Bioremediation of Petroleum Oil Sludge Polluted Brackish Water Ecosystem

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A B S T R A C T

Petroleum oil sludge resulting from crude oil storage, illegal crude oil refining and bunkering activities constitutes environmental hazards and pollution in the crude oil producing communities in the Niger Delta region of Nigeria. Biostimulation with N.P.K. fertilizer option C, bioaugmentation with indigenous hydrocarbon utilizing bacteria (HUB) option B, combination of biostimulation and bioaugmentation as option A and option D was without any bioremediation treatment were employed in the bioremediation of brackish water artificially polluted with petroleum oil sludge. Brackish water sample was obtained from Elechi Creek, Port Harcourt Rivers State. Petroleum oil sludge sample was obtained from Crude Oil Processing Plant in Obegi community, Rivers State. Bioremediation was monitored for 56 days using the percentage ratio of total petroleum hydrocarbon (TPH) losses for each period to TPH loss at day 0. The result of physicochemical analysis of the petroleum sludge showed that aliphatic hydrocarbon (n-alkanes) ranged from C\textsubscript{13} – C\textsubscript{35}, with concentrations ranging from 26.12–7,713.62ppm with TPH of 89,509.9ppm. The polycyclic aromatic hydrocarbon (PAH) range was 0.03–5.36ppm with total concentration of 24.21ppm. Heavy metal analysis showed; iron (49.42mg/kg), Zinc (3.79mg/kg), Nickel (4.53 mg/kg) and manganese (6.90 mg/kg). The average total heterotrophic bacterial (THB) and HUB counts for petroleum sludge were; 2.5 x 10\textsuperscript{5} cfu/g and 2.0 x10\textsuperscript{5} cfu/g and for the brackish water sample were 1.39 x 10\textsuperscript{6}cfu/ml and 1.1 x 10\textsuperscript{4} cfu/ml respectively. Statistical analysis (ANOVA) showed that the THB and HUB counts were significantly different at 5 percent levels (P<0.05) in the different treatment options during the bioremediation period. Changes in physico-chemical parameters showed that pH, alkalinity, conductivity, chemical oxygen demand, nitrate and phosphate were significantly different (P<0.05) while there were no significant differences (P>0.05) in the following parameter; salinity biochemical oxygen demand and total hydrocarbon continent.Using least significant difference (LSD), treatment option D and the control option E were different from treatments A, B and C. The phylogenetic analysis identification of the HUB isolates implicated in the degradation process revealed a closely related ness to the following organisms, Lysinibacillus sphaericus, Klebsiella pneumonia and Alcaligenes faecalis of different strains. The bacterial sequences submitted to Genbank were assigned Accession Number KX817218-KXV7225. The percentage losses in TPH from Gas Chromatography (GC) results showed the following; option A (91.8%), option B (92.5%), C (95%) D (57.8%) and option E control (39.5%) respectively. The results suggest that the application of biostimulation with N.P.K fertilizer, bioaugmentation with indigenous HUB or a combination of both will enhance the bioremediation of petroleum sludge polluted brackish water system in the Niger Delta of Nigeria.
Introduction

Petroleum sludge is made up of hydrocarbons, solids and other impurities and the remaining being water. Huge amount of petroleum sludge is formed during oil processing in refineries and oil processing as well as during illegal oil refining and bunkering in the creeks of oil producing communities. High demand for petroleum products has led to generation of large amount of oily wastes (Bhttacharyya and shekalar 2003). The petroleum oily sludge is attributed to two major factors; sedimentation of inorganic residues in the crude oil and the precipitation of paraffin wax, since wax precipitates are sparingly soluble in crude oil (Milne, 1998). Petroleum is capable to penetrate into ground and pollute ground water, surface water and the terrestrial environment if not properly treated and managed (Manning and Thompson, 1995). The components of petroleum sludge are toxic, mutagenic and carcinogenic and may persist in the environment for long period; posing environmental problem both to the aquatic and terrestrial ecosystems (Wu et al., 2008; Ayotamuno, et al., 2011, Balanchandran et al., 2012).

When hydrocarbon pollutants get into the aquatic systems, they may be biodegraded by indigenous microorganisms (Okpokwasili and Odukuma, 1990), though they may pose toxicity problems to indigenous microflora. Hydrocarbon contamination generally can cause damages to the aquatic vegetation (Krebs and Tanner, 1981). The young fish and aquatic invertebrates are the most threatened organisms in the aquatic environment (Calfee et al., 1999). Hydrocarbon toxicity due to the presence of PAHs has greater environmental and public health implication as it can pass on to human population. These effects will eventually lead to socio-economic impact of decline in food production, youth restiveness and community unrest.

The use of conventional techniques (mechanical removal, sediment relocation and application of chemical dispersants) are generally expensive and exposes personnel to health hazards. The ability of microorganisms to degrade hydrocarbon pollutants in the environment has been employed in the remediation of hydrocarbon contaminated sites. Several studies have reported on the abilities of microorganisms (bacteria, fungi and algae) to degrade petroleum hydrocarbons (Riser-Roberts 1992; Dean-Ross et al., 2002; Bundy et al., 2004; Chikere et al., 2009; Wang et al., 2011; Malik and Ahmed, 2012; Ahirwar and Dehariya, 2013; Macaulay, 2015). Bioremediation is the use of biological process and agents especially microbial, to degrade the environmental contaminants into less toxic forms (Vidali, 2010). Biodegradation transforms and mineralize organic compounds, though complete mineralization is often not realized. Only when environmental conditions permit microbial growth activity would the application be effective. Thus, manipulation of environmental parameters to achieve fast growth rate and optimal activities is a necessity (Mukred et al., 2008). Biostimulation and bioaugmentation are methods of bioremediation geared towards enhancing the process. Biostimulation is the injection of amendments (nutrients) into contaminated soil or water to stimulate indigenous microbial population already present to enhance the pollutant degradation (Obire and Akinde, 2004). Amendment may include oxygen, nutrient (organic or inorganic fertilizer), electron acceptors (Tyagi et al., 2011). Stimulation of the activity of indigenous microflora to remediate the target pollutant can also be accelerated by adjustment of physical process such as pH and moisture (Vidali, 2001). Bioaugmentation involves the addition of exogenous or indigenous bacterial cultures to the contaminated matrix to decontaminate it. It is
more commonly and successfully carried out by addition of large population of selected microorganisms grown in the laboratory removed from the contaminated sites (Vidali, 2001). Application of genetically engineered bacteria has been used for bioremediation trials. Genes could be introduced into native species using other genetic vectors such as plasmids (Crisafi et al., 2016) A combination of both biostimulation and bioaugmentation has also been employed in bioremediation process (Odokuma and Dickson, 2003; Mukred et al., 2008). This present study compared the biostimulation with N.P.K fertilizer, bioaugmentation with indigenous HUB isolates, combination of biostimulation and bioaugmentation as well as intrinsic bioremediation (natural attenuation) techniques in the bioremediation of petroleum sludge polluted brackish water ecosystem.

**Materials and Methods**

**Sample Collection**

Brackish water sample was collected from Elechi creek located in Port Harcourt Rivers stated behind Nigeria Agip Oil Company (NAOC) and Rivers State University, Nkpolu, Port Harcourt. The area lies on latitude 4˚47’37.6 “N” and longitude 6˚58’20.6 “E”. Sample bottle was rinsed trice with the river water before collection (ASTM, 1999). Water sample was collected by gradually lowering the bottle into the sub-surface (10-20cm of the river in direct sunlight). The bottle was opened and allowed to be filled and closed below the water. Water was collected into 4 liter plastic bottle and transported in ice-pack to the laboratory. Water sample was refrigerated at 4˚C and covered. The petroleum oily sludge was collected from the crude oil processing plant belonging to Total Exploration and Production, (Total E & P) Nigeria limited, located at Obegi community, Rives state. Petroleum oily sludge was collected at the base of crude oil storage tank during cleaning exercise with soil auger into sterile glass jar and covered. It was transported in ice pack to the laboratory and stored in refrigerator at 4˚c.

**Reagents**

All reagents employed in the study were of analytical grade and were obtained from Sigma-Aldrich chemical company, USA, and BDH chemical Ltd, Poole, England. All microbiological media used were products of Oxoidand Difco Laboratories England and Sigma-Aldrich, USA. Filter paper (whatman No.1) was obtained from WER Bauston Ltd, London. DNA extraction Kit was obtained from Inqaba Biotechnical Industries, South Africa. Bonny light crude oil used for HUB screening was obtained from Port Harcourt Refinery Company, Eleme, Rivers State, Nigeria. The NPK (Nitrogen, Phosphorus and Potassium) 20:10:10 NPK fertilizer used in this study was obtained from Indorama Eleme Petrochemicals Ltd, Port Harcourt, Nigeria.

**Experimental Set-Up**

The bioremediation experimental design consisted of five 2 liters Erlenmeyer flasks. The flasks were labeled A, B, C, D and E. To each flask 300ml of brackish water and 100g of petroleum sludge were added.

The different treatment options were constituted as follows: (Table 1)

| Option | Treatment |
|--------|-----------|
| A      | Addition of 5ml of 10% wt/v NPK fertilizer and 5ml of bacteria broth culture from the water and sludge samples. The isolates were sub-cultured into nutrient broth as mix culture and allowed to stand for 6h before inoculating into the test set up aseptically by use of sterile syringes. |
| B      | Addition of 5ml of bacterial broth culture. |

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Option C: Addition of 5ml 10% \text{ wt/v } NPK fertilizer.

Option D: No addition of fertilizer and bacterial broth culture.

Option E: Addition of 5g sodium azide biocide to eliminate microorganism). This served as control.

Each set up was plugged with cotton wool and allowed to stand at room temperature (28\pm 2\, ^{\circ}\text{C}) for 56 days. Repeated sampling procedures were carried out for microbiological and physico-chemical analysis at day 0 and subsequently at day 14, 28, 42 and 56 respectively.

**Enumeration of Microbial Population**

The total heterotrophic bacteria (THB) counts of water, petroleum sludge samples and bioremediation tests set up were performed in triplicates on nutrient agar (NA) oxoid using spread plate method (APHA, 1998). Plates were properly labeled and incubated at 37\, ^{\circ}\text{C} for 24h.

The HUB counts of water, petroleum sludge and bioremediation tests samples were carried out in triplicates on Mineral Salt Agar (MSA) of Mills et al., (1978) as modified by Okpokwasili and Odokuma (1990). Vapour phase transfer method (Amanchukwu et al., 1998) was employed by placing sterile Whatman No 1 filter papers saturated with filtered-Bonny light crude oil into the inside lids of each petri dish kept in an inverted position, incubated at 30\, ^{\circ}\text{C} for 3-7 days. The plates were examined for colony formation and enumeration. Identification and characterization of culturable HUB bacterial isolates were based on Grams reaction tests their morphological features and series of biochemical tests. When compared with the characteristics of known using the determination schemes of Chesbrough (2006) and Holt et al., (1994).

**Molecular Identification of the HUB Isolates**

**DNA Extraction**

DNA extraction was carried out by using a ZR fungal/bacterial DNA miniprep-extraction kit obtained from Inquaba, South Africa. Heavy growth of the pure isolates subcultured on MacConkey’s agar plates were suspended in 200 microlitre of isotonic into a ZR bashing bead lysis tubes, 75\, \mu l of lysis solution was added to the tubes. The tubes were held in position in a bead beater fitted with a zml holder assembly and processed at maximum speed for 5 minutes. The ZR bashing-bead lysis tubes were centrifuged at 10,000xg for 1 minute. Four hundred (400) \mu l of the supernatant were transferred aseptically with micropipette into zymo-spin IV spin filter (orange top) in a collection tube and centrifuged at 7000 \, xg for a minute, then 1200\, \mu l of DNA binding buffer was added to each filtrate in the collection tubes bringing the final volume to 1600\, \mu l. 800\, \mu l was afterwards twirled into zymo-spin IIC column in a collection tube and centrifuged at 10,000 \, xg for a minute, the flow through were discarded. The remaining volumes were wirled into the same zymo-spin and spun at 10,000\, xg for a minute, 200\, \mu l of the DNA pre-wash buffer were added to the zymo-spin IIC in a fresh collection tubes and spun at 10,000\, xg for a minute followed by the addition of 500\, \mu l of bacterial DNA, buffered and centrifuged at 10,000\, xg for a minute. The zymo-spin IIC column were transferred to clean fresh 1.5\, \mu l centrifuge tubes, 100\, \mu l of DNA elution buffer were added to the column matrix and centrifuged at 10,000\, xg for 30seconds to elute the DNA. The ultrapure DNA of each isolate properly labeled were then stored at -20\, ^{\circ}\text{C} for use. DENVILLE
260OD Brushless micro-centrifuge was used for the centrifugation process. After extraction, the DNA samples were quantified using NANODROP (ND1000).

**Agarose gel electrophoresis**

The extracted genomic DNA were resolved on a 1% agarose gel at 120v for 15 minutes and visualized on a UV transilluminator alongside with a 1kb ladder for size determination of the isolates DNA sizes.

**16S rRNA amplification**

The 16s RNA region of the rRNA genes of the isolates were amplified using the 27F and 1492R primers on a PCR System 9700 Applied Biosystem thermal cycler at a final volume of 25µl for 40 cycles. The PCR mix included: the x2 dream tag master mix supplied by Inqaba, South Africa (tag polymerase DNTPs, magnesium chloride (MgCl₂), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR condition were as follows: initial denaturation, 95°C for 4mins, denaturation, 95°C for 30seconds; annealing 52°C for 30 seconds; extension 72°C for 1minute for 40 cycles and final extension 72°C for 3mins. Than the products were resolved on a 1% agarose gel at 120V for 15mintes and visualized on a UV transilluminator (Ce’born et al., 2008).

**16SrRNA sequencing**

The amplified 16s products were sequenced on a 3500 genetic analyzer using the Bigdye termination technique by Inqaba, South Africa.

**Phylogenetic analysis**

The sequence were edited using the bioinformatics algorithm Bio edit, similar sequences were downloaded from the National Biotechnology Information Centre (NBIC) data base using BlastN. These sequences were aligned using clusta 1X. The evolutionary history of the isolates and relatedness were inferred following protocols described in Saitou and Nei (1987); Felsenstein (1985) and Thompson et al., (1994). The result of the bacteria sequences was submitted to GenBank for determination of accession numbers.

Physicochemical parameters of brackish water, petroleum sludge and bioremediation monitoring samples analysed included; pH, alkalinity, salinity, biological oxygen demand (BOD), chemical oxygen demand (COD), nitrate, phosphate, total hydrocarbon content (THC), sulphate, total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbons (PAHs).

They were determined using methods adopted from Stewart et al., (1974). Determination of THC was according to ASTM (1999) method D3921. The use of gas chromatographic Flame Ionization Detector (FID) were employed for TPH and PAH. The methods were based on (ASTM-D7678, 1999 and ASTM-D8270 (1999) respectively.

**Heavy Metal Analysis**

The petroleum sludge and condensate samples were analysed for the presence of iron, zinc, copper, vanadium, nickel, lead and manganese using G.B.C. Avanta Atomic Absorption Spectrophotometer (AAS) with detection limit of 0.05mg/kg.The process involves flame optimization. Prior to flame optimization, the water trap on the instrument was filled with distilled water as blank and the water level in the discharge container was reduced. It was ensured that the tip of the hose stays above the water level in the discharge container during running the AAS,
as well as ensuring that the burner head was clean, free from debris and confirming that aspirator was ducking properly.

Prior to analysis, the AAS was calibrated with standards of known concentrations to obtain curve for the individual metal. Concentration of each of the heavy metal was ascertained from the data generated by the AAS and expressed in ppm. At the end of the run, the displayed result was printed out. All gas pressures, used in the analysis were set to 70psi.

Determination of percentage losses in TPH in the various bioremediation treatment options were carried out by obtaining the difference in TPH values of GC results of the day 0 and that of TPH GC result of day 56. Calculation was percentage of ratio of TPH for day 0, 14, 2, 42 and 56 to TPH at day 0.

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\frac{(TPH\ at\ day\ 0 - TPH\ at\ day\ 56)}{TPH\ at\ day\ 0} \times 100\%
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Statistical Analysis

Analysis of variance (ANOVA) method and the least significant difference (LSD) test of 95% levels of confidence were employed with Statistical Package for Social Science (SPSS) to determine significant statistical differences in microbial counts and changes in physicochemical parameters between the different treatment options.

Results and Discussion

The physicochemical characteristics of the brackish water and petroleum sludge used in the study are presented in Tables 2 and 3 respectively. The brackish water sample had high salinity of 12,280.8mg/l and conductivity of 1,407us/cm. The high salinity and conductivity contents of the brackish water sample could be as a result of inflow of sea water and discharge of domestic and industrial waste water into the water body (Nester et al., 2001). The value of THC (0.85mg/l0 of the water body showed that there was no previous hydrocarbon contamination of the water body. The permissible limit of THC in natural aquatic systems is 10mg/l (DPR, 2002). The high values of BOD (448mg/l), COD (1,600.0mg/l), THC (915.0mg/l), TPH (89,509.9mg/l) and PAHs (24.21mg/l) of the petroleum sludge implies that it constitutes potential environmental hazard. The results of characterization of aliphatic hydrocarbon (n-alkanes) and PAHs in the petroleum sludge reveals that the n-alkanes ranged from carbon length of C\textsubscript{13} to C\textsubscript{37} with concentrations ranging from 26.7-7,713.63ppm, C\textsubscript{17} (Heptadecane) was the most significant n-alkane with highest concentration (7,713.63ppm) while C\textsubscript{37} (heptatriacontane) had the least concentration (26.12ppm) Table 5. The PAHs concentration indicated that Benzo (b) fluorenanthene had the highest concentration (5.36ppm) while anthracene was least (0.03ppm). Naphthalene, benzo (a) anthracene, chrysene, benzo (ghi) perylene and indeno (1,2,3-cd) pyrene were not detected (Table 6). The presence of these PAHs in the petroleum sludge is an indicator of high pollutant. The AAS concentration results of heavy metals in the petroleum sludge revealed high iron (Fe) content of 49.42ppm compared with other heavy metals investigated (Zn, Cu, V, Ni, Pb and Mn) which were relatively lower (Table 4). Many metals are essential for growth of microorganisms in lower concentrations, yet are toxic in higher concentrations. Many microorganisms have the ability to selectively accumulate metals by the process of biosorption which involves the building or adsorption of heavy metals to living or dead cells (Vijayadeep and Sastry, 2014). The concentrations of the heavy metals analysed in the petroleum sludge in this study may not
have affected the microbial growth in the overall biodegradation process.

The proportion of microbial population capable of hydrocarbon degradation in an aquatic habitat is influenced by a number of factors, one of which is the environmental conditions (Odokuma and Okpokwasili, 1993a; Odokuma and Okpokwasili, 1993b; Odokuma and Okpokwasili, 1997; Mona et al., 2015). The pH of the brackish water (7.27) and petroleum sludge (7.32) which showed pH near neutrality were ideal for biological functions (Nester et al., 2001). Changes in pH during the bioremediation period showed pH near neutrality. This favours most heterotrophic and HUB activities (Atlas, 1984). The pH changes during the monitoring period may be due to reduction in acidic compounds production and/or protons secretion. Generally, the pH of the various treatment options is a function of the chemical composition of the pollutant, water and microbial activities (Odokuma and Ibe, 2003; Delyan et al., 1990; Mayo and Noike, 1996).

The bacterial counts of the brackish water and petroleum sludge are presented in Table 7. It showed that the brackish water had higher THB count (1.39x10^6 cfu/ml) than the sludge (2.5x10^5 cfu/g) while the sludge had higher HUB count (2.0x10^5 cfu/g) than the brackish water (1.1x10^4 cfu/ml). The bacterial growth profile (THB and HUB) during the period are illustrated in Figures 1-2. They followed the same trend, except in the control option E, where an extremely low THB and HUB counts were observed as a result of the addition of biocide which eliminated microorganisms in the test systems (Figs. 1-2).

Statistical analysis results of growth profile of THB and HUB showed that there was significant difference in the treatment options at 5% confidence levels (P<0.05). This also indicated that the pollutant (petroleum sludge) was utilizable source of carbon and energy for the HUB cells (Milic et al., 2009; Hara et al., 2013; Singh and Chandra, 2014). The decline in bacterial counts from day 42 to 56 may be due to nutrient exhaustion with possible accumulation of toxic metabolites which gave rise to stationary and death phases (Nester et al., 2001). The relative few or no growth observed in the control option E, was due to the application of biocide (Odokuma and Akubuenyi, 2008). This led to low percentage loss in TPH (39.5%) Table 8. The observed % loss in TPH in the control option is attributable to environmental factors; natural attenuation process (auto-oxidation, evaporation, volatilization, emulsification, dispersion and sedimentation) other than biodegradation since microorganisms were eliminated.

Changes in physicochemical parameters during the period of bioremediation are illustrated in Figures 3-12. Statistical analysis (ANOVA) showed that there were significant differences at 5% level (<0.05) for pH, alkalinity, conductivity, COD, nitrate and phosphate, sulphate whereas there were no significant differences (P>0.05) in salinity, BOD and THC respectively. Least significant difference (LSD) analysis showed that treatments D and E were different from treatment A, B and C for THC and TPH.

Decreases in BOD in the various tests set up suggest that the amount of degradable organic materials were being degraded by the microorganisms. They showed the same trend of decrease from Day 0 to day 56 (Fig. 7). BOD represents the amount of oxygen required for microbial decomposition of organic matter in waste water sample, it is roughly proportional to the amount of degradable organic matter present in the water sample (Nester et al., 2001).
**Table 1** Bioremediation treatment options

| Options          | A            | B          | C          | D          | E          |
|------------------|--------------|------------|------------|------------|------------|
| BW+SL+BT+FT      | BW+SL+BT    | BW+SL+FT  | BW+SL+FT  | BW+SL+SA  |

Key: BW = Brackish water, SL = Sludge, BI = Bacterial Innoculum, FT = Fertilizer, SA = Sodium azide

**Table 2** Physicochemical characteristics of brackish water samples

| Parameters       | Values       |
|------------------|--------------|
| pH               | 7.27         |
| Salinity (mg/l)  | 12,280.8     |
| Conductivity (µS/cm) | 1407       |
| Alkalinity       | 32           |
| COD (mg/l)       | 46           |
| BOD (mg/l)       | 7.04         |
| Phosphate (mg/l) | Nil          |
| Nitrate (mg/l)   | 0.88         |
| Sulphate (mg/l)  | 0.69         |
| THC (mg/l)       | 0.85         |

**Table 3** Physicochemical characteristics of petroleum sludge sample used in the study

| Parameters                          | Values         |
|-------------------------------------|----------------|
| pH                                 | 7.32           |
| Conductivity (µS/cm)                | 5,230.0        |
| Salinity (mg/l)                     | 3,249.0        |
| Alkalinity                          | 1,900.0        |
| BOD (mg/l)                          | 448            |
| COD (mg/l)                          | 1,600.0        |
| Nitrate (mg/l)                      | 10.59          |
| Phosphate (mg/l)                    | 0.98           |
| Sulphate (mg/l)                     | 18.58          |
| Total hydrocarbon content (THC) (mg/l) | 915.0     |
| Total petroleum hydrocarbon (TPH) (mg/l) | 89,509.9  |
| Polyaromatic hydrocarbons (PAHs) (mg/l) | 24.21     |

**Table 4** Heavy metal content in petroleum sludge sample used in the study

| Parameters | Values (mg/kg) |
|------------|----------------|
| Iron       | 49.42          |
| Zinc       | 3.79           |
| Copper     | 3.32           |
| Vanadium   | 0.91           |
| Nickel     | 4.53           |
| Lead       | 2.59           |
| Manganese  | 6.90           |
Table 5 Characterization of aliphatic hydrocarbons (n-alkanes) of the petroleum sludge sample used in the study

| S/N | Number of carbon atoms | Name         | Conc. (ppm) |
|-----|------------------------|--------------|-------------|
| 1.  | C8                     | Octane       | ND          |
| 2.  | C9                     | Nonane       | ND          |
| 3.  | C10                    | Undecane     | ND          |
| 4.  | C11                    | Decane       | ND          |
| 5.  | C12                    | Dodecane     | ND          |
| 6.  | C13                    | Tridecane    | 259.07      |
| 7.  | C14                    | Tetradecane  | ND          |
| 8.  | C15                    | Pentadecane  | 3841.17     |
| 9.  | C16                    | Hexadecane   | 1807.55     |
| 10. | C17+                   | Heptadecane  | 4671.54     |
| 11. | C17                    | Heptadecane  | 7713.62     |
| 12. | C18                    | Octadecane   | 4292.26     |
| 13. | C18+                   | Octadecane   | 6474.51     |
| 14. | C19                    | Nonadecane   | 4125.91     |
| 15. | C20                    | Icosane      | 3948.02     |
| 16. | C21                    | Hericosane   | 5076.97     |
| 17. | C22                    | Decosane     | 3266.03     |
| 18. | C23                    | Tripicosane  | 4245.44     |
| 19. | C24                    | Tetracosane  | 4256.37     |
| 20. | C25                    | Pentacosane  | 5470.60     |
| 21. | C26                    | Hexacosane   | 3288.36     |
| 22. | C27                    | Heptacosane  | 4444.23     |
| 23. | C28                    | Octacosane   | 3648.16     |
| 24. | C29                    | Nonacosane   | 3015.70     |
| 25. | C30                    | Triacontane  | 4891.31     |
| 26. | C31                    | Hentriacontane | 2258.78   |
| 27. | C32                    | Dotriacontane| 498.90      |
| 28. | C33                    | Tritriacontane| 1706.33   |
| 29. | C34                    | Tetratriacontane| 1185.64  |
| 30. | C35                    | Pentatriacontane| 196.66     |
| 31. | C36                    | Hexatriacontane| ND         |
| 32. | C37                    | Heptatriacontane| 26.12      |
| 33. | C38                    | Octatriacontane| ND         |
| 34. | C39                    | Nonatriacontane| ND         |
| 35. | C40                    | Tetracontane | ND          |

**TOTAL** | 89,509.9 |

ND = Not Detected
Table 6 Characterization of Polycyclic aromatic hydrocarbons (PAHs) in petroleum sludge sample used in the study

| S/N | Name of Compound          | Conc. (ppm) |
|-----|---------------------------|-------------|
| 1   | Naphthalene               | ND          |
| 2   | Acenaphthylene            | 0.29        |
| 3   | Acenapthene               | 1.57        |
| 4   | Fluorene                  | 2.84        |
| 5   | Phenanthrene              | 5.12        |
| 6   | Anthracene                | 0.03        |
| 7   | Fluoranthene              | 2.14        |
| 8   | Pyrene                    | 0.19        |
| 9   | Benzo (a) anthracene      | ND          |
| 10  | Chrysene                  | ND          |
| 11  | Benzo (b) fluoranthene    | 5.36        |
| 12  | Benzo (k) fluoranthene    | 2.55        |
| 13  | Benzo (a) pyrene          | 4.12        |
| 14  | Benzo (ghi) perylene      | ND          |
| 15  | Indeno (1,2,3-cd) pyrene  | ND          |
|     | **TOTAL**                 | **24.21**   |

ND = Not Detected

Table 7 Bacterial Counts of Water and Petroleum sludge samples

| S/NO. | Type of Count | Brackish Water (cfu/ml) | Petroleum Sludge (cfu/g) |
|-------|---------------|-------------------------|--------------------------|
| 1     | THB           | 1.39 x 10⁶              | 2.5 x 10⁵                |
| 2     | HUB           | 1.1 x 10⁴               | 2.0 x 10³                |

Table 8 Percentage losses in TPH of various bioremediation options after 56 days in petroleum polluted brackish water

| Option | Percentage Loss (%) |
|--------|---------------------|
| A      | 91.8                |
| B      | 92.5                |
| C      | 95.1                |
| D      | 57.8                |
| E      | 39.5                |

Table 9 Identified Isolates with the GenBan Accession Numbers

| S/N | Name of Organism           | Accession Number               |
|-----|----------------------------|--------------------------------|
| 1   | Klebsiella pneumoniae strain B21 | SUB1917764B1 KX817218 |
| 2   | Klebsiella pneumoniae strain ICB-C183 | SUB1917764B2 KX817219 |
| 3   | Klebsiella oxytoca strain BCNA1 | SUB1917764B3 KX817220 |
| 4   | Klebsiella oxytoca strain BC4 | SUB1917764B4 KX817221 |
| 5   | Alcaligenesfaealis strain IOU PMR | SUB1917764B5 KX817222 |
| 6   | Alcaligenesfaealis strain AQ-1 | SUB1917764B6 KX817223 |
| 7   | Klebsiella pneumoniae strain ICB –C26 | SUB1917764 B7 KX817224 |
| 8   | Klebsiella pneumoniae strain B21 | SUB1917764 B8 KX817225 |
Fig.1 Growth Profile of THB in Sludge Polluted brackish water sample during the monitoring of various bioremediation options

KEY
A - APPLICATION OF BACTERIA AND FERTILIZER
B - APPLICATION OF BACTERIA ONLY
C - APPLICATION OF FERTILIZER ONLY
D - NO APPLICATION
E - ADDITION OF BIOCIDE
Fig. 2 Growth profile of HUB in sludge polluted brackish water sample during the monitoring of the various bioremediation options

KEY
A - APPLICATION OF BACTERIA AND FERTILIZER
B - APPLICATION OF BACTERIA ONLY
C - APPLICATION OF FERTILIZER ONLY
D - NO APPLICATION
E - ADDITION OF BIOCIDE
Fig. 3 Changes in pH level in sludge polluted brackish water sample during the monitoring of the various bioremediation options

KEY
A - APPLICATION OF BACTERIA AND FERTILIZER
B - APPLICATION OF BACTERIA ONLY
C - APPLICATION OF FERTILIZER ONLY
D - NO APPLICATION
E - ADDITION OF BIOCIDE
**Fig. 4** Changes in salinity level in sludge polluted brackish water sample during monitoring of the various bioremediation options

| Key | Description                                      |
|-----|--------------------------------------------------|
| A   | APPLICATION OF BACTERIA AND FERTILIZER           |
| B   | APPLICATION OF BACTERIA ONLY                     |
| C   | APPLICATION OF FERTILIZER ONLY                   |
| D   | NO APPLICATION                                   |
| E   | ADDITION OF BIocide                              |
Fig. 5 Changes in alkalinity level in sludge polluted brackish water sample during monitoring of the various bioremediation options.

KEY
A - APPLICATION OF BACTERIA AND FERTILIZER
B - APPLICATION OF BACTERIA ONLY
C - APPLICATION OF FERTILIZER ONLY
D - NO APPLICATION
E - ADDITION OF BIOCIDE
Fig.6 Changes in conductivity level in sludge polluted brackish water sample during monitoring of various bioremediation options

KEY
A - APPLICATION OF BACTERIA AND FERTILIZER
B - APPLICATION OF BACTERIA ONLY
C - APPLICATION OF FERTILIZER ONLY
D - NO APPLICATION
E - ADDITION OF BIOCIDE
Fig. 7 Changes in BOD level in sludge polluted brackish water sample during monitoring of the various bioremediation options

KEY
A - APPLICATION OF BACTERIA AND FERTILIZER
B - APPLICATION OF BACTERIA ONLY
C - APPLICATION OF FERTILIZER ONLY
D - NO APPLICATION
E - ADDITION OF BIocide
Fig. 8 Changes in COD level in sludge polluted brackish water sample during monitoring of the various bioremediation options

KEY
A - APPLICATION OF BACTERIA AND FERTILIZER
B - APPLICATION OF BACTERIA ONLY
C - APPLICATION OF FERTILIZER ONLY
D - NO APPLICATION
E - ADDITION OF BIOCIDE
Fig. 9 Changes in nitrate level in sludge polluted brackish water sample during monitoring of various bioremediation options

KEY
A - APPLICATION OF BACTERIA AND FERTILIZER
B - APPLICATION OF BACTERIA ONLY
C - APPLICATION OF FERTILIZER ONLY
D - NO APPLICATION
E - ADDITION OF BIOCIDE
Fig. 10 Changes in phosphate level in sludge polluted brackish water sample during monitoring of the various bioremediation options.

KEY

A - APPLICATION OF BACTERIA AND FERTILIZER
B - APPLICATION OF BACTERIA ONLY
C - APPLICATION OF FERTILIZER ONLY
D - NO APPLICATION
E - ADDITION OF BIOCIDES
**Fig. 11** Changes in sulphate level in sludge polluted brackish water sample during monitoring of the various bioremediation options

**KEY**

A - APPLICATION OF BACTERIA AND FERTILIZER
B - APPLICATION OF BACTERIA ONLY
C - APPLICATION OF FERTILIZER ONLY
D - NO APPLICATION
E - ADDITION OF BIOCIDES
Fig. 12 Changes in THC level in sludge polluted brackish water sample during monitoring of various bioremediation options

KEY
A - APPLICATION OF BACTERIA AND FERTILIZER
B - APPLICATION OF BACTERIA ONLY
C - APPLICATION OF FERTILIZER ONLY
D - NO APPLICATION
E - ADDITION OF BIocide
Fig. 13 Agarose gel electrophoresis results of the 16S gene bands of the isolates: L: represents the 1kb ladder, N: represents the negative control, 1-8 represents 16S gene bands of the isolates

Generally, changes in COD, nitrate, phosphate and sulphate showed different trend of reduction or increase during the remediation period in the different treatment options (Figs. 8-11). The relative reductions in nitrate, sulphate and phosphate levels in the treatment options indicate that the HUB degraders were actively utilizing the metallic salts of the anions as sources of nitrogen and sulphur (Odokuma and Akpokodje, 2004; Odokuma and Okara, 2005).

There were reductions in the values of THC from day 0 to day 56 in all the treatment options. There was slight reduction in THC in the control option E which is attributable to natural attenuation (Vidali, 2001). The reductions in the test options indicates that hydrocarbon degraders were utilizing the hydrocarbon in the pollutants as sources of carbon and energy for metabolic activities thereby reducing the content (Ayotamuno et al., 2011; Malik and Ahmed, 2012; Mona et al., 2015; Macaulay, 2015).

The results of % losses in TPH showed that treatment options C had the highest (95.1%) followed by option B (92.51%), option A (91.8%) and D (57.8%) respectively. This implies that biostimulation (additions of NPK fertilizer) could have stimulated and enhanced degradation rate by providing the limiting nutrients in the system required for cell growth such as nitrogen and phosphorus (Nester et al., 2001).

The result of % TPH loss in treatment option D (57.8%) which was more than in the control option E (39.5%) and less than in actual treatment options (A, B and C) suggests that microorganisms (HUB) played important role in the degradation process of the hydrocarbon pollutants in the aquatic system, since there was no nutrient supplement (biostimulation) and no addition of indigenous HUB (bioaugmentation). The differences in the % losses of TPH in the treatment options could be attributed to various factors, the microbial population of
the aquatic environment (Phulia et al., 2013), the physicochemical characteristics of the aquatic system, available nutrients, chemical composition and physical nature of the pollutant (petroleum sludge) and bioremediation technology employed. The results suggest that biostimulation with NPK fertilizer will enhance bioremediation of petroleum oily sludge polluted aquatic system more than bioaugmentation and a combination of the two. These observations from this study are in general agreement with various studies and reports regarding the use of biostimulation/bioaugmentation in clean-up of hydrocarbon polluted environments (Vidali, 2001; Odokuma and Dickson, 2003; Mukred et al., 2008; Ayotamuno et al., 2007; Ayotamuno et al., 2011; Mona et al., 2015; Macualey, 2015; Crisafi, et al., 2016; Wokem and Odokuma, 2016).

The agarose gel electrophoreses results of the 16S gene bands of the isolates are shown in Fig. 13. The bacterial sequences submitted to GenBank were assigned Accession Number KX8172218-KX817225 (Table 9). From the molecular identification of the isolates the following organisms were implicated in the overall hydrocarbon degradation namely; 

- *Klebsiella pneumoniae* B21, 
- *Klebsiella pneumoniae* ICB-C183, 
- *Klebsiella oxytoca* BCNAI, 
- *Klebsiella oxytoca* BC4, 
- *Alkaligenes faecalis* IOU PMR, 
- *Alkaligenes faecalis* AQ-1, 
- *Klebsiella pneumoniae* ICB-C26 and 
- *Klebsiella pneumoniae* B21F4 Table 9. 

*Alkaligenes faecalis* has been explored for use to promote biodegradation of petroleum hydrocarbons. *A. faecalis* produces biosurfactants which possessed high surface activity, decreasing surface tension adequately to allow for degradation of hydrocarbon (Igwo-Ezikpwe et al., 2009). Species identified in this study have also been implicated in crude oil degradation in other studies (Rodrigues et al., 2009; Chamkha et al., 2011; Olukunle, 2013; Chikere and Ekwuabu, 2014).

In conclusion, the findings in the present study indicate that petroleum oily sludge possess serious potential environmental hazard as a result of the high TPH and PAHs which are known to be major contaminants. The results of bioremediation of petroleum sludge polluted brackish aquatic system investigated in this study, suggests that the use of biostimulation with NPK fertilizer, the use of bioaugmentation with indigenous HUB or the combination of the two techniques will enhance and be effective in the bioremediation of petroleum sludge polluted aquatic system.

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