Original Research Article   https://doi.org/10.20546/ijcmas.2019.809.325

Bioremediation of Petroleum Oil Sludge Polluted Brackish Water Ecosystem

Vincent C. Wokem[*], Lucky O. Odokuma and Caroline N. Ariole

Department of Microbiology, University of Port Harcourt, P.M.B. 5323, Port Harcourt, Rivers State, Nigeria

*Corresponding author

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, bioargumentation 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 $C_{13} - C_{35,}$ with concentrations ranging from 26.12-7,713.62ppmwith 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 10^5cfu/g and 2.0 x10^5cfu/g and for the brackish water sample were 1.39 x 106cfu/ml and 1.1 x 10^4cfu/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 micoorganisms (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 applicationbe 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 biostimuation 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 regents 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 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.

Option B: Addition of 5ml of bacterial broth culture.

Option C: Addition of 5ml 10% $^{wt}/_{v}$ 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 ± 2^{0}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^{0}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^{0}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 Gramsreaction 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, 750$\mu$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) µl 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 1200µl of DNA binding buffer was added to each filtrate in the collection tubes bringing the final volume to 1600µl. 800µl 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 10,000xg for a minute. 200µl of the DNA pre-wash buffer were added to the zymo-spin IIC in a fresh collection tubes and spun at 10,000xg for a minute followed by the addition of 500µl of bacterial DNA, buffered and centrifuged at 10,000xg for a minute. The zymo-spin IIC column were transferred to clean fresh 1.5µl centrifuge tubes, 100µl of DNA elution buffer were added to the column matrix and centrifuged at 10,000xg for 30seconds to elute the DNA. The ultrapure DNA of each isolate properly labeled were then stored at -20°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 ($MgCl_2$), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR condition were as follows: initial denaturation, 95^0C for 4mins, denaturation, 95^oC for 30seconds; annealing 52^0C for 30 seconds; extension 72^0C for 1minute for 40 cycles and final extension 72^0C 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).

I6SrRNA 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.

$$\frac{(TPH\ at\ day\ 0 - TPH\ at\ day\ 56}{TPH\ at\ day\ 0} \times 100\%$$

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 1,407μs/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_{13} to C_{37} with concentrations ranging from 26.7-7,713.63ppm, C_{17} (Heptadecane) was the most significant n-alkane with highest concentration (7,713.63ppm) while C_{37} (heptatriacontane) had the least concentration (26.12ppm) Table 5. The PAHs concentration indicated that Benzo (b) fluoranthene 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, 2 49.0
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	Tripcosane	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	Pentatricacontane	196.66
31.	C36	Hexatricacontane	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	1.39×10^6	2.5×10^5
2	HUB	1.1×10^4	2.0×10^5

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

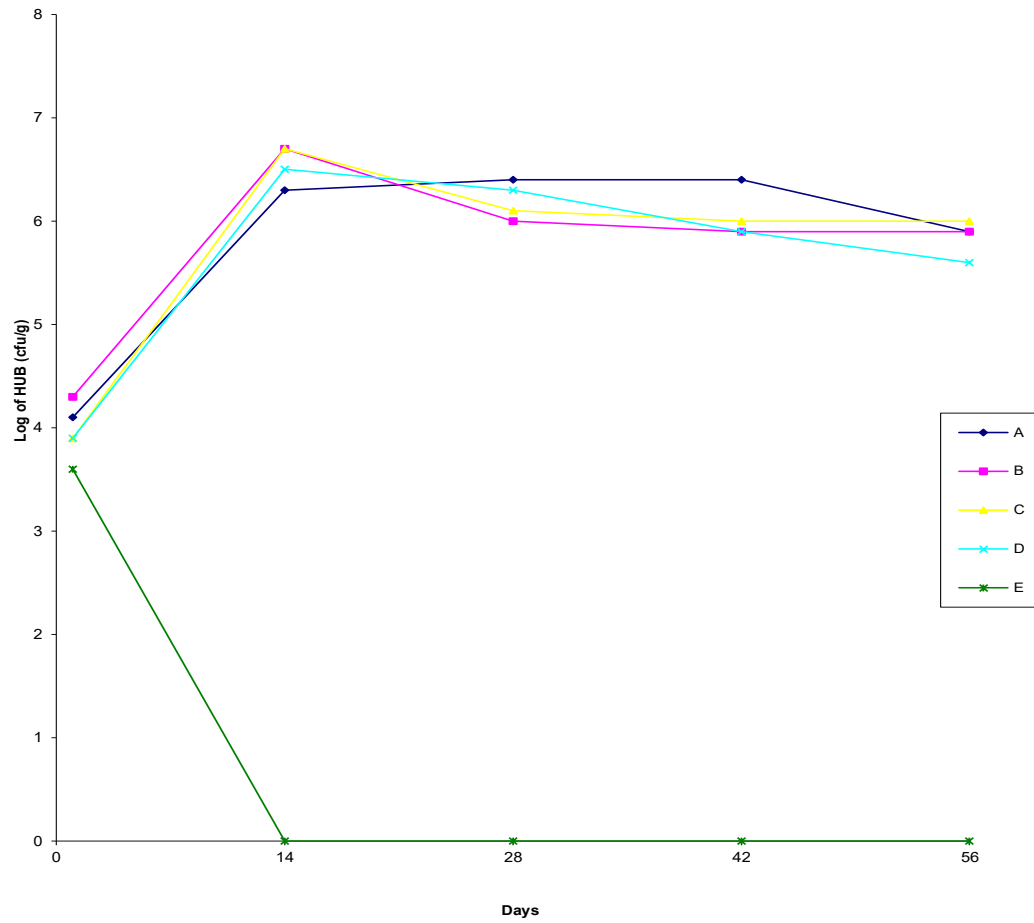| S/N	Name of Organism	Accession Number
1	*Klebsiella pneumoniae* strain B21	SUB1917764B1 KX817218
2	*Klebsiella pneumoniae* strain ICB-C183	SUB1917764B2 KX817219
3	*Klebsiellaoxytoca* strain BCNA1	SUB1917764B3 KX817220
4	*Klebsiellaoxytoca* strain BC4	SUB1917764B4 KX817221
5	*Alcaligenesfaecalis* strain IOU PMR	SUB1917764B5 KX817222
6	*Alcaligenesfaecalis* 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
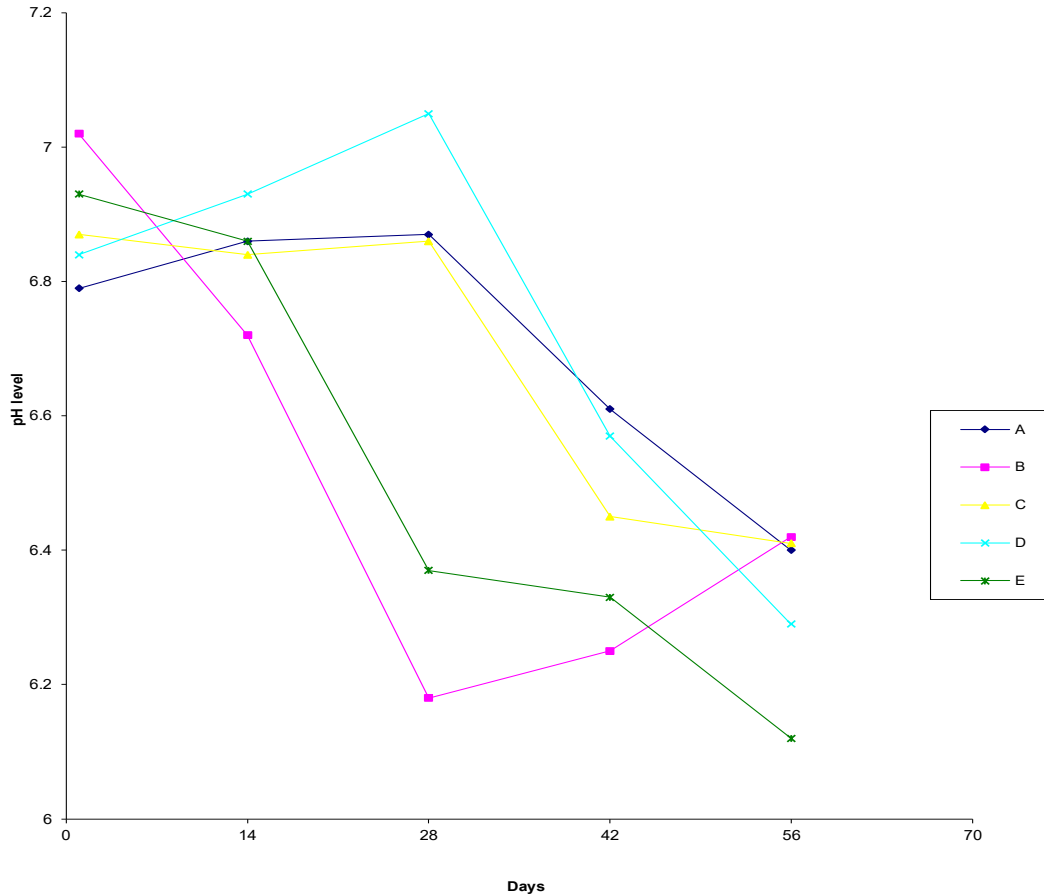

KEY

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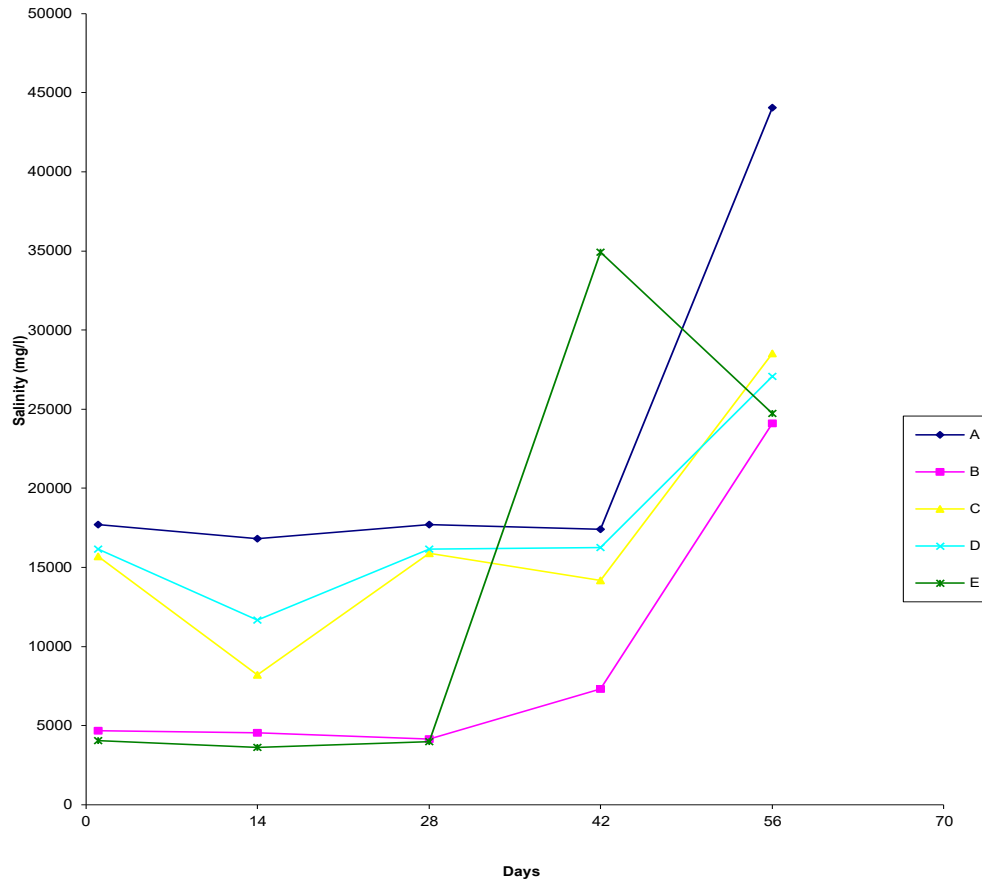D   -   NO APPLICATION
E   -   ADDITION OF BIOCIDE
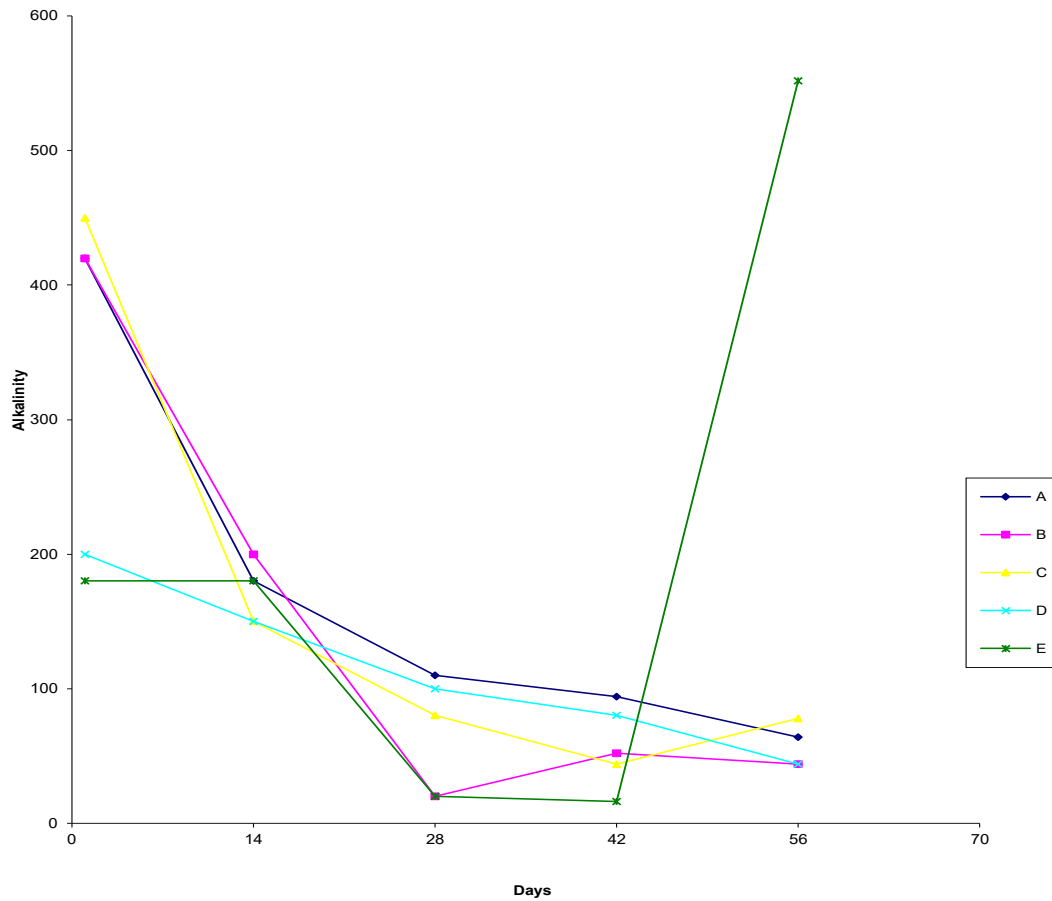
Fig.2 Growth profile of HUB in sludge polluted brackish water sample during the monitoring of the various bioremediation options

KEY

A - APPLICATION OF BACTERIA AND FERTILIZER
B - APPLICATION OF BACTERIA ONLY
C - APPLICATION OF FERTILIZER ONLY
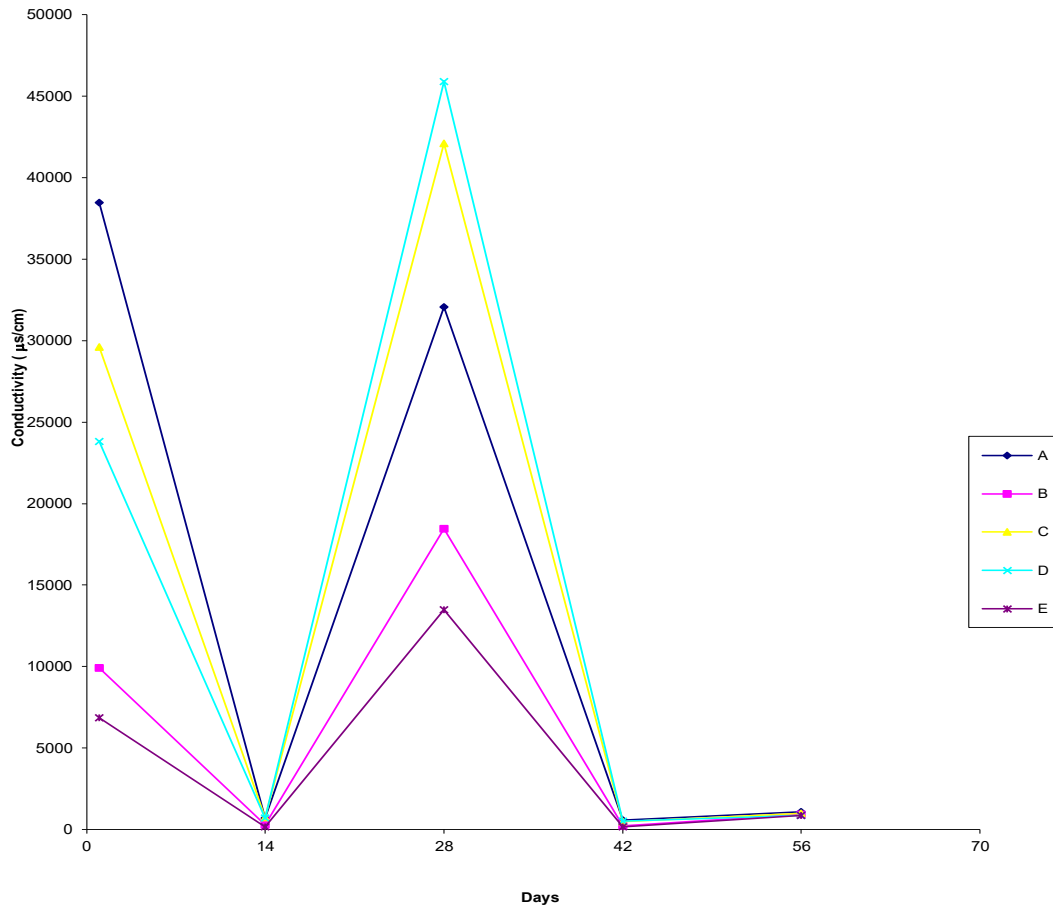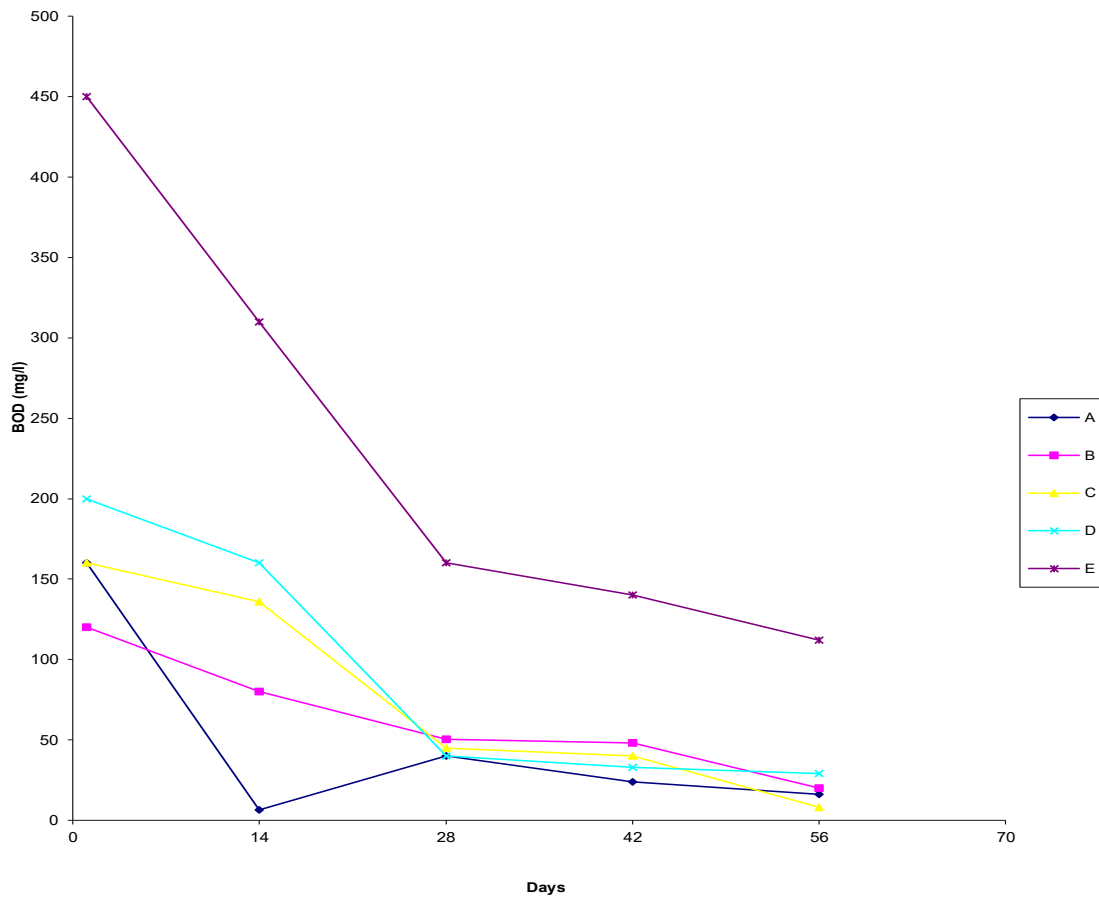D - NO APPLICATION
E - ADDITION OF BIOCIDE

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

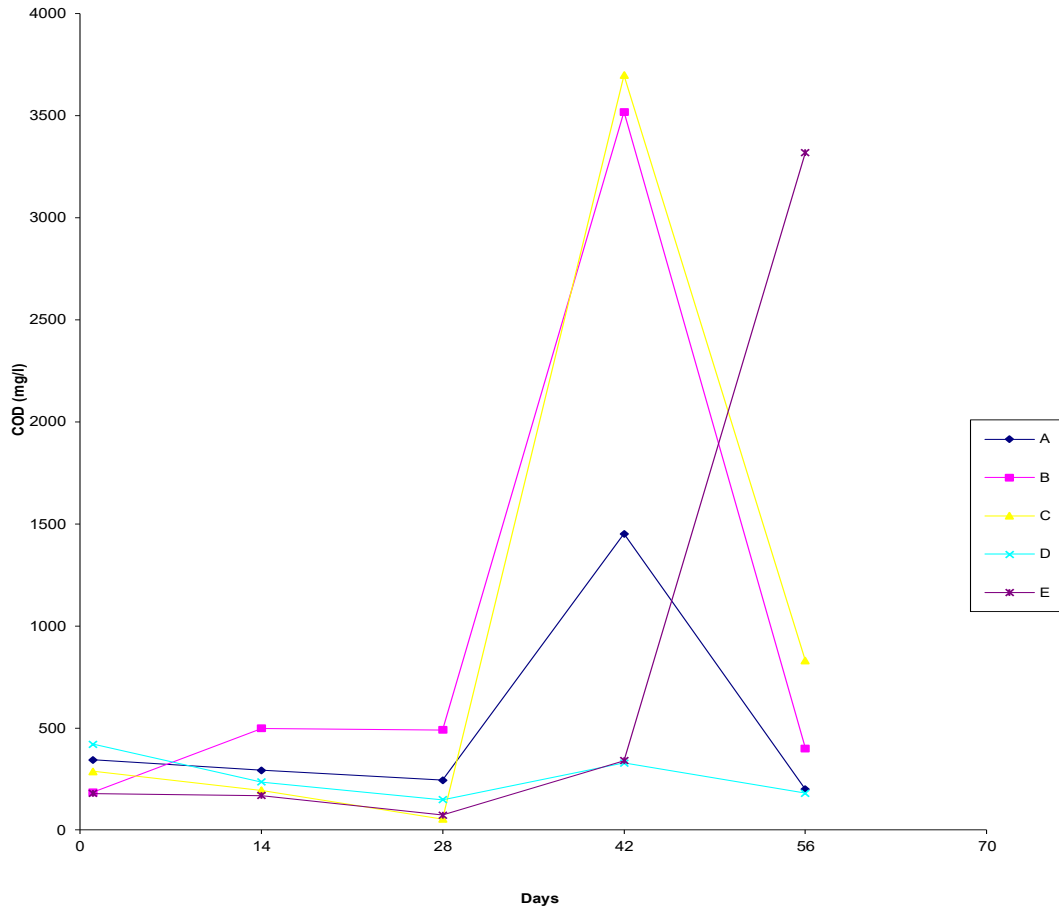|		
A	-	APPLICATION OF BACTERIA AND FERTILIZER
B	-	APPLICATION OF BACTERIA ONLY
C	-	APPLICATION OF FERTILIZER ONLY
D	-	NO APPLICATION
E	-	ADDITION OF BIOCIDE

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
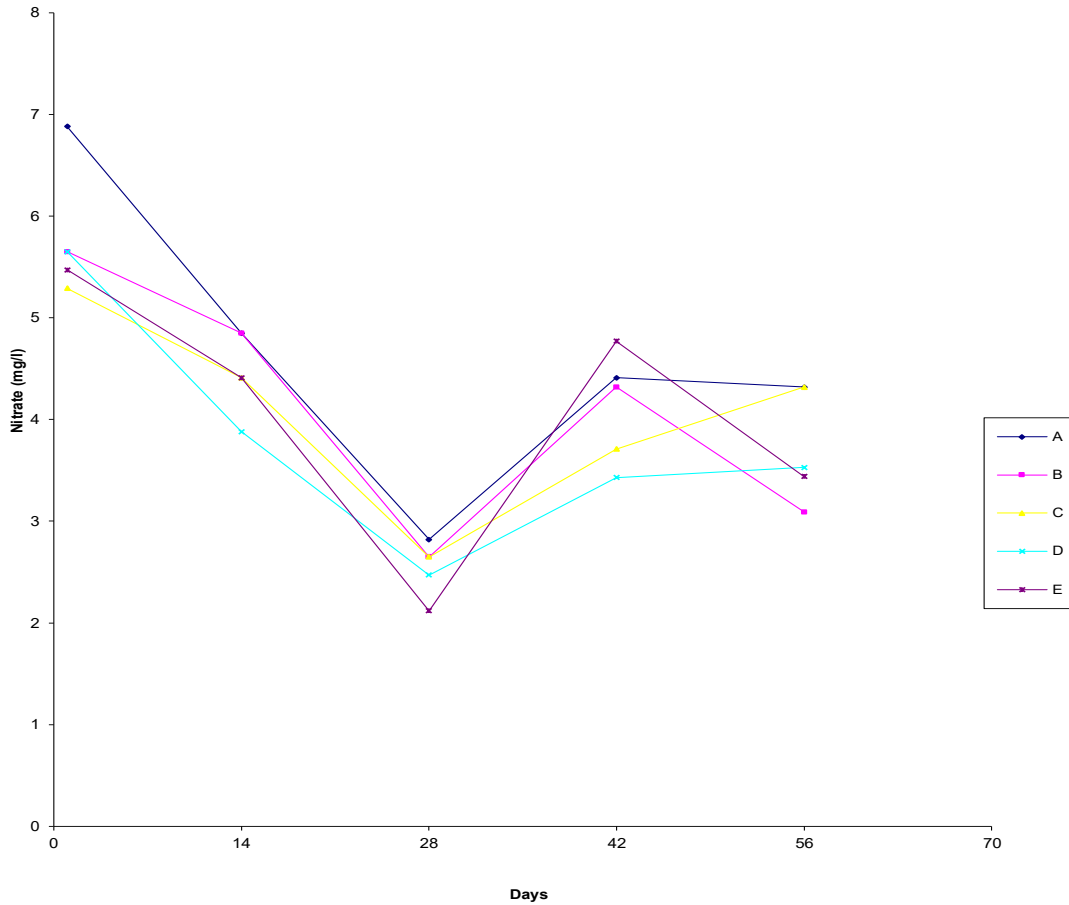

KEY

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
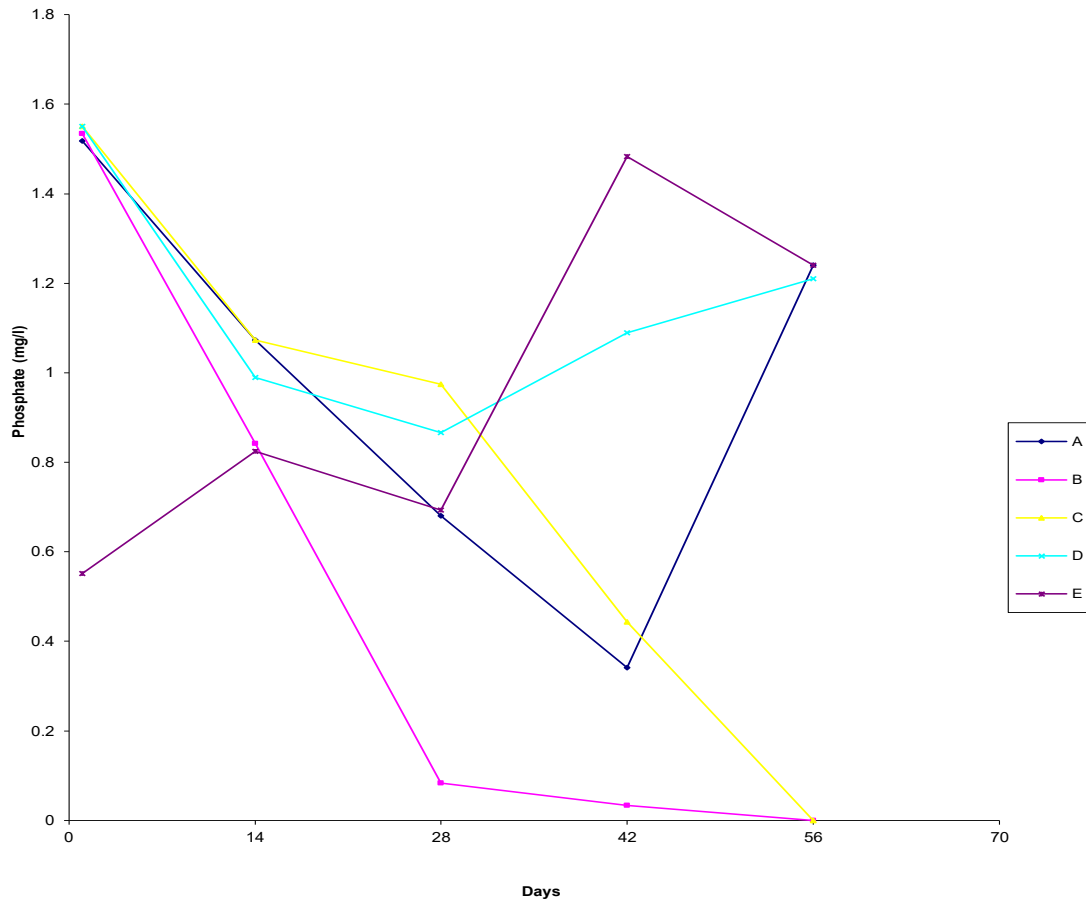

KEY
A - APPLICATION OF BACTERIA AND FERTILIZER
B - APPLICATION OF BACTERIA ONLY
C - APPLICATION OF FERTILIZER ONLY
D - NO APPLICATION
E - ADDITION OF BIOCIDE

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
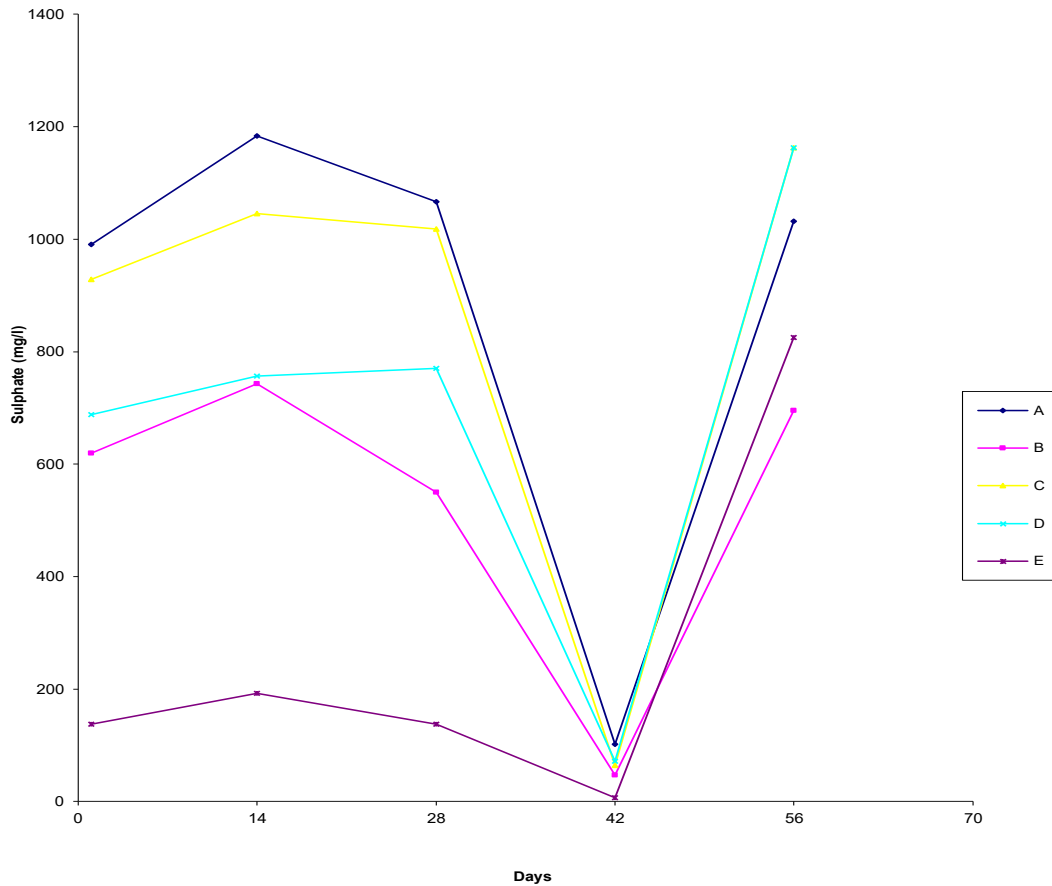

KEY

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
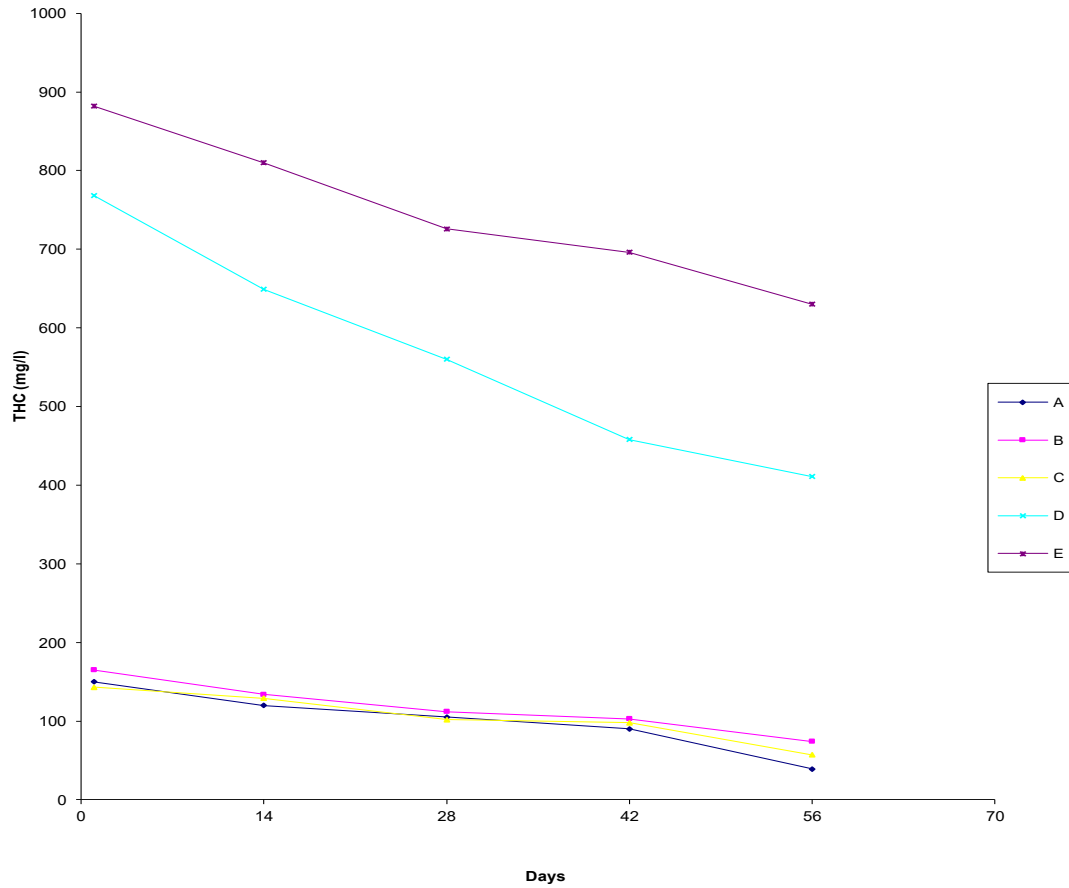

KEY

A	-	APPLICATION OF BACTERIA AND FERTILIZER
B	-	APPLICATION OF BACTERIA ONLY
C	-	APPLICATION OF FERTILIZER ONLY
D	-	NO APPLICATION
E	-	ADDITION OF BIOCIDE

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 BIOCIDE

Fig.12 Changes in THC level in sludge polluted brackish water sample during monitoring of various bioremediation options
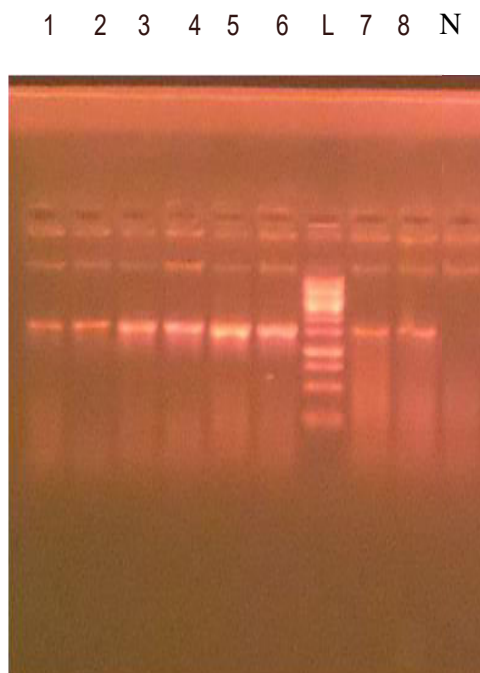

KEY
A   -   APPLICATION OF BACTERIA AND FERTILIZER
B   -   APPLICATION OF BACTERIA ONLY
C   -   APPLICATION OF FERTILIZER ONLY
D   -   NO APPLICATION
E   -   ADDITION OF BIOCIDE

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 metalbolic 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 bioagumentation 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, *Klebsiellaoxytoca* BC4, *Alkaligenes faecalis* IOU PMR, *Alacligenesfaecalis* 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 implicatedin 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.

References

Ahirwar, S and Dehariya, R (2013). Isolation and characterization of hydrocarbon degrading microorganisms from petroleum oil contaminated soil sites. *Bull. Environ. Sci.*

Amanchukwu, S. C., Okpokwasili, G. C. and Obafemi, A (1989). Factors affecting hydrocarbon degradation by *Schizosaccharomycespombe* isolated from palm wine. *Proceedings of 1987 Seminar on the petroleum Industry and the Nigerian Environment.* pp. 154-160. Department of Petroleum Resources (DPR) and Nigerian National Petroleum Corporation (NNPC), Lagos, Nigeria.

American Public Health Association- APHA (1998). Standard Methods for examination of water and waste water 20th ed. Washington DC: American works Association, water pollution control federation.

American Society for Testing Materials – ASTM D7678 (1999). Standard Test Method for total petroleum hydrocarbon in water and wastewater with solvent Extraction using Mid-IR laser Spectroscopy.

American Society for Testing Materials – ASTM D8270 (1999). Standard Test Method for total petroleum hydrocarbon-volatile organic compounds by Gas Chromatography.

Atlas, R. M. (1984). Microbial degradation of petroleum hydrocarbons: An environmental perspective. *Microbial Rev*. 54:180-209.

Ayotamuno, M. J., Okparanma, R. N. and Amadi, F. (2011).Enhanced remediation of an oily sludge with saline water.*Afri. J. Environ. Sci. Tech.* 5 (4): 262-267.

Ayotamuno, M. J., Okparanma, R. N., Ogaji, S. O. T and Robert, S.D (2007) Bioremediation of a Sludge Containing hydrocarbon. *J. Appl. Energy* 84 (9): 936-943.

Balachandran, C., Durapandiyan, V., Balakrisina, K and Ignacimuthu, S (2012). Petroleum and polycyclic aromatic hydrocarbons (PAHs) degradation and raphthalene metabolism in *Streptomyces sp.* (ERI-CPDA-1) isolated from oil contaminated soil. *Bioresour. technol.* 11: 83-90.

Bhahacharyya, J. K and Shekdar, A. V (2003).Treatment and disposal of refinery sludge; Indian Scenario.*Waste Mgt. Res.* 21 (3): 249-261.

Bundy, J. G., Paton, G. I., Campbell, C. D (2004).Combined microbial community level and single species biosensor responses to monitor recovery of oil pollutant soil.*Biol. Biochem.* 36 (7) 1149-11659.

Calfee, R. D., Little, E. E., Clevel, L and Barron, M. G (1999). Photo enhanced toxicity of a weathered oil to *ceriodaphinadubia* reproduction. *Environ. Sci. Pollut. Resour*. 6: 217-212.

Cébron, A., Norini, M.P., Beguiristain, T and Leyval C. (2008). Real-time PCR quantification of PHA- ring hydroxylatingdioxygenase (PAH-RHDa) genes from gram positive and gram negative bacteria in soil and sediment samples *J. Microbiol.Methods*. 73:148-159.

Chamkha, M., Trabelsi, Y., Mnif.S and Sayadi. S (2011) Isolation and characterization of *Klebsiellaoxytoca* strain degrading crude oil from Tunisian off-shore fields. *J. Basic Microbiol.* 5(6): 580-589.

Cheesbrough, M. (2006). *District Laboratory Practice* in Tropical countries, part 2, London, Cambridge University Press. Pp 58-100.

Chikere, C. B and Ekwwuabu, C. B. (2014). Culture dependent characterization of hydrocarbon utilizing bacteria in selected oil impacted sites-in Bodo, Ogoniland, Nigeria. *Afri. J. Environ. Sci. Technol.* 8 (6): 401-406.

Chikere, C. B., Okpokwasili, G. C and Chikere B. O. (2009). Bacterial diversity in a tropical crude oil polluted soil undergoing bioremediation. *Afri. J. Biotechnol.* 8: 2535-2540.

Crisafi, F. Genovase, M., Smedite, F., Russso, D., Cataliforna, M and Yokimov, M (2016). Bioremediation techniques for polluted sea-water sampled after an oil-spill in Taranto gulf (Italy): A comparison of biostimulation, bioaugmentation and use of a washing agent in microsom studies. *Marine Pollution Bulletin*.106 (1-2): 119.

Dean-Ross, D. Moody, J., Cemigla, C.E (2002). Utilization of mixtures of polycyclic aromatic hydrocarbons by bacteria isolated from contaminated sediment. FEMS. *Microb. Ecol.* 4 (1): 1-7.

Delyan, U. H., Harder, M. and Hopner, T. H (1990).Hydrocarbon biodegradation in sediments and soils.Systematic examination of physical and chemical conditions part II pH values.

WissechaftTechnik Sci. Technol. 43: 337-342.

Department of Petroleum Resources- DPR (2002). *Environmental Guideline and Standards for Petroleum Industry in Nigeria.* (EGASPIN). Revised edition, Department of Petroleum Resources, Abuja, Nigeria P: 314.

Felsentein, J. (1985). Confidence limits on phylogenies: An approach using bootstrap. *Evolution. 39: 783-791.*

Hara, E., Kurihara, M., Mormura, N., Nakajira, T and Uchiyama, H (2013). Bioremediation field trial of oil contaminated soil with food waste compost. *J. JSCE:* 1 (1): 125-132.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. and Williams, S. T. (1994). *Bergey's Manual of Determinative Bacteriology,* (9[th]ed). Williams and Wilkins Co., Baltimore.

Igwo-Ezikpe, M.N., Gbenle, O.G., Ilori, M.O., Okpuzor, J., and Osuntoki, A. A (2009) Evaluation of *Alcaligenesfaecalis* degradation of chysene and diesel oil with concomitant production of biosurfactant. *Res. J. Environ. Toxicol.*3:158-169.

Krebs, G. T and Tanner, G. E (1986).Restoration of oiled marshes through sediment stripping and spartina propagation. *Proceedings* of the 1981 oil spill conference, American Petroleum Institute API, Washington DC, 315-385.

Macaulay, B. M (2015).Understanding the behavior of oil-degrading microorganisms to enhance the microbial remediation of spilled petroleum. *Appld. Ecol. Environ. Resour*, 13 (1): 247-262.

Malik, Z. A and Ahmed, S (2012). Degradation of petroleum hydrocarbon by oil field isolated bacterial consortium. *Afri. J. Biotechnol.*, 11 (3): 650-658.

Manning, F. C. and Thompson, R. E (1995).*Oil filed processing crude oil*, Tulsa Pennwell books 2:5.

Mayo, A. W and Noike, T (1996).Effects of temperature and pH on growth of heterotrophic and bacteria in waste stabilization ponds.*Water Research* 30 (2): 447-455.

Milic, J., Beskoski, V., Ilic, M., Ali, S., Gojgic-Cvijovic, G and Vrvic, M (2009). Bioremediation of soil heavily contaminated with crude oil and its products: composition of the microbial consortium. *J. Serb. Chem. Soc.* 74 (4): 455-460.

Mills, A. C., Breuil, C. and Cowell, R. R. (1978).Enumeration of Petroleum degrading marine and estuarine microorganism by the Most Probable Number (MPN) method. *Canad. J. Microbiol.*, 25: 552-557.

Milne, B (1998). Composting of heavy oil Refinery sludge. *Environ. Programme* 17 (1): 17-24.

Mona, S. Z., Mohammed, M. N. A and Hossam, H. H.A (2015).Bioremediation of petroleum contaminants in aquatic environments. *Life Sci. J.*, 12 (5): 127-139.

Mukred, A. M., Hamid, A. A., Hamzah, A and Yusoff, W. M. W (2008).Development of three bacteria consortium for the Bioremediation of crude petroleum-oil in contaminated water.*Online J. Biol. Scis*. 8 (4): 1608-4217.

Nester, E.W., Anderson, D.G., Roberts, C.E., Pearsall, N.C and Nester, M.T (2001).*Microbiology: A human perspective*. 4th ed. McGraw. Hill Co. Inc, New York. Pp 781-793.

Obire, O and Akinde, S. B (2004). Poultry manure amendment of oil polluted soils for sustainable development in the Niger Delta *J. Nig. Environ. Soc.* 2 (2): 138-143.

Odokuma, L. O and Akpokodje, E. G (2004).Biodegradation of stimulated landfill leachate in tropical soil of depth 6.5 metres. *J. Appld. Sci. Technol.* 4 (1): 6-14.

Odokuma, L. O and Dickson, A. A. (2003). Bioremediation of crude oil polluted tropical mangrove environment. *J. Appld. Sci. Environ.* 7 (2): 23-29.

Odokuma, L. O and Okara, J. O (2005). Biodegradability of grounded cell phone recharge cards in two Niger Delta soils. *J. Appld. Sci. Technol.* 5 (1-2): 11-20.

Odokuma, L. O and Okporwasili G.C (1993a). Seasonal ecology of hydrocarbon-utilizing microbes in the surface waters of a river. *Environ. Monit. Assess.* 27:175-191.

Odokuma, L. O and Okporwasili G.C (1993b). Seasonal influences on inorganic Anion monitoring of the new Calabar River Nigeria. *Environ. Mgt* 17 (4): 491-490.

Odokuma, L. O. and Okporwasili G. C. (1997).Seasonal influence of organic pollution monitoring of the New Calabar River, Nigeria. *Environ. Monit. Assess.* 45: 43-56.

Odokuma, L. O. and Smith, V. A (2007).Bioremediation of a Nigerian crude oil by *chrococcus* and *chlorella species.Trop. Freshwater Biol.*, 16 (1): 17-30.

Odokuma, L. O., and Ibor, M. N (2003). Bioremediation of crude oil polluted tropical soil by nutrient supplementation. *Sci. Afri.* 1(2):1-5.

Odokuma, L.O and Ibe, C. O (2003).Bioremediation of paper and polyethylene using immobilized and free bacterial cells.*Nig. J. Microbiol.*, 17 (1): 17-25.

Okpowasili, G. C. Odokuma, and L. C. (1990). Effects of salinity on biodegradation of oil spill dispersants *Waste Mgt.* 10: 141-146.

Phulia, V., Jamwal, A., Saxena, N., Chadha, N. K., Muralidhar, A. P and Prusty, A. K. (2013).Technologies in aquatic bioremediation. Pp 65-91. In: Kumar, P., Zaki, M.S.A and Chauhan A. (Eds.), *Freshwater Ecosystem and Xenobiotics*. Discovering Publishing House PVT. Ltd., New Delhi, India.

Riser-Roberts, E (1992). Bioremediation of petroleum contaminated sites CK Smoley, Boca Raton.

Rodrigues, D. F., Sakate, S. K., Comassento, J. V., Bic, M. C and Pellizanri V. A. (2009). Diversity of hydrocarbon-degrading *klebsiella* strains isolated from hydrocarbon contaminated estuaries. *J. Appl. Microbiol.* 106 (2): 1304-1304.

Saituo, N and Nei, M (1987). The neighbor joining method: A new method for reconstructing phylogenetic trees. *Mole. Biol. Evol.,* 4:406-425.

Singh, K and Chandia S (2014). Treatment of petroleum hydrocarbon polluted environment through bioremediation: a review. *Pak. J, Biol. Sci.* 17 (1) 1-8.

Stewart, E. A., Maxcrimshaw, H., Parkinson, J. A and Quarmby, C. (1974).*Chemical Analysis of Biological Materials*.Blackwell Scientific Publication.Oxford London.

Thompson, J. D., Gibson, T. J., Plewniak, F., Janmeugin, F and Higgins, D. G (1994). The cluster IX windows interface flexible strategies for multiple sequence alignment aided by quantity analysis tools. *Nucleic Acid Resour.* 22:4673-4680.

Tyagi, M., Yafonseca, M. and Carvalho, C. (2011).Bioaugmnetation and Biostimulation strategies to improve the effectiveness of bioremediation process, *Biodegra*, 22.

Vidali. M. (2001). Bioremediation an Overview. *Pure Appld. Chem.*, 73 (7) 116-1172.

Vijayadeep, C. and Sastry, P. S. (2014).Effect of heavy metal uptake by *E. coli* and *Bacillus*sps. *J. Biorem. Biodegra.*5: 238.

Wang, X. B., Chi, C. Q., Nie., Y., Tang, Y. Q., Tan, Y., Wu., G and Wu., X. L (2011). Degradation of petroleum (C6-C40) and crude oil by a novel Dietzia strain *Bioresour. Technol.* 102 (7): 7755-7761.

Wokem, V. C and Odokuma L. O (2016). Bioremediation of condensate polluted freshwater Ecosystem. *Nat. Sci.* 14 (7): 96-102.

Wu, Y., Luo, Y., Zuo, D., Ni, J., Liu, W., Teng, Y and Li, Z (2008). Bioremediation of polycyclic aromatic hydrocarbons contaminated soil with *Monilinia sp.* Degradation and microbial community analysis. *Biodegra.* 19 (2): 247-257.