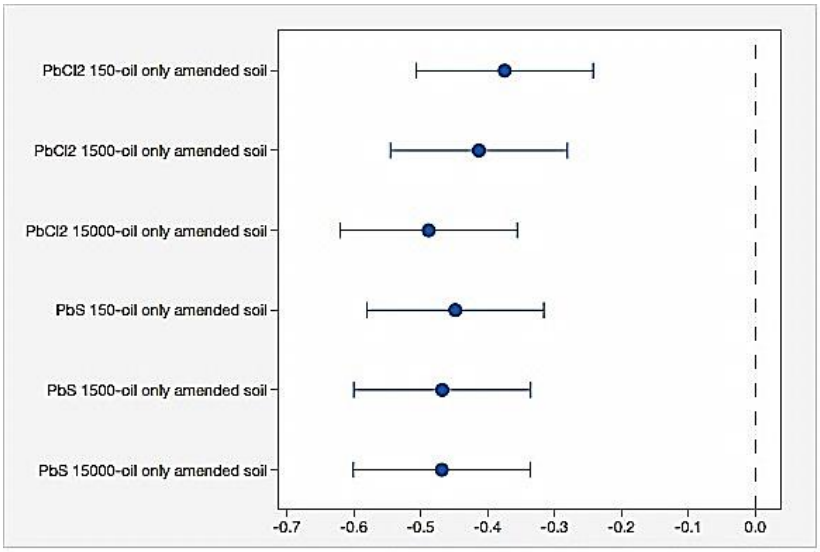


Figure 6.4: Comparative analysis of final cumulative CO_2 yield in $PbCl_2$ and PbS amended oil degrading soil microcosms at Pb concentrations of 150, 1500 and 15000 ppm relative to oil only amended control. The comparison was a Dunnett's multiple comparison analysis at 95% confidence interval. The values include the differences between the treatment mean and the control mean. A mean is not significantly different if its interval range encompasses zero (0). Y axis indicate sample comparison combination (all samples were compared with oil only amended soil control)

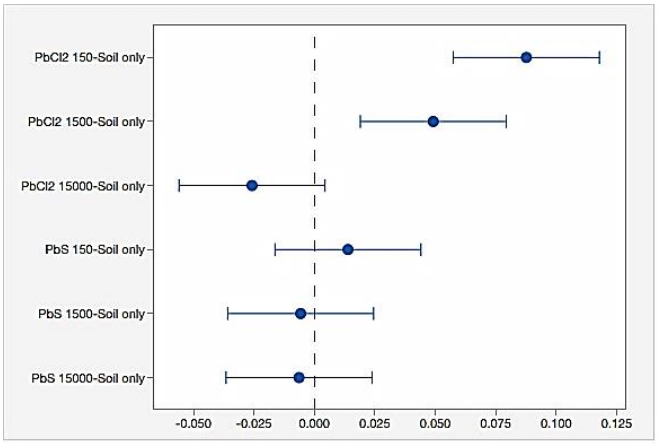


Figure 6.5: comparative analysis of cumulative CO_2 in $PbCl_2$ and PbS amended oil degraded soil microcosms at Pb concentrations of 150, 1500 and 15000 ppm relative to soil only (i.e unamended soil) controls. Analysis was by Dunnett's multiple comparison analysis at 95% confidence interval. The values include the differences between the treatment mean and the control mean. A mean is not significantly different if its interval contains zero (0). Y axis indicate sample comparison combination (all samples were compared with uncontaminated soil control).

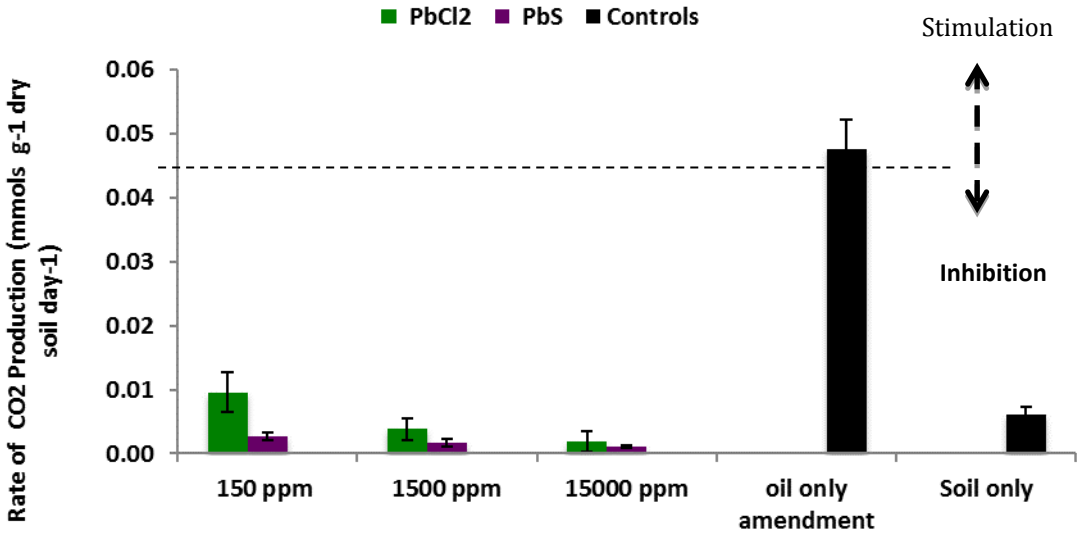


Figure 6.6: The maximal rate of CO_2 production in Pb and oil amended soils relative to oil amended and unamended controls. Each bar represents the mean maximal rate of triplicate experiments. The error bars represent the standard error of the means. The graph legend indicates the presence and chemical form of added Pb. The microcosm treatment indicated on the x-axis indicates the concentration of added Pb and the controls. The dotted line indicates the rate of oil-amended control; treatments with rates above the dotted lines are considered stimulatory while treatments with rates below the dotted lines are inhibitory.

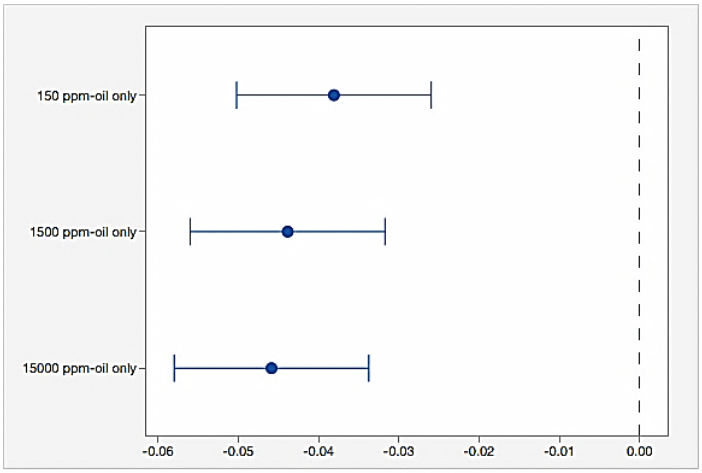


Figure 6.7: comparative analysis of maximal CO_2 production rates in the $PbCl_2$ amended oil amended soil microcosms at Pb concentrations of 150, 1500 and 15000 ppm relative to the oil only amended control. The analysis was by Dunnett's multiple comparison analysis at 95% confidence interval. The values include the differences between the treatment mean and the control mean. A mean is not significantly different if its interval contains zero (0) Y axis indicate sample comparison combination (all samples were compared with oil only amended soil control).

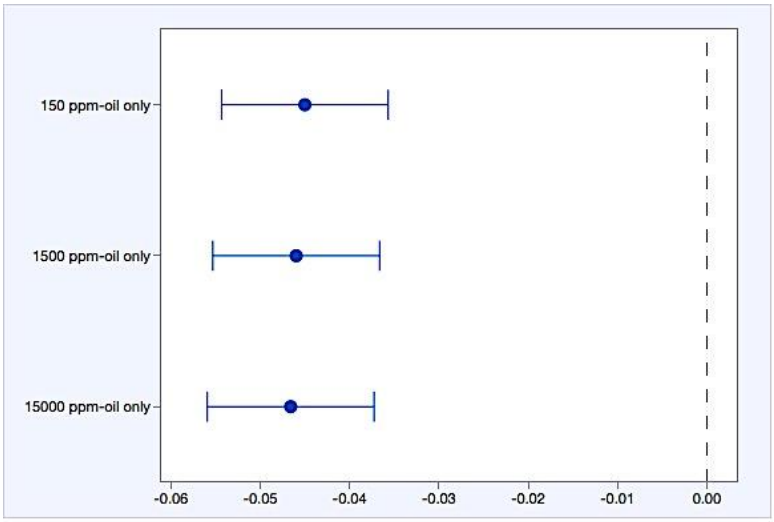


Figure 6.8: Comparative analysis of maximal CO_2 production rates in PbS amended oil degraded soil microcosms at Pb concentrations of 150, 1500 and 15000 ppm relative to oil only amended control. Analysis was by Dunnett's multiple comparison analysis at 95% confidence interval. The values include the differences between the treatment mean and the control mean. A mean is not significantly different if its interval contains zero (0). Y axis indicate sample comparison combination (all samples were compared with oil only amended soil control

6.4.2.2. Effect of long-term Pb contamination on petroleum degradation in soils

The effect of long-term Pb contamination on the petroleum degradation potential in soils was investigated using historically Pb contaminated soil amended with crude oil. The daily cumulative CO_2 production profiles in high and lower Pb contaminated soil amended with crude were compared to controls, which include oil only amended and unamended Nafferton farm soils as well as high and lower Pb soils from former Anthony works not amended with crude oil (see figure 6.9).

Firstly, the CO_2 profiles of the soils historically contaminated with Pb, without oil amendment, were analysed by comparing cumulative CO_2 production per observed day during degradation. Using ANOVA and Fisher's multiple pairwise comparison analysis, changes in cumulative CO_2 produced per day was compared. Interestingly, from the analysis, only short lag phases, if any,

were observed in these soils in comparison to the profile of oil-amended Nafferton soil control.

In soils contaminated with low concentrations of Pb, mean cumulative CO_2 changed from 0.0079 ± 0.0003 to 0.291 ± 0.006 mmol g^{-1} dry soil over the 15 days of incubation. Fisher's analysis indicated that there was a significant progressive change in cumulative CO_2 from day 0 to day 15. The change in cumulative CO_2 on day 0 was significantly different from that observed on day 2 ($p=0.05$). This implies that there was a very short, if any, lag phase observed in the soil. Subsequently, changes in cumulative CO_2 from one observed day to the other were significant. Furthermore, in high Pb contaminated soil, cumulative CO_2 changed from 0.011 ± 0.002 mmol g^{-1} dry soil on day 0 to 0.087 ± 0.006 mmol g^{-1} dry soil on day 15. Based on Fisher's analysis, there was a significant rise in cumulative CO_2 between day 0 and 2, indicating again a short, if any, lag-phase in the soil before the onset of any significant CO_2 production. This is followed by a period of reduced CO_2 production between days 2 and 13. Subsequently, a slight rise in cumulative CO_2 was observed between days 13 and 15. Compared to unamended Nafferton farm soil using ANOVA, the CO_2 production profile was significantly different in low the Pb soils. On the other hand, no significant difference in CO_2 production profile was observed in high Pb soils relative to unamended Nafferton farm soil.

On amendment of Pb soils with crude oil, the CO_2 production profile of the lower Pb soil was not found to be different relative to lower Pb soil without oil ($p=0.648$). However, the CO_2 production profile of high Pb soils was significantly different on the amendment with oil, relative to the high Pb soil not amended with crude oil ($p=0.0001$). Here, daily cumulative CO_2 changed from 0.027 ± 0.008 to 0.175 ± 0.005 mmol g^{-1} dry soil from day 0 to day 15. There was a significant rise in cumulative CO_2 between days 0 and 2. Subsequently, daily cumulative CO_2 production levelled off between days 2 and 15.

The daily cumulative CO_2 production profile of Pb soils amended with crude oil was compared with oil only amended Nafferton farm soil. ANOVA indicated that the CO_2 production profile in the high Pb soil amended with crude oil was significantly different relative to oil only amended Nafferton soil.

However, the daily cumulative CO_2 production profiles in the lower Pb soil amended with crude oil was not significantly different relative to oil only amended Nafferton farm soils. While a short, if any, the lag phase was observed in historically contaminated Pb soils, the lag phase observed during degradation in oil Nafferton oil-amended soil was ~6 days. Also, while there was a period of more rapid CO_2 production (linear-phase) with a subsequent period of lower rates of CO_2 production in oil-amended Nafferton soil and in lower Pb soil amended with crude oil, the high Pb soil amended with crude oil lacked these changes whereby CO_2 accumulated linearly throughout the incubation period.

Furthermore, the final maximum cumulative CO_2 yield in the historically Pb contaminated soils amended with crude oil was determined during crude degradation in soil microcosms and compared with that of the controls using ANOVA and Dunnett's pairwise comparison tests.

To understand the effect of crude oil amendment on the final maximum cumulative CO_2 yield in microcosms containing low and high Pb contaminated soils amended with crude oil were compared with the Pb soils not amended with crude oil. ANOVA indicated that there were no significant differences between lower Pb soils amended with crude oil relative to lower Pb soils not amended with crude oil ($p=0.160$). However, differences in the means of the maximum CO_2 yields was significant in a comparison of the high Pb soil amended with crude oil relative to the high Pb soil not amended with oil. This result signifies that while amendment of low-Pb soil with crude oil did not significantly influence the microbial activities in the soil, in high Pb soils, amendment with crude oil resulted in increased microbial activity.

Furthermore, Pb soils amended with crude oil were compared with Nafferton soil amended with crude oil to determine differences between maximum CO_2 produced in these different soils. ANOVA revealed that there were significant differences across treatments ($p=0.0098$). Pairwise analysis by Dunnett's test revealed that the mean of maximum CO_2 produced in High-Pb soils amended with crude oil was significantly lower relative to oil-amended Nafferton Farm soil by 0.339 ± 0.072 mmol g^{-1} dry soil ($p=0.006$). However, the mean maximum CO_2 produced in lower Pb soils amended with crude oil was not significantly lower relative to oil-amended Nafferton soil by 0.169 ± 0.072

mmol g^{-1} dry soil (p=0.098). Further analysis that compared high-Pb soils amended with crude oil with unamended pristine soil indicated that there were no significant differences in the mean maximum CO2 between the soils (p=0.191).

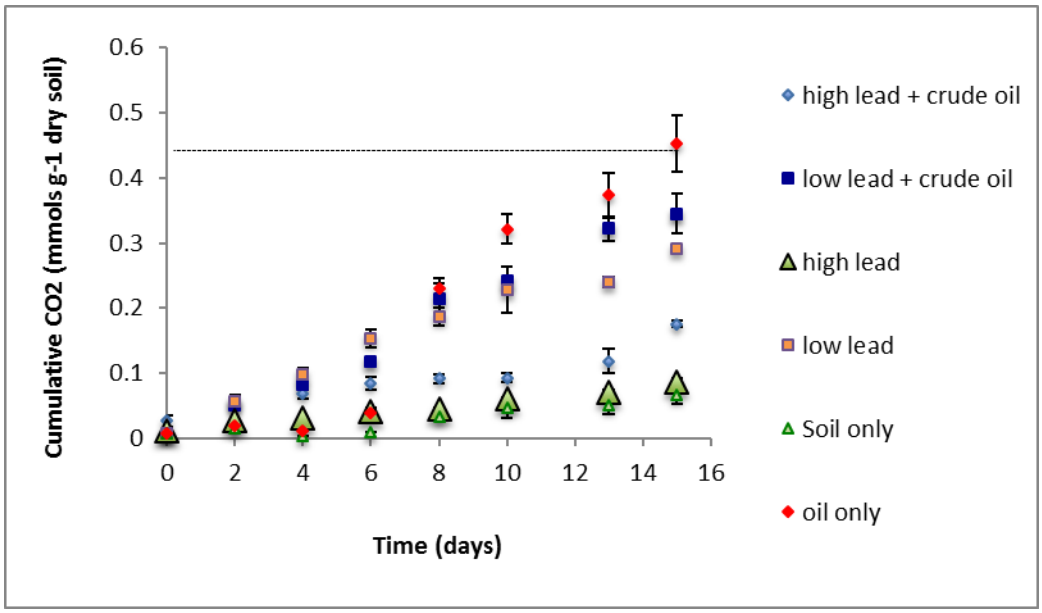


Figure 6.9: CO_2 production profiles of historically Pb contaminated soil (Anthony soil sample) amended with crude oil relative to historically Pb contaminated soils i.e Anthony soil (at high and lower Pb concentrations) not amended with crude oil and oil amended and unamended Nafferton Farm soil controls. The data points represent the mean cumulative CO_2 yields (mmols g^{-1} dry soil) from triplicate experiments. The error bars represent standard errors and the graph legend indicates the different treatment microcosms and the controls (unamended Nafferton Farm soil is indicated as soil while oil amended Nafferton soil is indicated as oil only). The dotted lines indicate the maximum CO_2 produced in crud oil only amended Nafferton farm soil.

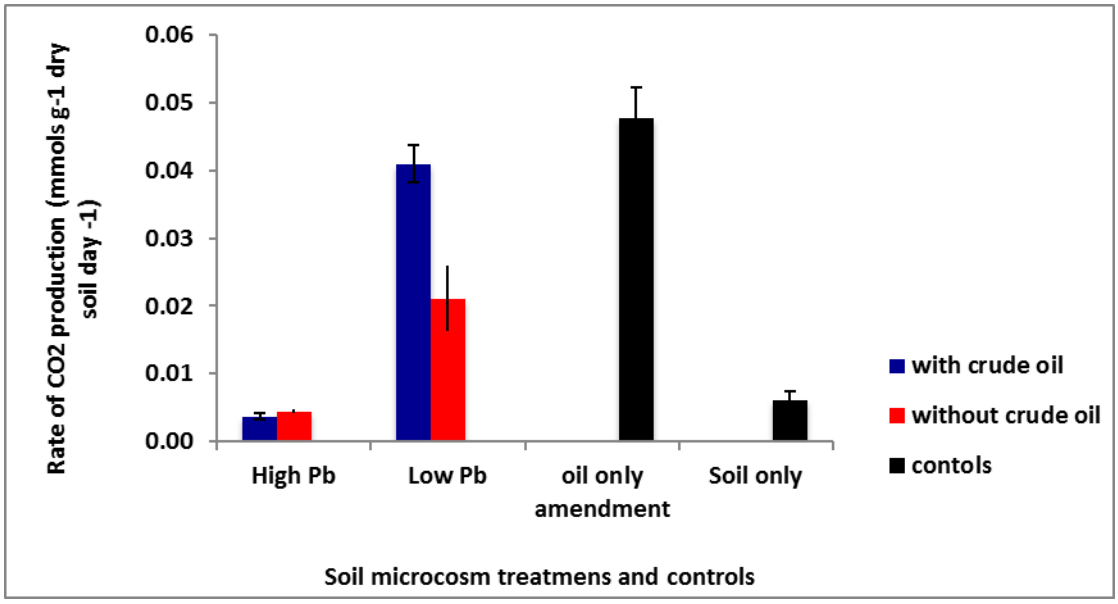


Figure 6.10: The maximal rate of CO_2 production in historically Pb contaminated soils i.e. Anthony soils (with high and lower Pb contaminations) amended with oil relative to oil amended and unamended Nafferton Farm soils and historically Pb contaminated Anthony soils (with high and lower Pb contamination) not amended with oil. Each bar represents the mean maximal rate of triplicate experiments. The error bars represent the standard error and the graph legend indicates the type of treatment and controls. The microcosm treatments on the x-axis indicate oil amended and unamended treatments and control

To determine if Pb contamination affected the rates of CO_2 production in soil microcosms the historically Pb contaminated soils amended with crude were compared to the same soil not amended with crude oil. Figure 6.10 shows the maximal rate of CO_2 production in treatment microcosm relative to control microcosms. From the figure, the rates of CO_2 production in Pb-crude oil contaminated soils were lower relative to the oil only control. ANOVA indicated that there were significant differences across treatment microcosms relative to oil only amended soils ($p<0.0001$). Figure 6.11 shows the Dunnett's pairwise comparison. The analysis indicated that the mean maximal rate of CO_2 production in the low-Pb soil amended with crude oil was not significantly different (differences in mean = 0.007 ± 0.005 mmol g^{-1} dry soil day^{-1}; $p=0.429$). On the other hand, the mean maximal rate in CO_2 production of high-Pb soils amended with crude oil significantly differed relative to oil only control by 0.044 ± 0.005 mmol g^{-1} dry soil day^{-1} ($p<0.0001$). In addition, the maximal rates of CO_2 production in the high and lower Pb soils amended with crude oil were compared with high and low lead soils not amended with

crude oil respectively. ANOVA revealed that there were no significant differences in mean maximal CO_2 production rates between crude oil-amended and unamended high Pb soils (p=0.1). On the contrary, the mean maximal CO_2 production rates in crude oil-amended and unamended soils differed significantly such that maximal rates were higher in the crude oil-amended lower Pb soil relative to unamended lower Pb soil by 0.019 ± 0.005 mmol g^{-1} dry soil day^{-1} (p=0.005).

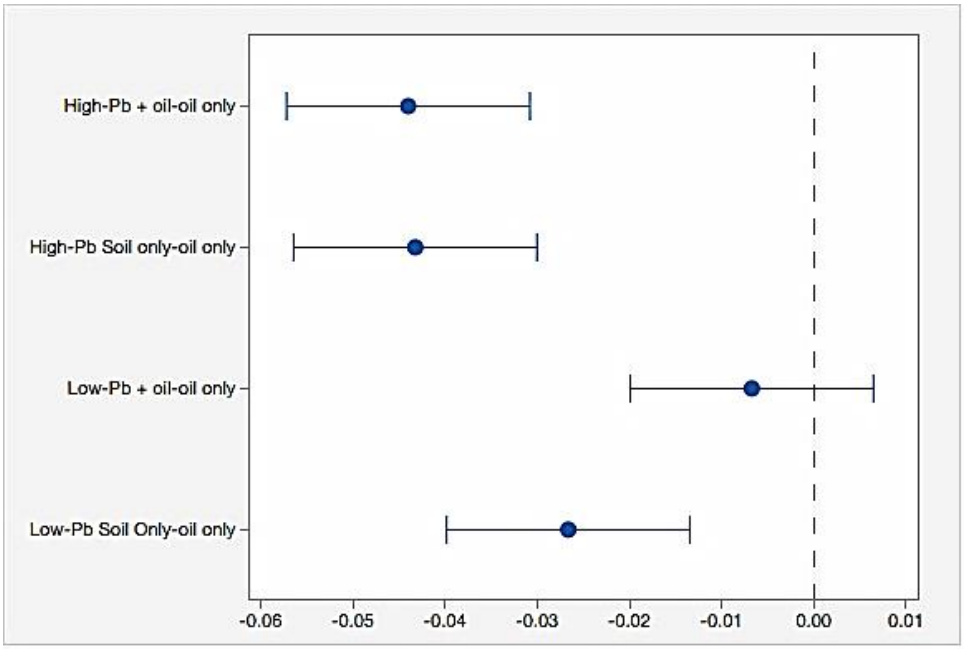


Figure 6.11: Comparative analysis of maximal CO_2 production rates in historically Pb contaminated soil amended and unamended with oil at low and high Pb concentrations relative to the oil only amended Nafferton soil control. Analysis was by Dunnett's multiple comparison analysis at 95% confidence interval. The values include the differences between the treatment mean and the control mean. A mean is not significantly different if its interval contains zero (0)

6.4.3. The fate and chemical association of Pb on addition to the farm soil microcosms

Pb amended soils were sequentially extracted to understand the chemical associations of the metal in soils. The aim of this analysis was to understand the fate of added Pb in these soils. To this end, triplicate soil samples were amended with PbS and $PbCl_2$ at a concentration of 1500 ppm and were incubates at room temperature (21 ^oC) for 7 days.

Figure 6.12 shows the apparent portioning of the added Pb determined by sequential extraction analysis. There are apparent differences in the sequestration of Pb influenced by the different Pb forms investigated. In the $PbCl_2$ amended soil, the mean concentrations associated with the soil exchangeable fractions, the carbonates and oxides of Fe and Mn 473.02 ± 64.34 ppm Pb (31.53 % of added Pb), 180.20 ± 11.83 ppm Pb (12.01 % of added Pb) and 638.57 ±49.07 ppm Pb (42.57 % of added Pb) respectively. For the organic and residual fractions, the mean concentrations were 111.44 ± 12.61 ppm Pb (7.43 % of added Pb) and 96.78 ± 96.78 ppm Pb (6.45 % of added Pb). Based on ANOVA, these concentrations of Pb associated with soil fractions are significantly different from each other ($p=0.0001$).

On the other hand, in PbS amended soils, the mean concentrations of Pb associated with exchangeables, the carbonates and oxides of Fe and Mn were 2.14 ± 0.79 ppm Pb (0.14 % of added Pb), 3.39 ± 0.81 ppm Pb (0.23 % of added Pb), 279.16 ± 41.89 ppm Pb (18.61 % of added Pb), respectively. Organics and residual fractions have mean concentrations of 239.96 ± 65.12 ppm Pb (16 % of added Pb) and 975.34 ± 106.61 ppm Pb (65 % of added Pb), respectively. A comparison of Pb partitioning relative to Pb form using ANOVA indicated that concentration of Pb associated with the exchangeables, carbonates and oxides of Fe and Mn were significantly lower ($p<0.0001$) in PbS amended soil relative to $PbCl_2$ amended soils by 470.88 ± 64.35 ppm ($p=0.0019$), 176.80 ± 11.86 ppm ($p=0.0001$) and 359.41 ± 64.52 ppm (0.0051), respectively. Interestingly, the differences in the mean concentrations of Pb associated with the soil organic fraction, which was 128.53 ± 66.33 ppm Pb, was not significant ($p=0.1247$) between forms of added Pb. However, the concentration of Pb associated with residuals was significantly higher by 878.6 ± 144 in PbS soils than in $PbCl_2$ soils.

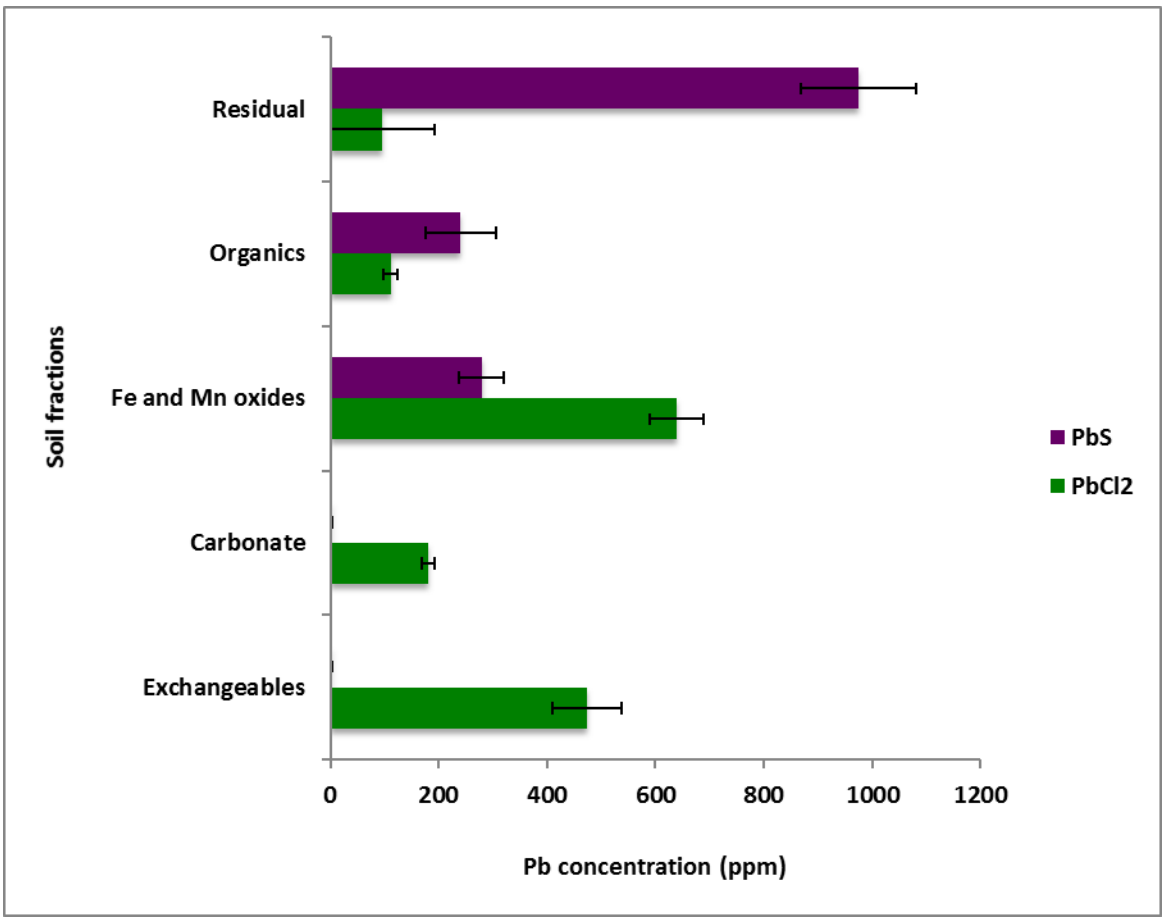


Figure 6.12: comparative analysis of the fate of added Pb in a farm soil by sequential extraction of Pb to determine Pb associated with exchangeable ions, carbonates, oxides of Fe and Mn, organics and residuals. The graph legend indicates the different chemical forms of Pb used for this study. Each bar represent the mean of the concentration of Pb associated with the soil fractions from triplicate amendment experiments. The error bars represent the standard error these mean values.

6.4.4. Degradation of the alkane component of oil added to the soil microcosms

At the inception of oil degradation experiment, treatment soil microcosms were amended with 100 mg crude oil. The residual petroleum hydrocarbons in the soil microcosms amended with crude oil were recovered after the degradation period and were analysed by GCFID. Specifically, the n-alkanes ranging from n-C_{11} to n-C_{32} (see figure 6.13) were isolated and quantified in microcosms and controls to ascertain the degree of petroleum degradation that had occurred. The masses of total n-alkanes were determined in microcosms

by the summation of individual n-alkane masses recovered from treatment soil microcosms, which were also compared with undegraded petroleum

6.4.4.1. Comparative analysis of total n-alkanes recovered from Pb-crude oil degraded soils relative to oil only amended soils and undegraded crude oil

The mean total n-alkane content of the undegraded crude oil was 7313.46 ± 718.16 µg per 10000 µg of oil mass. In contrast, the mean total n-alkane mass recovered from the oil only amended Nafferton farm soil control after 15 days of degradation was 2674.34 ± 495.92 µg. ANOVA followed by Dunnett's pairwise comparison analysis, indicated that mean total n-alkanes recovered from the oil only contaminated soil control was significantly lower by 4.64 ± 0.99 mg ($p=0.0011$) relative to the alkane content of the equivalent mass of undegraded crude oil. However, the mean total n-alkanes recovered from Pb contaminated soils irrespective of Pb forms, concentration or duration of contamination (long-term or short-term) was not significantly different relative to undegraded crude oil.

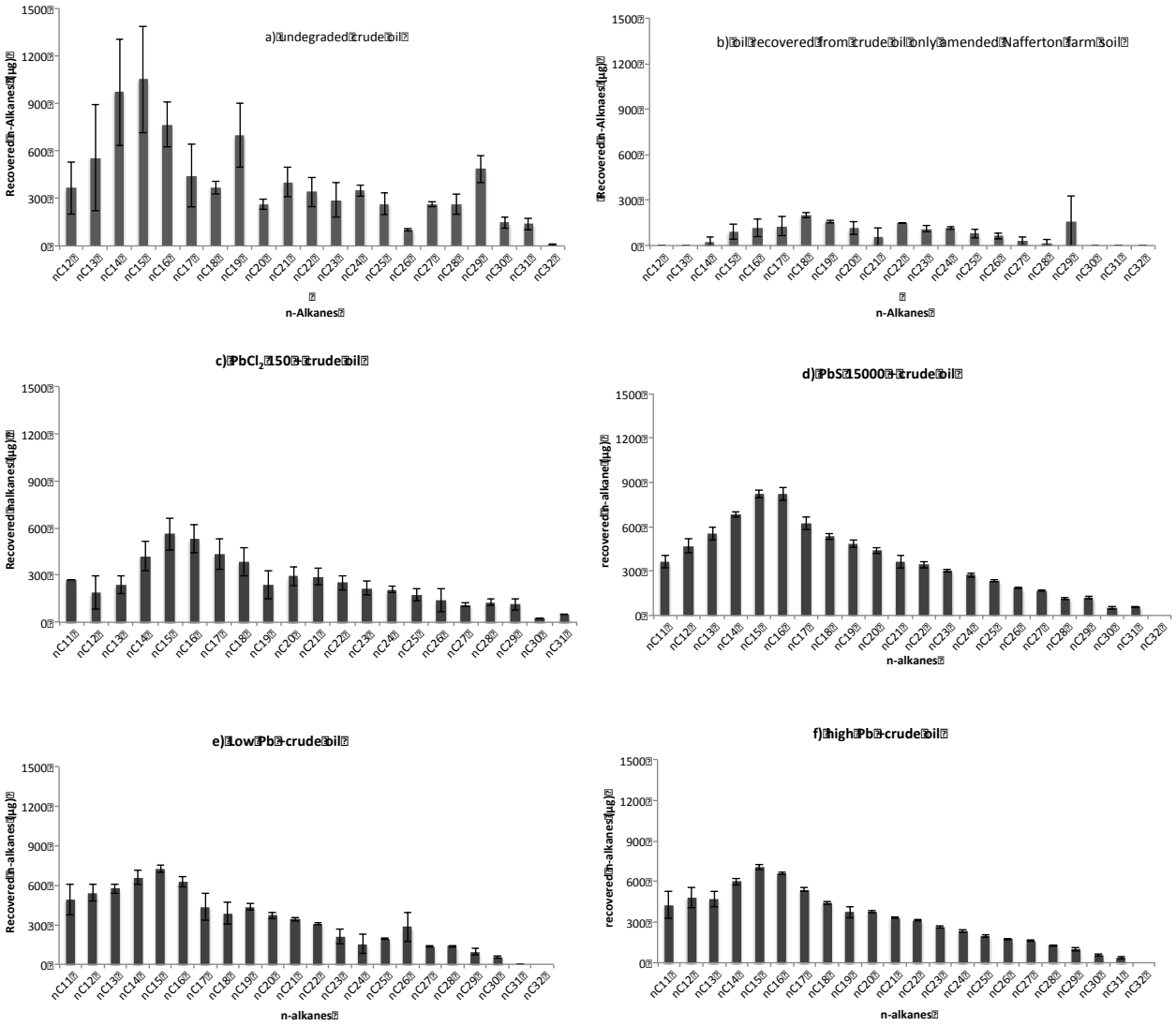


Figure 6.13: the profile of n-alkanes recovered from soils after degradation of crude oil in Pb contaminated soil microcosms indicating the degree of degradation of the whole petroleum in both the long-term and short-term Pb contaminated soils. Each bar indicates the mean weight of an individual n-alkanes (ranging from n-C11 to n-C32) in recovered oil after degradation period of 16 days.

To determine the effect of Pb on petroleum degradation, the mean total n-alkanes of treatment microcosms were compared with mean total n-alkanes of the oil only Nafferton amended soil control and the undegraded crude oil. The total n-alkanes recovered from the Pb-containing soils, irrespective of forms, concentration or duration of contamination (long-term or short-term) were higher relative to oil only amended control (see figure 6.14). ANOVA identified that there were differences in mean total n-alkanes across treatments. Dunnett's Pairwise analysis indicated that relative to the oil only control, the mean total n-alkane recovered from Pb-containing soils, irrespective of Pb form, concentration and duration of concentration, were significantly higher except for the mean total n-alkanes recovered from $PbCl_2$ amended soil at 150 ppm Pb, which was not significantly different (p=0.12).

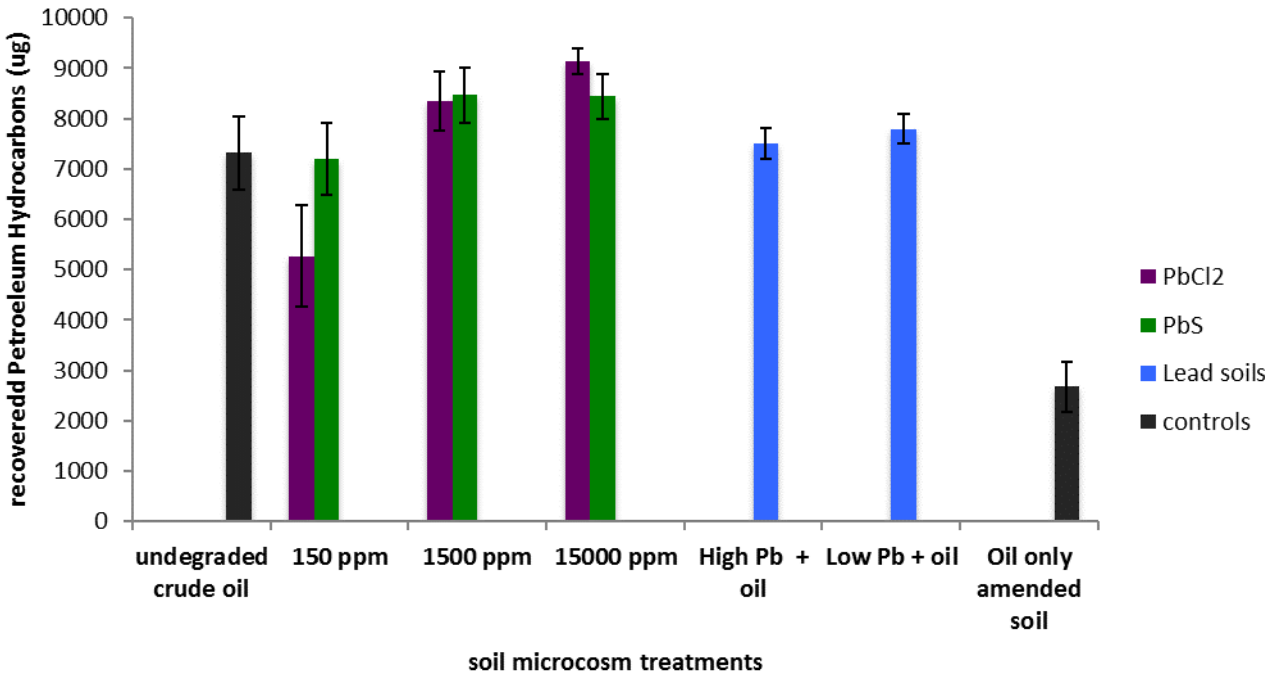


Figure 6.14: comparison of total n-alkanes (nC_{12}-nC_{32}) of microcosms contaminated with Pb and/or 100 mg oil after 15 days incubation. Each bar represents the mean total n-alkanes of triplicate samples. Error bars represent the standard error of triplicate samples.

6.4.5. Microbial diversity and Phylogeny.

Molecular biological methods were used to study the microbial community diversity and phylogeny in the experimental microcosms to understand the effect of Pb on hydrocarbon degrading soil communities. These methods included DGGE community fingerprint analysis and ion torrent based deep sequencing approaches. For the studies of the short-term Pb contamination experiments, microbial communities of crude oil containing soil microcosms amended with $PbCl_2$ and PbS at Pb concentrations of 150 and 15000 ppm were used. Microbial communities of historically contaminated Pb soils with high and lower Pb concentrations amended with crude oil were also analysed after incubation with oil. The diversities and phylogeny of soils were compared against oil only amended and unamended soil controls.

6.4.5.1. Denaturing gel electrophoresis indicating the microbial communities enrichment influenced by Pb contamination and the reproducibility of communities

DGGE analysis of the 16S rRNA gene was carried out to study the microbial diversity of communities. Figure 6.15 shows the community diversity profile of the 16S rRNA gene fragments on a polyacrylamide gel. Visual analysis of gel revealed that the communities were highly diverse, comprising of low G+C microbes as well as high G+C microbes. Also, a high reproducibility of communities from the treatment replicates was observed. In addition, selective enrichment of species in communities was observed in petroleum-contaminated soils (with or without Pb) and in soils with long-term Pb contamination (with or without petroleum). However, soil communities of 15000 ppm Pb amended Nafferton soil appear to show no selective enrichment of species and profiles and thus were comparable to communities of soil controls without oil.

Furthermore, ANOSIM was carried out on communities to statistically compare communities. With Global R (R) of 0.926, ANOSIM indicated significant differences across communities, which was influenced by the Pb forms and the duration of Pb contamination ($p=0.001$). In addition, community differences were significantly influenced by Pb concentrations ($R=0.912$; $p=0.001$).

Communities were compared with oil only amended soils to identify the effect of Pb contamination on the communities of degraded soils. A pairwise analysis of oil only amended soil community and communities of unamended soil identified a significant difference between communities (R= 1; p=0.018). This indicated that there was a change in community structure of soil due to amendment with crude petroleum, which may be because of selective enrichment of petroleum degraders in oil only amended soil communities. Furthermore, pairwise analysis between oil-amended short-term Pb contaminated soils and the oil only amended soil communities revealed that community diversities differed significantly (R=1; p=0.012 for the addition of $PbCl_2$ and PbS at 150 ppm). Although communities of oil only amended soil control indicated a good discrepancy from the petroleum crude oil-amended long-term Pb contaminated soils, this was not significant (R=1; p=0.1). However, communities of Long-term Pb contaminated soils without petroleum differed significantly relative to oil only amended soil (R=1; p=0.029 (lower Pb soil) and R=1; p= 0.01 (High-Pb soil)).

These observations imply that Pb influenced microbial community diversity in petroleum-degrading systems and Pb influence on microbial community diversity was dependent on Pb forms, concentrations and duration of contamination. Selective enrichment of petroleum degraders that resist Pb may have occurred in these Pb soil communities.

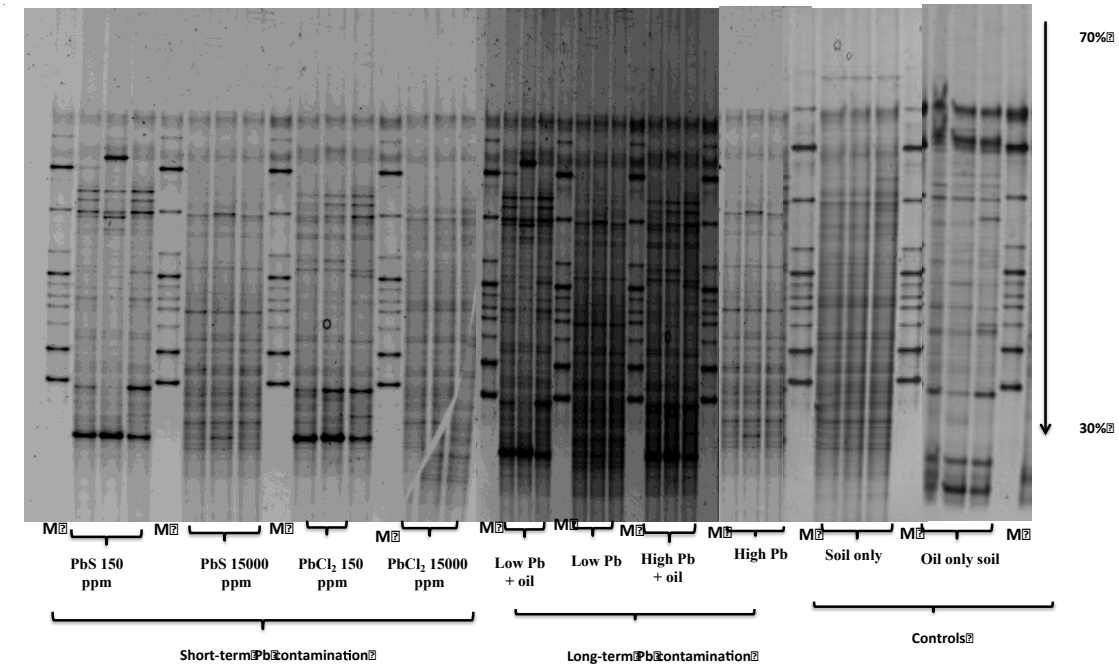


Figure 6.15: the 16S rRNA gene profile indicating the diversity of microbial communities and reproducibility of communities in triplicates of soil microcosms amended with petroleum crude oil and Pb. The treatments and controls were indicated and these included treatments for short-term Pb contamination and treatments for long term Pb contamination. The oil only amended indicates Nafferton farm soil amended with oil only control while soil control indicates unamended Nafferton farm soil. M indicates marker lanes.

Clustering analysis and MDS were also carried to identify similarities between communities. Figure 6.16 shows the MDS plot of the communities from Bray-Curtis similarity matrix overlaid with similarity contour generated from clustering analysis. MDS revealed that the diversities of all communities were similar, with a low percentage of 20%. This elaborated the clear separation of communities observed in the ANOSIM results. At 40% similarity, all communities of Pb soils (both long term and short term Pb soils) were clustered with soil only control. At 60% similarity, short-term contaminated soils separated from the long-term contaminated soils. In addition, high Pb soils separated from lower Pb soils. At 80% similarity, clustering of replicate communities was observed indicating a good reproducibility of communities. These observations corresponded with the ANOSIM results. Also, the Long-term Pb soils (with or without petroleum) were clustered at 80% similarity.

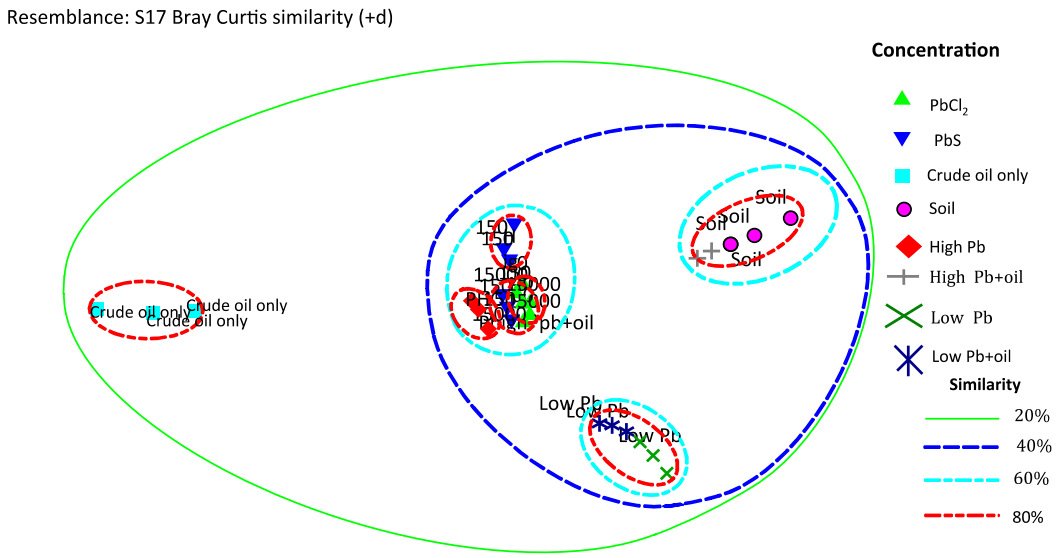


Figure 6.16: Non-metric MDS analysis of the bacterial communities present in Pb-crude oil contaminated soil communities, crude oil only amended, high and lower Pb soils not contaminated with oil and unamended soils (soil only) after 15 days of degradation period. MDS plots are representations of how different the communities are from each other based on clustering of like samples. Similarity contour lines from cluster analyses are superimposed on to the MDS plots. The legend indicate the Pb treatments (based on concentrations and duration of contamination) and the controls (i. e. oil only and soil only) as well as similarity contour lines

6.4.5.2. Qualitative analysis of sequencing data set used for the biodiversity study of microbial communities of Pb-contaminated crude oil degraded soil

Furthermore, studies based on sequencing analysis of amplified 16S rRNA gene were carried out to obtain more details of the microbial community structures. For this purpose, 16Sr RNA genes of communities were sequenced using the ion torrent PGM sequencing method. The sequence data obtained were analysed using the mothur software to trim sequence data, cluster sequence data into Operational Taxonomic Units (OTUs), assign taxonomic groups to OTUs and analyse community diversity (alpha- and beta- diversity of communities) to identify the differences in the microbial community structures. Due to the high reproducibility of communities identified from the

DGGE analysis, representative samples were used in this analysis. Tagged 16S rRNA samples from 17 soil samples consisting of metal (Ni, Cd and Pb) petroleum degraded soils and controls, which were the petroleum amended, and unamended soils made up the pooled clone library used for the ion torrent sequencing. Communities of the Pb-contaminated soils consisted of representative samples including PbCl2 amended soils at 150 and 15000 Pb-concentrations, PbS amended soils at 0.1 150 and 15000 Pb concentrations and historically contaminated Pb soil at high and lower Pb concentrations.

The ion torrent sequencing recovered high throughput sequence data set of 492574 sequences. Sequence data from Pb-contaminated communities accounted for 114835 sequences, i. e. approximately 23.31% of the sequence data from the clone library. The sequence reads of $PbCl_2$ soil communities amended with 150 and 15000 ppm Pb were 13409 and 14002 sequences, respectively. In addition, 26787 and 19081 sequence reads were obtained from PbS soil communities at 150 and 15000 ppm Pb, respectively. The sequence reads obtained from crude oil deranged historically contaminated soils at low and high concentrations of Pb were 21537 and 20019, respectively.

Sequence data were trimmed to recover sequences that had a minimum length of 300 bp, a maximum of 500 bp. and homopolymers lower than 7 bp were trimmed. This resulted in the omission of ~8.66% of the raw sequence data from the Pb-contaminated communities, reducing the sequence recovery to a total 104886 sequences.

For further analysis on mothur software, sequences were normalised to 8000 sequences per sample. Also, for QIIME analysis, sequences were normalised to 13500 sequences per sample, which equated to the lowest sequence count obtained for the17 samples analysed. Table 6.2 shows analysis of sampling based on the Chao estimate of the number of observed OTUs per sample based on 13500 sequences per treatment analysed in QIIME and 8000 sequences per treatment for analysis on the mothur platforms.

6.4.5.3. OTU-base studies on the influence of Cd on the community diversity of oil degraded soil microcosms

OTUs generated using an average neighbour clustering method were analysed based on a 97% similarity cutoff. Measures of diversity, which included

species richness Simpson's diversity (λ) and Shannon evenness (EH'), were evaluated using the mothur platform using 8000 16S rRNA sequences per sample (see table 6.3). Also, diversities of communities were compared relative to the oil only amended control and unamended soil control by determining the Yue and Clayton's dissimilarity coefficient (θ) (see table 6.4).

Table 6.2: the analysis of the 16S rRNA sequence sampling for the diversity and phylogenetic study of communities of Cd-oil amended soils

Samples	Chao estimator (13500 sequences)	Number of observed species (13500 sequences)	Chao estimator (8000 sequences)	Number of observed species (8000 sequences)
Pbcl2 150 ppm	8471.17	3966	4206.83	2042
Pbcl2 15000	5427.15	3224	2395.45	1733
Pbs 150	7999.89	4138	4434.31	2184
Pbs 15000	9087.23	4323	4288.75	2324
Low pb	6196.27	3780	6278.15	2775
High pb	7181.90	3907	5517.93	2261
Oil only	8754.93	3567	6104.18	1676
Soil only	9637.72	4567	7331.28	2457

The species richness was determined from the number of observed OTUs in communities. As has been pointed in chapter 4, 16382 OTUs were observed in the whole library analysed. The observed number of species in oil only amended and unamended soil communities were 1676 and 2457 OTUs, respectively. Hence, 68.21% of the number of species observed in the unamended soil was present in the hydrocarbon degrading oil-amended soil community. In the PbCl2 amended crude oil degraded communities, the number of observed OTUs at 150 and 15000 ppm Pb soils were 2042 and 1733 OTUs, respectively. This implies that relative to the soil only control, 83.11 % and 70.53% of the number of soil species were present in the PbCl2-amended soils at 150 and 15000 ppm Pb, respectively. Similarly, in PbS amended soils, the number of OTUs observed at 150 and 15000 ppm Pb were 2184 and 2324 OTUs respectively. These made up 88.89% and 94.58% of the unamended soil community. Also, in historically Pb contaminated soils amended with crude oil, the number of observed OTUs was 2775 and 2261 at

high and lower Pb, respectively and these are above 100% and 86.08% of the number OTUs observed in the unamended soil community.

These observations of species richness suggest that hydrocarbon amendment reduced the species richness in soil due to the dominance of enriched community of hydrocarbon degrading microbial species in oil only amended soils. This conclusion is supported by the appearance of brighter bands in the DGGE profiles of oil-amended soils. Also, Pb contamination, irrespective of Pb form, Pb concentration and duration of Pb contamination, affected dominance of hydrocarbon degraders such that there were slight changes in specie richness relative to oil only amended soil communities. Hence, when the hydrocarbon degraders are not inhibited i.e. in the soil amended with oil only, great drop in diversity due to the dominance is observed and this effect is lessened with metal contamination

Furthermore, the evenness of community species $E_{H'}$ was determined. $E_{H'}$ values are usually between 0 to 1, with 0 indicating perfect community species unevenness (i. e. a perfect dominance by a species) and 1 a perfect community species evenness (a perfectly heterogeneous community). The $E_{H'}$ of the oil-amended soil and unamended soil controls are 0.710628 and 0.874792, respectively. To clearly establish the effect of Pb on community species evenness, $E_{H'}$ in Pb contaminated soils were compared with the oil only amended soil control. The $E_{H'}$ of $PbCl_2$ amended soils were 0.793532 and 0.858115 at 150 ppm and 15000 Pb concentrations, respectively. Similarly, in PbS soils, $E_{H'}$ was 0.862031 and 0.877259 at 150 ppm and 15000 Pb concentrations, respectively. Also, similar $E_{H'}$ values were obtained in long-term Pb contaminated soils at high and lower Pb concentrations. Here the $E_{H'}$ values were 0.850906 and 0.860745 at low and high Pb concentrations, respectively. These observations imply that relative to oil only amended soils, communities of Pb contaminated soils irrespective of Pb form, the concentration or the contamination duration, have more species evenness, suggesting that selective dominance in these communities is less compared to oil only amended soil communities. A similar pattern was observed for λ.

Using Yue and Clayton's dissimilarity coefficient, θ (see table 6.4), communities were compared relative to oil only-amended soil and unamended soil communities. Here, communities are compared to identify similarities in diversity. Generally, the values of θ ranged from 0 to 1; with 0 indicating perfectly similar community diversity and 1 indicating perfect dissimilar community diversity. Analysis indicated that with θ values ranging from 0.939562 to 0.982061, communities of Pb contaminated soils, irrespective of Pb form, concentration or duration of contamination, were not similar to oil only amended soils. Also, relative to soil only control, no similarities in diversities were observed in Pb contaminated soils. Though, the lowest θ values of 0.478156 and 0.315519 were obtained in the community of crude oil degraded soil amended with $PbCl_2$ and PbS at 15000 ppm Pb respectively. This indicated that the diversities of these communities were more closely related to unamended soil.

Table 6.3: Community specie richness and diversity indices evaluated with 8000 16S rRNA sequences of Pb-crude oil contaminated soil microcosms

Samples	Number of Observed OTUs	Shannon's evenness (E_H)	Simpson's diversity index (λ)
PbCl2 150	2042	0.793532	0.034059
PbCl2 15000	1733	0.858115	0.00933
PbS 150	2184	0.862031	0.005849
PbS 15000	2324	0.877259	0.003285
High Pb-oil	2775	0.850906	0.014509
Low Pb-oil	2261	0.860745	0.005627
oil only	1676	0.710628	0.051148
soil only	2457	0.874792	0.003842

Notes

1. $0 \leq \lambda \leq 1$ where 0= perfect heterogeneous community and 1= perfect homogenous community

2. $0 \leq H' \leq 1$ where 0= perfect heterogeneous community and 1= perfect homogenous community

6.4.5.4. Community diversity study; Pb effects on the diversity of hydrocarbon degraders in oil degraded soil microcosms based on phylogenetic analysis

To further establish the effect of Pb on species dominance in crude oil degrading communities, the number of OTUs that made up 50% of the relative abundance of in oil only amended soil control was compared with the Pb and oil-amended communities. Firstly, 13 OTUs made up 50% of the relative abundance of the petroleum degrading microbial community in the oil only amended control. This is in contrast to 42 OTUs that occurred in soil only control. In addition, for the $PbCl_2$ amended soils, while 32 OTUs made up 50 % of the relative abundance of species in at 150 ppm Pb amendment, 48 OTUs occurred at 15000 ppm Pb. Similarly, for PbS soils 38 OTUs comprised 50% of the relative abundance of species at 150 ppm Pb amendment and 44 OTUs occurred with an amendment with 15000 ppm Pb. For the long-term Pb contaminated soils, 74 OTUs made up 50% of community relative abundance in lower Pb soil amended with oil while 58 OTUs occurred in high Pb soil. These observations indicated that species enrichment/dominance occurred most in the oil only amended soil communities and the presence of Pb inhibited selective enrichment of Pb sensitive petroleum degraders in the communities.

6.4.5.5. Comparative phylogenetic analysis of microbial communities of Pb- contaminated soils amended with crude oil relative to oil only amended soils and unamended soils

Using the Bayesian method of Wang et al, OTUs were assigned to taxonomic groups. This classification was accomplished by matching the sequences of OTUs against the Silva, RDP and green genes reference files on the mothur platform and QIIME. These analyses revealed that the most abundant 10 Phyla that dominated the Pb amended oil degrading communities belonged to 9 bacterial phyla and one Archaeal phylum. The bacterial phyla that predominated belong to the *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteriodetes*, Acidobacteria, *Chloroflexi*, *Verrucomicrobia*, *Planctomycetes* and *Gemmatimonadetes* in order of decreasing relative abundance (see figure 6.17). The archaeal phylum was *Creanarcheota* (see figure 6. 17).

Classification at the genus level revealed that the most predominant OTU in Pb and oil-amended soils was most closely related sequences belonging to an unknown *Bacillales* (see figure 6.18). Two OTUs, related to unknown *Bacillales* species strains were discriminated based on soil, duration of Pb contamination and Pb concentration and were found to differentially dominate in differing 'treatment' libraries. While OTU 2 predominated in the short-term Pb + oil soils and in the long-term high Pb soils amended with crude oil, OTU 5 predominated in long-term lower Pb soil amended with crude oil albeit OTU 2 was found to be present. BLAST and RDP sequence match identified that a bacterium *Scopulibacillus darangshiensis* originally isolated from rock (Lee and Lee 2009) shared 98% similarity, was the closest relative to OTU 5 (see figure 6.20). On the other hand, the sequence of OTU 2 was closely related to a metal tolerating bacteria, *Bacillus circulans* isolated from oil shale and crude oil contaminated soil (Yilmaz 2003), which indicated 99% similarity. Other close relatives that were between 95 to 97% similar to these OTUs, included hydrocarbon degraders, such as *Geobacillus sp* (Guazzaroni and Corte 2010) and *Bacillus benzoevorans* (Pichinoty et al. 1984), and metal tolerating bacteria, such as *Bacillus arsenicoselenates* (Switzer Blum et al. 1998) and *Lysinibacillus sphaericus* (Peña-Montenegro and Dussán 2013).

Table 6.4: Comparison of microbial community structure in soil microcosms based on 8000 16S rRNA gene sequences

Samples	Soil only (θ)	oil only (θ)
$PbCl_2$ 150	0.9821	0.937
$PbCl_2$ 15000	0.9396	0.4782
PbS 150	0.9618	0.6317
PbS 15000	0.9589	0.3155
High Pb + oil	0.9862	0.9444
Lower Pb + oil	0.9719	0.8129
Oil only	0.946	0
Soil only	0	0.946

Note: $0 \leq \theta \leq 1$ where 0= perfect similarity and 1= perfect dissimilarity

Also, extremophiles including; the halophilic *Bacillus chungangensis* (Cho et al. 2010), thermophilic *Anoxybacillus amylolyticus* (Poli et al. 2006), alkaliniphilic *Bacillus foraminis* (Tiago et al. 2006) and anaerobic *Vulcanibacillus modesticaldus* (L'Haridon et al. 2006) were closely related to the *Bacilllales* OTUs. In addition, the OTUs were related to bacteria isolated from industrial locations including *Bacillus gottheili* (Seiler et al. 2013) and *Geobacillus tepidamans*(Schäffer et al. 2004).

The relative abundance of OTU 2 was 1.92 % in unamended soils, which presumably means that it has a normal function in the pristine soil. The amendment of soil with crude oil and $PbCl_2$ increased the relative abundance to 17.30% at 150 ppm Pb and 6.09 % at 15000 ppm. Also, amendment of soil with PBS and crude oil increased the relative abundance of OTU 2 to 5.13% at 150 ppm. However, at 15000 ppm Pb relative abundance of OTU2 was slightly decreased to 0.82%. Although OTU 2 was not the most dominant in lower Pb crude oil amended soil, the relative abundance was 3.81 %. In the high Pb soil amended with crude oil, relative abundance of OTU2 was 4.03%. Interestingly, a different strain of *Bacillales* sp. dominated in lower Pb contaminated crude oil amended soil communities with a relative abundance to 11.58% relative. However, without an analysis of an oil free control for these long term contaminated soils it is not possible to say from this relative abundance data whether these organisms (OTUs 2 and 5) had actually been enriched.

6.4.5.6. Evidence of Pb-influence on selective enrichment of hydrocarbon degraders in soil

In addition to the finding that *Bacillales* strains were specifically enriched in Pb contaminated soils amended with crude oil, as was presented in section 6.4.5.5 above, classification of OTUs identified other evidence that the presence of Pb influenced community diversity profile. Comparison of the phylogeny of Pb-soils with oil only amended soils indicated that while strain of *Rhodococcus* sp. were most enriched in the oil only control it was not in the presence of Pb (see figure 6.19). Hence *Rhodococcus* strains enriched in the oil only amended soil might have been vulnerable to Pb contamination. Other

hydrocarbon degraders that were enriched in oil only amended soils included those with sequences related to *Mycobacterium*, *Comamonadaceae* and *Pseudomonas* (see figure 6.19). The relative abundances of these bacterial strains in oil amended Pb soils were very low while the relative abundance of *Rhodococcus* was 19.90 % in oil only amended soils its relative abundance was 0.075 % and 0.23 % in PbCl2 amended soils at 150 and 15000 ppm, respectively. In addition, *Rhodococcal* relative abundances were 0.11, 0.04, 0.11 and 0.10 in PbS soil at 150 ppm Pb, PbS soil at 15000 ppm Pb, high Pb soils amended with oil and lower Pb soils amended with oil, respectively. Furthermore, the relative abundances of *Mycobacterium*, unknown *Comamonadaceae* and *Pseudomonas* in oil only amended soils were 8.47 %, 3.50 % and 2.86 % respectively. In all Pb soils, relative abundance of *Pseudomonas* ranged from 0.002 % in lower Pb soils to 0.02 % in PbS soil amended at 15000 ppm Pb. Also, the relative abundances of *Mycobacterium* in the Pb containing soil communities ranged from 0.38 % in $PbCl_2$ amended soil at 15000 ppm Pb to 1.46 % in PbS amended soil at 150 ppm Pb. As for the unknown *Comamonadaceae*, in lower Pb soil, relative abundance (the lowest) was 0.79 %. In high Pb soil the relative abundance of unknown *Comamonadaceae* was 1.83 % (the highest).

Conversely, the strains of *Bacillales* enriched in Pb soil petroleum crude degraded soil communities were not enriched in oil only amended soil community (see figure 6.17). The relative abundances of the *Bacillales* strains closely related to OTUs 2 and 5 in oil only amended soils were 1.21 % and 0.24 % respectively. This is very low compared to abundances in Pb soils. This implies that these strains of *Bacillales* can only dominate in the presence of Pb.

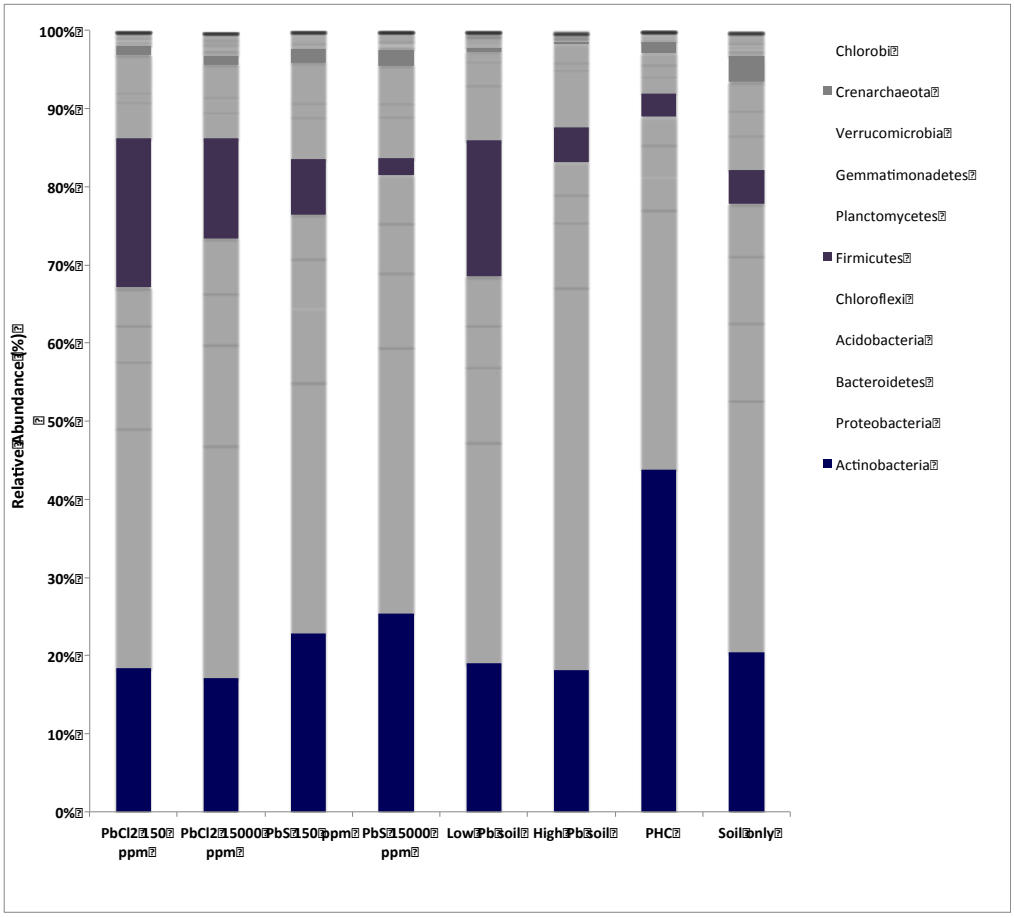


Figure 6.17: Comparative analysis of the relative abundances of phylogenic groups of 16S rRNA sequences recovered from representative Pb-crude oil contaminated soil microcosms relative to oil only amended soil and unamended soil (high and lower Pb soils not amended with oil were not included in this analysis). The relative abundance was determined using the QIIME and each treatment was normalized to 13500 16S rRNA sequences. The Legend indicate the different phyla represented in the microcosms. PHC indicated Nafferton farm soil contaminated with oil only. Soil only indicated unamended soil control.

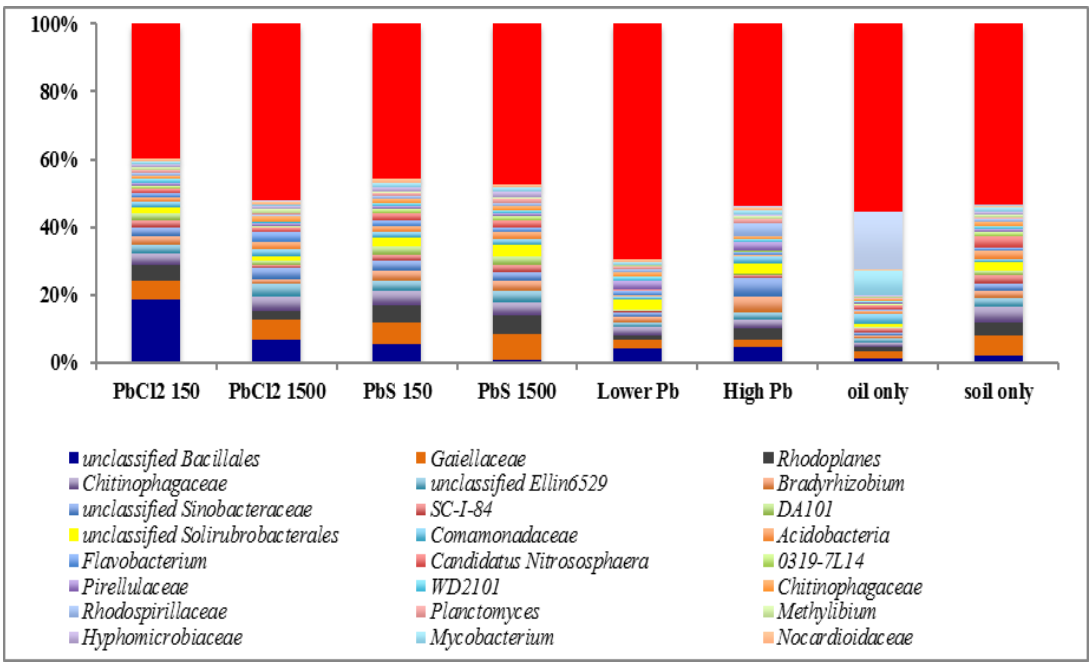


Figure 6.18: Comparative analysis of relative abundances of genera of 16S rRNA sequences recovered from representative Pb- crude oil amended soil microcosms relative to oil only amended soil and unamended soil. The relative abundance was determined using the QIIME and each treatment was normalized to 13500 16S rRNA sequences. The 50 most dominant genera were used in this analysis. The percentage sequences represented relative to the number of sequences per treatment is indicated in brackets. The Legend indicates the different genera represented in the microcosms.

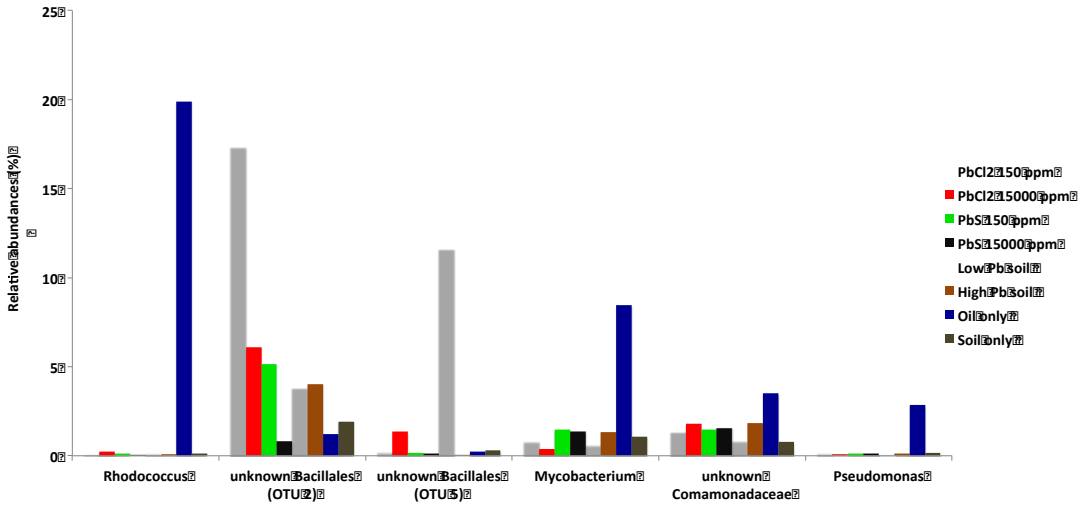


Figure 6.19: comparison of abundances of relatively enriched strains in oil amended Pb contaminated soil communities relative to oil only amended and unamended Nafferton farm soils. Bacterial sequence types represented here were those most dominant in the communities. Each bar represents the relative abundance of strains of communities in percentage. The legend indicates the soil treatments and controls.

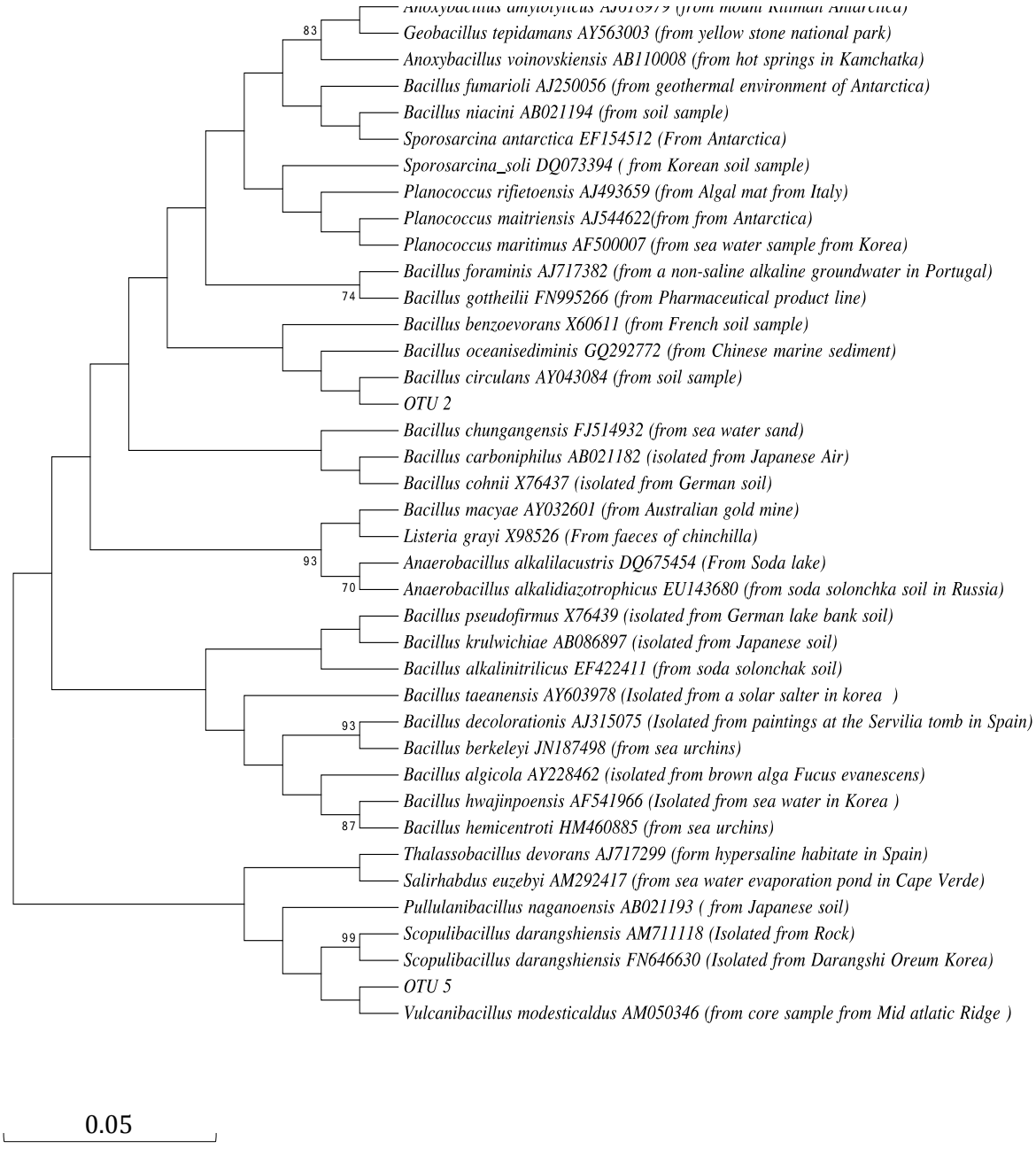


Figure 6.20: Comparison of the most dominant sequences recovered from Pb-crude oil contaminated soil community with closely related bacteria (mostly bacteria belonging to the phylum *Firmicute)*. The phylogenetic distance tree was based on the Neighbour-joining method. All sequences matched to the test sequence are typed 16S rRNA sequences. GenBank accession numbers for all sequences and a brief description of each bacterial strains origin are provided in brackets. The scale bar denotes 0.005 divergence and the values at the nodes indicate the percentage bootstrap trees that contained the cluster to the right of the node. Bootstrap values lower than 70 are not shown.

6.4.6. Absolute 16S rRNA gene abundances of general bacteria and *Rhodococcus* in the microbial communities of Pb contaminated crude oil degraded soil microcosms

Two strains of *Bacillales* were identified as the most apparently enriched taxonomic group in Pb-contaminated microcosms. To provide a clearer understanding of the Pb influence on species absolute abundances in the enrichment communities, qPCR assays specifically targeting the 16S rRNA gene of Bacteria and *Bacillales* was carried out to determine the absolute abundance of targeted 16S rRNA genes in the communities. Furthermore, the abundances of the 16S rRNA genes of treatment soils were compared with the controls to determine the absolute influence of Pb on 16S rRNA gene abundances in communities.

6.4.6.1 The influence of short-term Pb-contamination on general soil bacterial abundance in communities

qPCR analysis indicated that the absolute abundance of bacteria in soils amended with Pb and crude oil were lower relative to oil only control (see figure 6.21). ANOVA and Dunnett's pairwise analysis indicated that the mean 16S rRNA gene abundance of bacteria in microcosms was significantly lower across all treatment microcosms ($p=0.0015$). The mean 16S rRNA gene abundance of bacteria in oil only amended soil was 1.12×10^4 gene copies g^{-1} dry soil. This is significantly higher than the bacterial abundance in the unamended soil by $8.82 \times 10^6 \pm 2.89 \times 10^6$ gene copies g^{-1} dry soil indicating that oil amendment significantly increased bacterial abundance in soil (see figure 6.22). This could be attributed to the enrichment of hydrocarbon degraders (*Rhodococcus* species) in the oil-amended soil. General, in $PbCl_2$-crude oil amended soils, the mean 16S rRNA gene abundance of bacteria were lower across the concentrations, relative to oil only amended soil. The differences in mean included $1.35 \times 10^7 \pm 2.89 \times 10^6$, $1.45 \times 10^7 \pm 2.89 \times 10^6$ and $1.54 \times 10^7 \pm 2.89 \times 10^6$ gene copies g^{-1} dry soil at 150 ppm ($p=0.0015$), 1500 ppm ($p=0.0008$) and 15000 ppm ($p=0.0004$), respectively. Similarly, the mean 16S rRNA gene abundances of bacteria in PbS-crude oil amended soils were lower relative to oil only amended. The differences in means included $-1.38 \times 10^7 \pm 2.89 \times 10^6$, $1.21 \times 10^7 \pm 2.89 \times 10^6$ and $1.25 \times 10^7 \pm 2.89 \times 10^6$ gene copies g^{-1} dry soil at 150 ppm

(p=0.0012), 1500 ppm Pb (p=0.0041) and 15000 ppm (p=0.003), respectively. Furthermore, the 16S rRNA gene abundances in treatment microcosms were lower compared to the unamended soil. However, Dunnett's pairwise comparison indicated that the mean 16S rRNA gene abundances in treatment microcosms were not significantly different relative to unamended soil. These observations indicated that the presence of Pb might have inhibited the enrichment of some hydrocarbon degraders.

6.4.6.2 The influence of short-term Pb-contamination on *Bacillales* abundance in soil communities

As has been indicated earlier, the most abundant sequence in Pb contaminated soil is closely related to strain of *Bacillales* (OTU 2). A qPCR assay carried out to determine the absolute abundance of this *Bacillales* OTU indicated that its 16S rRNA gene abundance was higher relative in the Pb and oil treated soils relative to the oil only amended soil. ANOVA indicated that this was significant across all treatments ($p<0.0001$). Using Dunnett's analysis, treatment soil microcosms were compared with oil only amended control. This analysis revealed that the mean *Bacillales* 16S rRNA gene copies in $PbCl_2$-crude oil amended soil were higher relative to oil only control by $3.85 \times 10^6 \pm 5.79 \times 10^5$ gene copies and $2.54 \times 10^6 \pm 5.79 \times 10^5$ gene copies at 150 ppm ($p<0.0001$) and 1500 ppm ($p=0.0005$), respectively. However, at 15000 ppm, there was no significant difference in mean 16S rRNA gene abundance relative to oil only control.

6.4.6.3 Influence of long-term Pb-contamination on bacterial abundance in soil communities

ANOVA revealed that the mean 16S rRNA gene abundance of the bacteria varied significantly across soils historically contaminated with soils with or without crude oil ($p=0.0003$) (see figure 6.23). The mean bacterial 16S rRNA abundance in historically Pb contaminated soils at high and low concentrations of Pb amended with petroleum crude oil were 7.54×10^6 and 2.24×10^7 gene copies g^{-1} soil, respectively. In addition, in historically Pb contaminated soils at high and low concentrations of Pb not amended with petroleum crude oil, the mean bacterial 16S rRNA abundance were 3.53×10^5 and 1.31×10^7 gene copies g^{-1} soil, respectively. Hence, bacterial abundance

in the high Pb soil was greater by 7.19 X 10^{6} ± 4.22X 10^{6} gene copies g^{-1} soil (p=0.097) and similarly, the bacterial abundance in lower Pb soil was significantly more by 9.27 X 10^{6} ± 4.22X 10^{6} gene copies on amendment with crude oil (p=0.035) on amendment crude oil.

However, comparison of mean bacterial 16S rRNA gene abundances in the long-term contaminated Pb soils at high and lower Pb amended with crude oil relative to crude oil only control by Dunnett's analysis indicated bacterial abundance there were no significant differences between the treatment soils and the control. The mean bacterial 16S rRNA gene abundance was lower by 8.42 X 10^{6} ± 5.97 X 10^{6} gene copies g^{-1} soil in high Pb soil amended with crude oil (p=0.41). In addition, in lower Pb soil amended with crude oil the mean 16S rRNA gene abundance bacterial slightly higher by 6.43 X 10^{6} ± 5.97X 10^{6} gene copies g^{-1} soil (p=0.63) relative to oil only control. This implies that long-term Pb contaminated soils broadly sustained similar levels of bacterial cells. This may be attributed to the adaptation of bacterial strains to Pb presence over a long period.

To determine the influence of Pb concentrations, the mean bacterial 16S rRNA gene abundances in low and high Pb soils amended with crude oil were compared by ANOVA and analysis identified a significant difference between treatments. The mean bacterial 16S rRNA gene abundances was lower in high Pb soil amended with crude oil by 1.429 X 10^{7} ± 4.22 X 10^{6} gene copies g^{-1} soil (p=0.0012). This implies that despite the duration of contamination, higher concentration of Pb reduced bacterial abundance in soils.

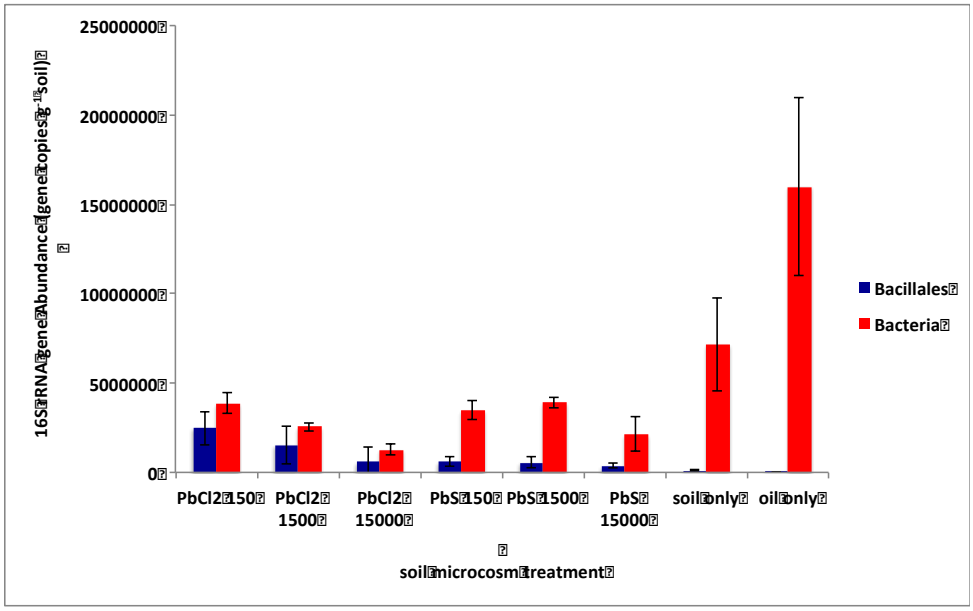


Figure 6.21: the 16S rRNA gene abundance of bacteria and *Bacillales* in short-term Pb and crude oil amended soil microcosms after incubation for 15 days. The 16S rRNA genes were quantified by specific qPCR assays that target 16S rRNA genes of bacteria and *Bacillales*. The X-axis indicates the treatment microcosms. Each bar represents the mean 16S rRNA gene abundance of bacteria or *Bacillales* of triplicate microcosms. The error bars are the standard error of means of triplicate microcosms. The graph legend indicates the target taxonomic group quantified.

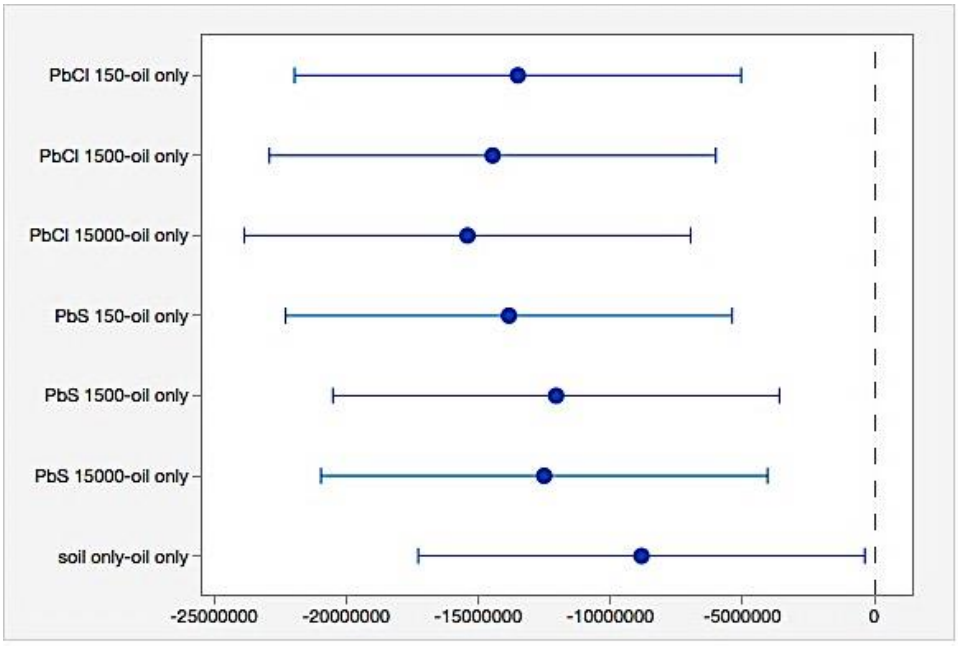


Figure 6.22: comparative analysis of the mean abundance of the Bacterial 16S rRNA gene of soils amended with Pb ($PbCl_2$ and PbS) and crude oil at Pb-concentrations of 150, 1500 and 15000 ppm relative to oil only amended soils. Analysis was by Dunnett's multiple comparison analysis at 95% confidence interval. The values include the difference between the treatment mean and the control mean. A mean is not considered significantly different if its interval encompasses zero (0).

6.4.6.4. Influence of long-term Pb-contamination on *Bacillales* abundance in soil communities

Bacillales enrichment occurred in historically Pb contaminated soils amended with crude oil. qPCR assay specifically designed to target 16S rRNA gene of *Bacillales* in the soil communities indicated that there the mean 16S rRNA gene abundance of *Bacillales* increased on amendment with crude oil (see figure 6.23). ANOVA that compared treatments showed that these differences in means were significant across treatments ($p=0.023$). The mean 16S rRNA gene of *Bacillales* in high and lower Pb soils amended with crude oil were $8.70 \times 10^{4} \pm 2.27 \times 10^{4}$ and $8.15 \times 10^{5} \pm 1.61 \times 10^{5}$ gene copies g^{-1} soil, respectively. Dunnett's pairwise comparison indicated that in high Pb soils, amendment with oil increased the mean 16S rRNA gene significantly by $8.26 \times 10^{4} \pm 1.27 \times 10^{4}$ gene copies g^{-1} soil ($p<0.001$). Conversely, although the mean 16S rRNA gene abundance in lower Pb soils amended with crude oil increased by $1.22 \times 10^{5} \pm 3.34 \times 10^{5}$ gene copies g^{-1} soil, this was not significant relative to lower Pb soil not amended with crude oil ($p=0.97$). Relative to the oil only amended soil control, the mean *Bacillales* 16S rRNA gene abundance was significantly higher in high Pb soil amended with crude oil by $7.58 \times 10^{4} \pm 1.79 \times 10^{4}$ gene copies g^{-1} soil ($p=0.0011$). Similarly, in lower Pb soils amended with crude oil, 16S rRNA gene abundance of *Bacillales* was significantly higher by $7.17 \times 10^{5} \pm 4.72 \times 10^{5}$ gene g^{-1} soil relative to oil only amended soil ($p=0.040$).

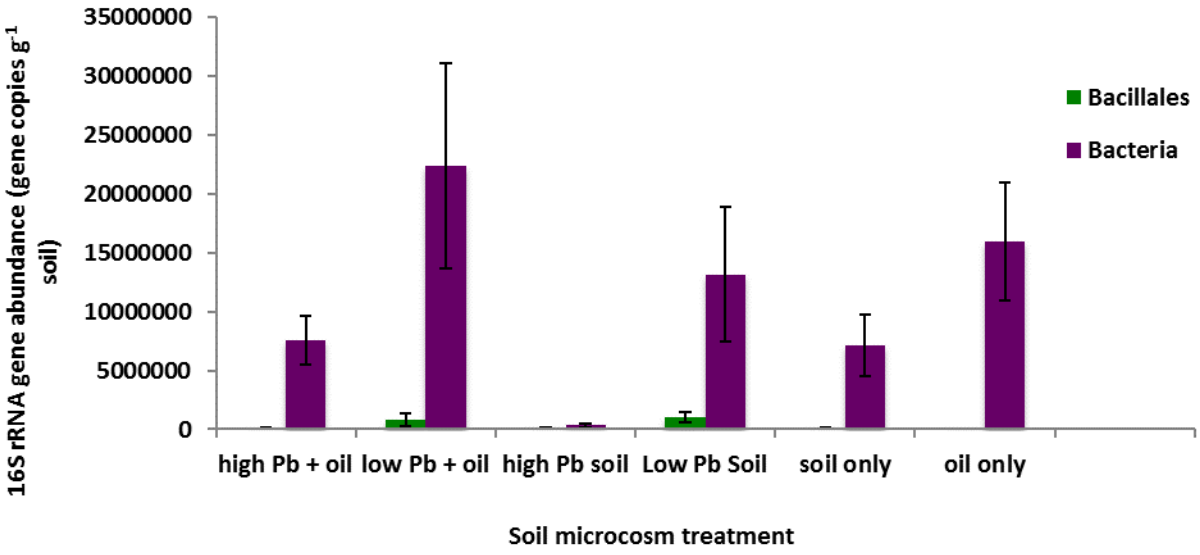


Figure 6.23: the 16S rRNA gene abundances of bacteria and *Bacillales* in historically Pb contaminated soil microcosms amended with crude oil after degradation for 15 days. The 16S rRNA genes were quantified by specific qPCR assays that target 16Sr RNA gene of general bacteria and *Bacillales*. The X-axis indicates the treatment microcosms. Each bar represents the mean 16S rRNA gene abundance of bacteria or *Bacillales* of triplicate microcosms. The error bars are the standard error of means of triplicate microcosms. The graph legend indicate the target taxonomic group quantified

6.4.7. Numerical relationships between cumulative CO_2, maximal CO_2 production rates, recovered n-alkanes after degradation period, Bacterial 16S rRNA Gene abundances and *Bacillales* 16S rRNA Gene abundances of soils co-contaminated with Pb and crude oil.

The geochemical and the microbiological data generated from the biodegradation of petroleum in long-term and short-term Pb contaminated soils have been presented separately in previous sections. In this section, a correlation analysis, which determined links between the biological and the geochemical data, is presented. Specifically a matrix based correlation analyses of cumulative CO_2, maximal CO_2 production rate, total n-alkanes recovered after degradation period, the bacterial 16S rRNA gene abundance and the 16S rRNA gene abundance of *Bacillales* was carried out. The Pearson's correlations were used to determine the strength of the relationships between variables (see table 6.4).

6.4.7.1. Correlation analysis of 16S rRNA gene abundance of bacteria relative *Bacillales* 16S rRNA gene abundance of soils co-contaminated with Pb and crude oil

Interestingly, the Pearson's correlation coefficient (r) between the bacteria and the *Bacillales* abundances in Pb-crude oil co-contaminated soils was -0.211 ($p=0.29$; $R^2= 0.044$) (see table 6.5 and figure 6.24). This implies that there is no significant relationship between the abundances of Bacteria and *Bacillales*. Hence, *Bacillales* enrichment in the degrading systems has no effect on the bacterial abundance.

6.4.7.2. Correlation of 16S rRNA gene abundances (Bacteria and *Bacillales*) relative to maximum cumulative CO_2 during the degradation of petroleum hydrocarbons in Pb contaminated soils

Correlation analysis (see table 6.5) indicated that there was no significant relationship between the bacterial abundance and the maximum cumulative CO_2 in the Pb-crude oil co-contaminated soils ($r=0.29$, $p=0.14$, $r^2=0.084$). However, with an R-value of 0.772 ($p<0.0001$; $r2=0.596$), the relationship between the *Bacillales* abundance and the maximum cumulative CO_2 is significantly strong and positive (figure 6. 25). Hence, an increase in the *Bacillales* abundance increased the cumulative CO_2 in soils contaminated with a combination of Pb and petroleum crude oil. The cumulative CO_2 as proxy for microbial activities in the soils, this interesting observation suggests that while the metabolic activities of most bacterial strains in the crude oil- Pb soil communities were inhibited, the metabolic activities of the *Bacillales* strains was not affected significantly. This is a strong evidence of the involvement of this organism in carbon turnover and possibly oil degradation hence, the identifying the important role of *Bacillales* in the in Pb-contaminated petroleum oil degrading soil communities.

6.4.7.3. Correlation of 16S rRNA gene abundances (Bacteria and *Bacillales*) with maximal rates of CO_2 production in soil microcosms during the degradation of petroleum hydrocarbons in Pb contaminated soils

The Pearson's correlation coefficient (see table 6.5) between the bacterial abundance and the maximal rate of CO_2 production indicated that there was no relationship between the bacterial abundance and the CO_2 production rate ($r=0.381$, $r^2=0.145$ $p=0.05$). However, a positive relationship was observed between the *Bacillales* abundance and the maximal rate of CO_2 production ($r=0.575$, $r^2=0.331$ $p=0.002$) (see figure 6.26). These observations suggest that the enrichment of *Bacillales* resulted from petroleum hydrocarbon mineralization in crude oil-Pb co-contaminated soils.

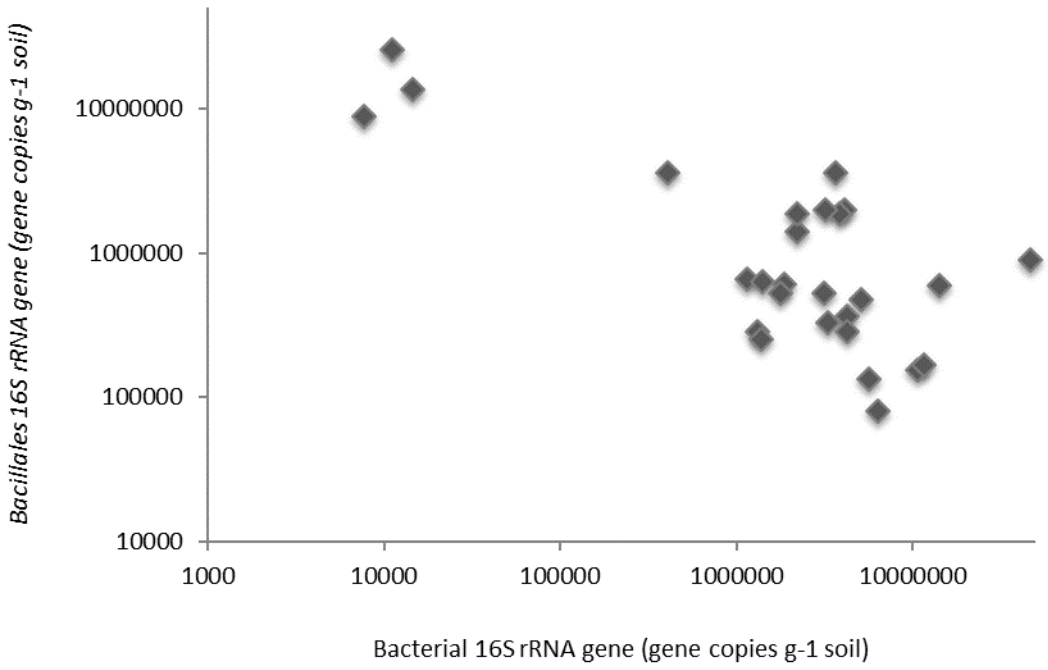


Figure 6.24: relationship between Bacterial and the *Bacillales* 16S rRNA gene abundances in Pb-crude oil co-contaminated soil microcosms. The coefficient of determination (r^2) is indicated.

Table 6.5: Pearson's correlation analysis describing the interaction between microbiological variables and geochemical variables determined from petroleum crude oil -Pb contaminated soils

		Bacteria	***Bacillales***	**Maximum cumulative CO2**	**Maximal Rates**	**n-alkanes**
Bacteria	Correlation Coefficient	1	-0.211	0.290	0.381	0.050
	Sig. (2-tailed)		0.291	0.142	0.050	0.806
	N	27	27	27	27	27
Bacillales	Correlation Coefficient	-0.211	1	0.772	0.575	-0.551
	Sig. (2-tailed)	0.291		<0.0001	0.002	0.003
	N	27	27	27	27	27
Maximum cumulative CO2	Correlation Coefficient	0.290	0.772	1	0.890	-0.591
	Sig. (2-tailed)	0.142	<0.0001		<0.0001	0.001
	N	21	21	21	21	21
Maximal Rates	Correlation Coefficient	0.381	0.575	0.890	1	-0.527
	Sig. (2-tailed)	0.050	0.002	<0.0001		0.005
	N	21	21	21	21	21
n-alkanes	Correlation Coefficient	0.050	-0.551	-0.591	-0.527	1
	Sig. (2-tailed)	0.806	0.003	0.001	0.005	
	N	21	21	21	21	21

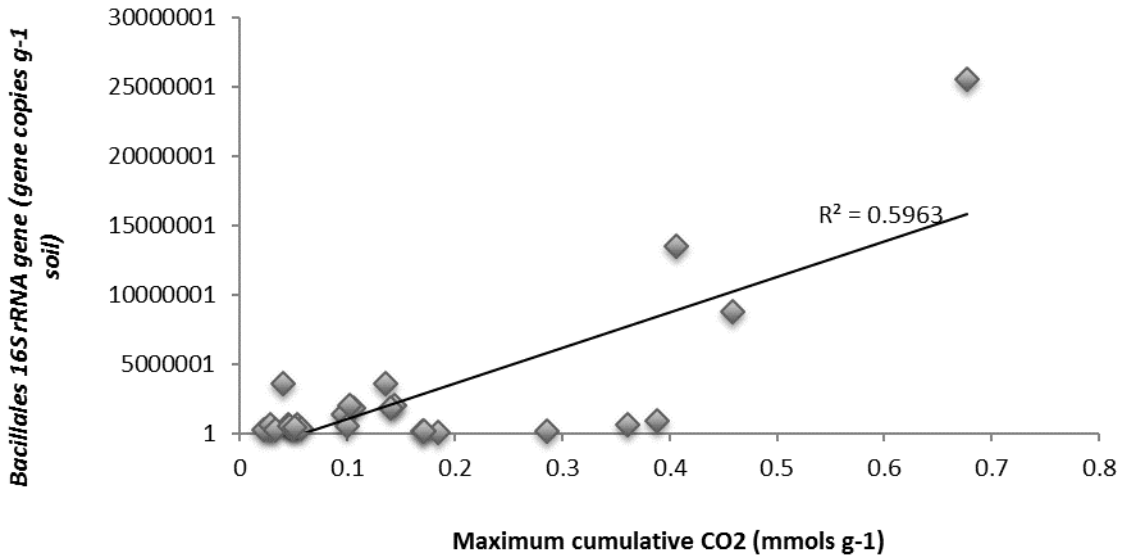


Figure 6.25: relationship between maximum cumulative CO_2 and the *Bacillales* 16S rRNA gene abundance in Pb-crude oil co-contaminated soil microcosms. The coefficient of determination (r^2) is indicated.

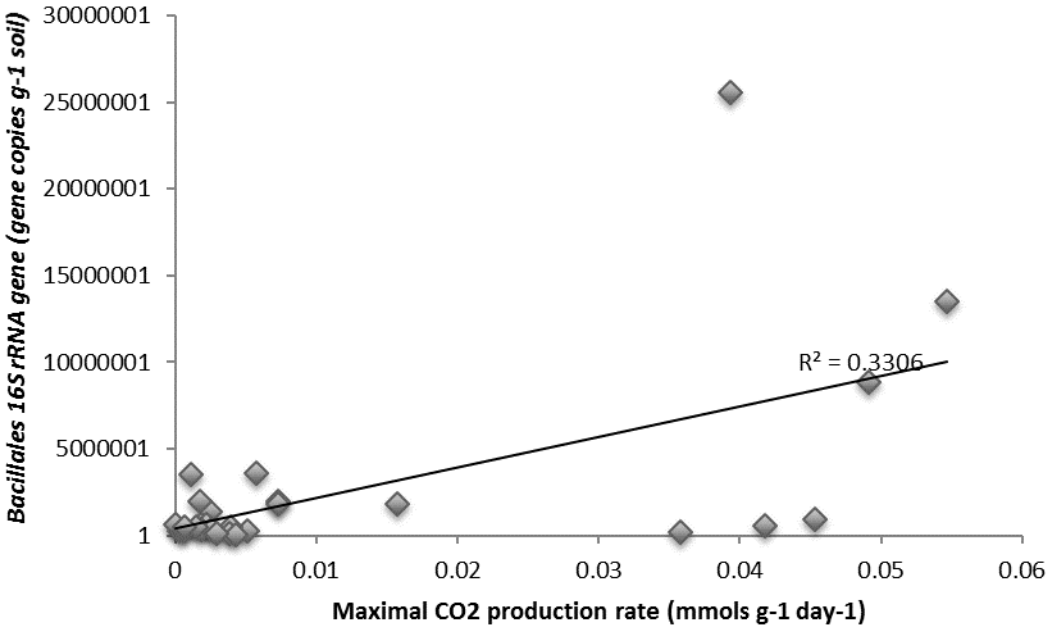


Figure 6.26: relationship between maximal CO_2 production rate and the *Bacillales* 16S rRNA gene abundance in Pb-crude oil co-contaminated soil microcosms. The coefficient of determination (r^2) is indicated.

6.4.7.4. Correlation of 16S rRNA gene abundances (Bacteria and *Bacillales*) with total n-alkanes during the degradation of petroleum hydrocarbons in Pb contaminated soils

Figures 6.27 and 6.28 show scatter plots of the total n-alkanes recovered from the microcosms after degradation relative to the bacterial and the *Bacillales* 16S rRNA gene abundances, respectively. From figure 6.27 and in addition to the r-value (see table 6.5), there was no significant relationship between bacteria and n-alkane quantity (r=0.050; r^2=0.003; p=0.806). Conversely, a negative relationship was observed between *Bacillales* abundance and total n-alkane recovered after degradation of petroleum in the Pb contaminated petroleum degrading soils (r=-0.551; r^2=0.304; p=0.003). Hence, increases in *Bacillales* abundance results in a decrease in the total n-alkanes recovered after degradation of petroleum in Pb-crude oil co-contaminated soils. These observations suggest that most bacterial strains of microbial communities of soils co-contaminated with Pb and petroleum crude oil were inactive due to the contamination. However, *Bacillales* was actively involved in the degradation of petroleum hydrocarbons in Pb-crude oil containing soils.

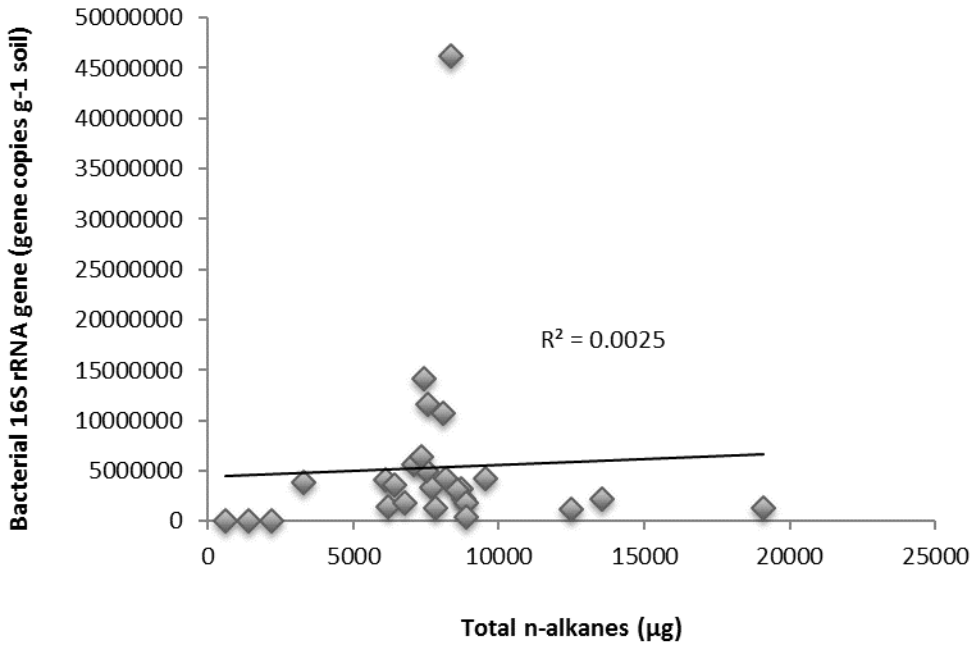


Figure 6.27: relationship between total n-alkanes recovered after a period of petroleum degradation and the bacterial 16S rRNA gene abundance in Pb-crude oil co-contaminated soil microcosms. The coefficient of determination (r^2) is indicated.

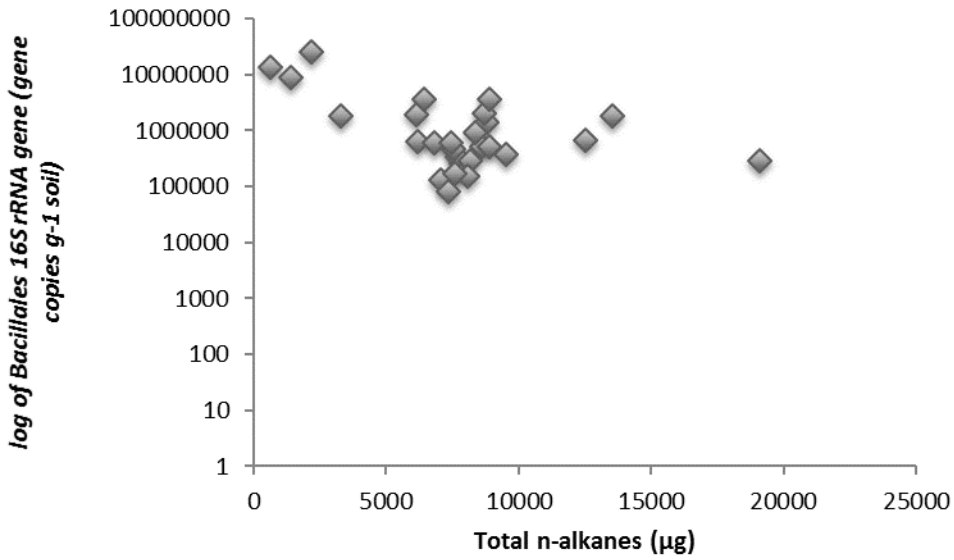


Figure 6.28: the relationship between total n-alkanes and the *Bacillales* 16S rRNA gene abundance in Pb-crude oil amended soil microcosms. The coefficient of determination (r^2) is indicated.

6.5. Discussion

6.5.1 The inhibitory effect of Pb on the microbial activities and biodegradation of petrol hydrocarbon

The maximum cumulative CO_2 and the rate of CO_2 production in short term Pb contaminated, petroleum degrading soil microcosms indicate that petroleum degradation was inhibited in the Pb-crude oil amended soil systems, irrespective of Pb form (PbS and $PbCl_2$) or concentrations (150, 1500 and 15000 ppm). Similarly, petroleum crude oil mineralization was inhibited in soil historically contaminated with high concentrations of Pb. Since CO_2 production is a proxy to metabolic activities in the soil, these observations suggest that the microbial metabolic activities were inhibited in the soil systems due to stress imposed by the presence of Pb in the systems, resulting in inhibition of important enzyme systems growth and even the death of cells. This inhibition of growth and possible cell death was corroborated by the abundance of *Bacillales* (the dominant taxonomic group) 16S rRNA genes in the soils, which significantly correlated positively with maximal rates of CO_2 production and the negative correlation with total bacteria. This result is similar to a previous research by Doelman and Haanstra (1979) who studied the effects of Pb on soil respiration (CO_2 production) and dehydrogenase

activities. Based on their studies, Pb amendment inhibited soil respiration and dehydrogenase activities in sandy and clay soils at Pb-concentration between 375 to 7500 ppm in soils. Also, Khan et al (2010) identified that addition of Pb to soil significantly decreased soil enzyme activities, microbial biomass carbon as well as the number of bacteria and *Actinomycetes* (the dominant taxonomic group). Pb like other heavy metals inhibit microbial activities by inducing oxidative stress in microbial cells leading to the loss of cell proliferation (Cao et al. 2012; Bussche and Soares 2011; Cabiscol et al. 2000). Oxidative stress is due to the production of reactive oxygen species, such as hydrogen oxide (H_2O_2) and super oxide radicals (O_2^-) in cells, which consequently impairs cell function and promote cell destruction (Harrison et al. 2007; Lemire et al. 2013; Cabiscol et al. 2000; Abha and Singh 2012; Nies 1992; Nies 1999; Sandrin and Maier 2003).

Interestingly, the maximum cumulative CO_2 and maximal rates of CO_2 production in the long-term contaminated low lead soil were not significantly different from the crude oil only amended soil control. This could be attributed to increased growth of Pb resistant hydrocarbon degraders in the soil system. The abundances of bacterial and *Bacillales* (the dominant taxonomic group) 16S rRNA gene indicated that there was significant increase in both bacteria and *Bacillales* due to amendment with crude oil. This observation is in line with the data on the maximum cumulative CO_2 and maximal rate of CO_2 production of lower Pb soil amended with crude oil. Most interestingly, the phylogenetic analysis indicated that the strain of *Bacillales*, which was dominant in the soil system, was different relative to other treatment soils. This indicates the possibility of a strain capable of concurrently resisting Pb and degrading petroleum hydrocarbons. It will be important to point out here that the long-term contaminated soils also have a history of polyaromatic hydrocarbon contamination, therefore, the possibility that Pb resisting hydrocarbon degraders dominating the soil microbial community is high. Metal resisting bacteria capable of hydrocarbon degradation have been reported in past studies (Oriomah and Adelowo 2015; Máthé et al. 2012; Riis et al. 2002; Barbieri et al. 1996; Lovley 1995). The biodiversity of the soil community and Pb resistance will be discussed in later sections.

However, in section 6.4.4.1 the total n-alkanes recovered from Pb-containing soils irrespective of Pb form, Pb concentration and duration of Pb contamination was not significantly different form undegraded crude oil. This demonstrated that little if any hydrocarbon degradation occurred in soils. One possible reason for non-degradation of petroleum hydrocarbon in Pb soil is the loss of bioavailability of hydrocarbons to the members of microbial community due to the alteration of hydrocarbon to organo-metallic complex. Metals such as Pb form complexes with organics, which renders organic substrates non-bioavailable to hydrocarbon degraders (Giller et al. 1998)(Biswas et al. 2015). Biwas et al 2015 demonstrated that the immobilization of heavy metal with Palmitic acid/stearic acid grafted clays increased the bioavailability of Poly aromatic hydrocarbon metal-PAH mixed contaminated soil.

5.5.2. Bioavailability of Pb and uptake by microbes

As has been acknowledged in earlier chapters that one factor that determines the susceptibility of microbes to metals is the bioavailability of the metals to the microbes. The sequential extraction indicated that a good percentage of the added metals are associated with exchangeable, carbonates, Fe and Mn and Organic fractions of the soil. The most mobile metal species are associated with the exchangeable fraction (Tessier et al. 1979), and these are readily available to soil biota (including soil microbes). Unsurprisingly, discrepancies were observed in the association of Pb added in different forms with soil fractions. While a relatively high concentration of Pb was associated with exchangeables, carbonates, Fe and Mn oxide and organics fractions when added as $PbCl_2$, Pb added as PbS was majorly associated with the organic fraction of soil. This could be attributed to the solubility of the Pb forms. $PbCl_2$ (with solubility in water of 10.8 g L^{-1} at 20 ^oC and a solubility product of 5.89×10^{-5} at 25 ^oC (World Health Organization International Agency for Reseach on Cancer 2006); Linke 1965) is considered slightly soluble in water. This is unlike insoluble PbS (World Health Organization International Agency for Reseach on Cancer 2006) (with solubility 6.8 X 10^{-13} g L^{-1} and solubility product of 9.04×10^{-29} (Linke 1965)). It has been documented in the past that the form of Pb determines the bioavailability of Pb, with the soluble Pb being more mobile then insoluble Pb (Canadian Council of Ministers of the

Environment 1999). Also, it appears that when Pb is bound to organic compounds (Jarosławiecka and Piotrowska-Seget 2014) it forms complexes that reduce the mobility of Pb in soils. At the same time, the formation of complexes also reduces the bioavailability of soil organic matter to soil biota (Giller et al. 1998). This, therefore, may have explained the observations in this study; Pb may have formed metal-organic complexes with the petroleum hydrocarbon thereby reducing the bioavailability of the hydrocarbon in hydrocarbon degrading microbial communities, hence, the reduced degradation of petroleum observed in degrading microcosms.

Generally, Pb is transported by binding of Pb ion to negatively charged groups on bacterial cell surface and progressive metabolism dependant intracellular uptake (Levinson et al. 1996). The mechanism of Pb uptake by microbes are through the transport pathways for divalent essential metals such as Fe, Zn and Mn (Lemire et al. 2013; Jarosławiecka and Piotrowska-Seget 2014). This way, Pb is transported as a divalent metal or complexed to organic molecules. For instance, Pb was transported into cell through CitMHS-family transporters which transports essential divalent trace metals such as Mn, Zn and Fe (Lensbouer et al. 2008; Krom et al. 2000; Blancato et al. 2006) in bacterial cell. Also, PbrT transport system, which belongs to the Iron/Lead transport system family, encodes the uptake of Pb in bacterial strains, including strains of *Bacillus* spp (Rensing and Mitra 2007).

6.5.3 Microbial community diversity of Pb-crude oil mixed contaminated soil

From this current study, combined contamination of soil with Pb and crude oil reduced the soil microbial diversity in short-term Pb contaminated soils. This can be attributed to selective enrichment of species, which are capable of growing in the presence of both petroleum hydrocarbon and Pb (notwithstanding the qPCR data on the bacteria that indicates cell death has occurred manifesting double effect in operation growth of one group and loss of lots of others). The enrichment of metal resisting hydrocarbon utilizers have been reported in previous studies (Roane and Kellogg 1996; Joynt et al. 2006a; Máthé et al. 2012; Thavamani et al. 2012; Oriomah and Adelowo 2015; Almeida et al. 2013; Shi, Becker, et al. 2002). Interestingly, DGGE and α-diversity studies revealed that Pb-crude oil mixed contaminated soil

communities of short-term Pb contaminated soil communities, irrespective of Pb form, have more diverse communities relative to oil only amended soil communities. More interesting is the fact that soils contaminated at high Pb concentration of 15,000 ppm Pb had more diversity than lower Pb concentrations of 150 ppm. Further comparison of communities (β-diversity, i.e the Yue and Clayton dissimilarity coefficient, θ) indicated that relative to oil only amended soil, community diversities of Pb soils are highly dissimilar. This observation coincided with the MDS plot of the DGGE profile of communities in which oil only amended communities were completely separated form other communities (see figure 6.18). These implied that the Pb contamination of soils (in short period) influenced the selective enrichment of species in the Pb containing soil communities. Furthermore, the diversities of communities of short-term Pb contaminated soils amended with crude oil were lower relative to unamended soil community. With lowest θ values, the communities of soils contaminated with Pb at 15000 ppm Pb (irrespective of form) were more similar to communities of unamended soil compared to other soil communities. This observation indicated that Pb concentrations influenced the microbial diversities in soils such that lower diversities occurred due to selective enrichment of species in communities at lower concentrations and little or no selective enrichment occurred at high concentrations of in short-term contaminated soils. This observation is similar to studies made by Thavamani et al (2012), who identified that the concentrations of both organic pollutant and metals differentiated the the soil microbial community structure of manufacturing gas plant soil, which was contaminated with Poly aromatic hydrocarbons (PAH), Pb, Cd and Zn. Their results indicated that diversity was more in soils with high concentrations of extractable metals and less in soils with high PAH occurrence. Furthermore, observations concurred with reports by Joynt et al, (2006), who studied the microbial community structure in soils co-contaminated with Pb, Cr and Petroleum hydrocarbon over a long period of time. But contrary to these observations, decrease in biodiversity due to high Pb- and Cr- presence in hydrocarbon co-contaminated soil community have been documented (Shi, Becker, et al. 2002). Also, the alteration of hydrocarbons to a less available form as a result of metal-organic complex formation, which rendered the organic hydrocarbons less bioavailable, and hence less sensitive to communities,(Giller et al. 1998) could be indicated in

the slight similarity between communities contaminated at 15000 ppm Pb and unamended soil control as indicated by the value of θ.

6.5.4. Microbial community phylogeny of Pb-crude oil mixed contaminated soils

In the Pb-crude oil amended soil communities, phylogentic studies carried out indicated that the most enriched taxonomic group that made up 50% of the microbial relative abundance in petroleum degrading systems with or without Pb, long term or short term Pb contaminateion, were members of the phylogenetic groups *Proteobacteria, Actinobacteria, Firmicutes, Bacteriodetes, Acidobacteria, Chloroflexi, Verrucomicrobia Plantomyceytes, , and Gemmatimonadetes* in order od descending relative abundance. In previous studies, *Proteobacterria* and *Firmicutes* were isolated from Pb-Cr-petroleum mixed contaminated soils (Joynt et al. 2006b). At the genus level, the most enriched organism was closely related to the order *Bacillales,* (which was unlike the oil only contaminated soil where a strain *Rhodococcus* species dominated the microbial populace). In addition, Correlation analysis indicated that the abundance of 16S rRNA gene of *Bacillales,* but not bacteria correlated significantly negatively with total n-alkanes recovered from microcosms after degradation and significantly positively with maximal rate of CO_2 and maximum cumulative CO_2. This implies that n-alkane degradation in Pb-contaminated soils is primarily as a result of *Bacillales* abundance in the microbial communities. Most interestingly, two different *Bacillales* strains were isolated from the lead soils. While one strain was predominant in short-term Pb contaminated soils and long-term high Pb contaminated soils with crude oil, the other was predominant in long-term lower Pb contaminated soils with crude oil. From BLAST and RDP analysis, while the former was closely related to *Scopulibacillus darangshiensis* (98% similarity) bacteria originally isolated from rock (Lee and Lee 2009), the later was closely related to *Bacillus circulans* (99% similarity) isolated from an oil shale and crude oil contaminated soil (Yilmaz 2003) as a metal tolerating hydrocarbon degrader. Other close relatives, which were 95 to 97 % similar were metal tolerating and hydrocarbon degrading strains including *Geobacillus spp* (Guazzaroni and Corte 2010), *Bacillus benzoevorans* (Pichinoty *et al*. 1984), *Bacillus arsenicoselenates* (Switzer Blum *et al*. 1998), halophilic *Bacillus*

chungangensis (Cho *et al.* 2010), thermophilic *Anoxybacillus amylolyticus* (Poli *et al*. 2006), alkaliniphilic *Bacillus foraminis* (Tiago et al. 2006)), anaerobic *Vulcanibacillus modesticaldus* (L'Haridon et al. 2006), *Bacillus gottheili* (Seiler *et al*. 2013) and *Geobacillus tepidamans* (Schäffer et al. 2004).

6.5.5. Pb resistance mechanisms

Although hydrocarbon degradation in the Pb-petroleum mixed contaminated soils studied was significantly lower than in soils not amended with Pb, there was the enrichment of soil microbial community by bacterial strains belonging to the taxonomic group, *Bacillales.* The abundances of *Bacillales* correlated with the measures of petroleum degradation in the soils, including the maximum cumulative CO2, the maximal rate of CO_2 emission and the total n-alkanes recovered after degradation. These could be attributed to the inherent resistance of the *Bacillales* strains to Pb. Bacterial Pb resistance is by bacterial exclusion of Pb, thereby limiting toxicity of Pb to resistant cells. Mechanisms of Pb resistance include adsorption of Pb on cell surface, Pb intracellular and extracellular sequestration and Efflux mechanisms (Jarosławiecka and Piotrowska-Seget 2014; Bruins et al. 2000; Nies 1999; Nies 2003).

The adsorption of Pb on cell surfaces immobilizes Pb and limits the mobility of Pb across cell envelopes. Usually, Pb binds to functional groups on cell surfaces which are usually macromoleculess carbonyl- and hydroxyl groups, bioligands such as amino groups and phosphates (Jarosławiecka and Piotrowska-Seget 2014). Bai et al. (2014) documented the immobilization of Pb by adsorption on cell envelop of *Bacillus subtilis* DBM, which reduced the transportation of Pb into the cell cytoplasm and hence, Pb toxicity to cell, in a heavy metal contaminated soil. Here, 61.5 % of sequestered Pb was isolated from the cell envelope as entrapped Pb, complexed/ precipitated Pb (with cell surface functional groups) or Pb exchanged with ions. Also, 91.7% of added Pb at concentration of 50mg L^{-1} was reported to bind to carbonyl, hydroxyl, amide and sulphamide groups of the cell surface of *Bacillus sp* ATS-2 (Cabuk et al., 2006). In addition, many microbes synthesize a sheath of extracellular polysaccharides on the cell envelope, which excludes heavy metals from the cell (Jarosławiecka and Piotrowska-Seget 2014 and rerences therein). The process of biosorption, a bioremediation strategy, is based on the capability of bacterial strains to absorb metals and has contributed to the reduction of metal

toxicity in environments. For instance *Bacillus spp.* PPS 03 and *Bacillus subtilis* PPS 04 isolated from sediment core of Paper mill effluent significantly reduced concentration of Pb (up to 85 and 86% reduction) in a bioreactor within 120 hours (Singh and Chopra 2014).

Extracellular and intracellular Pb sequestration is another mechanism by which Pb resistant *Bacillales* strains observed in this study may have reduced Pb toxicity. Pb resistant organisms secret extracellular molecules such as phosphates, siderosphores and extracellular polymeric substances that bind Pb resulting in precipitation and limitation of Pb, transport into cells. Also, the toxicity of Pb transported into bacterial cell cytoplasm is limited by intracellular sequestration by molecules such as phosphates and proteins (Jarosławiecka and Piotrowska-Seget 2014). For instance *Pseudomonas marginalis* isolated from metal contaminated soils indicated evidences of extracellular sequestration by extracellular polymeric substances (T.M. Roane 1999). Also, Levinson et al. (1996) identified intracellular sequestration of Pb which limited toxicity of Pb for up to 600 fold in resistant strain relative to wild strain of *Staphylococcus aureus*. In addition, sequestration of Pb by siderosphore-producing *Pseudomonas aeruginosa* strain 4EA have been documented in the past (Naik and Dubey 2011). Furthermore, Smt genes (SmtA and SmtB), which encode the biosynthesis of Metallothioneins (MT), that primarily sequester Zn, was expressed in *Bacillus cereus* due to the presence of Pb (Murthy et al. 2011) *and in addition* Homologue of MT protein was identified in Pb resisting *B. megaterium* (T. M. Roane 1999). MTs are cysteine-rich metal sequestering proteins for intracellular immobilization of heavy metals (Blindauer 2011). In addition, Genes bmtA which encodes metal-binding protein, lotheionein, was identified in Pb resistant *Pseudomonas aeruginosa*, strain WI-1 (Naik et al. 2012).

Another mechanism of Pb resistance by Pb- resisting bacteria involves active transportation of Pb out of the cell using the Efflux transport systems, which is energy dependant. The efflux transporter that excludes Pb from bacterial cells belongs to the protein family P1-type ATPases. In *Cupriavidus metallidurans* CH34, gene cluster *pbrTRABCD* encode transporter proteins, PbrA and PbrB, that were specific for Pb (Hynninen et al. 2009). The co-expression of PbrA and PbrB resulted to an efficient limitation of Pb in C.

metallidurans CH34. The proposed mode of action of the proteins is that PbrA non-specifically exports Pb, Zn and Cd out of cell cytoplasm and exported Pb is sequestered with inorganic Phosphate produced by PbrB (Hynninen et al. 2009). Also, Plasmid born gene, pbtTFYRABC, encodes proteins, which are similar to the products of *pbrTRABCD* of C. *metallidurans* CH34, when expressed conferred increased resistance of Pb and Cd in *A. xylosoxidans* A8 (Hložková et al. 2013).

Conclusions

The aim of this study was to understand biodegradation of petroleum hydrocarbon in long-term and short-term Pb contaminated soils. Results from studies indicated that maximal rate of petroleum degradation (determined from the maximal rate of CO_2 production) was significantly reduced in crude oil degrading short-term Pb, contaminated soils, irrespective of Pb form or concentration. However, in long-term Pb contaminated soils, while maximal rates of petroleum degradation reduced at high-Pb concentration, no effect was observed at lower lead concentration. This was attributed to the possibility of soil microbial community adaptation to the harsh environment over the long duration of Pb-contamination. Also, the n-alkane recovered from degraded soil microcosms indicated that degradation of petroleum in soil microcosms contaminated with Pb (short-term or long-term) was not significant, irrespective of Pb concentration, form or duration of Pb contamination, implying that Pb may have impaired biodegradation in the soils. The interesting aspect of this study is that despite this chemical data the other analysis (microbiological analysis) support that hydrocarbon degradation still occurs to some extent albeit at reduced rates.

The microbiological diversity studies indicated that amendment of petroleum degrading soil with Pb confers differences in the soil microbial community diversity. In addition, community diversities of Pb soils differed relative to the diversity of unamended soil control. The further phylogenetic analysis identified that Pb inhibited the growth of most hydrocarbon degraders in the soil community. While Rhodococcus dominated in metal free crude oil-amended soils, a strain of Bacillales dominated the soil communities contaminated with Pb. two Bacillales strains were isolated. While one

dominated short-term Pb contaminated soils and long-term Pb soil at high concentration, the other was isolated from long-term Pb soil contaminated at a lower concentration. While the former was closely related to an isolate from rock Scopulibacillus darangshiensis (98%), the later was closely related to hydrocarbon degrader Bacillus circulans (99% similarity).

The clear proliferation of Bacillales in the Pb soils was also indicated in the results obtained from the qPCR assay specifically designed to target the 16S rRNA gene of Bacillales of communities. While the abundance of Bacillales 16S rRNA gene significantly correlated positively with maximum cumulative CO_2 and maximal CO_2 production rate, and negatively with the weight of recovered n-alkanes, no correlation of parameters was observed with the 16S rRNA gene abundance of bacteria.

Chapter 7: Conclusions and recommendations

The aim of this project was to investigate heavy metal effects on hydrocarbon degradation in complex natural systems (soils) using a microbial ecological approach combined with rigorous geochemical analysis of the fate of individual hydrocarbon components and metals during the degradation of whole oils. Specifically, the objectives were to determine the effect of the different concentrations of the Ni, Cd and Pb on the rate of biodegradation of petroleum hydrocarbon in soil for each metal by:

1. Estimating biodegradation of petroleum hydrocarbon through the monitoring of CO_2 production in soil microcosms using GC-MS;

2. Determining the differences in the effect on biodegradation of petroleum hydrocarbon of different concentrations and forms of added heavy metals (organic complexed and inorganic; soluble or insoluble;

3. Determining the fate of added metals in the petroleum hydrocarbon contaminated soil system by sequential extraction analysis;

4. Determining the extent of degradation of the whole petroleum by identifying and quantifying n-alkanes recovered from microcosms after degradation;

5. Determining the microbiological community structures and the pattern of the microbial population enrichment under different metal type and concentration in the complex soil systems during biodegradation using molecular microbiological techniques.

The choice of metals (Ni, Cd, and Pb) reflected metals that might be present in co-contaminated sites, which are either essential or non-essential for microbial growth and each encompass different concentrations where they are putatively toxic.

7.1. Effects of heavy metals on the petroleum biodegradation in soil microcosms

The study on the effects of Ni on petroleum biodegradation in soils suggests that Ni at lower concentrations, has stimulatory or no effect on biodegradation of crude oil in soils depending on the chemical form of added Ni. Critically,

the stimulatory effect was observed in the Ni-porph amended soils and declined with increasing Ni concentration. However, in NiO soils, no effects occurred at low concentrations and increased concentration of Ni resulted in increased inhibition of biodegradation. This is unlike $NiCl_2$ amended soils where Ni effects on biodegradation were neutral irrespective of Ni concentration. These results are only explicable when it is considered that Ni can be linked indirectly to the release of metabolic enzymes, which are important during biodegradation in aerobic environments (involved in mitigation of oxygen stress) but also may require the Ni to be in complexed (chaperoned) form to reduce the inherent toxic effects. Therefore, observations made in this study suggest that Ni could be a limiting factor during the biodegradation of crude oil in the soil and that this limitation might not be easily remedied simply by adding the metal indiscriminately concerning form and concentration. These results also suggest that providing a suitable supply of Ni in the correct form and concentration could have effects on biodegradation akin to that observed for N and P addition.

Unlike Ni, Cd inhibited oil biodegradation in soils, irrespective of Cd form or concentrations and, critically, even at concentrations below those recommended by soil guideline values of the UK environmental agency and below the intervention value of Dutch list. The inhibitory effect increased with increasing concentration. In addition, the n-alkanes recovered from microcosms after the incubation period indicated that very little petroleum was degraded in the Cd amended microcosms. This effect is likely due to the toxicity of Cd binding to the active site of enzymes to reduce enzyme potency, interacting with enzyme-substrate complex, affecting the synthesis of enzyme within cells and inducing the production of ROS thereby imposing oxidative stress and consequently leading to the damages of DNA, Lipids and proteins.

Although the n-alkane recovered from Pb contaminated oil degraded soil microcosms indicated that degradation of oil in soil microcosms contaminated with Pb (short-term or long-term) was not statistically significant, irrespective of Pb concentration, form or duration of Pb contamination, the hydrocarbon degradation rates were significantly reduced in the crude oil degrading short-term Pb contaminated soils, irrespective of Pb form or concentration. However, in long-term Pb contaminated soils, while maximal rates of petroleum

degradation were reduced at very high-Pb concentration, no effect was observed at the lower lead concentration. It should be noted here that this lower Pb concentration was still relatively high in comparison to the pristine soil used in the amendment experiments. This activity in the long-term Pb contaminated soils was attributed to the possibility of soil microbial community adaptation to the harsh environment over the long duration of Pb-contamination.

7.2. Effects of heavy metals on soil microbial community diversity

There was evidence from community fingerprinting analysis of selective enrichment of metal resistant hydrocarbon degraders in microbial communities of heavy metal contaminated soils. Specifically, DGGE analysis indicated that enrichment microbial community structures of soils varied significantly in relation to Ni-form and concentration. Furthermore analysis of community diversity and structure using an OTU-based approach, where indices of diversity (specie richness, λ and $E_{H'}$ and θ) were determined, also indicated variation in microbial community diversity. Dominance by hydrocarbon degraders prominent at lower concentrations decreased with increased Ni concentration. Interestingly, no change in community diversity was observed in NiO contaminated soil at 350 ppm Ni. In Cd contaminated soils, disparity in microbial diversity was based on Cd forms. Unlike Ni, analysis using OTU-based approach indicated for Cd contaminated soil communities little dominance by hydrocarbon degraders in the community consistent with the reduced levels of hydrocarbon degradation. In Pb contaminated soils, DGGE indicated disparity in community structure, which was dependent on the length of Pb contamination, Pb concentrations and the chemical form of Pb. Further analysis using the OTU-base approach indicated slight dominance by a Pb-resistant hydrocarbon degrader. Also, θ-value indicated great differences of community structure of Pb-soils relative to oil only amended control and unamended soil control.

7.3. Effect of heavy metals on the phylogeny of microbial community

Phylogenetic studies indicated that the most dominant organisms in the Ni and Cd amended soil community after hydrocarbon degradation enrichment belonged the phylogenetic groups *Actinobacteria, Firmicutes,* α-, β-, γ-

Proteobacteria, Verrucomicrobia, Bacteriodetes, Chloroflexi Acidobacteria and *Crenarcheota* in the order of decreasing dominance. At the genus level, the dominant microorganisms were *Rhodococcus, Mycobacterium, unclassified Comamonadaceae, Nocardia and Pseudomonas* in order of decreasing dominance. The most dominant OTU in both Ni and Cd amended soils was closely related to a strain of *Rhodococcus* (100% similarity). Close relatives included strains of *Rhodococcus* that were isolated from hydrocarbon contaminated environments (*R. erythropolis* DN1, *Rhodococcus sp*. YE1, *Rhodococcus sp.* Pi71, *Rhodococccus sp.* SQ8,*R. qingshengii djl-6* and *R. jialingiae* strain djl-6-2), metal contaminated soil (uncultured bacterium resistant to uranium) and stains Isolated form extreme environments (*R. erythropolis* and *Rhodococcus sp*. Q15), However, in Pb amended soils, phylogentic studies indicated that the most abundant taxonomic groups were *Proteobacteria, Actinobacteria, Firmicutes, Bacteriodetes, Acidobacteria, Chloroflexi, Verrucomicrobia Plantomyceytes, , Gemmatimonadetes* and *Crenarcheota* in order of decreasing dominance. At the genus level, the two dominant OTUs (OTUs 2 and 5), were related to strains of *Bacillales.* Close relatives of OTU2 is *Bacillus circulans* (99%) isolated from oil shale and crude oil contaminated soil while OTU5 is closely related to a bacteria originally isolated from rock *Scopulibacillus darangshiensis* strain (98%).

7.4. The effect of heavy metals on the abundance of bacteria and dominant hydrocarbon degraders in the soil communities

qPCR assays that specifically targeted bacterial 16S rRNA genes was designed to quantify the general bacterial abundance in the heavy metal – hydrocarbon contaminated soils. Also, qPCR that specifically targeted 16S rRNA genes of *Rhodococcus* and *Bacillales* was designed to ascertain the abundances of the dominating strains in the soils.

The abundances of bacterial 16S rRNA was not significantly different across treatments in Ni and Pb contaminated soils. However, in Cd contaminated soils, bacterial abundances decreased with increased concentration of Cd. Furthermore, the abundance of *Rhodococcus* decreased significantly with increased concentration of Ni, irrespective of Ni-form. These effects, however, were not significant relative to oil only control except at 350 ppm Ni in NiO contaminated soils. In Cd contaminated soils, *Rhodococcal* enrichment

decreased logarithmically with increased metal concentrations and the *Rhodococcal* enrichment were significantly lower relative to the oil only control at all metal concentrations. In addition, in short-term Pb contaminated soils, *Bacillales* was enriched significantly in terms of abundance but enrichment of *Bacillales* decreased with increased Pb concentrations irrespective of forms. Similar observations were made in long term Pb contaminated soils.

In Ni contaminated soils, the abundance of bacteria and *Rhodococcus* correlated positively and significantly, indicating that most of the bacterial population growths in the system were from the growth of strains of *Rhodococcus*. Furthermore, bacteria as well as *Rhodococcus* correlated positively with maximum cumulative CO_2 yields produced and maximal rates of CO_2 production. However, a negative correlation was observed between the total n-alkane and bacterial as well as *Rhodococcal* abundances. Likewise, in the Cd contaminated soil, again, bacterial abundance correlated positively with the abundance of *Rhodococcus* which themselves correlated positively with maximum cumulative CO_2 yields, maximal rates of CO_2 production and negatively with total n-alkanes. In the Pb contaminated soils, there was no correlation observed between the abundances of bacteria and *Bacillales*. However, the abundance of *Bacillales* correlated positively with the maximum cumulative CO_2 and maximal rates of CO_2 production and negatively with the total n-alkanes.

7.5. General synthesis of findings and Recommendations for future research

From this study, Cd and Pb inhibited biodegradation of crude oil, which is expected given the toxic nature of these metals. However, this systematic study has determined that this inhibition occurred at threshold levels below that regarded as harmful by regulators a finding, which has profound amplifications for the assessment of the vulnerability of soils with moderate to low levels of contamination by such non-essential metals. However, despite the observed inhibition the short term additions of these metals also indicated the rapid adaptive response of a previously uncontaminated soil to

the different selective pressures imposed namely that the two metals selected for entirely different groups of dominant hydrocarbon degraders which were clearly both present in the original microbial community. While this adaptability in terms of hydrocarbon degrader selection is clear it was also found that within an individual metal gradient further selection did not take place whereby higher and lower concentrations selected for different populations with putatively higher and lower tolerances to the metal. It was interesting to note that the overall selection pattern i.e. *Bacillales* for Pb contamination compared to *Rhodococcus* more generally is backed up by the longer term Pb soil which were both present in the original microbial community. It remains to be determined whether longer-term experiments would eventually select for greater metal tolerances.

In contrast to the studies of the non-essential metals described above a more varied picture was observed in the response of the hydrocarbon degrading communities exposed to Ni. For instance, the unexpected large stimulatory effect observed when Ni was added as Ni-Porphyrin is potentially an important outcome of this work, which warrants further study. Namely, the possibility of enhancing Ni concentrations and more to the point its availability/reduced toxicity to mitigate Ni limitation during biodegradation of crude oil. These findings when applied more generally to the role of other essential metals in terms of the mechanism by which they might be supplied to stimulate degradation opens up a wide field of study.

Another, related issue that is highlighted in this study is that when contamination studies are undertaken it is not just the bulk concentrations of metals that should be considered but the chemical associations of these metals. This is strongly indicated especially by the Ni studies but to a lesser extent.

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Appendix I: Reagents for the preparation of DNA Denaturing Gels used for the ecological diversity study of the microbial communities

Reagent	0% denaturant	40% denaturant	70% denaturant
40% BisAcrylamide (ml)	50	50	50
50X TAE Buffer (ml)	4	4	4
Deionized Formamide (ml)	0	32	56
Urea (g)	0	33.6	58.8
dH2O (make up to) (ml)	200	200	200

Appendix II: Enlarged diagram of St. Anthony's Lead works concentration of Lead at the surface (mg/kg)