Journal of Applied & Environmental Microbiology, 2016, Vol. 4, No. 1, 1-20
Available online at http://pubs.sciepub.com/jaem/4/1/1

DOI:10.12691/jaem-4-1-1

Microbial Community Profiling of Active Oleophilic Bacteria involved in Bioreactor-based Crude-oil Polluted Sediment Treatment

Chioma Blaise Chikere*, Amara Ukamaka Okoye, Gideon Chijioke Okpokwasili

Department of Microbiology, Faculty of Biological science, College of Natural and Applied Sciences, University of Port Harcourt, Rivers State, Nigeria, P.M.B. 5323, East-West Road, Choba, Port Harcourt, Rivers State, Nigeria
*Corresponding author: ujuazed@yahoo.com

Abstract Petroleum hydrocarbon pollution has been a major environmental challenge in the coastal areas, Niger-Delta, Nigeria. In this study, culture dependent and molecular techniques were used to monitor bioremediation over a-64 day period in seven microcosms setup in 2.5 L stirred tank bioreactors with each tank containing either Poultry droppings (BPOUT), NPK fertilizer (BNPK), Cow dung (BCD) or Urea fertilizer (BUREA). One bioreactor (BAUG) was bioaugmented while two others served as unamended (BUNa) and heat-killed (BHKD) controls. A decrease in petroleum hydrocarbon concentration and a concomitant increase in biomass was observed in all treatments at varying levels. BNPK (97.2%; 97.1%) showed highest reduction percentage while BHKD (82.34%, 81.3%) was the least for total petroleum hydrocarbon and polycyclic aromatic hydrocarbon amongst all treatment. Screening of isolates for aromatic hydrocarbon ring cleavage functional gene (catechol 2,3-dioxygenase) revealed that catechol 2,3-dioxygenase (*C23D0*) gene was detected in the following genera: *Pseudomonas* spp. (3), *Rhodococcus* sp. (2), *Bacillus* spp.(2)., *Achromobacter* sp., *Serratia* sp., *Aeromonas* sp., *Micrococcus* sp. and *Acinetobacter* sp. Sequences obtained from amplification of 16S rRNA gene gave a total number of 24 hydrocarbon utilizing bacterial species which showed 96-100% similarity with those deposited in GenBank and are identified as *Brevundimonas naejangsanensis*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas* spp. (6), *Aquitalea magnusonii*, *Achromobacter* sp., *Halomonas lutea*, *Pseudomonas aeruginosa* (8), *Shewanella* sp, *Achromobacter* sp., *Gordonia* sp., *Sphingobacterium* sp. and *Bacillus* sp. Our result revealed that these extant indigenous bacterial population in the crude oil-polluted sediment habour the relevant aromatic hydrocarbon ring cleavage genes (catechol 2,3-dioxygenase) and may have a key role in bioremediation of crude oil-polluted sediment.

Keywords: *bioreactor-based, hydrocarbon, microbial community, oleophilic bacteria, sediment*

Cite This Article: Chioma Blaise Chikere, Amara Ukamaka Okoye, and Gideon Chijioke Okpokwasili, "Microbial Community Profiling of Active Oleophilic Bacteria involved in Bioreactor-based Crude-oil Polluted Sediment Treatment." *Journal of Applied & Environmental Microbiology*, vol. 4, no. 1 (2016): 1-20. doi: 10.12691/jaem-4-1-1.

1. Introduction

The Niger-delta's environment in Nigeria is an incredibly well-endowed ecosystem which contains one of the highest concentrations of biodiversity on the planet. In addition to supporting abundant flora and fauna, arable terrain that can sustain a wide variety of crops, lumber or agricultural trees, and diverse species of seafood, this oil-rich region provides more than 80% of crude-oil derived revenue for Nigeria. Accidental spills of crude oil and its refined products in Niger Delta region, which is the centre of petroleum production activities in Nigeria occur on a frequent basis during operations by the increased oil exploration and exploitation, transportation, storage, refining and distribution. This has caused devastating effect on the aquatic ecosystem in this area and the devastating consequences of crude-oil spill in the Niger Delta region in Nigeria are colossal on man, fauna, flora and the totality of the ecosphere. People in this area are exposed to petroleum hydrocarbons, sometimes at very elevated concentrations, in outdoor air and drinking water. The adsorption of hydrocarbons in the sediments makes them more stable and less accessible to biological degradation and these pollutants can persist in the sediments several months even several years. After adsorption on the sedimentary particles, hydrocarbon is accumulated, and reach concentrations higher than the concentrations of hydrocarbon found in the water column.

United Nation Environmental Programme (UNEP) [1] report revealed that contaminated sediments can also act as reservoirs for pollution, releasing hydrocarbons when disturbed (e.g. by the propeller action of a motorboat) into the aquatic environment long after the original source of pollution has been removed and people in this area are also exposed through dermal contacts from soil, sediments and surface water. Most of the crude oil contamination in this area exceeds Nigerian national standards, as set out in the Department of Petroleum Resources (DPR), Environmental

Guidelines and Standards for the Petroleum Industries in Nigeria (EGASPIN) [2]. Previous investigation carried out by [1] on the polycyclic aromatic hydrocarbons (PAHs) concentrations in some Niger Delta sediments revealed elevated values of these priority pollutants in the sediments studied.

Microbial remediation which is the first line of defense against oil pollution in the [3.4] hydrocarbon contaminated site depends utmost on the microbial community structure.

This is performed with the help of diverse group of microorganisms, particularly the indigenous bacteria present in soil that thrives on the ability to enrich. maintain microbial populations and activities within the target environment [4,5]. Microorganisms relies on the use of the diverse metabolic capabilities or their parts for the degradation and removal of many environmental pollutants notably petroleum hydrocarbons [6,7,8]. One of the main interest of bioremediation is its compatibility with the major natural biogeochemical cycles and recycling routes of the earth and marine ecosystems [7].

Slurry-phase bioreactor is very useful in monitoring bioremediation because it provides an optimal controlled environment. The conditions in the bioreactors are controlled to eliminate most of the rate-limiting/variable factors such as oxygen supply, optimal pH, temperature and creates the optimum environment for microorganisms to degrade the contaminants and specific nutrient formulations associated with bioremediation. The optimal controlled environment also allows management of volatile organic compounds (VOCs) by creating reactor conditions which accelerate the process of bioremediation of these VOCs. Various studies have examined the ability of hydrocarbon degrading bacteria to degrade different petroleum products and the results have been encouraging. For instance, in the study of assessment of biodegradation efficiency of some isolated bacteria from oil-polluted contaminated sites in solid and liquid media containing oil-compounds, report showed that many species of bacteria obtain both energy and tissue building material from petroleum, literature data also indicates that a large number of bacteria capable of degrading a wide range of PAHs have been isolated from contaminated sites [9,10,11,12]. Many bacteria have evolved or acquired the ability to utilize hydrocarbon as sources of carbon and energy [13,14]. Krishna *et al*., [15] reported that bacteria posses diverse metabolic pathways to utilize most recalcitrant petroleum hydrocarbons and need the appropriate catabolic genes in order to be a degrader of a compound.

Screening and assessment of the hydrocarbonoclastic bacteria involves sampling, isolation of potential hydrocarbon utilizers / degraders [16]. The combination of cultivation-independent, PCR amplification, microbial community profiling techniques and identification of catabolic genes are ways to elucidate the composition, functions and interactions of microbial communities during bioremediation [17]. A functional gene array that includes DNA from a wide range of genes involved in anaerobic and aerobic degradation of pollutants provides opportunities to associate the occurrence of particular catabolic pathways in microbial communities with removal of different classes of compound during the course of bioremediation. [17].

The fate of polycyclic aromatic hydrocarbons (PAHs) is of great interest due to their recalcitrant hydrophobic nature, potential for bioaccumulation and many are toxic and/or carcinogenic to humans and wildlife [18,19]. Diversity of Polycyclic aromatic hydrocarbon (PAH) degrading genes in bacteria in the hydrocarbon polluted environment is assumed to be huge and can be the key determinant of the fate of certain aromatic compounds in the environment. Catechol 2,3 dioxygenase (*C23DO*) genes play important role in the degradation of aromatic compounds and are excellent targets on which to base catabolic screening assay of aromatic hydrocarbon degraders because *C23DO* genes have a well characterized phylogeny that allows for the systematic design of dioxygenase-specific primers. [18]. Knowledge of the genes coding for dioxygenase enzymes that catalyze the primary step of PAH degradation by incorporating molecular oxygen into the aromatic nucleus is an essential prerequisite to revealing the contributions of microbial population networks to transformation, assimilation, and degradation of hydrocarbons.

In the degradation pathways of aromatic hydrocarbons, aromatic ring cleavage is a key step for the complete mineralisation of the compounds. Ring cleavage is catalysed by different types of dioxygenases, such as the *ortho*- or the *meta*-cleavage dioxygenases. The most widespread ring cleavage enzymes are catechol 2,3-dioxygenases (*C23DO*) and is an extremely important and common intermediate in aromatic hydrocarbon catabolism which cleave the aromatic ring outside two adjacent hydroxyl groups (*meta*-cleavage dioxygenases) [6,12,20,21]. In order to elucidate the degradative genes in putative hydrocarbon degraders from petroleum-contaminated soil, primers specific for hydrocarbon-degrading enzymes are used in PCR and other fingerprinting methods [22].

The purpose of this study was to monitor a 64-day bioremediation process carried out in seven 2.5 L stirred tank slurry bioreactors and to acquire insight in the effectiveness of bioreactors in monitoring petroleum hydrocarbon degradation. We ascertained the bacterial community structure of indigenous bacteria capable of degrading hydrocarbon compounds and evaluated the effectiveness of biostimulation and bioaugmentation on the attenuation of crude oil hydrocarbons by natural microbial communities. We also characterized bacterial isolates using PCR of 16S rRNA gene marker as benchmarked with functional genomics and molecular detection of aromatic hydrocarbon ring cleavage functional gene (catechol 2,3 dioxygenase enzyme coding gene) using *C23DO*-specific primers targeting the genes that make up the I.2.A subfamily of *C23DO* genes coded by catechol 2,3-dioxygenases in bacterial isolates and degenerate primers targeting other dioxygenases (degenerate primers are used to amplify DNA in situations where only the protein sequence of a gene is known, or where the aim is to isolate similar genes from a variety of species).

2. Materials and Methods

2.1. Sampling Site/Sample Collection

Crude oil-polluted sediment from Bodo creek in Ogoniland, Niger Delta, Rivers state, Nigeria was collected from a depth of 30 cm with Eckman grab (Wild Life Supply Co., NY) into a sterile container, while

seawater was collected with sterile 10 L container. All samples were transported to the laboratory within 6 h at 4°C for analysis.

2.2. Bioslurry Bioreactors

Seven microcosms was set up using 2.5 L stirred tank bioreactors, four bioreactors were designated and biostimulated with either cow dung (BCD), poultry litter (BPOUT), NPK fertilizer (BNPK) or Urea fertilizer (BUREA). One bioreactor (BAUG) was bioaugmented with previously identified hydrocarbon utilizing bacteria [20ml of bacteria cell ($10^{-8}ml^{-1}$)] while two others served as unamended [designated BUNa] and heat killed controls (designated [BHKD]). Each of the bioreactors received 1 kg (wet weight) of sediments, 1 L of seawater and spiked with 20 ml of crude oil and 20 mg of anthracene. Four bioreactors were amended with appropriate amount of nutrient sources such as urea, NPK fertilizer (20:10:10), urea fertilizer (BUREA), poultry droppings (BPOUT) and cow dung (BCD) to stimulate the extant autochthonous bacteria. For the controls, the unamended treatment was only spiked with the hydrocarbons without nutrient addition to determine whether the indigenous bacteria in the sediments have the natural propensity to degrade petroleum hydrocarbons as shown in the experimental setup (Table 1). For the heat-killed (killed by autoclaving sediments and seawater at 121°C for 15 min at 15 psi on 2 consecutive days) control was set up to measure the role of abiotic factors in the loss of petroleum hydrocarbons. The bioreactors were continuously stirred (by 2 impellers) in 150 rpm agitation rate at 0.05 L/min air flow rate throughout the 64-day experimental period. The bioreactor and interior of the bioreactors with the accessories is shown in Figure 1a-Figure 1b. Filtered air was supplied to the bioreactors from the air compressor through hoses running in and out of them. They were sealed with Teflon to prevent the ingress of atmospheric air and egress of the slurry and were operated at room temperature (28°C) throughout the experimental period. pH in the 7 bioreactors at day zero ranged from 7.3 to 7.9 after adjustment using method of [22].

Table 1. Experimental set up for bioreactor

Bioreactor code	Test experiment (amended)
BPOUT	1kg of sediment + 1 litre of sea water + 20ml of crude oil + 0.2g PAH (anthracene) + 20g of poultry droppings.
BCD	1 kg of sediment + 1 litre of sea water + 20ml of crude oil + 0.2g PAH (anthracene) + 20g of Cow dung
BUREA	1 kg of sediment + 1 litre of sea water + 20ml of crude oil + 0.2g PAH (anthracene) + 20g of Urea.
BNPK	1 kg of sediment + 1 litre of sea water + 20ml of crude oil + 0.2g PAH (anthracene) + 20g of NPK (20:10:10).
BAUG	1 kg of sediment + 1 litre of sea water + 20ml of crude oil + 0.2g PAH (anthracene) + 20ml of bacteria cell ($10^{-8}ml^{-1}$)
Bioreactor code	Control experiment (unamended)
BUNa	1 kg of sediment + 1 litre of sea water + 20ml of crude oil + 0.2g PAH (anthracene)
BHKD	1 kg of heat killed sediment + 1 litre of heat killed sea water + 20ml of crude oil + 0.2g PAH (anthracene)

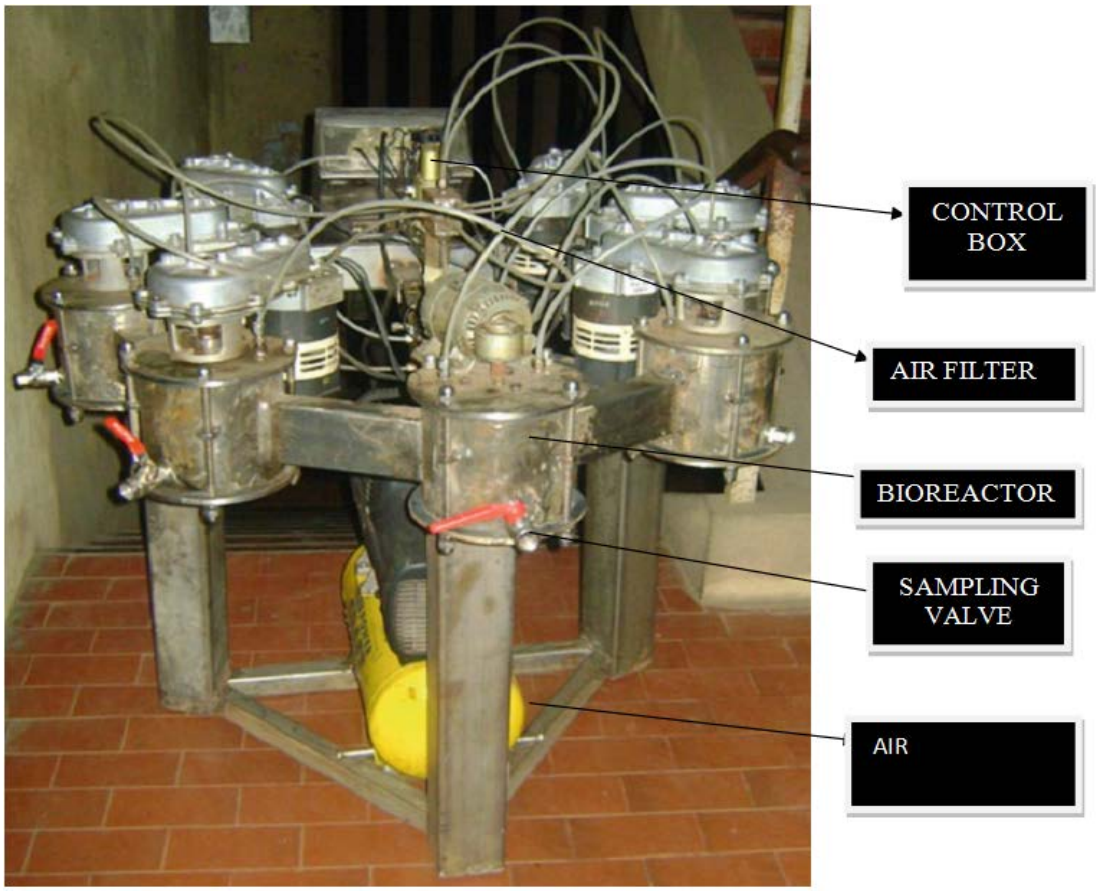


Figure 1a. The seven 2.5 L bioreactors (Source: Chikere *et al.* 2012)

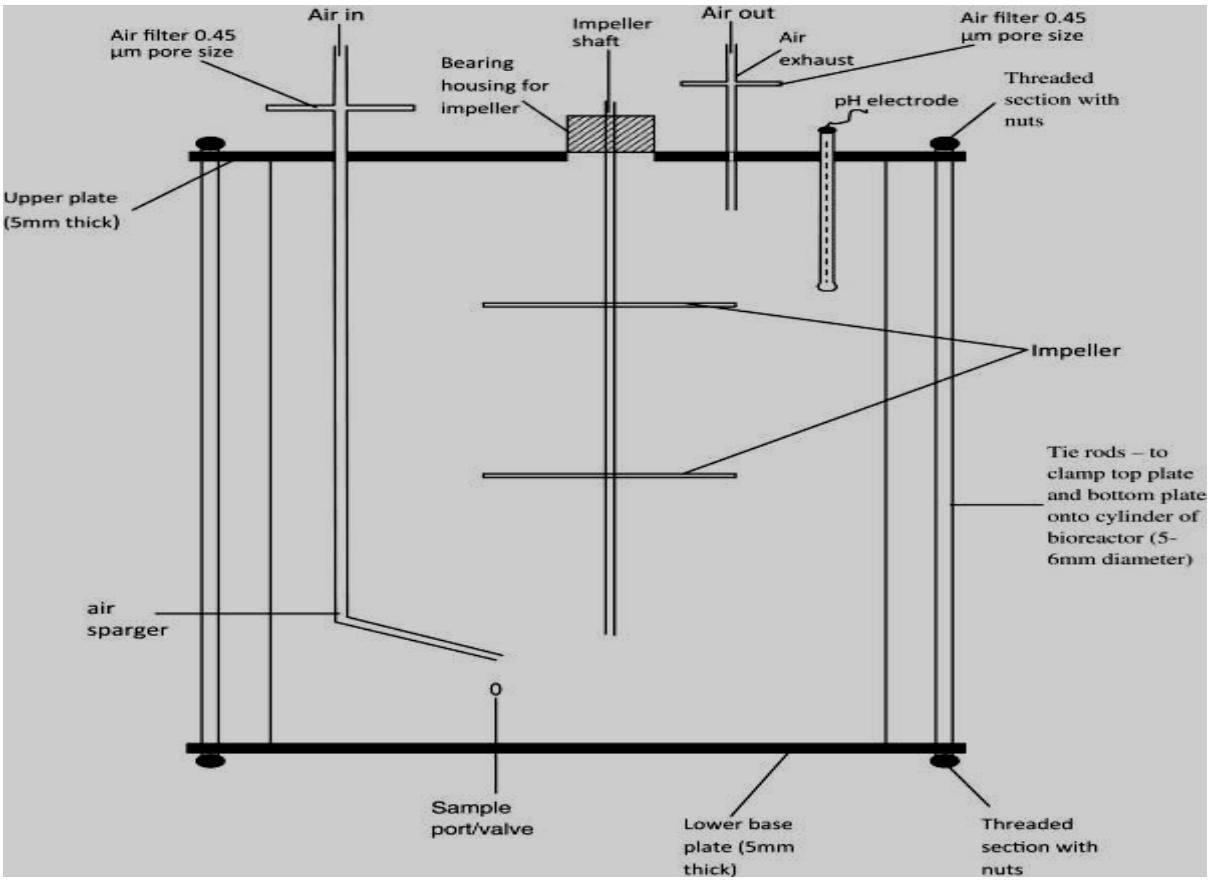


Figure 1b. The interior design of the 2.5 l bioreactors (Source: Chikere *et al*. 2012)

2.3. Physico-chemical Characterization

The pH of the samples was determined using a digital pH meter (Jenway 3015 UK) during experimental setup. This was to determine the negative logarithm of the hydrogen ion concentration. The Electrical conductivity of the sediment sample was measured with a conductivity meter, (Jenway 4010 UK). This was determined by means of a mercury thermometer calibrated in 0.2°C units from 0°C to 100°C. Electrical Conductivity (EC) was measured in a 1:4 sediment to water aliquot after filtration using a field portable conductivity meter. Total organic carbon was done using wet oxidation technique. The Brucin method was used to analyze the nitrate content. The ascorbic acid method was used to determine the phosphate content.

2.4. Chromatographic Analysis

Residual total petroleum hydrocarbons (TPH) and polycyclic aromatic hydrocarbons (PAHs) were extracted from the samples and quantified using gas chromatograph-mass spectrometry (GC–MS). The hydrocarbons in the sediment samples for each treatment in the bioreactors were quantified using an Agilent 6890GC, Agilent technologies, Wilmington, USA GC equip with 5975B MSD and MSD chemstation version D. 03.00. Helium gas was used as carrier gas at constant flow rate of 75 Kpa and the column with catalogue number HP-5 (19091J-413) and had the following dimensions: Column—30-m × 0.25-mm ID × 0.25µm film thickness silicone-coated fused-silica capillary column.

2.5. Microbiological Analysis

2.5.1. Enumeration of Total Culturable Heterotrophic Bacteria

Total heterotrophic bacteria (TCHB) counts were determined on nutrient agar using pour plate method using [22] method. In each bioreactor,1 g (wet weight) of sediment was homogenized in 0.85% of normal saline. Decimal dilutions (10-fold) of the suspensions were plated out in duplicate on Plate Count Agar (Merck, Germany) modified with 10% NaCl and incubated at 30°C for 24 h. The colony forming units were afterwards enumerated.

2.5.2. Enumeration of Total Culturable Hydrocarbon Utilizing Bacteria

Hydrocarbon utilizing bacteria (TCHUB) were enumerated by a method adopted from [22] which involved the dilutions of appropriate appropriate dilutions of sediment suspensions from each bioreactor (1 g wet weight of sediment homogenized in 0.85% of normal saline) were plated out in duplicate on Bushnell-Haas agar (Sigma-Aldrich, USA) modified with 10% NaCl. Hydrocarbons were supplied through the vapour phase to putative hydrocarbon utilizers by placing sterile Whatman No.1 filter papers impregnated with 5 ml Okono crude oil on the lids of the inverted plates and incubated for 14 days at 30°C.

2.5.3. Purification and Characterization of Hydrocarbon Utilizing Bacteria

Discreet colonies of different HUB were randomly picked using a sterile inoculating wire loop and sub

cultured for purification by streaking on nutrient agar plates and incubated at 30°C for 24 h. The Phenotypic tests were carried out as described in [23,24].

2.6. Turbidometry Test

The hydrocarbon utilizing bacteria from all treatments, were further screened for oil degrading capability under aerobic conditions by inoculating a calibrated loop full of 18h old culture of each isolate into 9mls of Bushnell Haas Broth containing 1ml of Okono medium crude oil as the sole carbon source. Biodegradation was scored by the turbidity of Bushnell Haas Broth after 0-14 days incubation at 30^{O}C with a concomitant visual gradual reduction in the oil layer. The growth of the bacterium was measured by taking the optical density (OD) reading at 600nm with SP-OPTIMA Spectrophotometer (Optima Japan) every 2 days interval for 14 days against a negative control setup which contained only the Bushnell Haas broth and Okono medium crude oil using methods of [25].

2.7. Molecular Analysis

2.7.1. DNA Extraction

DNA from pure cultures of putative hydrocarbon utilizing bacterial isolates were extracted at the time of subculturing using Zymo Research microbe DNA extraction kit™ (Zymo Research Corp. USA) Bio 101 FP-120 FastPrep cell disruptor (Qbiogene, Inc. Canada) in accordance with the manufacturers' instructions. The supernatant of extracted DNA from the bacterial isolates were quantified using NanoDrop ND-2000 spectrophotometer.

2.7.2. PCR-amplification Targeting Bacterial 16S rRNA Using Primer set pA8f-GC/ KPRUN518r

The DNA extracted was amplified using 16S rRNA universal primer sets, pA8f-GC (5'-CGC-CCG-CCG-CGC-GCG-GCG-GCG-GGC-GGG-GCG-GGG-GCAGGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG-3') and KPRUN518r (5'-ATTACCGCGGCTGCTGG-3') to amplify a partial fragment (500bp) of the 16S rRNA. Amplification of the template DNA was performed using 2 µl of the extracted DNA with Eppendorf thermal cycler (Germany). The 50 µl PCR mixture contained 5µl of deoxy nucleoside triphosphates (dNTPs) mixture (2.5 µM) (Promega, USA), 5 µl of 5X Green Go Taq Flexi buffer (Promega, USA), 3.5 µl of 25 mM MgCl2 (Promega, USA), 2 µl each of 10 pmol of both forward and reverse primers pA8f-GC (5'-CGC-CCG-CCG-CGC-GCG-GCG-GCG-GGC-GGG-GCG-GGG-GCAGGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG-3') and KPRUN518r (5'-ATTACCGCGGCTGCTGG- 3'), 0.25 µl of 5U/µl hot start Go Taq DNA polymerase, 2.5 µl of 20 mg/ml of bovine serum albumin and 27.75 µl of sterile water. DNA amplification was performed in a PCR thermal cycler (Eppendorf made in Germany) using the following programme: denaturing at 95°C for 3 min, 33 cycles of 30 sec at 95°C, annealing for 30 sec. at 55°C, extension at 72°C for 1 min and final elongation at 72°C for 7 mins. A reaction without template DNA was included as negative control. PCR was done according to method of [26]. Amplified DNA was examined by electrophoresis in 1% agarose gel with 2 µl aliquots of PCR products in 1X Tris-Acetate-EDTA buffer and viewed using UV Light transilluminator Photogel.

2.7.3. PCR-amplification of aromatic Hydrocarbon Ring Cleavage Dioxygenases

PCR-amplification of dioxygenase gene from extracted bacterial DNA samples was performed with 50 µl PCR mixture contained 5µl of deoxy nucleoside triphosphates (dNTPs) mixture (2.5 µM) , 5 µl of 5X Green Go Taq Flexi buffer , 3.5 µl of 25 mM $MgCl_2$, 2 µl each of 10 pmol of both the primer sets 23CAT-F…5'-CGACCTGATCTCCATGACCGA-3' and 23CAT-R ...5'-TCAGGTCAGCACGGTCA-3 targeting the genes that make up the I.2.A subfamily of C23DO genes. The thermal cycling conditions was 50µl reaction with the programme: 5min initial denaturation step at 95^{o}C; 33 cycles of 94°C for 1 min, annealing at 55^{o}C for 1min; elongation at 72°C for 2min and final 10-min extension at 72°C. Ten microliters of each PCR product will be run on a 1% agarose gel and visualized under UV Light transilluminator Photogel. for the detection of 238-bp dioxygenase amplicons. All PCRs had a negative control without template DNA to check contamination. PCR-amplification of other dioxygenase genes using

DEG-F..5'CGACCTGATC(AT)(CG)CATGACCGA-3' and DEG-R...5'-T(CT)AGGTCA(GT)(AC)ACGGTCA-3' are degenerate primers for dioxygenases (letters in parentheses indicate positions of degenerate bases) was used to amplify other dioxygenase genes. Primers DEG-F and DEG-R are identical to 23CAT-F and 23CAT-R except for five positions where degenerate bases were used to account for primer-target mismatches with known *Pseudomonas* spp. that harbour aromatic ring cleavage dioxygenases. The template DNA was performed using 2 µl of the extracted DNA, 0.25 µl of 5U/µl hot start Go Taq DNA polymerase, 2.5 µl of 20 mg/ml of bovine serum albumin and 27.75 µl of sterile water. samples would be performed in a 50µl reaction with the programme and the thermal cycling conditions were: 5min initial denaturation step at 95°C; 33 cycles of 94°C for 1 min, annealing at 55°C for 1min; elongation at 72°C for 2min and final 10-min extension at 72°C. 10 µl of each PCR product will be run by electrophoresis in 1 % agarose gel and visualized under UV for the detection of 250-bp dioxygenase amplicons. A reaction tube without template DNA was included as negative control and was done using method of [27].

2.8. Sequence Determination of PCR Amplified Product

The isolates were further confirmed by 16S rRNA sequence using amplified product from universal primer set pA8f and KPRUN518r and determined with ABI 3130 XL genetic analyzer incorporating the ABI Big Dye Terminator cycle sequencing kit version 3.1.Sequencing was performed by GATC Biotech AG, European custom sequencing center, Germany according to Sanger sequencing protocol. Electrophoregrams of the sequences generated were inspected with Finch TV software (Geospiza). The 16S rRNA sequences were aligned with Blast search of National Centre for Biotechnology information (NCBI), which was deposited in the GenBank and assigned accession numbers for the nucleotide sequences.

2.9. Statistical Analysis of Data

Statistical analysis was performed on the data generated from the bacterial counts and hydrocarbon concentrations for the different treatments using one way ANOVA and Tukey's Multiple Comparison Test. The software Graph-Pad Prism (GraphPad Software, CA, USA) for Windows version 5.01 was used to do the analysis.

3. Results and Discussion

3.1. Baseline Characterization of Sediment Sample

The values of the physicochemical parameters (potassium, conductivity, Temperature, pH, nitrate, phosphate, and total organic carbon contents), baseline bacterial counts (total culturable heterotrophic and hydrocarbon utilizing bacteria) and gas chromatographic analysis of total petroleum hydrocarbons (TPH) and polycyclic aromatic hydrocarbons (PAHs) in the sediment sample prior to bioremediation are presented in Table 1. The total culturable heterotrophic bacterial counts (TCHB) was 7.0 x 10^7cfu/g and culturable hydrocarbon utilizing bacterial counts (TCHUB) was 6.5 x 10^7cfu/g. Both TCHB and TCHUB counts of the sediment were in the same range of 10^7 Cfu/g which indicates the fact that the bacterial community was capable of utilizing hydrocarbon. This phenomenon occurs when an environment is chronically exposed to hydrocarbon from anthropogenic sources [13]. The pH of the sediment was 7 and previous studies have demonstrated that the optimal pH range for biodegradation to occur is 6-7 [28]. The baseline characteristics of the sediment prior to bioremediation were 19ppm and 3.1 ppm for TPH and PAH respectively. The low concentrations of the TPH and PAHs in the sediment also showed that there is a metabolically active bacterial community in the sediments and the bacterial community probably uses the hydrocarbons as source of carbon and energy.

Table 2. Baseline Characteristics of Sediment

Parameters	Concentrations
Total petroleum hydrocarbon (TPH)	19ppm
Polycyclic aromatic hydrocarbon (PAH)	3.1ppm
pH	7
Nitrate	88mg/kg
Phosphate	20mg/kg
Temperature	27.6^{0}C
Conductivity	3521µS/cm
Total organic carbon (TOC)	3.3%
Total culturable heterotrophic bacterial count (TCHB)	7.0x10^7cfu/g
Hydrocarbon utilizing bacterial count (HUB)	6.5x10^7cfu/g

3.2. Enumeration of Total Culturable Heterotrophic and Hydrocarbon Utilizing Bacteria

The degree of remediation was monitored over a 64-day period using physicochemical and microbiological indices to score degradation under laboratory conditions in the amended, bioaugmented and control treatments using bioreactors. Physico-chemical, gas chromatographic-mass spectrometry analyses were carried out on the nutrient-amended, bioaugmented and control samples on each sampling day as the experiment progressed. Presence of microbial activity was determined by enumeration of total culturable heterotrophic and hydrocarbon utilization bacteria in nutrient-amended, bioaugmented and control samples on each sampling day (0, 16, 32 and 64) period as the experiment progressed. There was general increase in bacterial count for all treatments (BUNa, BNPK, BUREA, BPOUT, BCD and BAUG). Total culturable hydrocarbon utilizing bacterial (TCHUB) counts in all treatments during the 64-day bioremediation increased from 10^4 cfu/g at day 0 to 10^9 cfu/g at day 64 while total culturable heterotrophic bacterial (TCHB) increased from 10^7cfu/g by day 0 to 10^9 cfu/g by day 64 when the experiment ended. This increase in count could be attributed to the diverse bacterial populations in the oil-polluted sediment and addition of nutrients which enhanced bacterial growth. Figure 2a-b illustrates the total culturable heterotrophic bacterial (TCHB) and hydrocarbon utilizing bacterial (TCHUB) counts during the 64-day bioremediation with the Error bars. BUREA recorded the highest TCHUB counts throughout the experimental period with a value of 8.5 x 10^9 cfu/g and TCHB total culturable hydrocarbon utilizing bacterial (TCHUB) with a values of 19.5 x 10^7 cfu/g. In the study, biostimulation by addition of organic and inorganic nutrients supplied limiting nutrients to indigenous hydrocarbon degraders and ensures that microbial activity occur in pollutants..

Similar investigation of bioreactor-based treatment and amendment of crude oil polluted sediments with inorganic and organic sources of nitrogen and phosphorus (NPK fertilizer and Urea fertilizer, Poultry droppings) by [29] showed that nutrient amendment caused more proliferation of crude oil degrading bacteria and enhanced microbial degradation of crude oil in sediment. Similar result was reported by [22], that biostimulation by addition of organic and inorganic nutrients to hydrocarbon polluted matrics supplies limiting nutrients to indigenous hydrocarbon degraders and ensures that microbial activity occur in pollutants. The presence of nutrients, high organic matter concentration and other ecological factors attributed to the high counts recorded in the sediment samples from the bioreactor. Some recent studies have converged upon the idea that increased biodegradation efficiency can be reached when a mixed bacterial culture is used [9].

BUNa control was only spiked with the hydrocarbons without nutrient addition to determine whether the indigenous bacteria in the sediments have the natural propensity to degrade petroleum hydrocarbons. From our result, it was clear that there was increase in bacterial count in the unamended control (BUNa). From this, it can be inferred that the indigenous bacteria in the sediment have the natural propensity to degrade petroleum hydrocarbons and were already acclimatized to hydrocarbons since there was also loss in TPH and PAHs in this control as bioremediation progressed. There was increase in bacterial count for all treatments except the heat killed control (BHKD) because the oil-polluted sediment and sea water was autoclaved at 121°C for 15 min at 15 psi on 2 consecutive days and all forms of microbial life was terminated. The heat killed (BHKD) control was to check the effect of abiotic factor in hydrocarbon degradation.

The total culturable heterotrophic and hydrocarbon utilizing bacterial (TCHB and TCHUB) counts obtained from the nutrient-amended and unamended slurries when compared with those from the oil-contaminated heat-killed control were statistically significant at $P < 0.05$ using one way ANOVA and Tukey's multiple comparison test.

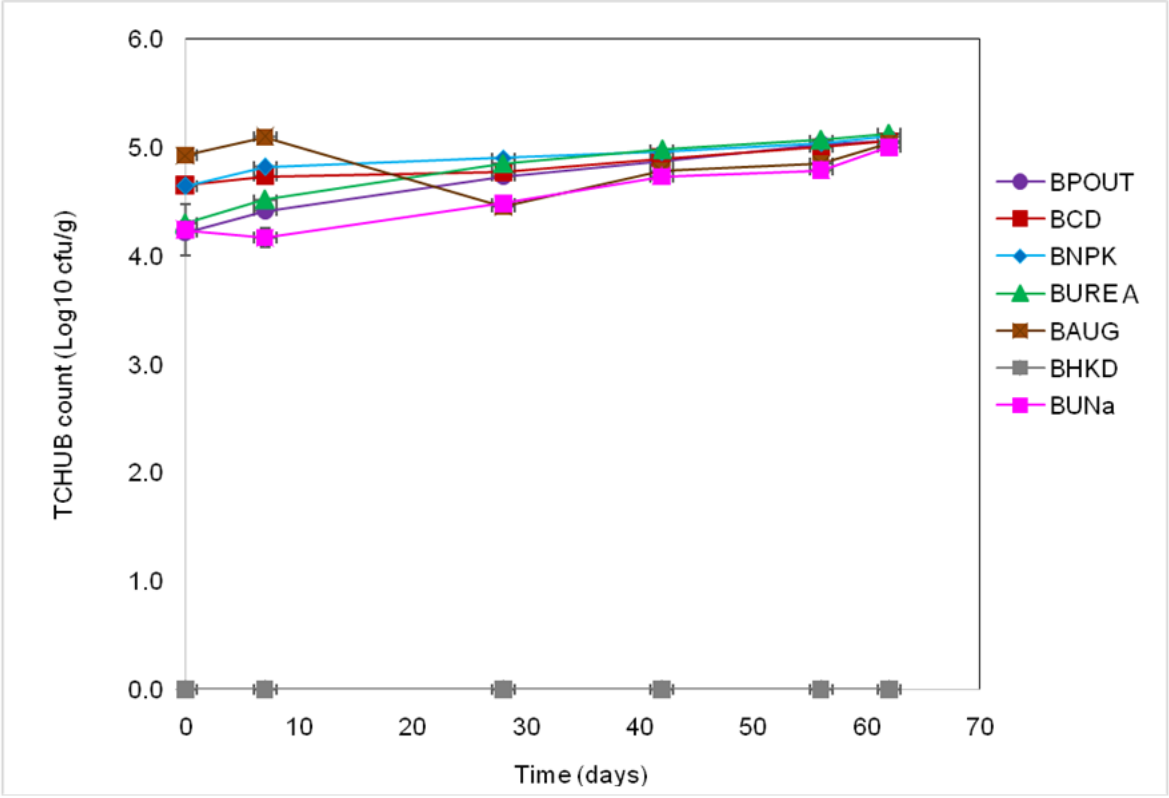


Figure 2a. Total (TCHUB) Culturable Hydrocarbon Utilizing counts during the 64-day bioremediation

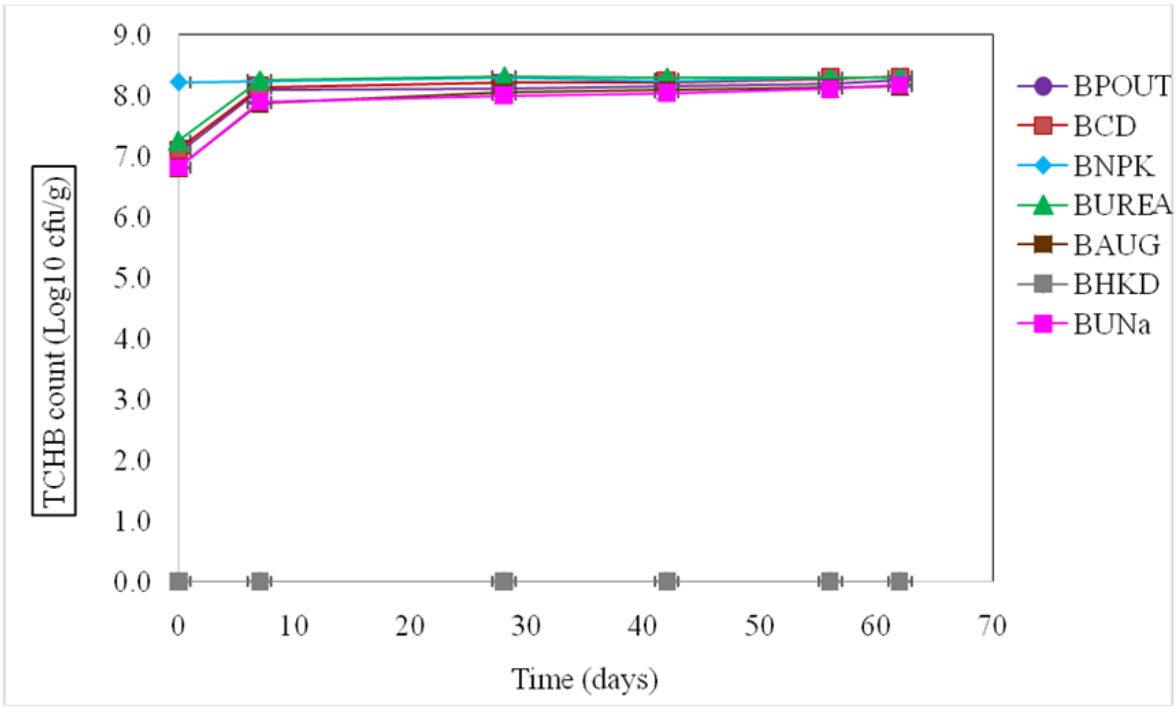


Figure 2b. Total Culturable Heterotrophic Bacterial (TCHB) counts during the 64-day bioremediation

3.3. Chromatographic Analysis

Measurement of residual hydrocarbons using GC-MS showed significant reduction in the concentrations of the PAHs and TPHs in the amended treatments, bioaugmrnted and controls on each sampling day as the study progressed. The chromatography-mass spectrometry (GC-MS) analysis for residual total petroleum hydrocarbons (TPH) was 97.04 ppm - 105.05 ppm across all treatments on day 0 and reduced to range of 2.84 ppm - 8.40 ppm by day 64. PAHs decreased appreciably from range of 57.09 ppm -

61.03 ppm to range of 1.62 ppm - 5.1 ppm. The residual concentrations of total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon (PAH) in the sediment samples amended with different nutrient sources, the bioaugmented and the biotic and abiotic controls (BUNa and BHKD) on the sampling days (0, 32 and 64) during the 64-day bioremediation are shown in Figure 3a-b . There was reduction in TPHs and PAHs in all treatments. By day 64, the percentages of degradation for PAHs, as measured with GC–MS were BNPK (97.1%), BUREA (94.52%), BPOUT (94.08%), BCD (94.01%), BAUG (93.06%), BUNa (82.7%) and BHKD (81.3%) and TPHs degradation rates were as follows: BNPK (97.2%), BUREA (95.05%), BUNa (86.05.%), BHKD (82.34%), BPOUT (96.8%), BCD (96.2%), and BAUG (94.2%). BNPK (97.2%; 97.1%) showed highest reduction percentage while BHKD (82.34%, 81.3%) was the least for total petroleum hydrocarbon and polycyclic aromatic hydrocarbon amongst all treatment. Figure 4 shows the percentage degradation of polycyclic aromatic hydrocarbon (PAH) and total petroleum hydrocarbon (TPH) during the 64-day bioremediation.

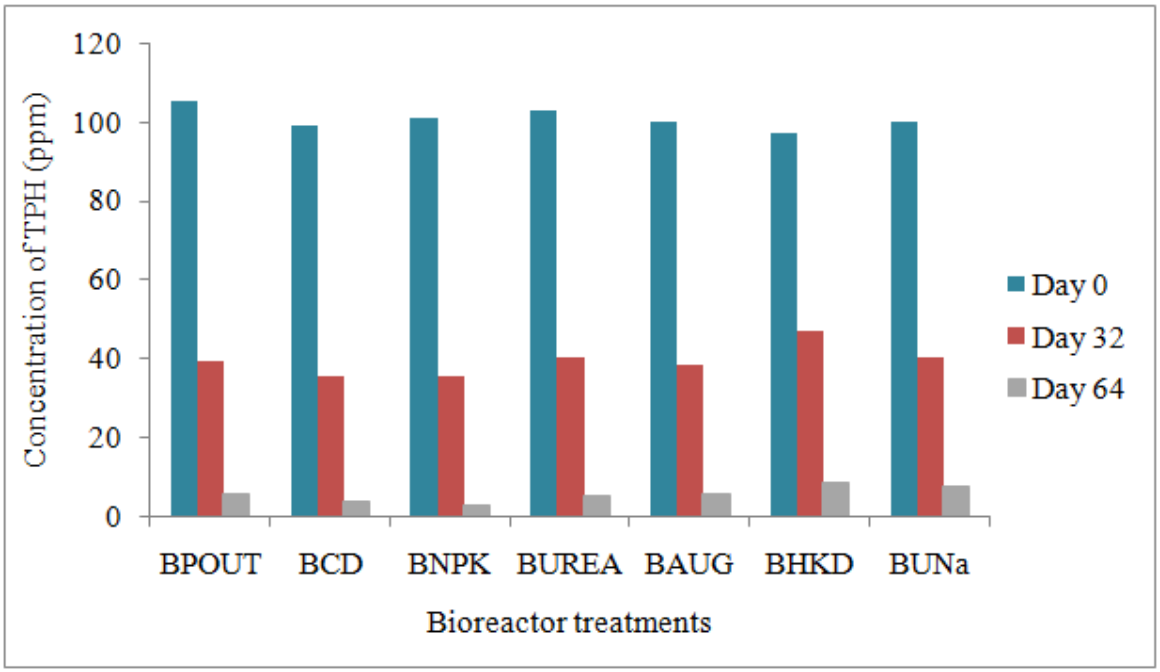


Figure 3a. Residual concentrations of total petroleum hydrocarbon (TPH) in different treatments during the 64-day bioremediation

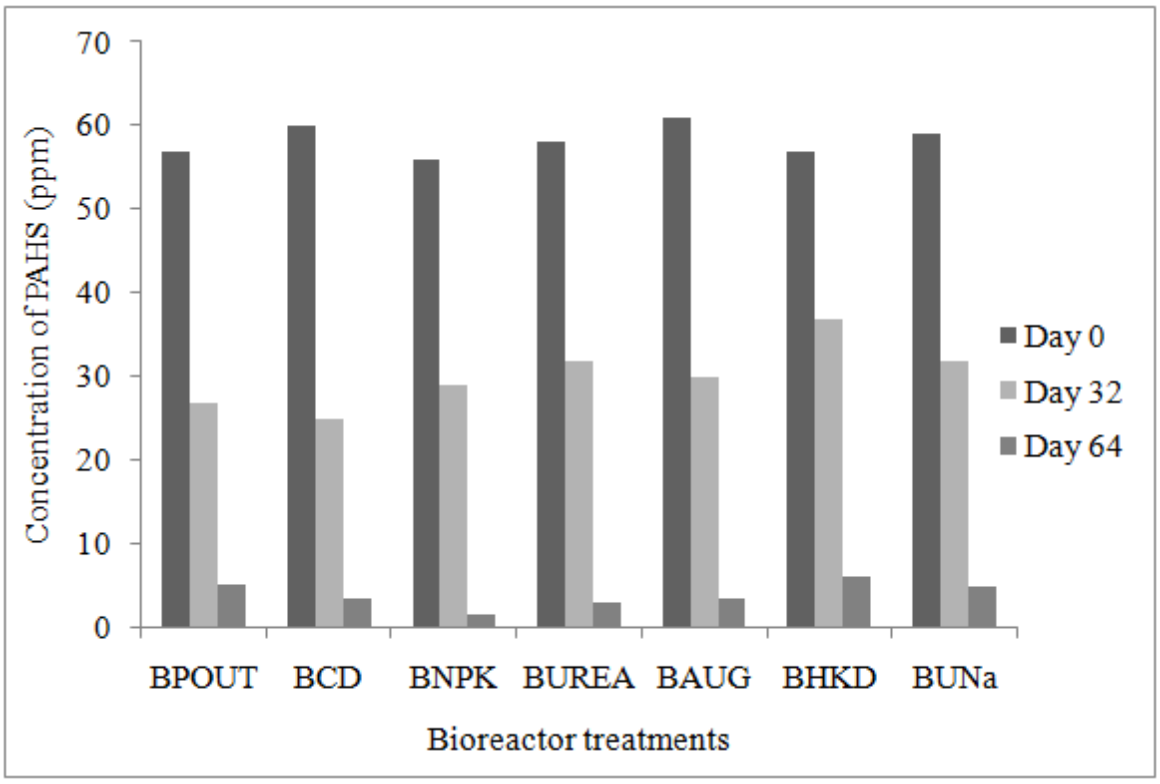


Figure 3b. Residual concentrations of Polycyclic aromatic hydrocarbon (PAH) in different treatments during the 64-day bioremediation

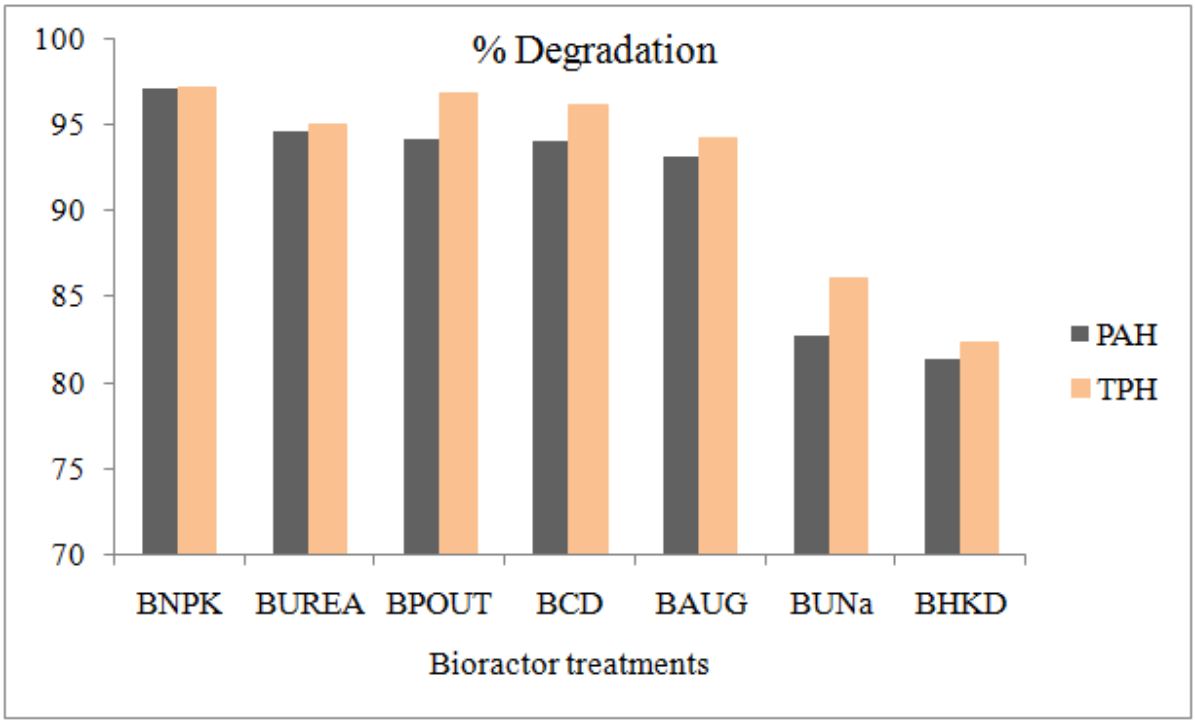


Figure 4. Percentage degradation of polycyclic aromatic hydrocarbon (PAH) and total petroleum hydrocarbon (TPH) during the 64-day bioremediation

The reduction in TPH and PAH in amended treatments and bioaugmented shows there is metabolically active bacterial community that probably uses the hydrocarbons as source of carbon and energy in the treatment samples. We observed higher disappearance of PAHs fractions in the BNPK treatment, as compared with other treatments.

There was slightly lower loss of hydrocarbon in the unamended (BUNa) and heat killed (BHKD) than the amended treatments. Disappearance of hydrocarbon in the BUNa (unamended) and BHKD (heat killed) controls was due to both biotic (complete mineralisation, production of biomass, and production of metabolites) and abiotic (volatilisation) processes. The reduction in TPH and PAH in the unamended (BUNa) shows that the indigenous microorganisms adapt to the presence of the hydrocarbon contaminants and bring about their degradation to reduced level of contamination [30,31]. The hydrocarbon loss in the heat killed (BHKD) control is attributed to abiotic factor, signifying that abiotic factors could as well contribute to hydrocarbon attenuation in the environment. Abiotic factor play significant role in biodegradation of hydrocarbons by directly affecting the chemistry of the pollutants as well as affecting the physiology and diversity of the microbial flora [30,31]. The bioreactor had a controlled environment and this loss was achieved by continuous agitation and stirring of the bioreactors, since no evaporation or leaching of volatile fraction occurred [32]. Stirring (by 2 impellers) in 150 rpm agitation rate at 0.05 L/min air flow rate throughout the 64-day experimental period. Stirring increases the contact between the reagents (Hydrocarbon, Oxygen, and Biomass) thus enhancing the mass transfer and biodegradation rate in amended, bioaugmented as well as the controls (heat killed [BHKD] and unamended [BUNa]). Air flow rate and agitation rate are important factors affecting the efficiency of biodegradation in a slurry-phase bioreactor. In the bioreactor, oxygen concentration is directly dependent on the air flow rate. These parameters influenced the structure and physiology of microbial communities and changed the physical and chemical properties of the pollutants [26]. Statistical study showed that the rate of degradation of both TPH and PAHs in the amended, bioaugmented, unamended and heat killed controls by day 64 were not significant at $P > 0.05$ using one way ANOVA and Tukey's multiple comparison. This observation meant that biodegradation of hydrocarbons in the amended, bioaugmented, unamended and heat killed control sediment slurries was taking place at similar rates. In addition, the reasons for the slight differences detected can also be explained in the light of the pH values variation. pH in the 7 bioreactors ranged from 7.3 to 7.9 after adjustment.

GC/MS analysis of TPH revealed hydrocarbon components with carbon numbers of C_6 - $C_{30..}$ The gas chromatography-mas spectrometry (GS-MS) analysis of PAHs resulted in different fractions being detected which were acenaphthylene, anthracene, benzo(α)pyrene, benzanthracene, 1, 12, benzoperylene, chrysene, 1, 2, 5, 6 dibenzanthracene, fluoranthene, fluorene, indeno (1, 2, 3) pyrene, naphthalene, phenanthrene, pyrene. Measurement of residual fractions of polycyclic aromatic hydrocarbon (PAH) in all treatments during the 64-day bioremediation is illustrated in Figure 5a-c. Many polycyclic aromatic hydrocarbons (PAHs) are known to be toxic and carcinogenic for humans, and their contamination of soils and aquifers is of great environmental concern. Over 94% of PAHs in the amended treatments were degraded, showing that nutrient amendment enhanced PAHs degradation because it increased the biomass and a concomitant decrease in petroleum hydrocarbon concentration.

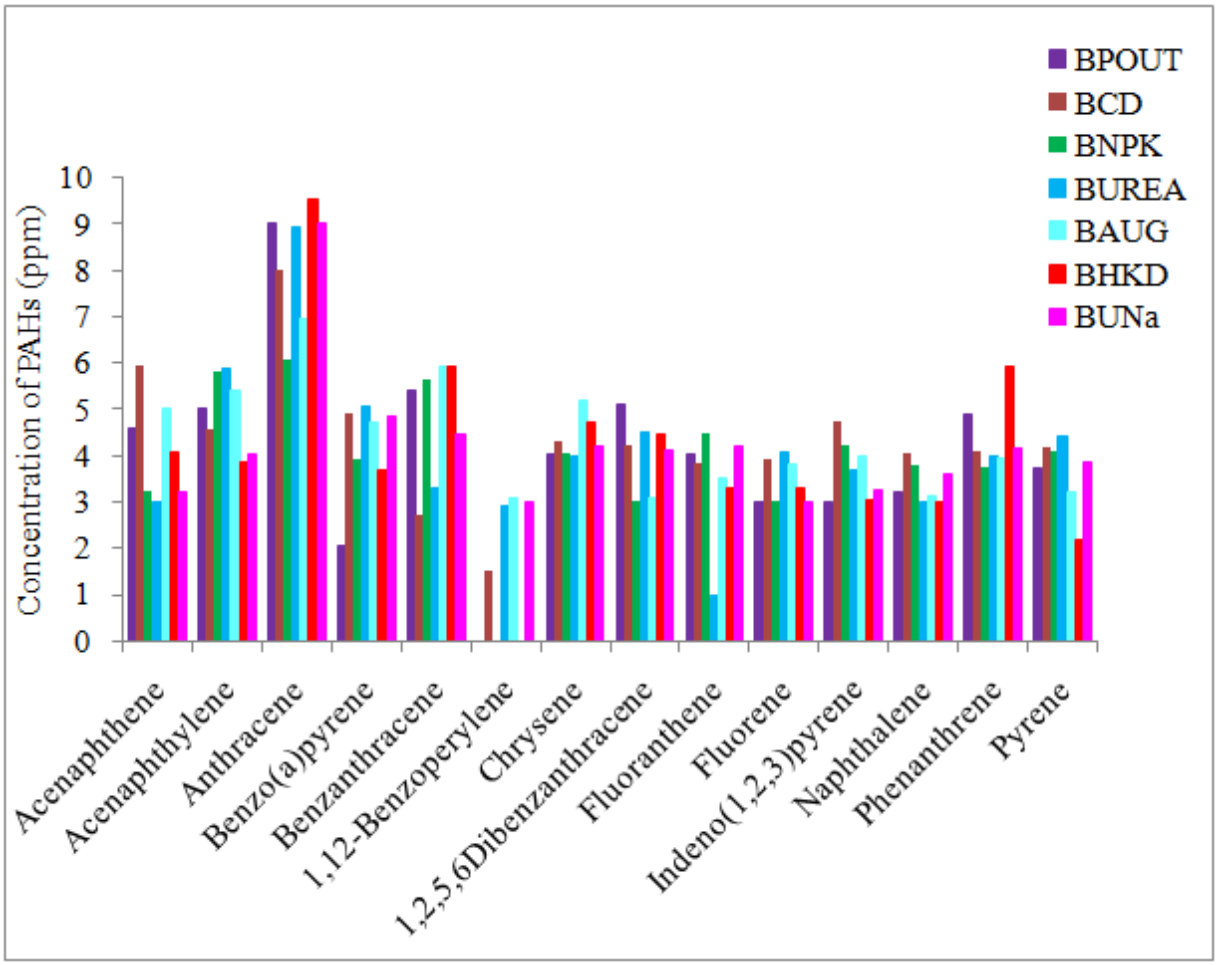


Figure 5a. Measurement of residual fractions of polycyclic aromatic hydrocarbon (PAH) in all treatments on day 0 of bioremediation

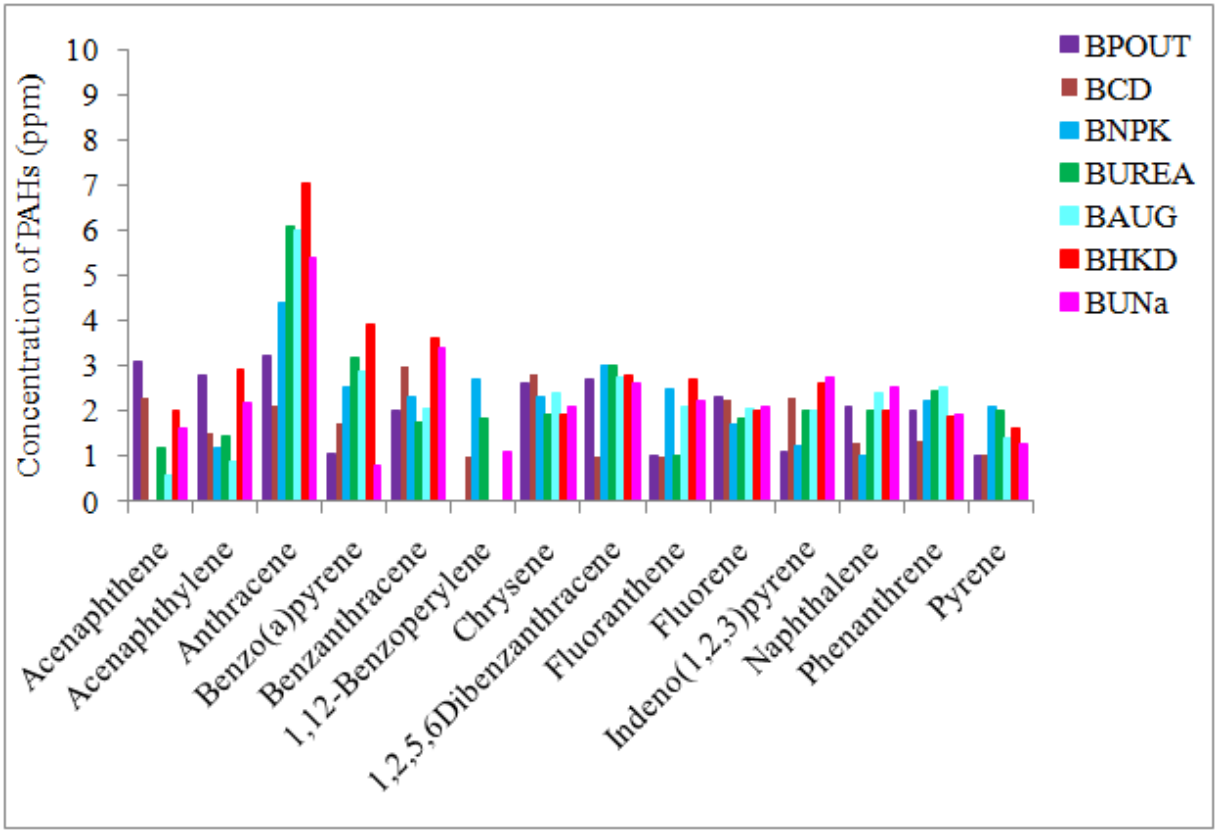


Figure 5b. Measurement of residual fractions of polycyclic aromatic hydrocarbon (PAH) in all treatments on day 32 of bioremediation

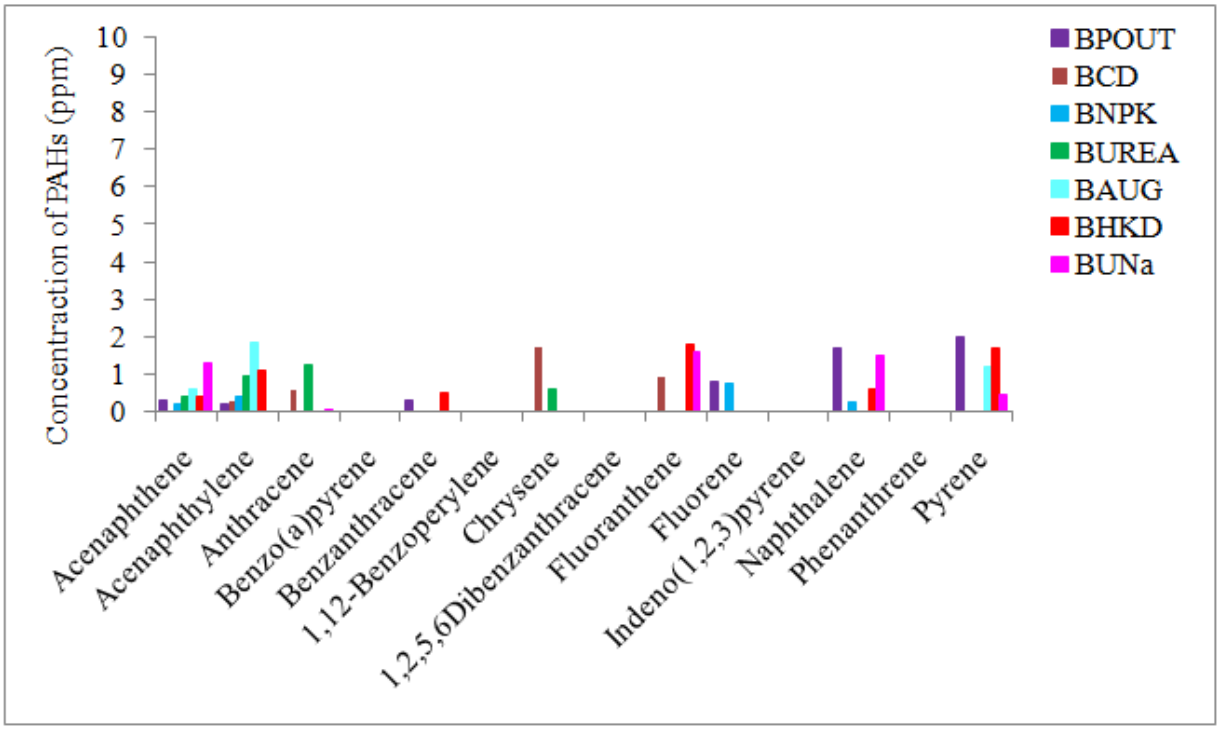


Figure 5c. Measurement of residual fractions of polycyclic aromatic hydrocarbon (PAH) in all treatments on day 64 of bioremediation

The low-molecular weight and the high-molecular weight PAHs fractions, showed rapid depletion in all mesocosms indicating the stored potential to degrade PAH contaminants and presence of functional genes is widespread in Bodo Creek sediment. The following PAHs fractions, benzo(α)pyrene, 1, 12, benzoperylene, 1, 2, 5, 6 dibenzanthracene, indeno (1, 2, 3) pyrene and phenanthrene were degraded below detectable limit (BDL) in all treatments. These high molecular weight compounds, are major contaminant classes of concern in oil spills because they are known for their mutagenic, teratogenic properties and are toxic and/or carcinogenic to humans and wildlife and are often recalcitrant to degradation in environment for example, benzo(α)pyrene metabolites are mutagenic and highly carcinogenic, and it is listed as a Group 1 carcinogen by the IARC (The International Agency for Research on Cancer).

3.4. Characterization of Hydrocarbon Utilizing Bacteria (HUB)

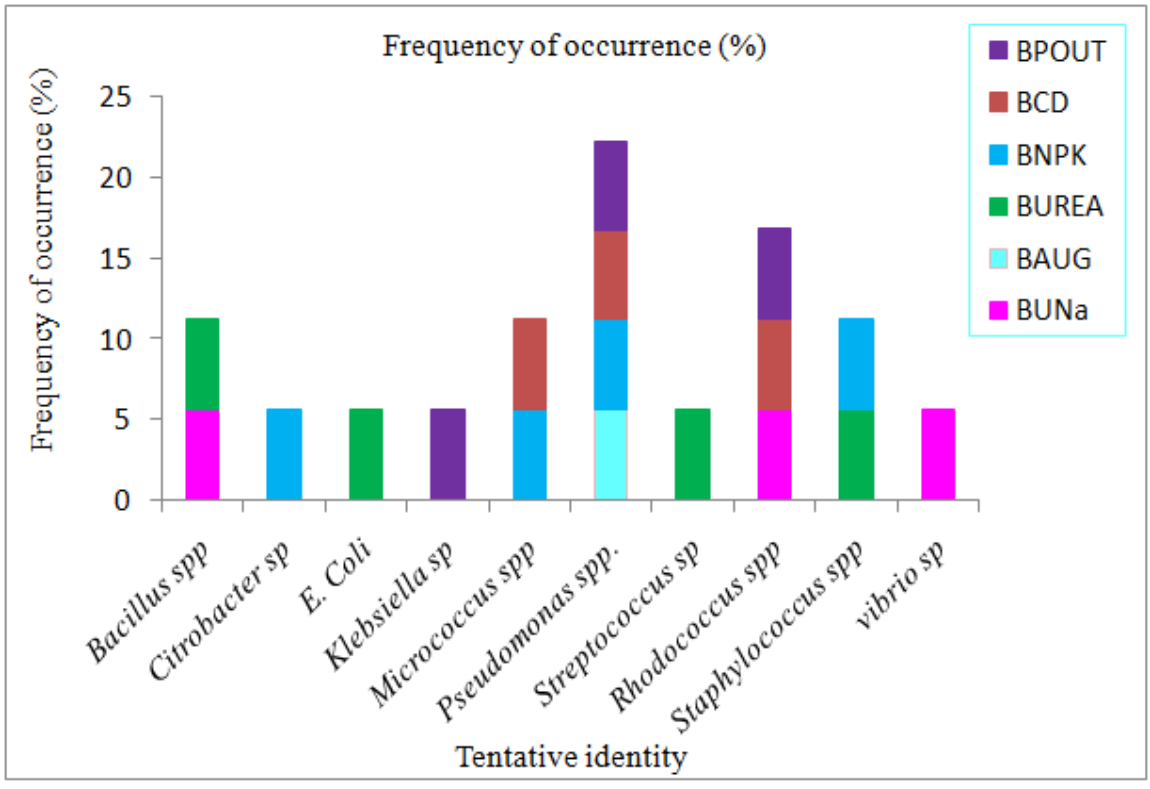


Figure 6a. Frequency of occurrence for tentative bacterial isolates on day 0

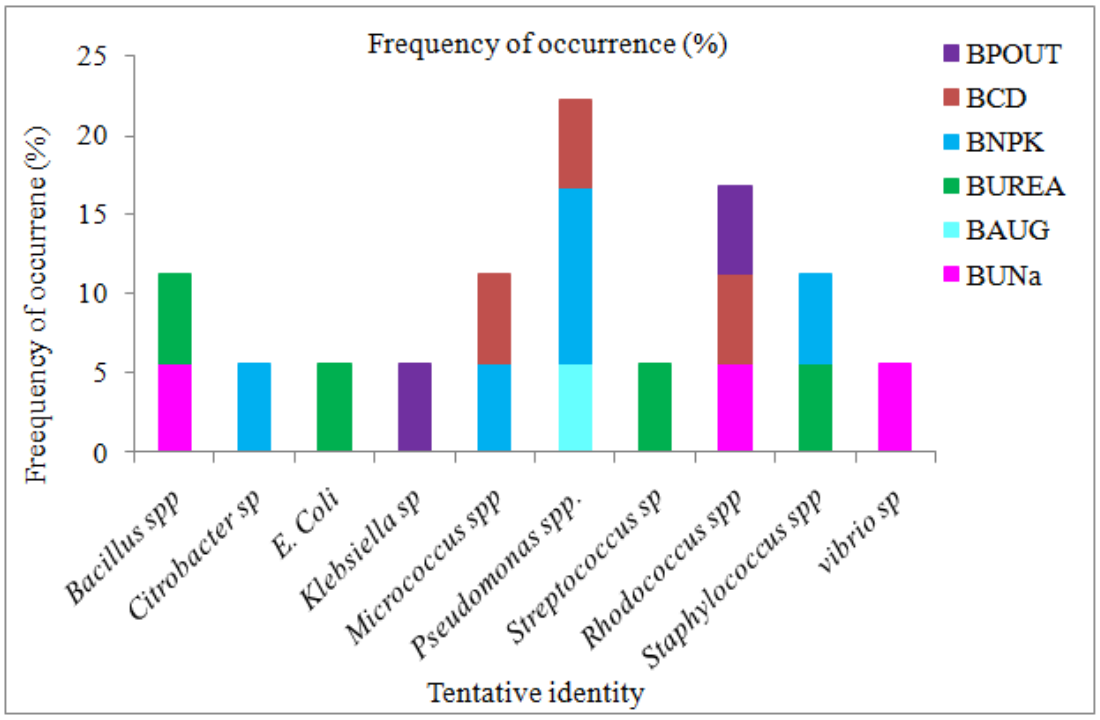


Figure 6b. Frequency of occurrence for tentative bacterial isolates on day 16

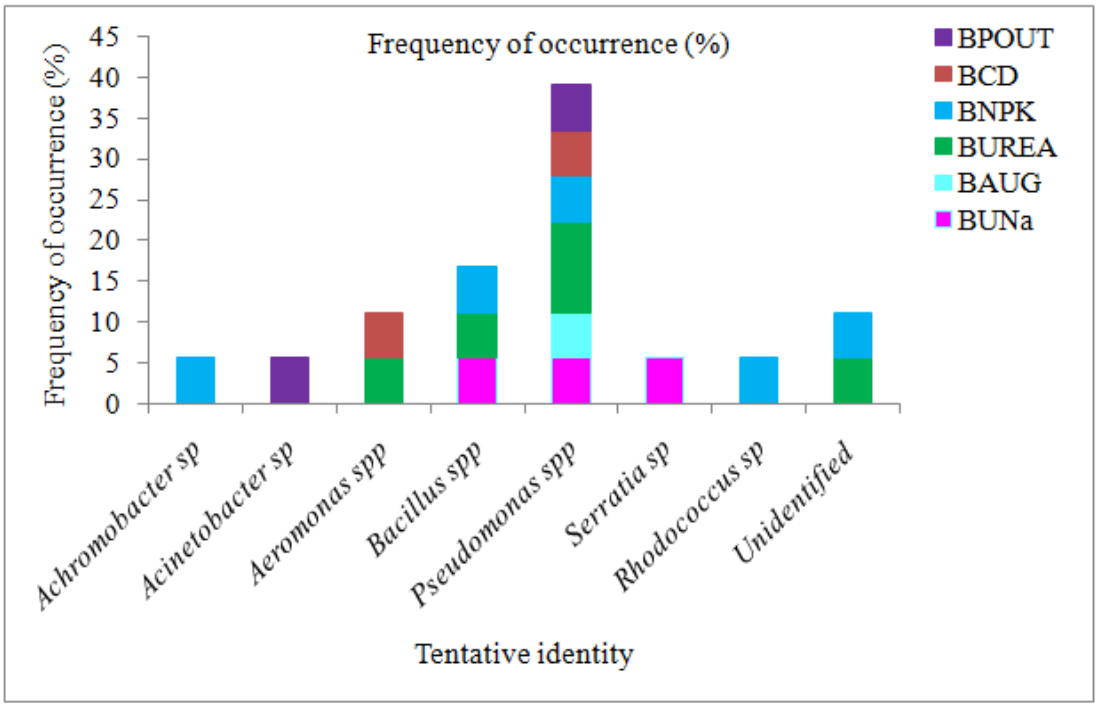


Figure 6c. Frequency of occurrence for tentative bacterial isolates on day 32

During the 64-day bioremediation study, variety of bacteria genera known to have the ability to degrade petroleum hydrocarbons were isolated from the nutrient-amended (BUREA, BNPK, BCD, BPOUT, BAUG) and unamended (BUNa) control. Result revealed diverse bacterial population in the amended treatments consisting of Gram positive and Gram negative bacteria. Seventy-two bacterial isolates was obtained and characterized as Gram positive and Gram negative bacteria. Bacterial genera obtained were characterized as *Corynebacterium* spp., *Pseudomonas* spp., *Citrobacter* spp., *Bacillus* spp., *Proteus* sp., *Micrococcus* spp., *Providencia* sp., *Vibrio* spp., *Aeromonas* spp., *Serratia* sp., *E. coli*, *Proteus* sp. *Staphylococcus* spp., *Streptococcus* spp., *Salmonella* sp., *Acinetobacter* spp., *Achromobacter* sp., *Klebsiella* sp. and *Rhodococcus* spp. Five bacterial isolates could not be given tentative identities and were designated unidentified bacteria. Figure 6a-d shows the frequency of occurrence for tentative bacterial isolates on day 0, 16, 32 and 64.

Our result revealed presence of diverse extant bacterial population. Several culture-dependent studies of oil-polluted marine environment have shown that some of these bacteria are rapidly and strongly selected when hydrocarbon degradation is stimulated by addition of organic and inorganic nutrients

The dominant genera isolated and appeared from the baseline to day 64 are *Pseudomonas* and *Bacillus*. These group of organisms have been implicated in hydrocarbon degradation, particularly *Pseudomonas* by several researchers. In this study, *Pseudomonas* sp had the highest frequency of occurrence. [33,34,35,36] found that *Pseudomonas* plays key role in hydrocarbon degradation in oil polluted sites and [36,37] reported *Pseudomonas* as the dominant polycyclic aromatic hydrocarbon-degrading bacteria isolated from Antarctic soils and they illustrated the important role this genera plays in PAH biodegradation in Antarctic soils. BNPK had more bacterial population than other treatments, this could have attributed to the high hydrocarbon loss of TPH and PAHs than other treatments. The unamended revealed presence of diverse extant autochthonous bacterial population. The bacterial isolates in the BUNa had the natural propensity to degrade hydrocarbon and this attributed to the hydrocarbon loss in the unamended (BUNa) even without nutrient addition. *Bacillus* sp., *Pseudomonas* sp., and *Rhodococcus* sp were isolated in all bioreactor treatments. There was diverse bacterial population in the treatments. The degradation of both crude and refined oils seems to involve a consortium of microorganisms, including both eukaryotic and prokaryotic forms. Hundreds of bacterial taxa rapidly proliferate in the presence of hydrocarbon and have been reported by several researchers to be capable of decomposing a variety of oil hydrocarbons but their relative abundance changes as the chemical composition of the oil is modified by the microbial community.

3.5. Turbidometry Test (Degradation Screening)

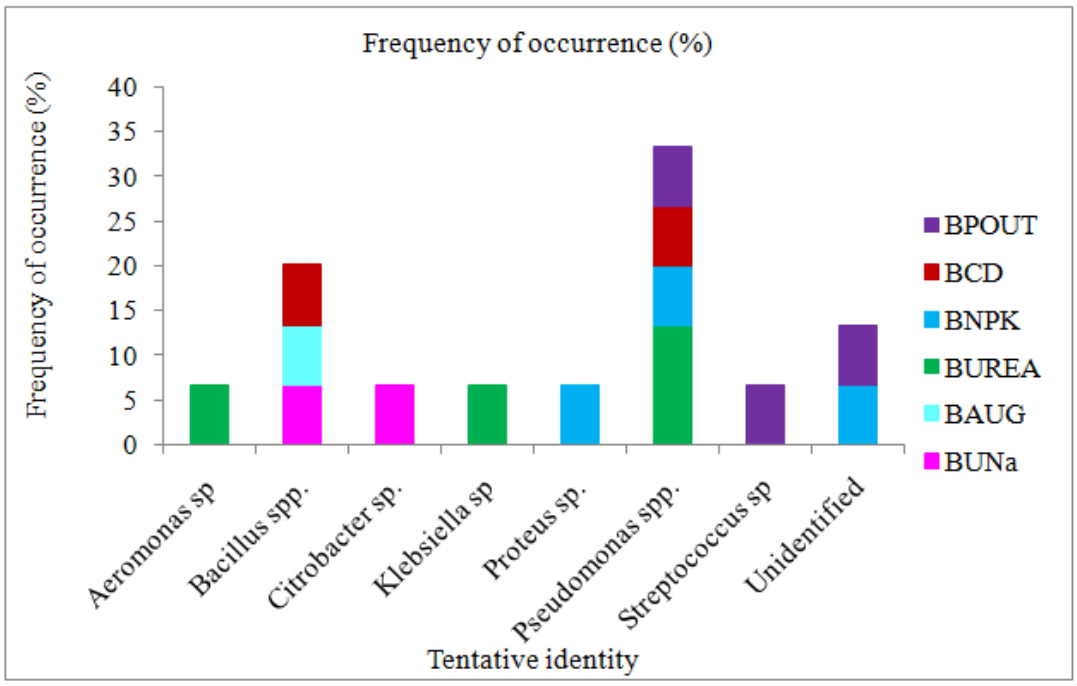


Figure 6d. Frequency of occurrence for tentative bacterial isolates on day 64

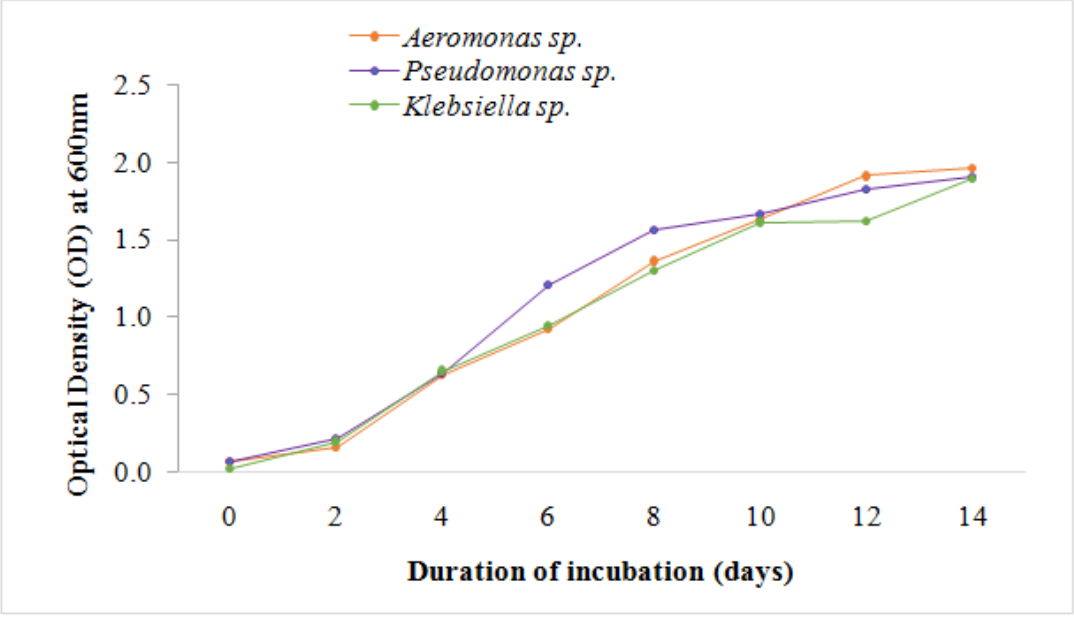


Figure 7a. Graph showing growth curve optical density (O.D) values of bacteria in Bushnell Haas broth + Okono crude oil for a period of 14 days

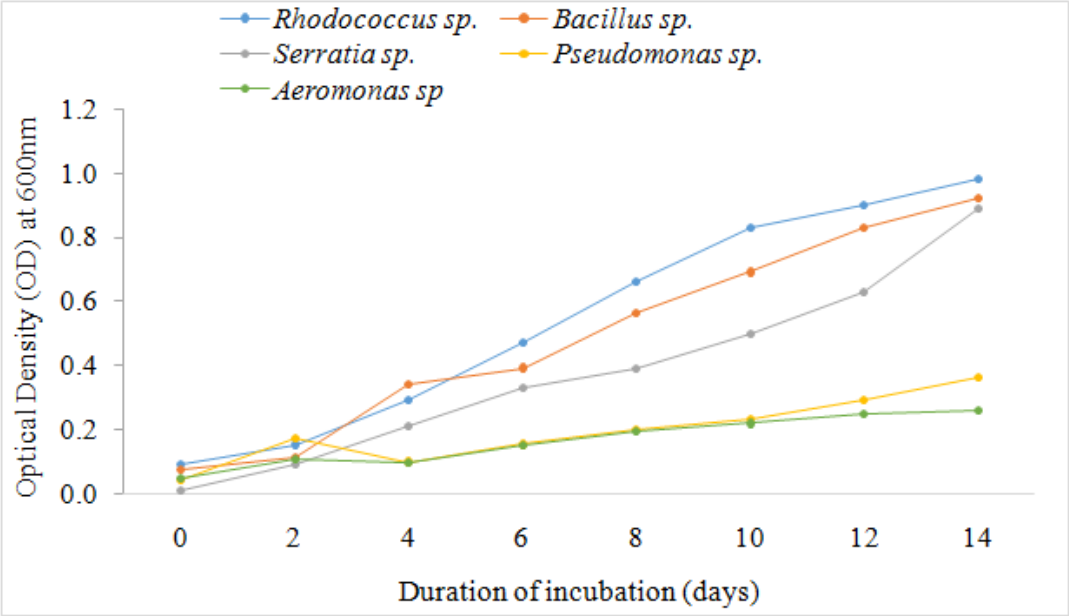


Figure 7b. Graph showing growth curve optical density (O.D) values of bacteria in Bushnell Haas broth + Okono crude oil for a period of 14 days

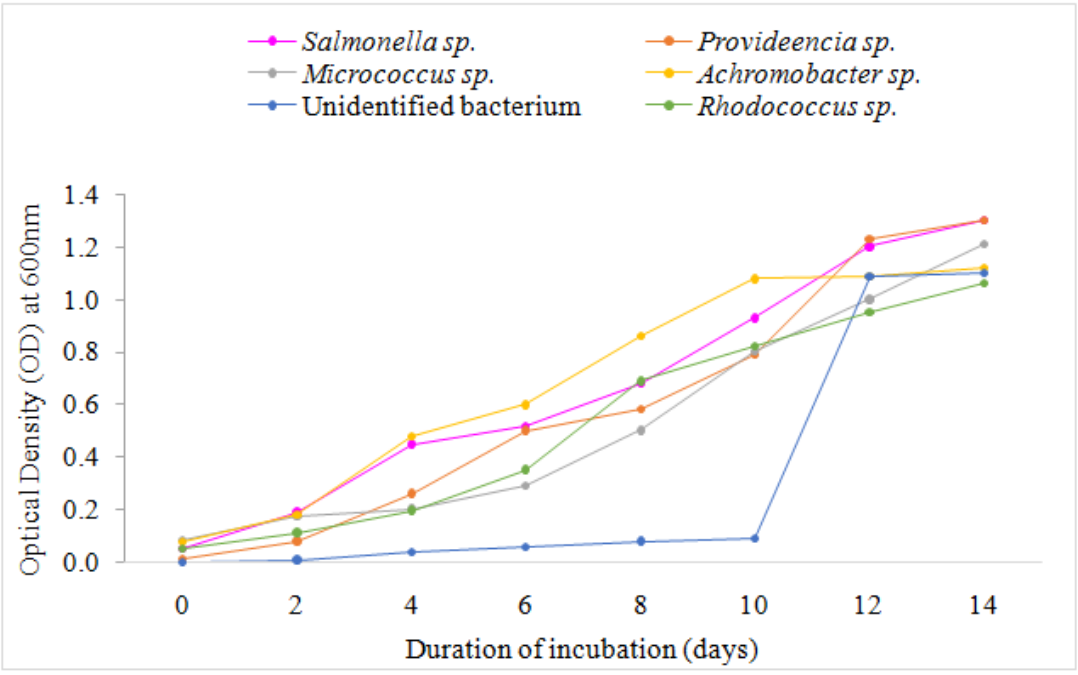


Figure 7c. Graph showing growth curve optical density (O.D) values of bacteria in Bushnell Haas broth + Okono crude oil for a period of 14 days

Biodegradation screening (Turbidometry test) was used to assess the degradation ability of individual isolate. It was assessed at optical density readings of 600nm wavelength at regular intervals of two days. Result revealed fifty hydrocarbon utilizing bacteria (HUB) out of the seventy-two isolated bacteria. Their degradation ability and the level of hydrocarbon utilization differs from one bacteria to another (due to difference in their growth curve). The optical density (O.D) values showed an increasing growth pattern in individual bacteria with time. We observed gradual increase in the concentration of the broth (turbidity) especially between 5-12 days and gradual decrease by 13-14. *Pseudomonas* sp., *Klebsiella* sp and *Aeromonas* sp. (Figure 7a) had the highest O.D values and ability to degrade crude oil. The graph based on the O.D readings at various time intervals of incubation period on the degradation activity of the oil-degrading bacteria are illustrated in Figure 7a-c. Our result shows that these organisms utilized the crude oil when supplied as the sole carbon and energy source. The bacteria with the least degradation ability was *Aeromonas* sp. (Figure 7b). The degrading organisms are *Pseudomonas* sp., *Bacillus* sp., *Corynebacterium* sp., *Vibrio* sp., *Klebsiella* sp, *Micrococcus* sp., *Rhodococcus* sp., *Staphylococcus* sp., *Citrobacter* sp., *Aeromonas* sp. The level of utilization differs from one microbe to another (due to difference in their growth curve).

3.6. Molecular Characterization

Bacterial 16S rRNA was used to characterize the hydrocarbon utilizing bacteria. Molecular fingerprinting of isolates was done using polymerase chain reaction (PCR) of template DNA targeting bacterial 16S rRNA. For the

past two decades, microbiologist have primarily relied on it for identification and classification of isolated pure cultures and estimation of bacterial diversity in environmental samples without culturing through metagenomic approaches [38]. PCR amplification using universal primer set (pA8f and KPRUN518r), resulted in amplification of 65 bacterial isolate amplified out of seventy-two hydrocarbon utilizing bacteria isolates obtained and yielded PCR product of 500kb which were assigned tentative identities as *Corynebacterium* sp., *Pseudomonas* spp., *Citrobacter* spp., *Bacillus* spp., *Proteus* sp., *Micrococcus* spp., *Providencia* sp., *Vibrio* spp., *Aeromonas* spp., *Serratia* sp., *E. coli*, *Staphylococcus* spp., *Streptococcus* spp., *Salmonella* sp., *Acinetobacter* spp., *Achromobacter* sp., *Klebsiella* sp., *Rhodococcus* spp. Gel electrophoresis showing amplified 16S rRNA fragments of bacterial DNA (1-72) is presented in Figure 8a-c.

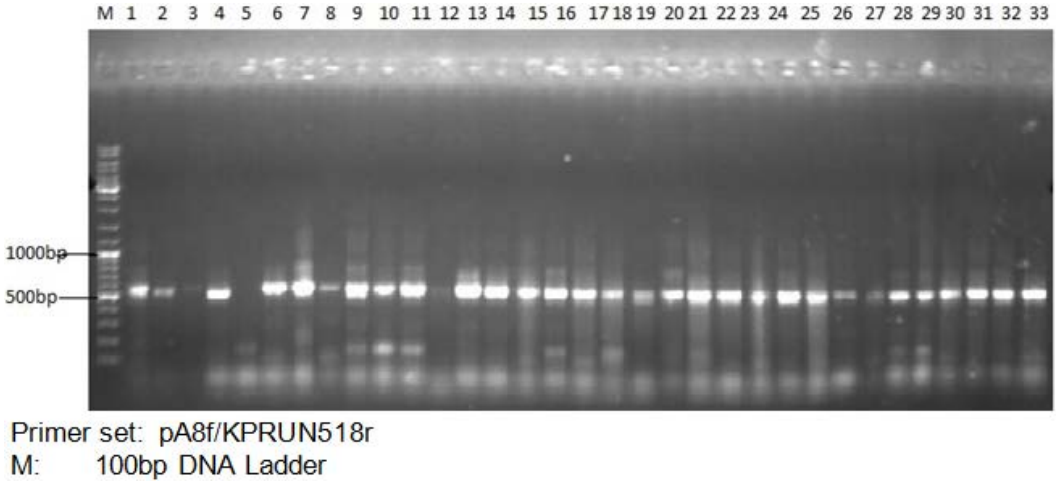


Figure 8a. Gel electrophoresis showing amplified 16S rRNA fragments of bacterial DNA (1-33).

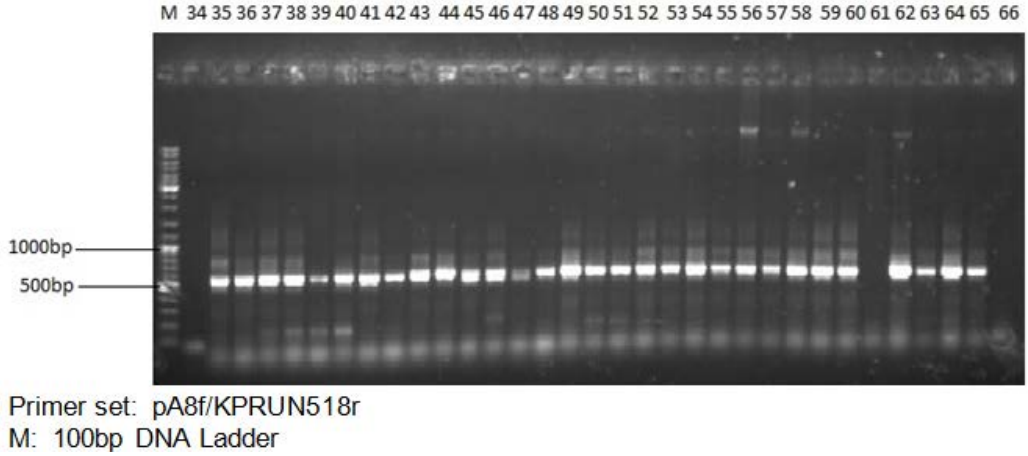


Figure 8b. Gel electrophoresis showing amplified 16S rRNA fragments of bacterial DNA (34-66)

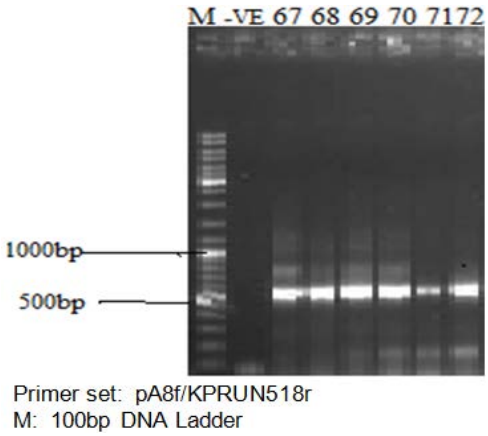
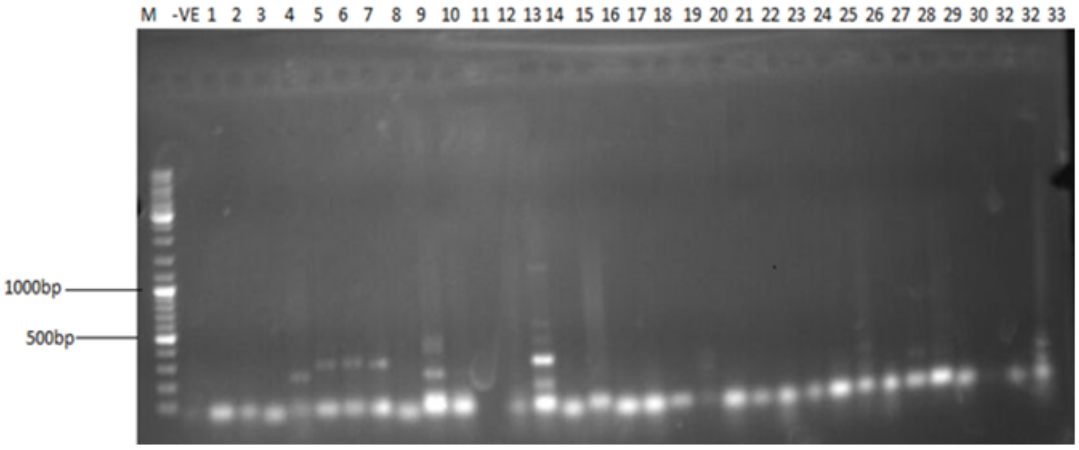


Figure 8c. Gel electrophoresis showing amplified 16S rRNA fragments of bacterial DNA (67-72)

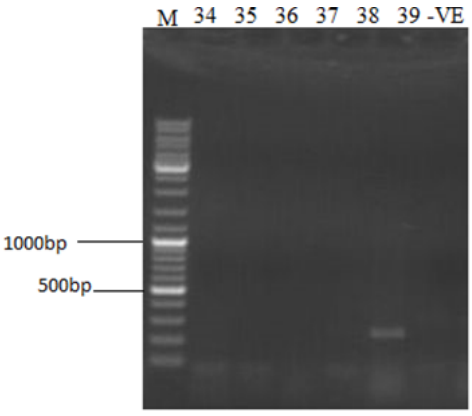
The fifty hydrocarbon utilizing bacterial (HUB) isolates from the turbidometry test were further screened for Catechol 2, 3-dioxygenase genes and other dioxygenases using degenerate primers. Amplification of bacterial DNA sample with specific primer sets 23CAT-F…5'-CGACC TGATCTCCATGACCGA-3' and 23CAT-R ...5'-TCAGG TCAGCACGGTCA-3 targeting catechol dioxygenases resulted in DNA fragment of 238bp-250bp. Figure 9a-c represents gel electrophoresis showing amplified DNA fragments of catechol 2,3- dioxygenases. Catechol 2,3-dioxygenases (*C23DO*) gene was assessed by polymerase chainreaction (PCR). Each PCR product was run on a 1% agarose gel in 1X TAE buffer and visualized under UV Light transilluminator Photogel for the detection of dioxygenase amplicons betweeen 238bp-250bp. The following genera were found to posses the functional gene (catechol 2, 3-dioxygenases): *Pseudomonas* spp. (3), *Rhodococcus* sp., *Achromobacter* sp., *Serratia* sp, *Citrobacter* sp. and *Bacillus* sp. Screening of bacterial isolates using degenerate primer DEG-F.. 5' CGACCT GATC(AT)(CG)CATGACCGA-3' and DEG-R... 5' T(CT)

AGGTCA(GT)(AC)ACGGTCA-3' targeting bacterial dioxygenase gene of 250bp. DEG F and DEG R are degenerate primers for dioxygenases (letters in parentheses indicate positions of degenerate bases) was used to amplify dioxygenase genes. Primers DEG-F and DEG-R are identical to 23CAT-F and 23CAT-R except for five positions where degenerate bases were used to account for primer-target mismatches with known *Pseudomonas* spp. that habour aromatic ring cleavage dioxygenases. The *C23DO*-Specific primers and DEG-specific primer are capable of detecting bacteria that can degrade various aromatic hydrocarbons. The following genera *Rhodococcus* sp., *Micrococcus* sp., *Acinetobacter* sp. and *Aeromonas* sp. showed positive results for DEG. Dioxygenase genes was assessed by polymerase chain reaction (PCR). Each PCR product was run on a 1% agarose gel in 1X TAE buffer and visualized under UV Light transilluminator Photogel for the detection of 250bp dioxygenase amplicons. Figure 10a-b represents gel electrophoresis showing positive PCR bands for DEG. Three genera, *Rhodococcus* sp., *Micrococcus* sp and *Bacillus* sp. showed dioxygenase amplicon. Degenerate primers may be used to amplify DNA in situations where only the protein sequence of a gene is known or where the aim is to isolate similar genes from a variety of species. Garcia *et al.*,(39) used degenerate primers to investigate dioxygenase enzyme activity during biodegradation of various aromatic compounds. In addition, *Mycobacterium*, *Pseudomonas* and strains *Rhodococcus* which could degrade higher molecular weight PAH were detected with the degenerate primer in previous study by [40]. There is no amplification in the rest of the isolates indicating the absence of catechol 2, 3-dioxygenases enzyme activities for the hydrocarbon degradation. Catechol 2,3-dioxygenases constitute a group of enzymes that are considered crucial for degradation of a wide range of aromatic compounds by indigenous hydrocarbon-degrading bacteria in contaminated environment because they play important role in natural attenuation process of hydrocarbon contaminated environment. [41].



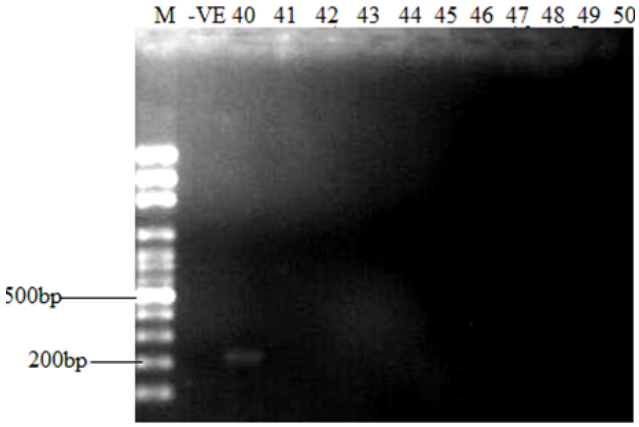
Lane 4: *Pseudomonas* sp. Lane 5: *Rhodococcus* sp.
Lane 6: *Pseudomonas* sp. Lane 7: *Pseudomonas* sp.
Lane 9: *Achromobacter* sp. Lane 13: *Serratia* sp.
M: 100bp DNA Ladder
Primer set: 23CAT-F and 23CAT-R (-VE, 1-33)
-VE: Negative control:

Figure 9a. Gel electrophoresis showing amplified DNA fragments of catechol 2,3- dioxygenases (Lane 1-33).



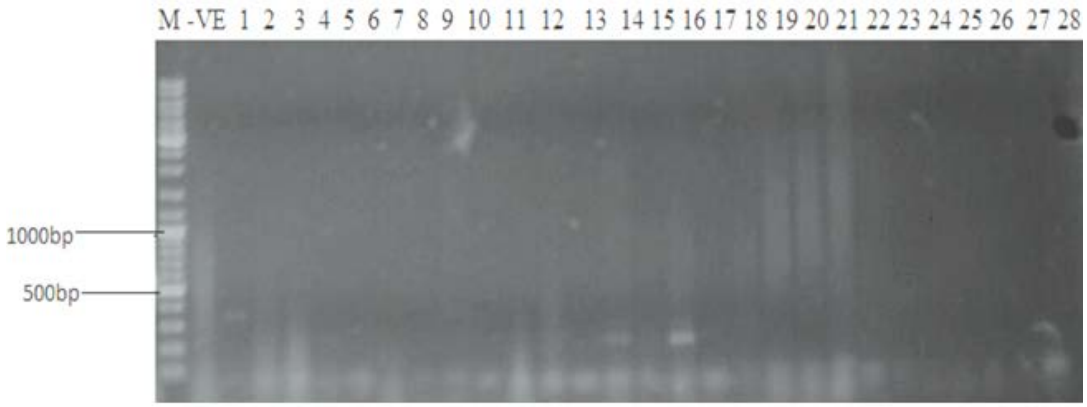
lane 39: *Bacillus* sp.
M: 100bp DNA Ladder
Primer set: 23CAT-F and 23CAT-R (34-39, -VE)
-VE: Negative control:

Figure 9b. Gel electrophoresis showing amplified DNA fragments of catechol 2,3 dioxygenases (Lane 34-39)



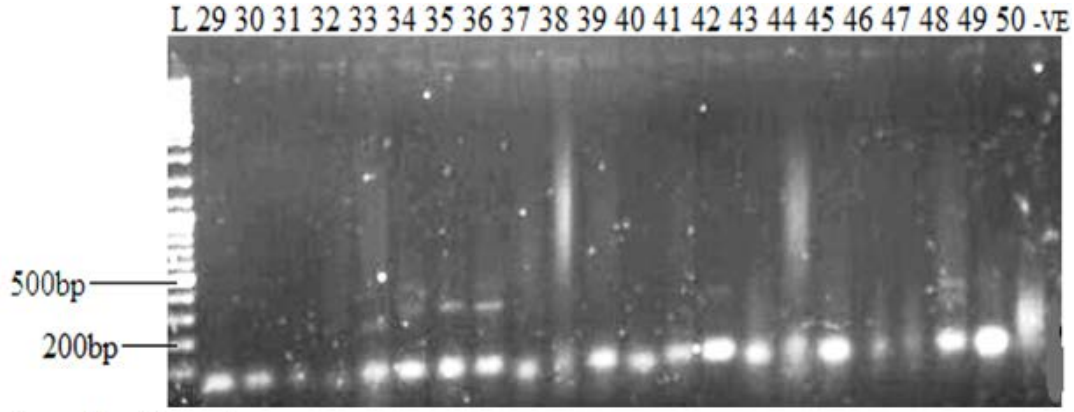
Lane 40: *Citrobacter* sp.
M: 100bp DNA Ladder
Primer set: 23CAT-F and 23CAT-R (-VE, 40-50)
-VE: Negative control:

Figure 9c. Gel electrophoresis showing amplified DNA fragments of catechol 2,3- dioxygenases (Lane 40-50)



Lane 13: *Rhodococcus* sp.
Lane 15: *Micrococcus* sp
DEG-F and DEG-R (-VE, 1-28)
M: DNA LADDER 100bp
-VE: Negative control

Figure 10a. Gel electrophoresis showing amplified DNA fragments of DEG (Lane 1-28)



Lane 35: *Acinetobacter* sp.
Lane 36: *Aeromonas* sp.
L: DNA LADDER 100bp
-VE: Negative control

Figure 10b. Gel electrophoresis showing amplified DNA fragments of DEG (Lane 29-50, -VE)

Several reports have assessed the degradation of hydrocarbons by environmental microflorae and reported that it involves having specialized metabolic capabilities and detection of hydrocarbon degradative genes like aromatic ring cleavage dioxygenases (*C23DO*) genes. The ability of an organism to degrade a specific substrate is a clear evidence that it genome harbors the relevant degrading gene The result from our study gives credence to the fact that these bacterial genera (*Pseudomonas* spp. (3)., *Rhodococcus* sp.(2), *Achromobacter* sp., *Serratia* sp, *Citrobacter* sp., *Bacillus* sp, *Micrococcus* sp., *Acinetobacter* sp. and *Aeromonas* sp. which showed positive bands betweeen 238bp-250bp for detection of dioxygenase are well adapted to the polluted environment because they possess the dioxygenase gene (D23DO) genes. The presence of slightly lower (<250 bp) positive bands in lanes 4, (*Pseudomonas* sp.), 9 (*Achromobacter* sp.) is an indication these organisms are capable of transforming and degrading different fractions of PAHs. This detection is a direct evidence that *C23DO* genes, can be amplified from the indigenous bacteria in this environment and is a good marker gene for monitoring bioremediation in oil-polluted environment. For example [27] found that *Pseudomonas* is present in contaminated environment and posses this catabolic gene. Some microorganisms, mainly from the genera *Pseudomonas*, *Achromobacter*, *Klebsiella*, *Bacillus*, *Acinetobacter* and *Mycobacterium*, were found to be capable of transforming and degrading PAHs. As discussed by [33] reported the following *Achromobacter*, *Pseudomonas*, *Klebsiella*, *Bacillus*, *Acinetobacter* as bacterial genera that contain PAHs-degrading species

Molecular detection of same was observed by [42], they used degenerate primers to investigate dioxygenase enzyme activity during biodegradation of various aromatic compounds. The detection of the relevant degradative gene (catechol 2,3-dioxygenase) in the extant indigenous bacterial populations in this oil-impacted area shows that they have the natural propensity to degrade hydrocarbons. Our findings are consistent with several other studies showing the detection of functional gene (catechol 2, 3-dioxygenases) in numerous Gram-negative (*Pseudomonas*, *Sphingomonas*, *Acinetobacter*, *Ralstonia*, *Burkholderia*,

Stenotrophomonas) and Gram-positive (*Nocardia*, *Rhodococcus* and *Bacillus*) bacteria [19, 25].

3.7. DNA Sequencing and Nucleotide Sequence Accession Numbers

The isolates were further confirmed by sequencing amplified PCR products of universal primer set pA8f and KPRUN518r using GATC biotech AG, European custom sequencing center. Electrophoregrams of the sequences generated were inspected with Finch TV software (Geospiza) Germany according to Sanger sequencing protocol. Identification of 16S rRNA sequenced was aligned with BLAST search facility of National Centre for Biotechnology information (NCBI). Molecular identities of bacterial isolates is shown in Table 3. The use of molecular techniques allowed the identification of 24 isolates to specie level and one (1) band uncultured bacterium which was submitted to the FASTA GenBank and assigned accession numbers for the nucleotide sequences. Majority of the successfully sequenced isolates belonged to the genus *Pseudomonas* and are related to *Pseudomonas aeruginosa* and *Pseudomonas* sp. which exhibited similarity of (96%-100%). Our findings are consistent with several other studies showing that bacteria from the *Pseudomonadaceae* family respond to degrade oil. *Gordonia, Brevundimonas, Achromobacter, Chromohalobacter* are halo tolerant organisms and play key role in degradation of mono and poly-aromatic compounds.

Table 3. Molecular identities of bacterial isolates

Phylum	Identification	% Similarity	Accession number
Actinobacteria	*Gordonia* sp. strain=BH1	96	KR261407
α-proteobacteria	*Brevundimonas naejangsanensis* strain=BH2	99	KR261408
γ-proteobacteria	*Pseudomonas* sp. strain=BH3	97	KR261409
γ-proteobacteria	*Pseudomonas* sp. 1 strain=BH4	100	KR261410
γ-proteobacteria	*Pseudomonas pseudoalcaligenes* strain=BH5	99	KR261411
γ-proteobacteria	*Pseudomonas aeruginosa* strain=BH6	99	KR261412
γ-proteobacteria	*Shewanella* sp. strain=BH7	99	KR261413
β-proteobacteria	*Achromobacter* sp. strain=BH8	96	KR261414
γ-proteobacteria	*Pseudomonas* sp. 2 strain=BH9	96	KR261415
Bacteroidetes	*Sphingobacterium* sp. strain=BH10	97	KR261416
γ-proteobacteria	*Pseudomonas* sp. 3 strain=BH11	99	KR261417
γ-proteobacteria	*Pseudomonas aeruginosa* strain=BH12,	99	KR261418
Firmicutes	*Bacillus* sp. strain=BH13	99	KR261419
γ-proteobacteria	*Pseudomonas aeruginosa* strain=BH14	96	KR261420
γ-proteobacteria	*Pseudomonas aeruginosa* strain=BH15	100	KR261421
γ-proteobacteria	*Pseudomonas aeruginosa* strain=BH16	100	KR261422
γ-proteobacteria	*Pseudomonas* sp. 4 strain=BH17	100	KR261423
β-proteobacteria	*Aquitalea magnusonii* strain=BH18	97	KR261424
γ-proteobacteria	*Pseudomonas aeruginosa* strain=BH19	100	KR261425
β-proteobacteria	*Achromobacter* sp. strain=BH20	100	KR261426
γ-proteobacteria	*Pseudomonas aeruginosa* strain=BH21	99	KR261427
γ-proteobacteria	*Halomonas lutea* strain=BH22	96	KR261428
	Unidentified bacterium strain=BH23	96	KR261429
γ-proteobacteria	*Pseudomonas* sp. 5 strain=BH24	100	KR261430
γ-proteobacteria	*Pseudomonas aeruginosa* strain=BH25	100	KR261431

We noticed a phenomenon named "gamma-shift", which consists of having high number of hydrocarbons degrading bacteria belonging to γ-*Proteobacteria*, caused by increase in the nutrients from the presence of oil (contamination source) which provides a major source of carbon and electrons in an otherwise nutrient-starved marine environment. The shift is a deviation in bacterial community structure/diversity towards γ-*Proteobacteria* which are known class of bacteria associated with hydrocarbon degradation. The shifts in community composition in response to oil presence in Bodo Creek sediment were manifested at the strain to family level. This results emphasize that the embedded oil exerted a strong selective pressure on the bacterial community. The present study, along with past studies, shows that the γ-*Proteobacteria* predominates the bacterial communities of marine sediment ecosystems following exposure to oil hydrocarbons [12,42]. Members of this group have been described as major bacterial degraders of PAHs (phenanthrene and pyrene) in soil and may represent a good bioindicator for the potential biodegradation of 2-, 3- and 4-ring PAHs in a coal-tar contaminated. [43]. Research by [44], in the study of New Hydrocarbon Degradation Pathways in the Microbial Metagenome from Brazilian Petroleum Reservoirs reported that members of *Pseudomonas aeruginosa* and *Pseudomonas putida* are hydrocarbonoclastic. *Pseudomonas* sp. have been successfully used to treat hydrocarbon contaminated sites. Previous study by [45] on biodegradation of oil tank bottom sludge using microbial consortia isolated and identified *Pseudomonas pseudoalcaligenes* as hydrocarbon degrader from petroleum polluted soil. However, [45] reported *P. pseudoalcaligenes*, a bacterium in the *P. aeruginosa* group, capable of degrading a range of PAHs. *Shewanella oneidensis and Bacillus* sp. have been reported to degrade all PAHs [46]. Other studies have demonstrated that bacterial genera *Gordonia* isolated from petroleum contaminated soil proved to be one of the potential organisms for hydrocarbon degradation and three strains of *Gordonia* sp., to be capable of degrading anthracene, nephthalene, pyrene, phenathrene, benzo [a] pyrene and flouranthene as sole carbon and energy source [46]. *Halomonas* species can degrade complex hydrocarbons such as diesel fuel and crude oil under

psychrotrophic conditions [26]. The degradation of both crude and refined oils seems to involve a consortium of microorganisms, including both eukaryotic and prokaryotic forms. In our study, gamma shift points to a succession of bacterial populations associated in oil-contaminated marine environments.

4. Conclusion

The outcome of this study showed that bioreactors can be effectively employed in monitored-petroleum hydrocarbon degradation. It was observed that Bodo creek sediment harbour diverse bacterial populations and an appreciable population of indigenous hydrocarbon utilizing bacteria population from known and very versatile genera such as *Pseudomonas* and other γ-*Proteobacteria* which can cope in multiple pollutants and can be monitored, enhanced to increase the bioremediation rate in this crude oil-polluted site. This study provides insight into the effectiveness of biostimulation in petroleum hydrocarbon degradation. The ability of these extant indigenous bacterial populations in this oil-impacted sediment to degrade hydrocarbon is a clear cut evidence that their genome harbours the relevant degrading gene (catechol 2,3-dioxygenase). This study is of great importance because the presence of aromatic hydrocarbon-degrading genes in bacteria isolated from Bodo Community, Niger delta in Nigeria, indicates that metabolic characteristics of interest for applications in environmental biotechnology may be retrieved from such environment. Therefore information on the diversity and distribution of aromatic hydrocarbon-degrading bacteria would be instrumental in determining the amenability of oil-polluted ecosystems to bioremediation. Further studies need to be carried out in details on the genetics of the hydrocarbon degrading bacteria in this Bodo Creek, Bodo community, Niger Delta, Nigeria marine sediments and to ascertain other degradative genes/enzymes they posses.

List of Abbreviations

GC-MS:-	Gas Chromatography-Mass Spectrometry
PCR :-	Polymerase Chain Reaction
TCHB:-	Total Culturable Heterotrophic Bacteria
TCHUB:-	Total Culturable Hydrocarbon Utilizing Bacteria
TPHs :-	Total Petroleum Hydrocarbons
µS/cm :-	Micro Siemens per centimetre,
mg/kg :	Milligram per kilogram
ppm :	Parts per million
cfu/g:	Cell forming unit per gram.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

Acknowledgement

Funding for this study was provided by a Grant (W/4263-2F) given to Dr.(Mrs.) Chioma Blaise Chikere, one of the authors from the International Foundation for Science (IFS) Stockholm, Sweden.

Authors' Contribution

CBC designed the whole study and AUO carried out the experiment, helped in manuscript preparation and was guided/ revised it critically for important intellectual content by CBC and GCO. All authors read and approved the final manuscript.

References

[1] United Nation Environmental Programme (UNEP0 (2011). Environmental setting in Ogonilannd and Niger Delta. Environmental Assessment of Ogoniland. UNEP, Nairobi, Kenya.

[2] Environmental Guidelines and Standards for Petroleum Industries in Nigeria (EGASPIN) (1992). Issued by the Department of Petroleum Resources, Nigeria. (Revised edition, 2002).

[3] Youssef, M., El-Taweel G. E., A. Y. Naggar, S. E. El-Hawary, El-Meleigy, M. A. and Ahmed, S. A. (2010). Hydrocarbon Degrading Bacteria as Indicator of Petroleum Pollution in Ismailia Canal, Egypt. *World Applied Sciences Journal* 8 (10): 1226-1233.

[4] Varjani S. J;. Rana Dolly P.; Bateja, S. and Upasani, V. N. (2013). Isolation and Screening for Hydrocarbon Utilizing Bacteria (HUB) from Petroleum Samples. *Internal Journal of Current Microbiology and Applied Science* 2(4): 48-60.

[5] Das, N. and Chandran, P. (2011) Microbial degradation of petroleum hydrocarbon contaminants – an overview. *Biotechnology Research International* (11):1-13.

[6] Paniagua-Michel, J. and Rosales, A. (2015) Marine Bioremediation - A Sustainable Biotechnology of Petroleum Hydrocarbons Biodegradation in Coastal and Marine Environments. *Journal Bioremediation and Biodegradation* 6:273.

[7] Godleads, O. A.; Prekeyi T. F.; Samson E. O. and Igelenyah E. (2015). Bioremediation, Biostimulation and Bioaugmention: A Review *International Journal of Environmental Bioremediation & Biodegradation* 3 (1): 28-39.

[8] Moscoso, F.; Teijiz, I.; Deive F. J. and Sanromán, M. A. (2012). Efficient PAHs biodegradation by a bacterial consortium at flask and bioreactor scale. *Bioresource Technology, Elsavier* 119: 270-276.

[9] Sebiomo, A.; Bankole, S. A. and Awosanya, A. O. (2010). Determination of the ability of microorganisms isolated from mechanic soil to utilise lubricating oil as carbon source. *African journal of microbiology research* 4(21): 2257-2264.

[10] Chikere, C. B.; Okpokwasili, G. C. and Ichiakor, O. (2009) Characterization of hydrocarbon utilizing bacteria in tropical marine sediments. *African Journal of Biotechnology* 8: 2541-2544.

[11] Rojo, F. (2009). Degradation of alkanes by bacteria. *Environ Microbiol* 11:2477-2490.

[12] Yakimov, M. M.; Timmis, K. N. and Golyshin, P. N. (2007) Obligate oil degrading marine bacteria. *Current Opinion on Biotechnology*. 18:257-268.

[13] Gertler, C; Gerdts G; Timmis, K. N. and Golyshin, P. N. (2009). Microbial consortia in mesocosm bioremediation trial using oil sorbents, slow release fertilizer and bioaugmentation. *FEMS Microbiology Ecology*. 69:288-300.

[14] Atlas, R. M. and Hazen, T.C. (2011).Oil biodegradation and bioremediation: A tale of the two worst spills in U.S. history. *Environmental Science Technology*,45:6709-6715.

[15] Krishna, K..K.; Keryn, L.S.; Pawel, P.S.; Robert, B.M. and Andrew, S.B. (2012). A complementary approach to identifying and asssessing the remediation potential of hydrocarbonoclastic bacteria. *Journal of Microbiological Methods* 88:348-355.

[16] Chikere, C.B. and C. C. Azubuike (2013). Catechol 2,3-dioxygenase screening in putative hydrocarbon utilizing bacteria. *International Research Journal of Microbiology* 4 (1): 1-6.

[17] Guo-Chun, D.; Holger, H.; Sebastian, Z., Michael, S.; Geertje Johanna, P.; Katja Ko¨gel-Knabner, H. I. and Smalla, K. (2010). Soil Type-Dependent Responses to Phenanthrene as Revealed by Determining the Diversity and Abundance of Polycyclic Aromatic Hydrocarbon Ring-Hydroxylating Dioxygenase Genes by Using a

Novel PCR Detection System. Polish Journal of Environmental Studies. 12 (1) :15-25.

[18] Anthony, D. K.; YinWei, .; Ryan, J.; Newton, I.; van Nostrand, J. D. and Jizhong Zhou, Sandra.L. McLellan and Krassimira. R..H.(2014). The polycyclic aromatic hydrocarbon degradation potential of Gulf of Mexico native coastal microbial communities after the Deep water Horizon oil spill. *Frontiers in Microbiology.* 5 (205):1-12.

[19] Ma, Y.; Wang, L. and Shao Z (2006). *Pseudomonas*, the dominant polycyclic aromatic hydrocarbon-degrading bacteria isolated from Antarctic soils and the role of large plasmids in horizontal gene transfer. *National Center for Biotechnology Information*, 8 (3):455-65.

[20] Tullio, B.; Sara ,B.; Fabrizio, F.; Claudia, S.; Cesare, C. and Daniele, D. (2001) Aromatic hydrocarbon degradation patterns and catechol 2,3-dioxygenase genes in microbial cultures from deep anoxic hypersaline lakes in the eastern Mediterranean sea *Microbiology Research.* 156, 49-57.

[21] Van Elsa, J. D.; Jansson, J. K. and Trevors, J. K. (2007) *Modern Soil Microbiology*. 2nd ed. New York: *CRC Press,*. p. 387-429.

[22] Chikere, C. B., Chikere , B. O and Okpokwasili, G. C.(2012). Bioreactor-based bioremediation of hydrocarbon-polluted Niger Delta marine sediment, Nigeria *3 Biotechecnology* 2:53-66.

[23] Bergey. D. H. and Holt, J. G. (1994). *Bergey's Manual of Determinative Bacteriology*. 9th edition. Williams and Wacket, Baltimore, Washington DC.

[24] Luis, M.; Maria, T. P.; Janet, S.; Grace, T. and Eilana (2013). *Colour Atlas of Medical Bacteriology*. ASM Press, Washigton DC.

[25] Jyothi k., Surendra B., Nancy CK., Anita k.(2012) Identification and Isolation of Hydrocarbon Degrading Bacteria by Molecular Characterization. *Helix*. 2: 105-111.

[26] Mnif, S.; Chamkha, M. and Sayadi, S. (2009). Isolation and characterization of *Halomonas* sp. Strain C2SS100, a hydrocarbon degrading bacterium under hypersaline conditions. *Journal of Applied Microbiology*. 107:785–794.

[27] Mesarch, B. M.; Nakatsu, C. H. and Niles, L.(2000): Development of catechol 2,3-dioxygenase-specific primers for monitoring bioremediation by competitive quantitative PCR. *Applied Environmental Microbiology*. 66: 678-683.

[28] Aparna, A.; Srinikethan, G. and Smitha, H. (2012). Production and characterization of biosurfactant produced by a novel *Pseudomonas* sp. 2B. Colloids Surf B. *Journal of Environmental Science and Pollution Research.* 95: 23-29.

[29] Quatrini, P.; Scaglione, G.; De Pasquale, C.; Reila, S. and Puglia, A.M. (2008) Isolation of Gram-positive n-alkane degraders from a hydrocarbon contaminated Mediterranean shoreline. *Journal of Applied Microbiology* 104:251-259.

[30] Atlas, R.M. and Philip. J.(2005). *Bioremediation: Applied Solution for Real-World Environmental Clean-up*. American Society for Microbiology (ASM). 75-105.

[31] Collina. E., G.; Bestetti, P.; Gennaro, D.; Franzetti, A.; Gugliersi, F.; Lasagni, M. and Pitea. D. (2005) Naphthalene biodegradation kinetics in an aerobic slurry-phase bioreactor, *Environmental international* 31:167-171.

[32] van Hamme J. D.; Singh, A. and Ward, O. P. (2003) Recent advances in petroleum microbiology. *Microbiology Molecular Biology* Review 67:503-549.

[33] Singh, A. K; Sherry, A.; Gray, N. D.; Jones, D. M.; Bowler, B. F. J. and Head, I. M. (2014). Kinetic parameters for nutrient enhanced crude oil biodegradation in intertidal marine sediments. *Frontier in Microbiology*. **5**:160.

[34] Li, D.; Midgley, D.J.; Ross, J. P.; Oytam, Y.; Abell, G. C. and Volk, H..(2012). Microbial biodiversity in a Malaysian oil field and a systematic comparison with oil reservoirs worldwide. *Arch.Microbiol.* 194:513-523.

[35] Zhang, F.; She, Y. H.; Chai, L. J.; Banat, I. M.; Zhang, X. T. and Shu ,F.C.,(2012). Microbial diversity in long-term water-flooded oil reservoirs with different *in situ* temperatures in China. *Sci.Rep*. 2:760.

[36] Berdugo-Clavijo, C. and Lisa, M.G. (2014) Conversion of crude oil to methane by a microbial consortium enriched from oil reservoir production water .*Petroleum Microbiology Research Group,Department of Biological Sciences*, 10: 3389-3342.

[37] Rajendhran, J. and Gunasekaran, P. (2011). Microbial phylogeny and diversity: Small subunit ribosomal RNA sequence analysis and beyond. *ScienceDirect Microbiological Research* 166: 99-110.

[38] Magali, S. M.; Mariana, L.; Walter, D.J.; Marzio, D.I. and Hebe, M. (2012). Abundance, Dynamic and Biographic Distribution of seven polycyclic Aromatic Hydrocarbon Dioxygenase Gene variant in coastal sediment of Patagonia. *Applied and Environmental Microbiology* 78(5): 1589-2514.

[39] Garcia, M. T.; Ventosa, A. and Mellado, E. (2005) Catabolic versatility of aromatic compound-degrading halophilic bacteria. *FEMS Microbiol. Ecol.* 54, 97-109.

[40] Agrawal, N. and Shahi, K. S. (2015). Review Article: An Environmental Cleanup Strategy Microbial Transformation of Xenobiotic Compounds. *Int. Journ. Curr. Microbiol. App. Sci*. 4(4):429-461.

[41] Garcia-Sierra, I. N.; Correa, A., J.; Pantaroto de Vasconcellos, S.; Pereira de Souza, A.; dos Santos Neto , E.V. and Anete Pereira de Souza. (2014). New Hydrocarbon Degradation Pathways in the Microbial Metagenome from Brazilian Petroleum Reservoirs. *PLoS ONE* 9 (2): 1371-1382.

[42] Kostka, J. E.; Prakash, O.; Overholt, W.A.; Green, S. J.; Freyer, G. and Canion, A. (2011). Hydrocarbon-degrading bacteria and the bacterial community response in Gulf of Mexico beach sands impacted by the Deepwater Horizon oil spill. *Applied and Environmental Microbiology.* 77:7962-7974.

[43] Niepceron, M.; Martin-Laurent, F.; Crampon, M,; Portet-Koltalo, F, Akpa-Vinceslas, M.; Legras M.; Bru, D.; Bureau, F. and Bodilis, J. (2013). GammaProteobacteria as a potential bioindicator of a multiple contamination by polycyclic aromatic hydrocarbons (PAHs) in agricultural soils. *Environmental Pollution. Elsevier*. 180. 199-205.

[44] Gallego, J. L. R..; Garcia-Martinez, M. J.; Llamas, J. F.; Belloch, C.; Pelaez, A.I. and Sanchez, J. (2007). Biodegradation of oil tank bottom sludge using microbial consortia. *Biodegradation*. 18 (3) : 269-281.

[45] Surekha, K. S.; Arun, G. K.; Prashant, K. P.; Ibrahim, M. B .and Balu, A. C. (2010). Methods for investigating biosurfactants and bioemulsifiers: a review. *Critical Reviews in Biotechnology*. 30 (2): 127-144.

[46] Chaillan. F.; Le Flèche, A.; Bury, E.; Phantavong, Y. H.; Grimont, P.; Saliot A, and Oudot, J. (2004). "Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms," *Research in Microbiology*, 155 (7):587-595.