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Culture-Independent Analysis of Bacterial Community Composition during Bioremediation of Crude Oil-Polluted Soil

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Authors' contributions

This work was carried out by the author during her research visit as part of her PhD. Author designed the study and prepared the manuscript.

Research Article

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ABSTRACT

Aim: To use cultivation-independent techniques based on DGGE of PCR-amplified 16S rRNA gene and to evaluate bacterial community composition during bioremediation of crude oil-polluted soil.
Study Design: Molecular fingerprints of bacterial populations involved in the active phase of crude oil biodegradation were generated with DGGE after 16S rRNA gene amplification.
Place and Duration of Study: Department of Microbiology and Plant Pathology, University of Pretoria, South Africa, between March and August 2008.
Methodology: Crude oil-degrading bacteria in soil microcosms contaminated with 4% crude oil and then biostimulated with nitrogen-phosphorus-potassium inorganic fertilizer (NPK: designated PN soil), calcium ammonium nitrate (designated PU soil) and poultry droppings (designated PP soil) respectively were characterized with PCR of the gene for the small subunit (SSU) of the bacterial ribosome. Total culturable heterotrophic and hydrocarbon utilizing bacteria were enumerated using plate count and Bushnell Haas media. Total organic carbon content was measured throughout the study period to indirectly determine the effect of microbial activity on carbon content in biostimulated treatments as against controls. Gas chromatography was used to monitor hydrocarbon degradation with time while electron microscopy examined community richness during hydrocarbon degradation. Reamplified dominant DGGE bands (550bp) were cleaned up and sequenced using an ABI 3130XL genetic analyzer. Electropherograms were inspected with Chromas

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Lite 2.01. Sequence identification was performed using BLAST.
Results: Dendogram of the DGGE bands constructed using Jaccard coefficient algorithm revealed that communities from PU and PP-amended soils each formed distinct clades whereas PN treated soil showed less association when compared with PU and PP respectively. Fifty distinct bands were excised, reamplified by PCR and sequenced. Sequence analysis revealed the presence of phylogenetically distinct known hydrocarbon degrading bacteria like *Corynebacterium* spp., *Dietzia* spp., *Janibacter* sp. low G+C Gram positive bacterial clones *Nocardioides* spp., *Rhodococcus erythropolis* and uncultured bacterial clones. Forty successful sequence data obtained from the excised DGGE bands were submitted to GenBank database under accession numbers GU451069 to GU451108. Chromatograms of the residual hydrocarbons in test treatments and controls showed that biodegradation occurred markedly in treated soils in this order PN>PU>PP while no significant loss was observed in the oil-contaminated control on days zero and 42 respectively. Bacterial counts increased significantly in PN, PU and PP treatments and not in controls PC and OC. Total organic carbon increased appreciably in PN, PU and PP respectively from day zero to day 28. Electron micrographs of microbial consortia in the nutrient-amended soils revealed presence of active populations induced by biostimulation as against the sparsely populated controls.
Conclusion: The results suggest that nutrient amendment stimulates and selects indigenous soil bacteria that are able to degrade petroleum hydrocarbons.

Keywords: Sequences; DGGE; crude oil; biodegradation; bioremediation; electron micrographs.

1. INTRODUCTION

Crude oil and other petroleum hydrocarbons are chemically heterogeneous and almost ubiquitous in the environment. Not only are they found at the site of oil pollution, but chemical analysis has revealed the presence of both aliphatic and aromatic hydrocarbons, in most pristine soils and sediments (Atlas and Philp, 2005; Quatrini et al., 2008). The probable origins of these low concentrations of hydrocarbons in pristine environmental media are seepage from natural deposits and biosynthesis by plants and microorganisms (Atlas and Philp, 2005; Jiménez et al., 2007). It is therefore not surprising that hydrocarbon utilizing bacteria (HUB) are widely distributed in nature. Several investigations have demonstrated an increase in numbers of HUB in oil-polluted habitats undergoing bioremediation (Rosenberg et al., 1992; Rosenberg and Ron, 1996; Rosenberg et al., 1996; Bouchez-Naitali et al., 1999; Macnaughton et al., 1999; Williams et al., 1999; Odokuma and Ibor, 2002; Bordenave et al., 2004; Chikere and Chijioke-Osuji, 2006; Hamamura et al., 2006; Margesin et al., 2007; Rojas-Avelizapa et al., 2007; Quatrini et al., 2008; Ruberto et al., 2009). However, previous and recent works have suggested that despite an increase in the HUB percentage, the biodiversity of the bacterial community may be dramatically reduced since the presence of hydrocarbons in the environment often leads to selective enrichment of HUB, to the relative detriment of biodiversity (Leahy and Colwell, 1990; Rosenberg and Ron, 1996; Abed et al., 2002; Evans et al., 2004; Atlas and Philp, 2005; Hamamura et al., 2006; Popp et al., 2006; Quatrini et al., 2008; Rodrigues et al., 2009; Alonso-Gutierrez et al., 2011). In order to achieve hydrocarbon utilization by bacteria, a number of rate limiting nutritional requirements need to be provided. Hydrocarbons as their name implies are composed of hydrogen and carbon; therefore there is a need to supply all other elements essential for growth in the growth medium (Philp et al., 2005). These growth factors include molecular oxygen for the

oxygenases, nitrogen, phosphorus, sulphur and metals like K^+ and Na^+ (Leahy and Colwell, 1990; Atlas and Bartha, 1998; Rosenberg et al., 1998; van Hamme et al., 2003; Ollivier and Magot, 2005). Biostimulation by addition of organic and inorganic nutrients to hydrocarbon-polluted matrices has been shown to supply limiting nutrients to indigenous hydrocarbon degraders to ensure that microbial activity is enhanced for biodegradation of pollutants to occur (Chikere et al., 2012a and b). Hydrocarbon pollution usually results in nutrient depletion and destruction of viable microorganisms causing a shift in the microbial community composition of such sites.

Microbial communities can be extremely diverse. This is especially the case for soil microbial communities (Delmont et al., 2011). It is likely that not all microbes will be accessible as defined cultures in the laboratory by only employing traditional culture-dependent techniques. Unfortunately, only a fraction of the microorganisms involved in the biodegradation of pollutants such a crude oil hydrocarbons in soil can currently be cultured *in vitro* (Theron and Cloete, 2006). It has been observed that fast growing strains best adapted to particular culture conditions grow preferentially than those which are not, and therefore do not accurately represent the actual microbial community composition during aerobic biodegradation of crude oil (Malik et al., 2008). In the environment, multiple species of microorganisms share one ecological niche (e.g. degradation of a pollutant); among these, one population (or a few) that best adapts to the ecosystem conditions becomes predominant, while others exist as minorities. Molecular ecological approaches may be able to detect the dominant species, while traditional culture-dependent methods may fail to do so (Watanabe and Hamamura, 2003; Sleator et al., 2008; Christoserdova, 2010). A shortfall of culturing may not be unconnected to the fact that microorganism utilize various intra and inter species cooperative interactions for their growth in the ecosystem which include syntrophic interaction and cell-to-cell communication (Atlas and Bartha, 1998; Zengler, 2008).

The use of 16S rRNA-based approaches have proven to be powerful tools for comparing and exploring the ecology and metabolic profiling of complex microbial communities involved in pollutant degradation (Atlas and Philp, 2005; Chikere et al., 2011a; Morales and Holben, 2011; Simon and Daniel, 2011). For the past two decades, microbiologists have relied on 16S rRNA gene sequences for the identification, classification and estimation of bacterial diversity/dynamics in environmental samples through PCR and DNA sequencing (Simon and Daniel, 2009; Delmont et al., 2011; Rajendhran and Gunasekaran, 2011). The development of universal 16S rRNA gene primers and high throughput next-generation sequencing techniques have been very useful in monitoring bioremediation and developing field evidence in support of microbe-driven hydrocarbon degradation (Jones et al., 2011). In this research, bioremediation of a crude oil-polluted soil was monitored using 16S rRNA gene as a molecular marker with PCR, DGGE and sequencing to elucidate the bacterial community composition during hydrocarbon degradation. Hydrocarbon loss was monitored with gas chromatograph equipped with a flame ionization detector (GC-FID). Electron microscopy was also used to examine the structure of the bacterial consortia during the bioremediation experimental period.

2. MATERIALS AND METHODS

2.1 Bioremediation Setup

Four kilograms each of soil experimentally polluted with 4% Arabian light crude oil (sulphur:1.86m/m%; C2:0.01m/m%; C3:0.25m/m%; pour point:-27ºC; IC4:0.15m/m%; NC4: 0.94m/m%) were placed in four different 5L pots with five 1cm diameter openings at the base while the fifth 5L pot received 4kg of pristine soil. The pots were in triplicates to represent five different treatment regimens namely NPK amended soil (PN), calcium ammonium nitrate amended soil (PU), poultry droppings amended soil (PP), oil-contaminated control (OC) and pristine control (PC). All treatments except PC were contaminated with 4% (w/v) of Arabian light crude oil. The oil contaminated soil samples were thoroughly mixed with a hand trowel sanitized with 70% ethanol. For the nutrient amended soils, 25g of NPK, calcium ammonium nitrate or poultry droppings was dissolved in 200ml sterile distilled water and mixed with the contaminated soil to mix the crude oil and nutrients with the soil particles and also to enhance aeration. Microcosms were kept at room temperature in a greenhouse; nutrient treated soils were regularly watered weekly with 200ml sterile distilled water to substitute for evaporated water and also mixed every other day for aeration. The oil-contaminated and pristine controls were left undisturbed throughout the 6-week experimental period in order to underscore the role of oxygenation in hydrocarbon biodegradation. The microcosms were sampled at days zero, 7, 14, 21, 28, 35 and 42. Triplicate microcosms were sampled for each treatment and bulked to obtain a composite sample.

2.2 Enumeration of Total Heterotrophic Bacteria (THB) and Hydrocarbon Utilizing Bacteria (HUB)

Bacterial growth in the treatments and controls were determined with appropriate aliquots of soil suspensions inoculated onto Petri dishes containing plate count agar for THB and Bushnell Haas agar for HUB with crude oil supplying carbon source through the vapour phase transfer on the lids. Plates were incubated at 30ºC for 24h (THB) and 7 days (HUB) respectively.

2.3 Determination of Total Organic Carbon (TOC) Content and Crude Oil Composition

Total organic carbon (TOC) was determined during the 6-week experimental period for the oil-contaminated control and nutrient-amended soils according to Standard Soil Testing Methods for Advisory Purposes compiled by Soil Science Society of South Africa (1990) while the composition of Arabian light crude oil sample used was determined by Sasol South Africa.

2.4 Total Soil Community DNA Extraction

Total microbial community DNA from each microcosm corresponding to the five treatments was extracted from 0.25g of soil using the Zymo Research Soil Microbe DNA kit™ (Inqaba Biotech, SA) and the Bio 101 FP-120 FastPrep cell disruptor (Qbiogene, Inc. Canada).

2.5 Polymerase Chain Reaction

PCR of template DNA targeting bacterial 16S rRNA gene was conducted using 2µl volume (ca.50ng/µl) of the extracted DNA with BIO RAD MJ Mini thermal cycler. The 50µl PCR mixture contained 5µl of deoxy nucleoside triphosphates (dNTPs) mixture (2.5µM) (Promega, USA), 5µl of 5X Green Go Taq Flexi buffer (Promega, USA), 3.5µl of 25mM $MgCl_2$ (Promega, USA), 2µl each of 10pmol of both forward (primer M) and reverse (primer K) primers pA8f-GC(5'-CGC-CCG-CCG-CGC-GCG-GCG-GCG-GGC-GGG-GCG-GGG-GCA-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG-3') and KPRUN518r (5'-ATTACCGCGGCTGCTGG-3') (Maila et al., 2006; Surridge, 2007), 0.25µl of 5U/µl hot start Go Taq DNA polymerase, 2.5µl of 20mg/ml of bovine serum albumin and 27.75µl of sterile water. A reaction tube without template DNA was included as negative control. The PCR programme was as follows: denaturing step at 95ºC for 3 min, followed by 33 cycles of 30 sec at 95ºC, annealing for 30 sec at 55ºC and extension at 72ºC for 1 min, followed by a final extension at 72ºC for 7 min and then held at 4ºC. Amplified DNA was examined by electrophoresis in 1.4% agarose gel with 2µl aliquots of PCR products in 1X Tris-Acetate-EDTA buffer.

2.6 Denaturing Gradient Gel Electrophoresis and Sequencing

PCR products were subjected to DGGE according to the method described by Muyzer et al. (1993) using BioRad Dcode Multi Mutation Detection System. DNA fragments from bands were then subjected to PCR as described above. PCR products of excised bands were sequenced using an ABI 3130XL genetic analyzer (Applied Biosystems, Foster City, CA) incorporating the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA) by Inqaba Biotechnical Industries Pty. Ltd. South Africa. Electropherograms of the sequences generated were inspected with Finch TV software (Geospiza). Sequences were edited using BioEdit software prior to phylogenetic analysis while multiple sequence alignment (MSA) was done using MAAFT programme. Sequence identification was performed using the BLAST facility of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/). Each sequence was subjected to a basic local alignment search tool (BLAST) analysis on the GenBank database and matching hits, with e-values closest to 0.0 indicating a statistically plausible match, were selected for alignment.

2.7 Electron Microscopy

Electron microscopy was carried out with JEOL JSM-840 scanning electron microscope (Advanced Laboratory Solutions, South Africa). A suspension of the soil sample (1g) for each treatment and control was prepared with 9ml of normal saline (0.85%) by vortexing to disperse the bacterial cells. The soil suspension was aspirated using a syringe with a 0.2 µm Millipore membrane filter (Millipore Corporation). Afterwards, the suspension was flushed out of the syringe leaving 1ml of it. Then the suspension was fixed with 1ml of 25% glutaraldehyde leaving it for 30 min. The glutaraldehyde was discarded and sample was treated with 2ml of 0.15M sodium phosphate buffer by uncoupling the syringe and filling it again with the buffer. The buffer was run out of the syringe leaving 1ml of the buffer with the membrane filter still at the base of the syringe. The sample was left for 15min. Buffer treatment was repeated 3 times and left for 15 min after each step. Then sample was dehydrated in sequence with 50%, 70% and 90% ethanol and left after each dehydration step for 15min. Final dehydration was done with 100% ethanol and left for 15 min in between

each treatment. Membrane filters with bacterial cells were fixed onto metal coupons for gold treatment prior to electron microscopy.

2.8 Gas Chromatography

Residual hydrocarbons were extracted from PN, PU, PP and OC soils weekly starting from the zero day with 40µl of n-pentane (HPLC grade) to which 32µl of Cumene (Isopropyl benzene) was added as internal standard. The analysis was carried using a Varian 1440 GC-FID (California, USA).

3. RESULTS AND DISCUSSION

3.1 Bacterial Counts

The trends in the bacterial counts for the six-week period are shown in Figs. 1 and 2. It was observed that the nutrient amended pots PN, PU, PP peaked in the THB counts by day 21. These remained fairly stable till day 35 after which the counts started decreasing. The THB counts for the OC increased slightly by day 7, dropped by day 21, increased slowly and remained stable between days 28 and 35. After this period, the counts started decreasing. The THB for PC remained fairly stable from day zero till day 42 (Figs. 1 and 2).

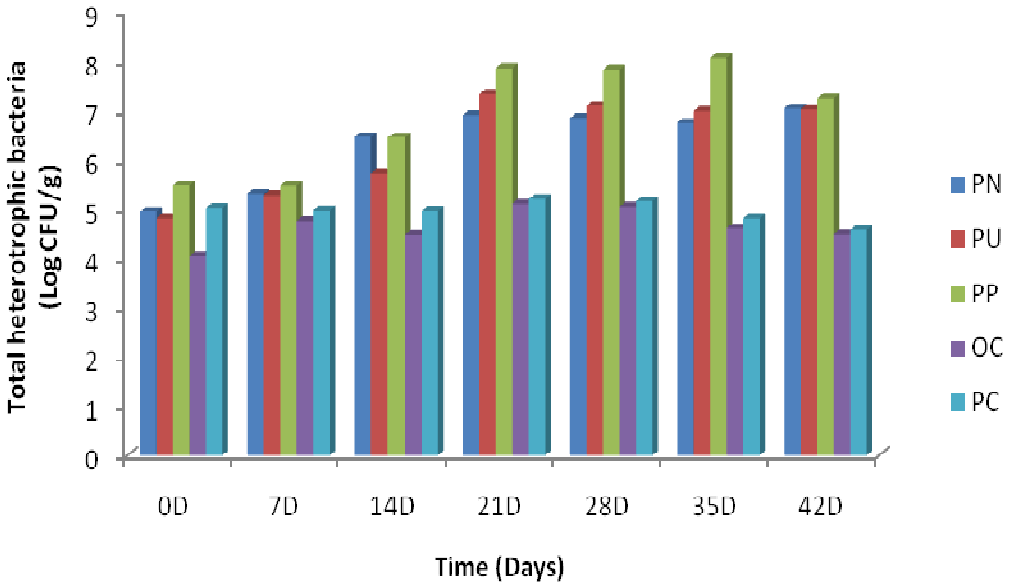


Fig. 1. Total heterotrophic bacterial count

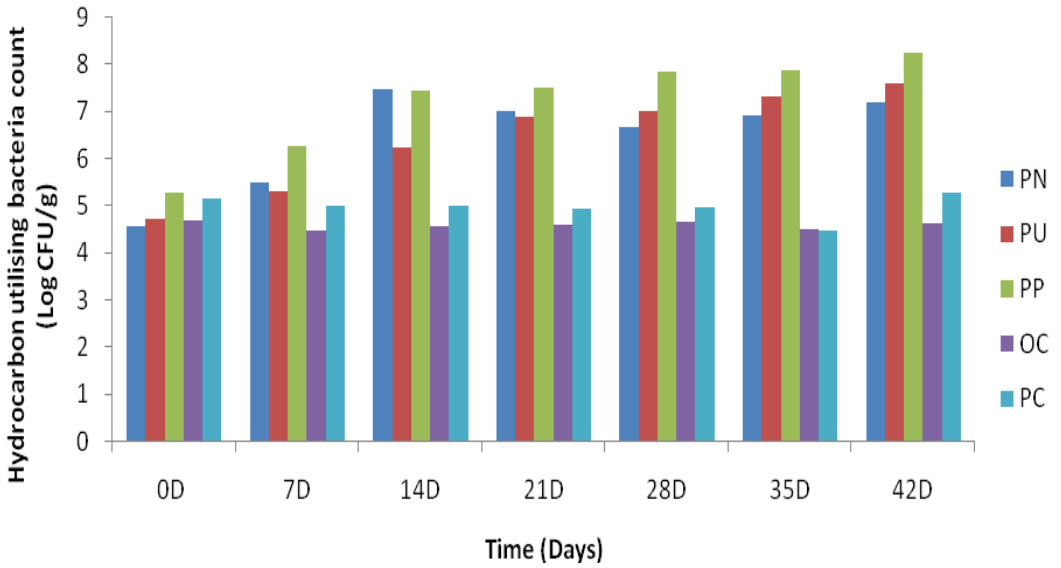


Fig. 2. Hydrocarbon utilizing bacterial count

The HUB counts for the nutrient amended pots peaked appreciably by day 14 for PN and PP while PU peaked by day 21. This period as was seen in the chromatograms for the amended treatments corresponded with the highest degradation of the hydrocarbons. However PN treatment showed the greatest loss of the pollutants by day 14 followed by PU and PP by day 21 respectively. It was observed that almost all the hydrocarbons in the amended pots were lost three weeks post oil contamination. By day 42 the heights of the hydrocarbon peaks were all reduced significantly in the nutrient amended pot in this order, PN>PU>PP (Fig. 9).

Throughout the 42 day period, PN showed marked degradation of the crude oil as shown by the chromatograms and seems to be the best treatment that significantly stimulated the extant indigenous bacteria in the soil. The bacterial counts however did not exactly reflect these trends since PP showed the highest counts for both THB and HUB counts. A similar trend was observed in the total organic content (TOC) across treatments and controls as presented in Table 2. TOC in PC was slightly stable throughout the experiment indicative of the pristine nature of the soil devoid of any hydrocarbon perturbation. TOC increased for PN, PU and PP treatments within days 0 to 7. However it decreased slightly a week later as indicated on day 14 in all the nutrient-amended treatments probably as a result of microbial activity. After day 14 TOC increased appreciably up till day 28 after which it started decreasing till day 35. It slightly increased in the treatments a week later as recorded on day 42 the end of the experiment. Between days 14 and 28 as observed in both the chromatograms and DGGE gel marked an active phase of hydrocarbon degradation and bacterial community activation. In oil-contaminated control, TOC maintained a stable trend from start to finish of the experiment indicative of reduced microbial activity probably because of the paucity of bioaccessible and bioavailable nutrients (Stroud et al., 2007).

3.2 Polymerase Chain Reaction (PCR) Products

PCR amplification of the 16S rRNA gene fragment was obtained for all soil extracts indicating that DNA was successfully extracted and inhibition of the PCR had not occurred. The extracted DNA samples all yielded PCR products of *ca*. 550bp. The PCR products were visualized in 1.4% tris acetate EDTA (TAE) agarose gel with ethidium bromide (Fig. 3).

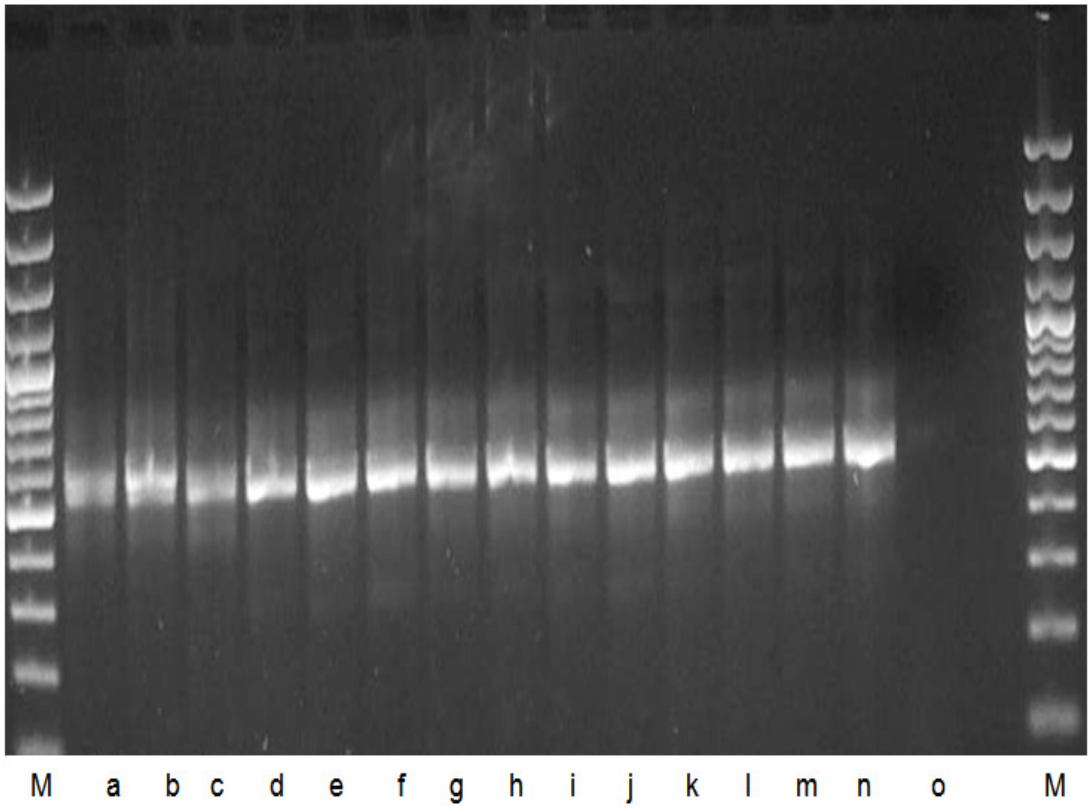


Fig. 3. Gel electrophoresis of PCR products amplified from DNA extracted from soil samples in 1.4% agarose

(M) 100 bp ladder; (a) pristine control baseline; (b) PN 0D; (c) PU 0D; (d) PP 0D; (e) OC 0D; (f) PN 14D; (g) PU 14D; (h) PP 14D; (i) PU 21D; (j) PP 28D; (k) PN 42D; (l) PU 42D; (m) PP 42D; (n) OC 42D; (o) negative control. PN – NPK treatment; PU – calcium ammonium nitrate treatment; PP – Poultry droppings treatment; OC – oil-contaminated control.

3.3 Denaturing Gradient Gel Electrophoresis (DGGE)

Molecular fingerprints of the bacterial populations associated with crude oil biodegradation as generated using DGGE is presented in Fig. 4. DGGE yielded a gel showing clear multiple banding, forming a fingerprint in each lane. The biostimulated treatments namely PN, PU and PP showed the presence of distinguishable bands representing operational taxanomic units (OTU) dominant during hydrocarbon attenuation as at the days of sampling while none appeared in the oil-contaminated (OC) and pristine (PC) controls respectively. Fifty bands were excised from the gel and further sequenced to ascertain their identity using BLAST algorithm in the NCBI database. Due to the basis of DGGE, PCR fragments of the same size

are separated into bands according to their sequence, the resulting fingerprint pattern being indicative of species diversity. From the gel a graphic cluster representation (Fig. 5) of the band pattern was generated using the software Gel2K (Norland, 2004). The programme does this by estimating band peak intensity along a lane. Dominant sequences per lane are indicated as dark prominent bands across the lane.

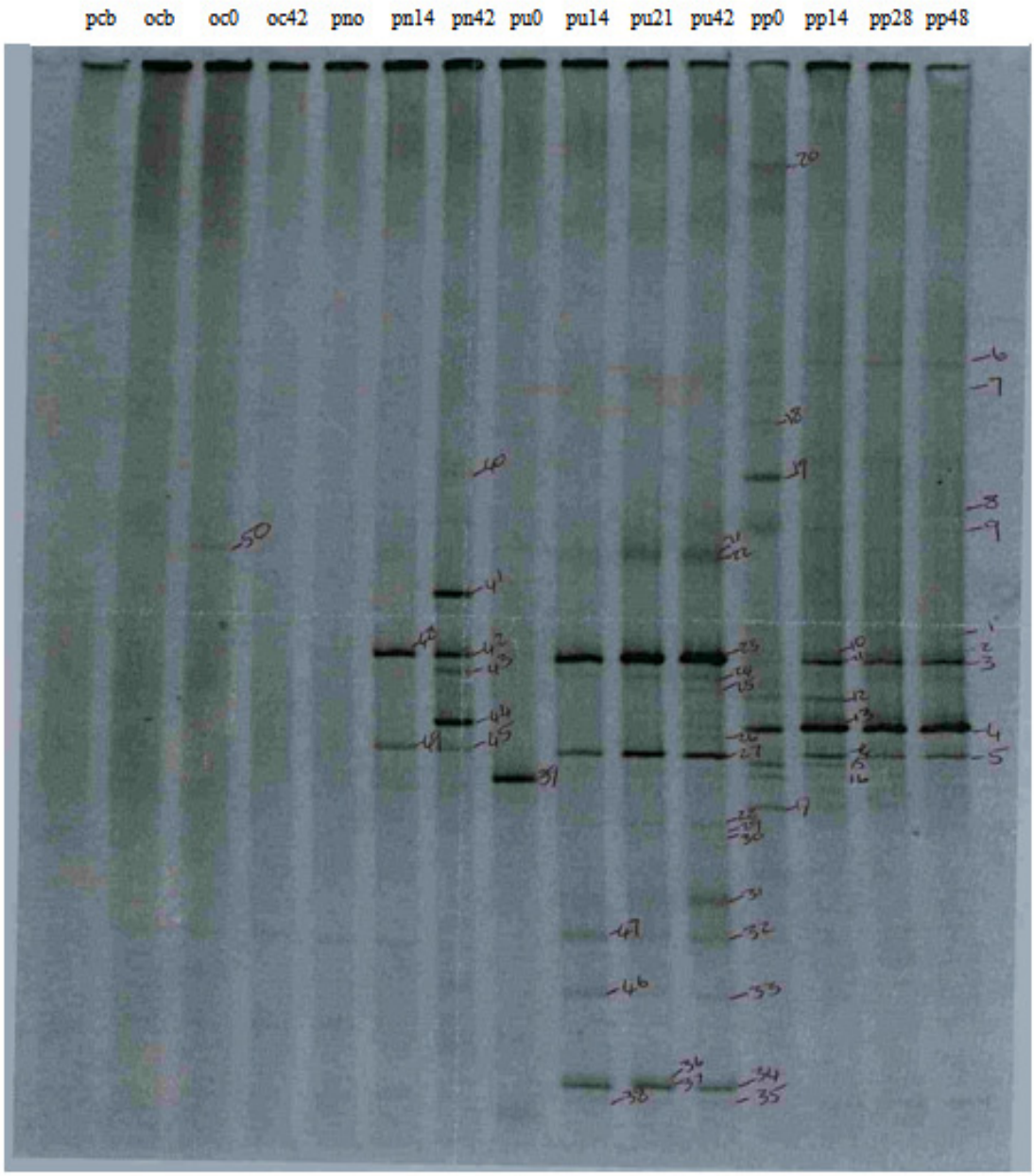


Fig. 4. DGGE Bands 1-50 across the different treatments and controls excised for sequencing and BLAST

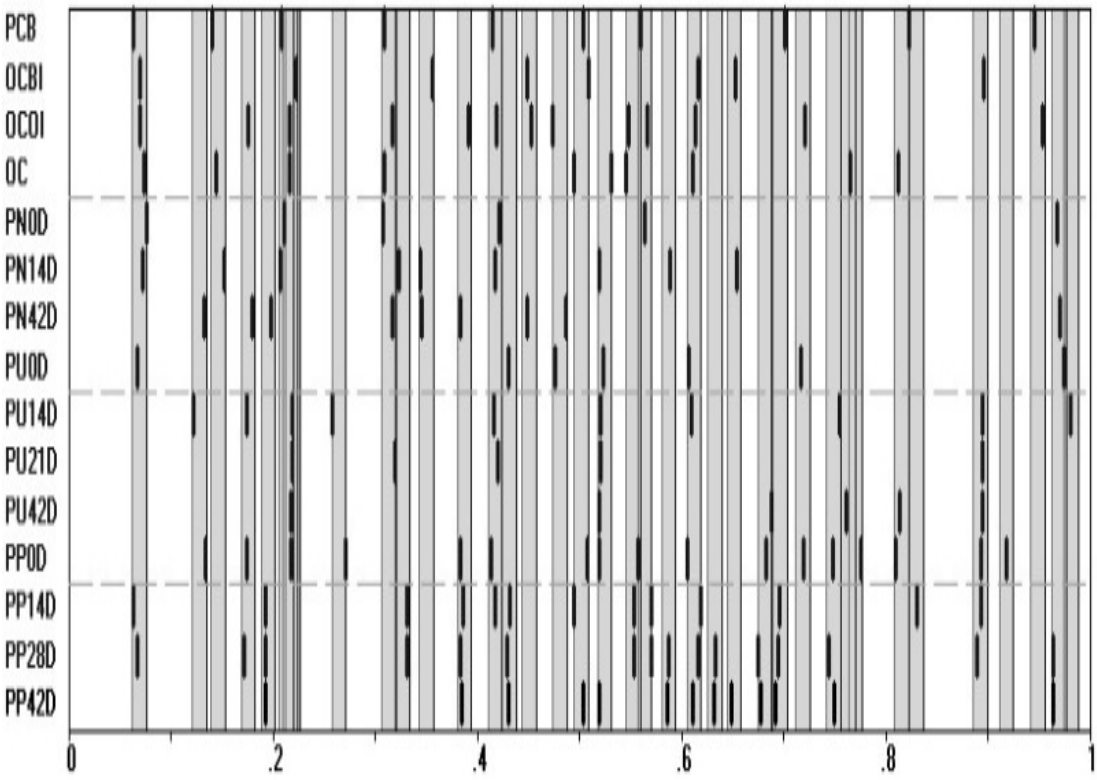


Fig. 5. Graphical representation of the DGGE gel using a denaturing gradient of 25-55%, 16S rRNA gene PCR products separated based on base-pair sequence differences indicating community richness and bacterial diversity in soil microcosms during bioremediation

PCB: pristine control baseline; OCB: oil-contaminated control baseline (immediately after crude oil contamination); OCOI: oil-contaminated control day 0; OC42: oil-contaminated control day 42; PN0D; NPK amended soil day 0; PN14D: NPK-amended soil day 14; PN42D: NPK-amended soil day 42; PU0: calcium ammonium nitrate amended soil day 0; PU14D: calcium ammonium nitrate amended soil day 14; PU21D: calcium ammonium nitrate amended soil day 21; PU42D: calcium ammonium amended soil day 42; PP0D: poultry droppings amended soil day 0; PP14D: poultry droppings amended soil day 14; PP28D: poultry droppings amended soil day 28; PP42D: poultry dropping amended soil day 42.

Following this, species diversity and to certain extent species richness, were derived from the DGGE gel by compiling a dendogram (cluster analysis) using Jaccard coefficient – group average setting (Fig. 6) of the CLUST software (Norland, 2004). The programme CLUST is based on Shannon index algorithms and groups the profiles of the species in each sample according to how similar in community composition the samples are. Thus, samples from similar treatments would be expected to display analogous communities and group together in the CLUST dendogram. Using Jaccard coefficient- group average setting, two main clusters were formed with four sub clusters which were (1) PCB, PN0D, PN14D and OC42D; (2) OCBI, PU14, PP0D, PU21D and PU42D; (3) OC0I and PN42D; (4) PU0D, PP14D, PP28D and PP42D. The dendogram revealed that PU and PP amended soils formed distinct clusters indicating that each of the treatments selected similar bacterial communities as biodegradation progressed whereas PN treated soil showed less association when

compared with PU and PP respectively. It could be inferred from this that biostimulation and time course had a great effect on the nutrient-amended soils relative to the oil-contaminated and pristine controls respectively.

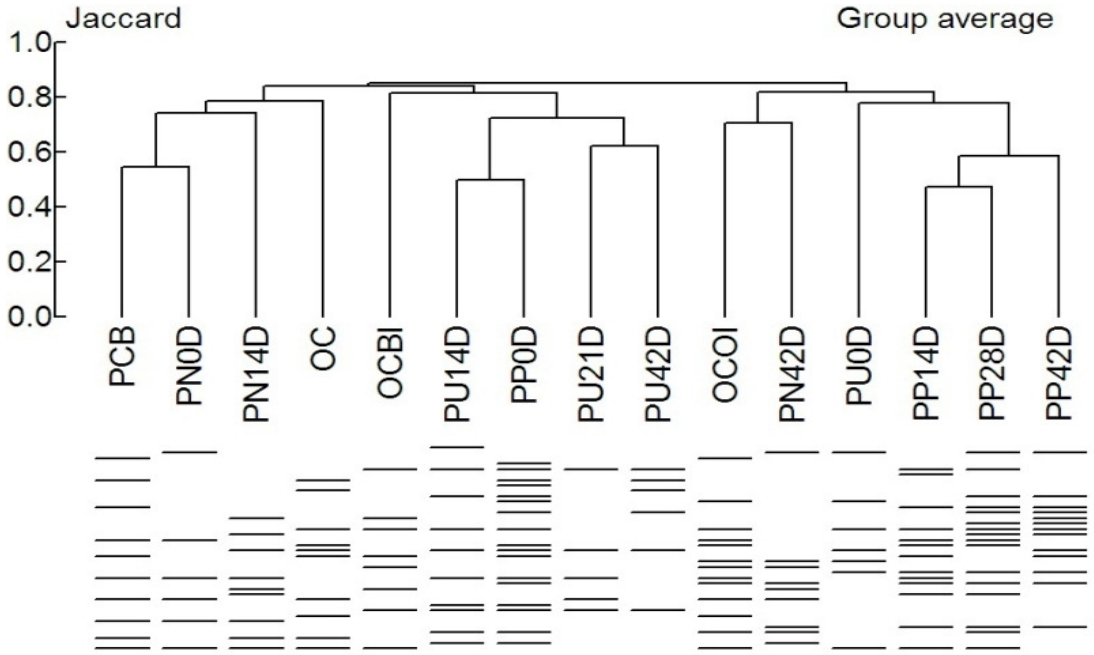


Fig. 6. Cluster analysis of the banding pattern in Fig. 2 using Jaccard coefficient group average setting to separate communities according to species sequence differences

PCB: pristine control baseline; OCB: oil-contaminated control baseline (immediately after crude oil contamination); OCOI: oil-contaminated control day 0; OC42: oil-contaminated control day 42; PN0D; NPK amended soil day 0; PN14D: NPK-amended soil day 14; PN42D: NPK-amended soil day 42; PU0: calcium ammonium nitrate amended soil day 0; PU14D: calcium ammonium nitrate amended soil day 14; PU21D: calcium ammonium nitrate amended soil day 21; PU42D: calcium ammonium amended soil day 42; PP0D: poultry droppings amended soil day 0; PP14D: poultry droppings amended soil day 14; PP28D: poultry droppings amended soil day 28; PP42D: poultry dropping amended soil day 42.

3.4 Sequencing

Fifty bands were excised from the DGGE gel as indicated in Fig. 4 and sequenced. Phylogenetic affiliations of the 16S rRNA gene sequences obtained from the study that had 85% to 100% similarity with those already deposited in GenBank were used to assign identities to them and as such considered close relatives. Forty out of the fifty DNA bands yielded good sequences which have been deposited in GenBank with accession numbers GU451069 to GU451108 as shown in Table 1. Sequencing revealed that dominant bacterial populations in the biostimulated treatments were mainly Gram positive bacteria which comprised *Corynebacterium* spp., *Dietzia* spp., Low G+C Gram positive bacteria, uncultured bacterial clones, *Janibacter* sp., *Nocardioides* spp. and *Rhodococcus* sp all belonging to the Actinobacteria and Firmicutes groups.

Table 1. Sequence identification of DGGE bands 1 – 50

DGGE band	Accession no.	Phylogenetic group	Closest GenBank relative	Source of GenBank relative	Maximum identity
1	GU451069	Firmicutes	Low G+C Gram positive bacterium T135	Compost	99%
2	GU451070	Firmicutes	Low G+C Gram positive bacterium T135	Compost	98%
3	na	na	na	na	na
4	GU451071	Firmicutes	Low G+C Gram positive bacterium T135	Compost	99%
5	GU451072	Actinobacteria	*Corynebacterium terpenotabidum*	Squalene degrading bacterium	91%
6	GU451073	Firmicutes	Low G+C Gram positive bacterium T135	Compost	96%
7	GU451074	Actinobacteria	*Corynebacterium* CNJ954 PL04	Marine sediments	97%
8	GU451075	Firmicutes	Low G+C Gram positive bacterium T135	Compost	97%
9	GU451076	na	Uncultured bacterium	Solid waste compost	98%
10	GU451077	Actinobacteria	*Corynebacterium* sp. IC10	Oil-contaminated site	95%
11	GU451078	Actinobacteria	*Corynebacterium* sp. CNJ954 PL04	Marine sediments	93%
12	GU451079	Firmicutes	Low G+C Gram positive bacteria T135	Garbage compost	99%
13	GU451080	Firmicutes	Low G+C Gram positive bacteria T135	Garbage compost	99%
14	GU451081	Actinobacteria	*Corynebacterium* sp. IC10	Oil-contaminated site	95%
15	GU451082	“	“	“	95%
16	GU451083	“	“	“	94%
17	na	na	na	na	na
18	GU451084	Firmicutes	Uncultured bacterium clone OB21	Compost	98%
19	GU451085	Firmicutes	Uncultured bacterium clone OB22	Compost	97%
20	na	na	na	na	na
21	na	na	na	na	na
22	na	na	na	na	na
23	GU451086	Actinobacteria	*Corynebacterium* sp. IC10	Oil-contaminated site	99%

24	GU451087	"	"	"	99%
25	na	na	na	na	na
26	GU451088	na	bacterium TI71	na	90%
27	GU451089	na	uncultured bacterium	naked mole-rat faeces	100%
28	GU451090	Actinobacteria	*Dietzia* sp. P27-19	marine sediments	85%
29	GU451091	Actinobacteria	*Nocardioides* sp. A-3	Dioxin polluted site	85%
30	GU451092	Actinobacteria	*Rhodococcus erythropolis* strain 51T7	tetradecane-degrading strain	85%
31	GU451093	Actinobacteria	*Nocardioides* sp. RS3-1	Polychlorinated dioxin polluted soil	94%
32	GU451094	Actinobacteria	*Dietzia* sp. 100N22-3	Deep sea sediments	93%
33	GU451095	Actinobacteria	*Dietzia* sp. AL410	Oil reservoir	99%
34	GU451096	Actinobacteria	*Dietzia* sp. CNJ898 PL04	Marine sediments	100%
35	GU451097	Actinobacteria	*Dietzia* sp. Ice-oil-101	Petroleum hydrocarbon-polluted sea	89%
36	GU451098	Actinobacteria	*Dietzia* sp. A14101	Oil reservoir	100%
37	GU451099	Actinobacteria	*Dietzia* sp. KUA-5	cyclohexylacetic acid-contaminated site	99%
38	GU451100	Actinobacteria	*Janibacter* sp. 20/6G5	Diesel contaminated site	94%
39	GU451101	Chloroflexi	*Kouleothrix aurantica*	Activated sludge	88%
40	na	na	na	na	na
41	na	na	na	na	na
42	GU451102	Actinobacteria	*Corynebacterium* sp. IC10	Oil contaminated site	97%
43	na	na	na	na	na
44	GU451103	Firmicutes	low G+C Gram-positive bacterium T135	Compost	98%
45	GU451104	Actinobacteria	*Corynebacterium* sp. CNJ954 PL04	Marine sediments	95%
46	GU451105	Actinobacteria	*Dietzia* sp. OB5	Diesel-contaminated site	96%
47	GU451106	Actinobacteria	*Dietzia* sp. JTS6048-306	Deep sea	88%
48	GU451107	Actinobacteria	*Corynebacterium* sp. IC10	Oil contaminated site	99%
49	GU451108	"	"	"	98%
50	na	na	na	na	na

[na-] not available

Table 2. Total organic carbon (TOC) content across treatments and controls

Sample ID	PC	OC	PN	PU	PP
Days					
0	0.37	1.90	2.10	1.92	2.01
7	0.36	2.17	2.29	2.15	2.24
14	0.26	1.91	2.15	2.10	2.2
21	0.21	2.08	2.26	2.33	2.37
28	0.27	1.81	2.44	2.37	2.51
35	0.22	1.71	2.11	2.09	1.67
42	0.29	1.87	2.11	2.20	2.07

PC: pristine control; OC: oil-contaminated control; PN: NPK-treated soil; PU: calcium ammonium nitrate –treated soil; PP: poultry droppings-treated soil

3.5 Electron Microscopy

The electron micrographs of the bacterial populations in the controls (PC and OC) and those that responded to biostimulation in the amended treatments (PN, PU and PP) are shown in Figs. 7 to 10. The two controls harboured few bacterial populations as shown in Fig. 7 when compared with the biostimulated treatments which evidenced robust consortia involved in crude oil degradation following nutrient amendment (Figs. 8 to 10). In the DGGE gel as well, molecular fingerprints observed in the two controls showed no prominence and changes in banding pattern during the experimental period. Conversely, the populations seen in the electron micrographs for the biostimulated treatments were clearly due to nutrient addition which stimulated increased microbial activities in the consortium. For PN treatment (Fig. 8a and b), *Bacillus* spp. and *Corynebacterium* spp.-like bacilli were the most dominant bacterial groups seen in the consortium. From the sequencing done, sequences of low G+C bacteria (this group comprises two genera *Bacillus* which are aerobic and *Clostridium* which are anaerobic) and *Corynebacterium* spp. with maximum identity of 95-100% with NCBI database closest relatives were obtained and this corroborates the electron microscopy results. PP treatment as shown in the electron micrographs in Fig. 9 contained consortium mainly of *Corynebacterium* spp. and *Bacillus* spp.-like organisms as well. Spiral-shaped bacteria were also observed in the consortium which could not be identified. These may probably be amongst uncultured bacterial clones that their sequences were obtained in the PP treatment. PU treatment as shown in the electron micrograph in Fig. 10 contained consortium comprising *Corynebacterium* spp., *Dietzia* spp.-like organisms and flagellated bacteria which may likely be Gram negative bacterial species *Burkholderia*. Sequences of these bacteria were obtained in the first DGGE after the gel gradient was optimized to obtain a better banding pattern. *Corynebacterium* spp. were the dominant bacteria in the electron micrographs of all the biostimulated treatments (PN, PU and PP) and also *Corynebacterium* sequences were recovered across all amended treatments.

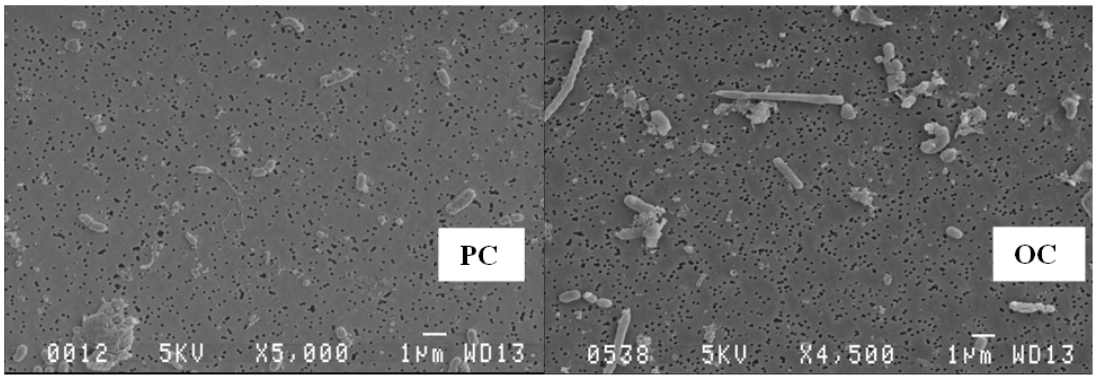


Fig. 7. Electron micrograph of the consortium in pristine control (PC) and oil-contaminated control (OC)

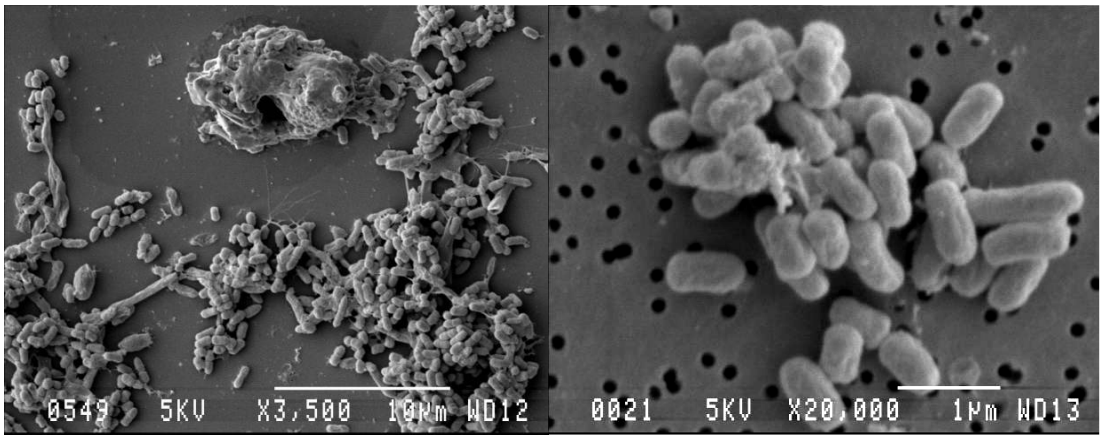


Fig. 8a. Consortium in NPK amended (PN) treatment

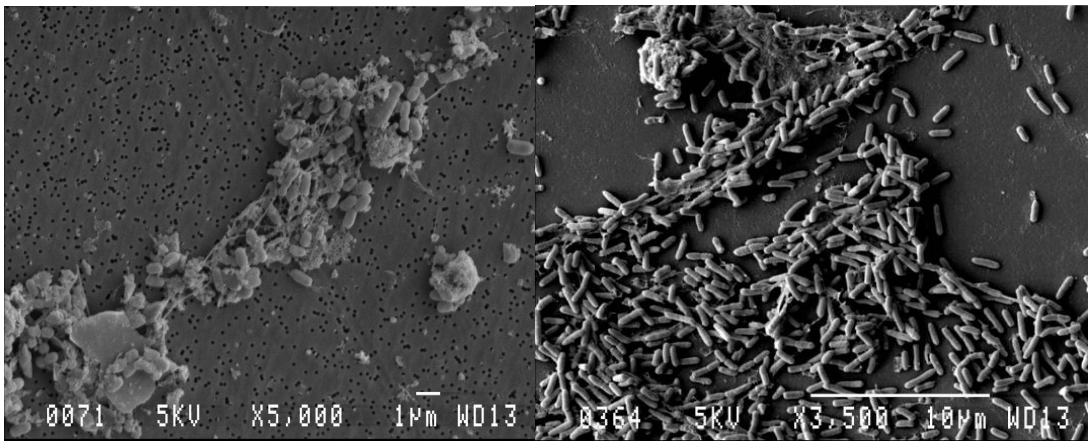


Fig. 8b. Consortium in NPK amended (PN) treatment

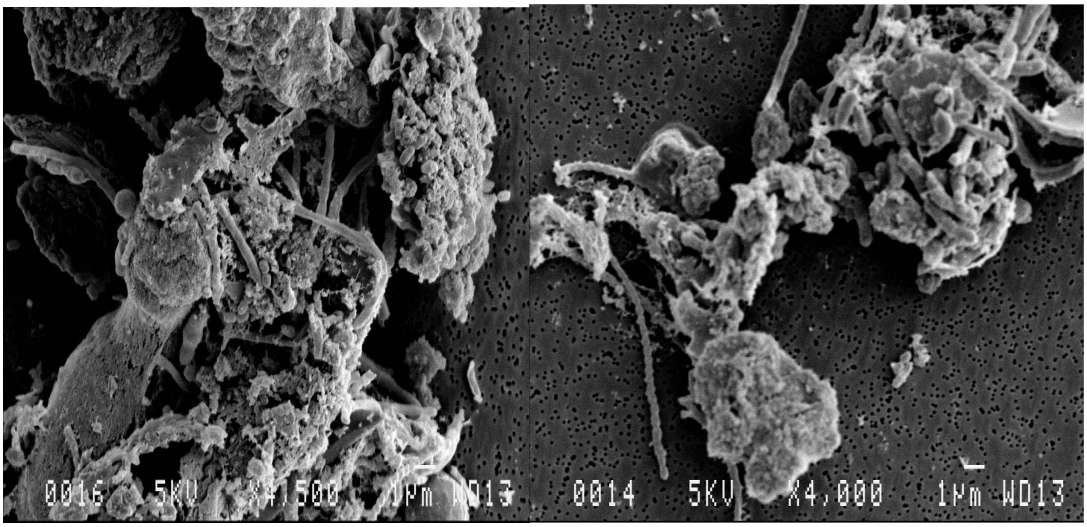


Fig. 9. Consortium in poultry droppings (PP) amended treatment

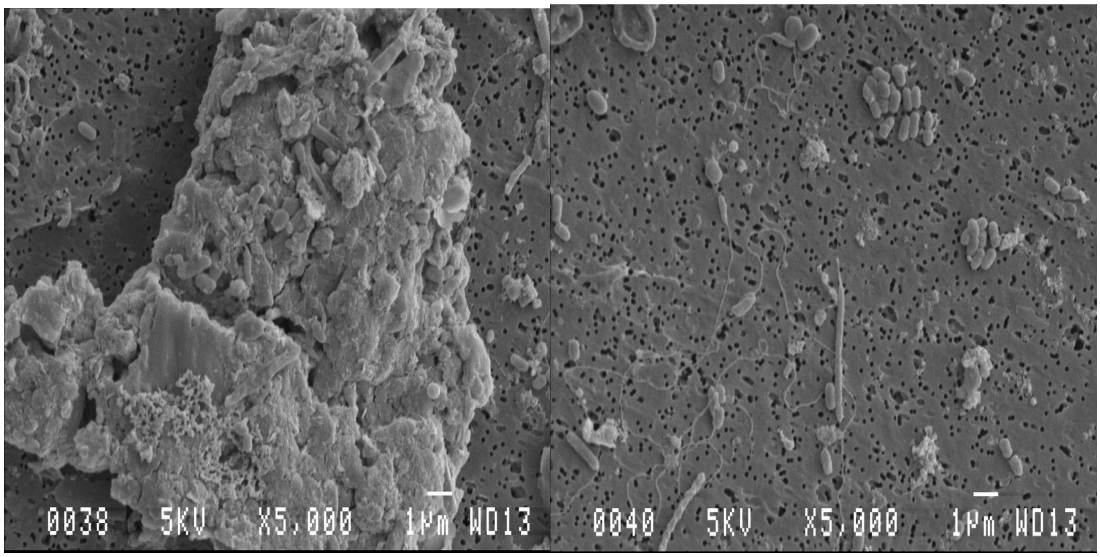
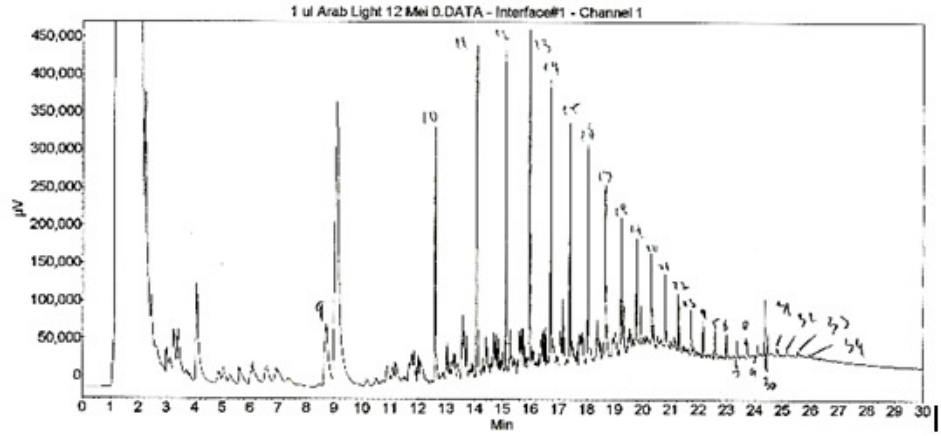
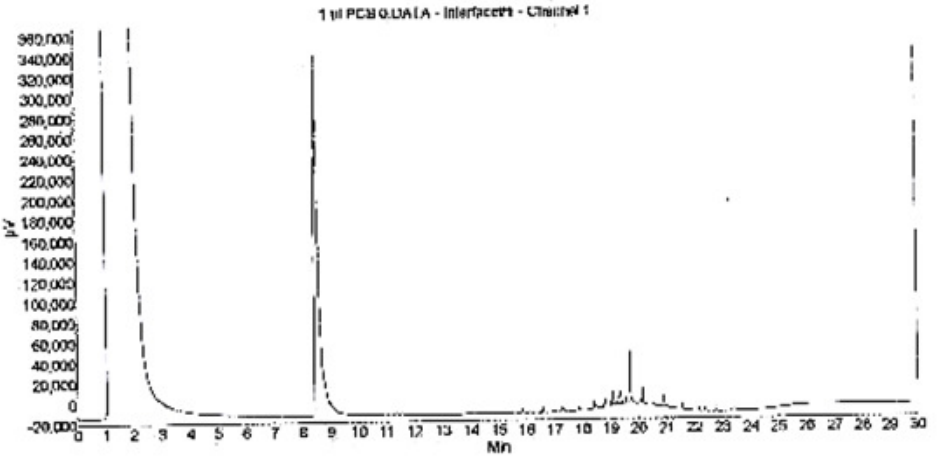


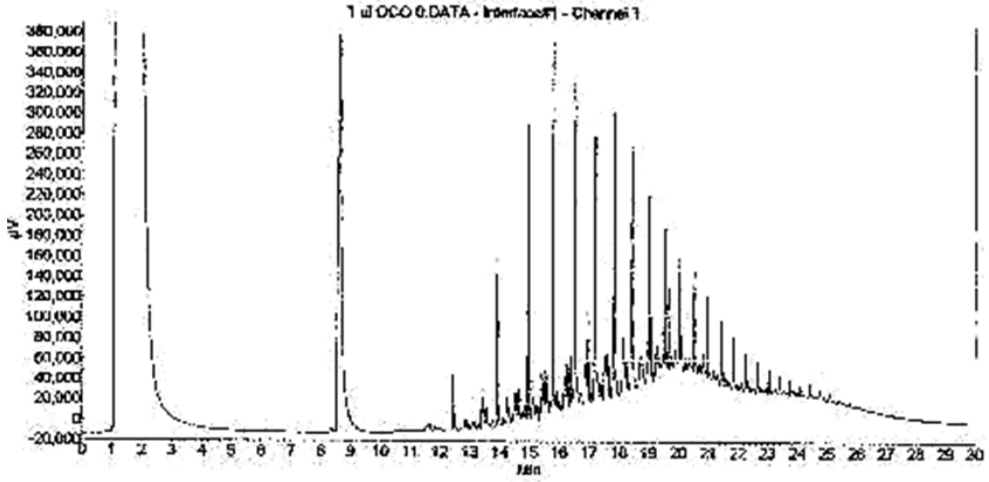
Fig. 10. Consortium in urea amended (PU) treatment



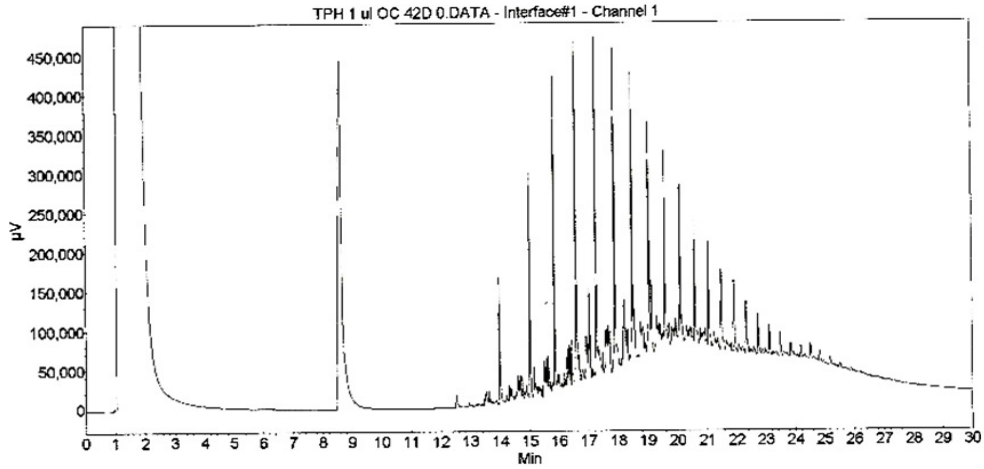
Crude oil sample



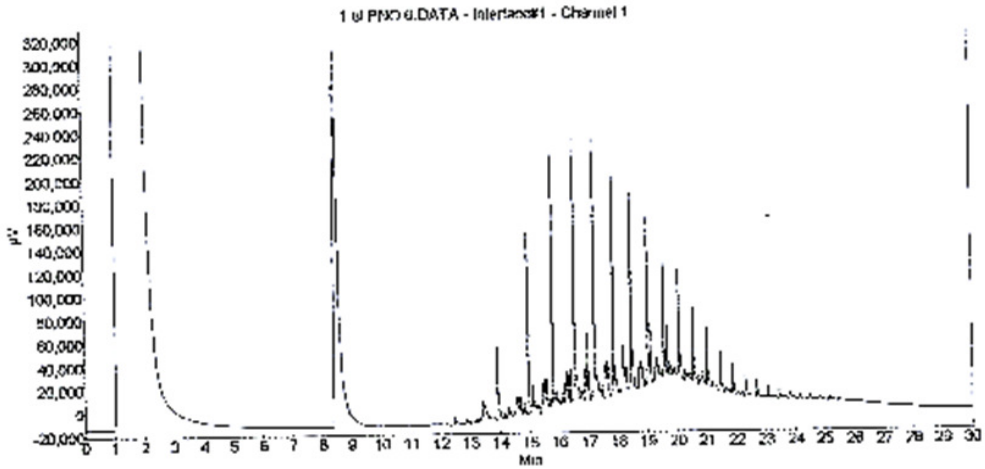
PCB



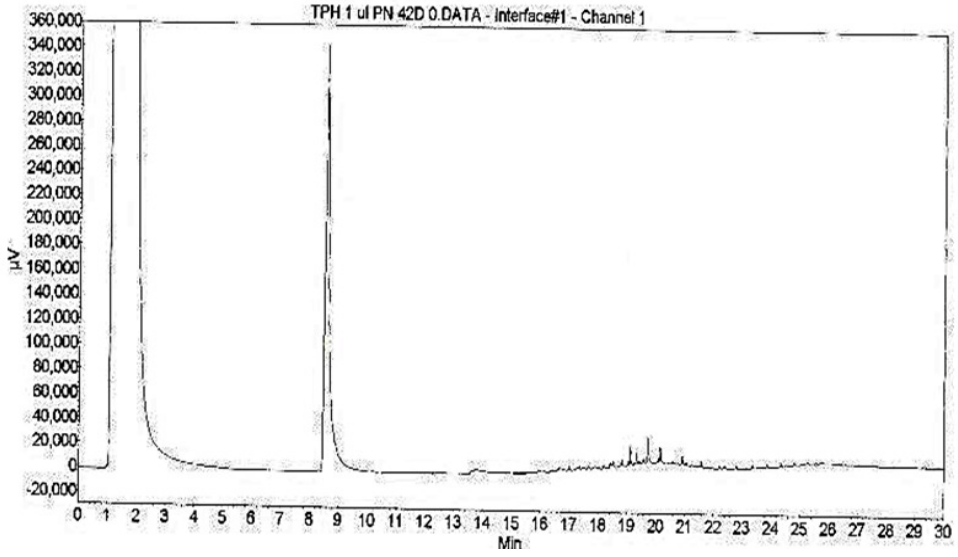
OC0



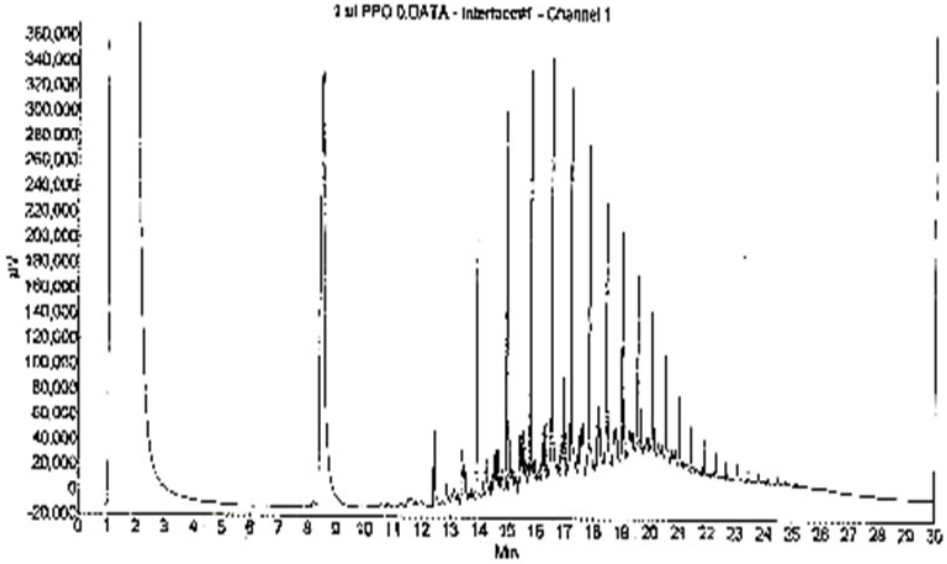
OC42



PN0



PN42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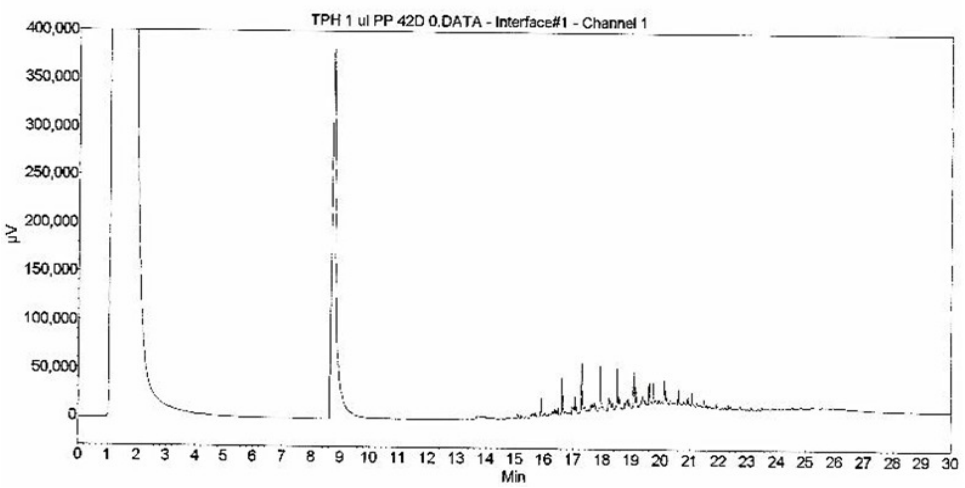
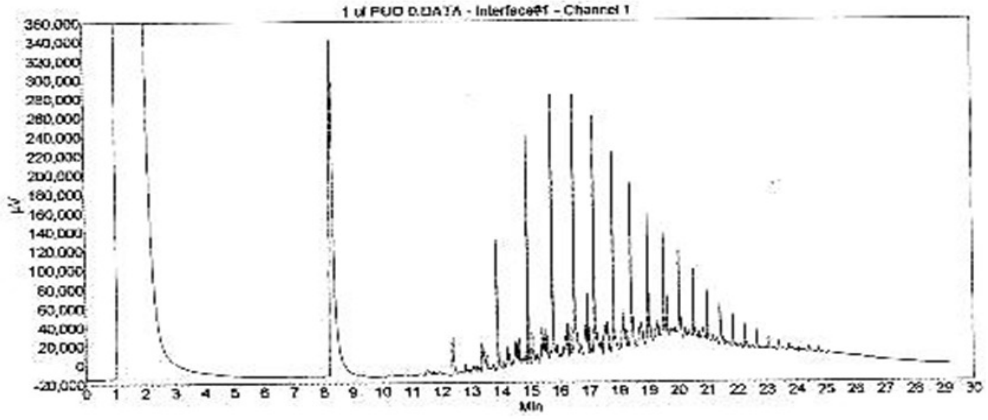
µV
Min

PP0


TPH 1 ul PP 42D 0.DATA - Interface#1 - Channel 1
µV
Min

PP42


1 ul PU0 0.DATA - Interface#1 - Channel 1
µV
Min

PU0

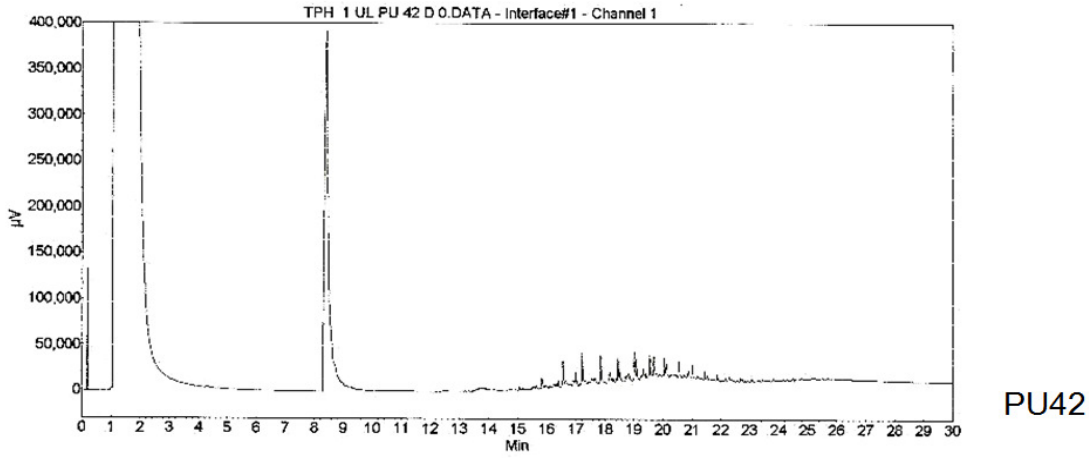


Fig. 11. Chromatograms showing hydrocarbons in crude oil sample and pristine soil (PCB); residual hydrocarbons extracted from oil-contaminated control days 0 and 42, PN, PU and PP treatments days zero and 42

3.6 Gas Chromatographic Analysis to Monitor Hydrocarbon Attenuation

Chromatograms of the hydrocarbons extracted from the crude oil sample used in spiking the soil samples, pristine soil, oil-contaminated control on days zero and 42 (OC0 and OC42), NPK-treated soil (PN), calcium ammonium-treated soil (PU) and poultry droppings-treated soil (PP) all on days zero and 42 are presented in Fig. 11. The crude oil sample contained up to C-34 chain length while there were no significant presence of hydrocarbons in the pristine soil which confirmed that the peaks of hydrocarbons observed in the nutrient-amended soil samples PN, PU and PP on day zero were as a result of the crude oil spiking. By day 42, almost all the hydrocarbon peaks reduced markedly in PN, PU and PP treatments while the OC control showed no significant hydrocarbon loss. Hydrocarbon degradation involves oxygen-dependent enzymes called the oxygenases and as such the controls that were not aerated could not support significant microbial-driven hydrocarbon degradation. The initial step in the aerobic degradation of saturated, aliphatic hydrocarbons (n-alkanes) involves the enzymes that have a strict requirement for molecular oxygen, that is monooxygenases (mixed function oxidases) or dioxygenases while key step required for the degradation of aromatic hydrocarbons is cleavage of the aromatic ring. This step is carried out by dioxygenase enzymes with molecular oxygen as the reactant (Chikere et al., 2011b). This showed that hydrocarbon degradation was biological rather than abiotic since biostimulation enhances microbial activity by providing limiting nutrients required for biodegradation to indigenous degraders.

The exploration of bacterial diversity in hydrocarbon-polluted soil undergoing bioremediation requires molecular approaches that can comprehensively extract DNA from the soil microbial community sufficient enough to capture majority of the species that are dominant (Simon and Daniel, 2009; 2010). In the present investigation, PCR amplification of bacterial 16S rRNA from total soil DNA and further DGGE and sequencing revealed that species (or operational taxanomic unit [OTU]) diversity decreased as hydrocarbon degrading bacterial populations increased after biostimulation. Changes in the band intensities were also detected over time in the biostimulated soils and not in the undisturbed controls, indicating

that the dynamics observed in the bacterial community structure were predominantly induced by nutrient addition and aeration rather than oil spiking alone since the crude oil used for contamination was applied at the same concentration of 4% in all soil microcosms. Similar observation was also made by Zucchi et al. (2003) and Evans et al. (2004). Hydrocarbon pollution has been shown to affect soil microbial diversity by selecting for those bacterial species capable of surviving the perturbation and at the same time utilizing the contaminant as source of carbon and energy (Quatrini et al., 2008; Jones et al., 2011). DGGE revealed the same banding pattern for PU and PP treatments while PN treatment showed a dissimilar pattern throughout the study period. Dendogram constructed with Jaccard coefficient was also consistent with the DGGE banding pattern as PU and PP DGGE lanes formed distinct clusters for each of the treatments while in PN, lanes PN0 and PN14 clustered together whereas lane PN14 formed a different cluster with lane OC0. PU and PP soils showed a stable bacterial community profile starting from day 14 to day 42, the last day of the experiment, unlike PN soil where population succession was observed throughout the period. This could suggest that PU and PP soils may habour special populations that possess alkane degradation pathways with broad hydrocarbon specificity as also observed by Hamamura et al. (2006) in a related study. In PN treatment, sequences affiliated with low G+C Gram positive bacteria and *Corynebacterium* spp. were the dominant OTUs selected when NPK was used to biostimulate the extant indigenous bacteria. On the other hand, uncultured bacterial clones, *Dietzia* spp., *Nocardioides* spp. *Janibacter* sp., *Corynebacterium* sp. and *Kouleothrix aurantica* were the prominent sequences selected when calcium ammonium nitrate (PU treatment) was used to amend the oil-polluted soil. However *Dietzia* spp. were the most abundant sequences associated with hydrocarbon degradation in this treatment. In PP treatment, low G+C Gram positive bacteria, an uncultured bacterial clone and *Corynebacterium* spp. were the prominent sequences selected when the soil was amended with poultry droppings. Most of the sequences that affiliated with Actinobacteria group with high percentage identity to closest GenBank relatives which were originally obtained from hydrocarbon-polluted sites, for instance *Corynebacterium* sp. IC10 sequence obtained from an oil-contaminated site had 94%-99% similarity with sequences in DGGE bands 10, 14, 15, 16, 23, 24, 42, 48 and 49. In the same vein, three out of the six *Dietzia* spp. sequences selected in PU treatment had 89%, 99% and 100% similarity with closest GenBank relatives all of which were originally obtained from petroleum-hydrocarbon polluted environments. Alonso-Gutierrez et al. (2011) isolated an alkane degrading *Dietzia* sp. H0B from Prestige oil spill site which gives credence to the fact that these bacteria are associated with hydrocarbon degradation. Still in the PU treatment, *Janibacter* sp. 20/6G5 sequence originally obtained from diesel contaminated site had 94% similarity with sequence in DGGE band 38. Out of the forty successful sequences obtained from the present investigation, sixteen affiliated with sequences obtained from petroleum hydrocarbon-polluted environments, fifteen affiliated with sequences from either compost or other contaminated environments while the remaining nine sequences affiliated with those from marine environment. The most striking pattern observed in the present study was the emergence of *Corynebacterium* sp. IC10-like bacteria in all nutrient-amended soils (Fig. 2, PN: bands 42 and 48; PU: band 23 and PP: bands 10 and 14) treated with different organic and inorganic nutrients. Wide distribution of these bacterial species in the biostimulated soils could suggest that *Corynebacterium* spp. may not be unconnected in hydrocarbon degradation since such sequences were not obtained from the pristine and oil-contaminated controls. Other independent studies have underscored the involvement of *Corynebacterium* spp. in hydrocarbon degradation (Chaillan et al., 2004; Jimenez et al., 2007). All other sequences obtained from the study which affiliated with *Dietzia* spp., *Rhodococcus erythropolis*, low G+C Gram positive bacteria, *Nocardioides* spp. and *Janibacter* sp. may as well be hydrocarbon degraders as other researchers have demonstrated hydrocarbon

degradation in strains of these bacteria using nucleic acid-based or metagenomic approaches (Hamamura et al., 2006; Quatrini et al., 2008; Chikere et al., 2011a).

4. CONCLUSION

In the present study, bacterial counts increased significantly in nutrient-amended treatments as against the controls. Hydrocarbon attenuation was also appreciable in biostimulated soils as measured by gas chromatography. Total organic carbon increased and reduced sequentially in biostimulated soils indicative of microbial activity and biochemical reactions associated with hydrocarbon biodegradation. Sequences of Gram positive bacteria in Actinobacteria group were seen to be the dominant microbial species selected after biostimulation of the crude oil-polluted soil by organic and inorganic nutrient amendment. Members of the species selected are known hydrocarbon degraders and could as well be associated with crude oil biodegradation in the polluted soil following nutrient amendment.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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