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BIOREMEDIATION OF A CRUDE OIL POLLUTED TROPICAL RAIN FOREST SOIL

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ABSTRACT

A combination of options including Biostimulation with agricultural fertilizers, Bioaugumentation and physical processes were evaluated in-situ in the clean-up of crude oil polluted tropical rain forest soil for a period of nine weeks. Soil physicochemical parameters such as moisture (19% to 13%), pH (6.34 to 4.5) and organic carbon (85% to 1.5%) dropped during the remediation treatment. Total Nitrogen increased (0.021% to 0.081%) with increase in period of remediation. There was an increase in the total heterotrophic bacterial (THB) and hydrocarbon utilizing bacterial (HUB) counts in all the remediation options throughout the period. The control site O (no treatment employed) revealed no significant hydrocarbon loss (2%) within the study period. The hydrocarbon losses (73% to 88 %) experienced in five other options were significantly different from the control. Option A (a combination of Biostimulation and Bioaugumentation with indigenous HUB, hot water washing and tilling) recorded a hydrocarbon loss of 83%. Option B (a combination of mixing uncontaminated soil from a different location with polluted soil, tilling and Bioaugumentation with indigenous hydrocarbon utilizing bacteria) produced the lowest level of hydrocarbon loss (73%). Option C (a combination of Biostimulation, Bioaugumentation with indigenous HUB) recorded the highest level of hydrocarbon loss (88%). Option D (a combination of Bioaugumentation with genetically engineered strains of hydrocarbon utilizing *Pseudomonas*, Biostimulation and tilling) produced a hydrocarbon loss of 82%. Option D^1 (a combination of Biostimulation with tilling) produced a hydrocarbon loss of 86%. These results suggest that Biostimulation with tilling (nutrient enhanced in-situ bioremediation) and or the combination of Biostimulation and Bioaugumentation with indigenous hydrocarbon utilizers would be effective in the remediation of crude oil polluted tropical soils.

Keywords: Bioremediation, Bioaugumentation, Biostimulation, Hydrocarbon, Fertilizer

INTRODUCTION

Biodegradation of organic waste is becoming an increasingly important method of waste treatment(Atlas, 1984). The advantages of this option include inexpensive equipment, environmentally friendly nature of the process and simplicity(Nadeau *et al.* , 1983). However, one disadvantage of this process is its relative slow speed in achieving results. Bioaugumentation and Biostimulation are methods of biodegradation (bioremediation) geared towards enhancing and speeding the process. (Lee *et al.* , 1993). Bioaugumentation involves the addition of external microbial populations (indigenous or exogenous) to the waste. Sometimes they are genetically engineered (Okpokwasili *et al* 1986). Biostimulation involves the addition of appropriate microbial nutrients to a waste stream. This may either occur in-situ or ex-situ

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(Lee and Levy 1987,1989,1991). The objective of this process is to stimulate the indigenous microbial flora of the waste to bring about its degradation.

The biostimulation (nutrient enhancement of microbial organic waste breakdown) option is relatively new in the tropics thus there is a dearth of information in this area. In the tropics, however, bioremediation is used in conjunction with ecological rehabilitation to clean-up oil spills. Ecological rehabilitation involves the replanting and /or the re-stocking of a severely damaged area with vegetation. The objective of this study was to evaluate a combination of options, biostimulation, bioaugumentation, hot water washing, mixing with uncontaminated soil and tilling for the remediation of a crude oil polluted tropical soil.

MATERIALS AND METHODS

Study area description

The experimental plots were located in the University of Port Harcourt. This is a seasonal rainforest zone. The coastal plain sand geological formations where the area is situated is characterized by sand and clay deposits. The top soil is usually sandy loam and the vegetation cover is the tropical rainforest.

The soil is moist of the year round due to the excessive rainfall of about 2700 mm. This volume of rainfall provides great amounts of surface run-offs rivulets and occasional streams which may carry substances like crude oil to nearby lands and rivers.

C	D^1	B	A	D^1	O
A	B	C	D	O	D

Figure 1Experimental layout of the site

Cell A: Hot water washing followed by additions of fertilizer, indigenous hydrocarbon -utilizing bacteria and tilling

Cell B: Sand mixing (uncontaminated soil) followed by additions of fertilizer indigenous hydrocarbon-utilizing bacteria and tilling

Cell C: Addition of indigenous hydrocarbon-utilizing bacterial followed by fertilizer application and tilling

Cell D^1: Fertilizer application an tilling

Cell D: Addition of genetically engineered hydrocarbon-utilizing bacteria followed by fertilizer application and tilling

O Treatment: This is control without any remedial treatment.

Experimental design

The remediation study took place from November, to February. The soil was divided in to eight treatment cells(options) as presented in Figure 1.Each cell was 1m x 1m.

Soil treatment

Twenty litres of Bonny light crude oil was poured on each treatment plot (including control). The objective was to simulate conditions of a major spill. The plots were left undisturbed for three days. After three days, the top soil (3cm depth) containing some oil was removed manually to simulate emergency clean-up conditions (after clean-up). The treatment options were then applied.

Soil sampling

Ten random spots 15cm apart to a depth of 30cm were augured, and bulked together (composite soil samples) and put in well labeled polyethene bags. This procedure was done three times to form three replicates. The bags were immediately transferred to the laboratory for analysis

Detailed description of treatment options

(A) HOT WATER WASHING:

The procedure for hot water washing involved excavating soil in the affected cells to a depth of 30cm into large vats containing water at a temperature of 100°C. The objective was to remove hot water soluble fractions of crude oil from the soil before bioremediation. The mixture of water and soil was maintained at the above temperature for 1hr. The hot water soluble fractions and some insoluble fractions were removed by decantation. The soil was then taken back to the cell .

(B) TILLING

The relevant cells were tilled five times a week with spades or shovels to provide maximum aeration, and adequate mixing of nutrients and microbes with the contaminated soil.

(C) FERTILIZER APPLICATION

A 15:15:15 NPK fertilizer was applied broadcast to the relevant cells and worked into 30 cm depth at each site. About 400kg/ha of the fertilizer was applied once a week for four weeks and then, once in every two weeks for another four weeks, applying a total of 2.4 tons of the fertilizer per hectare to the plot for the period. This quantity of fertilizer supplied about 360kg each of N,P and K per hectare to the plot for the 9-weeks remediation period.

(D) BACTERIA ADDITIONS

Bacteria used in this study were of two types: (a) indigenous hydrocarbon- utilizing bacterial populations and (b) a genetically engineered hydrocarbon-degrading bacterial population. The genetically engineered bacteria was a strain of *Pseudomonas*. It was obtained from the Federal Ministry of Environment Lagos Nigeria, while the indigenous bacterial populations were obtained from the soil within the area. The hydrocarbon-utilizing bacterial population consisted of *Pseudomonas, Bacillus, Proteus, Serratia, Aeromonas, Klebsiella* and *Micrococcus* .Both genetically engineered and indigenous hydrocarbon-utilizing bacteria were applied in this area. The bacterial deposits obtained during scaling-up process were transferred into a sterile 4 litre jerry-can containing 4000 ml of normal saline. This was shaken vigorously to ensure proper mixing of the contents. The contents were then poured onto the soil of appropriate treatment cell. The culture was poured all-round the cell to ensure even spread within the cell.

Microbial analysis

(A) RECONSTITUTION OF FREEZE DRIED GENETICALLY ENGINEERED (GEM) BACTERIAL STRAIN.

The GEM obtained from Federal Ministry of Environment, Lagos Nigeria was a freeze- dried strain hence the need for re-constitution. This process involved adding the freeze-dried organism in peptone broth, incubating for 48hr

and sub-culturing into fresh medium and incubating for 48hr. This process was repeated four times. This was followed by inoculation by streaking on the peptone agar and incubating for 48hr. All aseptic techniques were observed. Discrete colonies were picked sub-cultured into peptone slants and observed microscopically for their purity. Gram positive rods with biochemical characteristics of *Pseudomonas* were observed.

(B) SCALING UP OF HYDROCARBON-UTILIZING MICROORGANISM FOR INOCULATION INTO TREATMENT CELLS.

The right species and number of microorganism must be present before any bioremediation programme could be effective. Hydrocarbon-utilizing bacterial population of study area was generally very low before experimentation. So it became necessary to increase their population. The process of scaling up was used.

In the process, nutrient agar slants of all the hydrocarbon-utilizing bacterial genera isolated from the site were prepared. Nine milliliters of sterile normal saline (0.85% NaCl) was transferred into a 20ml-sterile test-tube. A sterile inoculating wire loop was used to scrape organism from these slants into the normal saline. The suspension was then transferred into a conical flask containing 190ml of sterile mineral salt medium (minimal medium C) of Mills *et al.,* (1978) containing 1ml of Bonny medium crude oil. This mixture was placed on a shaker and incubated at room temperature of 30 + 2°C for 7 days. One hundred and ninety milliliters of this culture was then transferred aseptically into a 3 litre Erlenmeyer flask containing 1, 800ml of mineral salt medium and 10ml of Bonny medium crude oil. The flask was placed on a rotary shaker. The contents of this flask were then transferred into 20 litre plastic jerrycan that had been sterilized with butanol. The jerrycan contained 17, 900ml of mineral salt medium and 100ml of Bonny medium crude oil. The culture was incubated for seven days. The jerrycan was shaken thrice daily to ensure aeration. The pH of each of the cultures at all stages was monitored daily and maintained at 7.2 by adjusting with standard phosphate buffer (APHA, 1985). Aliquots of the final culture were centrifuged. The deposits were collected and weighed. The process (centrifugation) was repeated until the weight (wet) desired for deposition on treatment cells was achieved. This deposit served as inoculum for treatment cells. The microbial load applied per cell was 100kg/ha.

(C) ENUMERATION OF TOTAL HETEROTROPHIC BACTERIA

The total heterotrophic bacterial count was performed on nutrient agar (Oxoid) using the spread plate method (APHA, 1985). Total viable counts of aerobic heterotrophic bacteria were also obtained by this method.

(D) DETERMINATION OF PHYSICOCHEMICAL PARAMETERS OF SOIL

Physicochemical parameters such as moisture content, pH, total organic carbon, nitrogen, phosphorus and hydrocarbon were determined using methods adapted from Black *et al.* , (1965).

(E) SOURCE OF MATERIALS

All chemicals used in the study were of analytical grade and were purchased from Sigma Chemical Company, St Louis Missouri USA. The fertilizer used was obtained from the National Fertilizer Company of Nigeria (NAFCON) Limited, Port Harcourt, Nigeria. Ply wood used to construct the treatment cells (Fig. 1) was obtained from the Timber market Port Harcourt, Nigeria. Crude oil 'Bonny medium crude oil' was obtained from the Nigerian National Petroleum Corporation (NNPC) Port Harcourt Nigeria.

(F) STATISTICAL ANALYSIS

Analysis of Variance (ANOVA), Least Significant Difference (LSD) and Correlation Coefficient

methods (Finney 1978) were employed to analyze data.

RESULTS AND DISCUSSION

The soil characteristics (moisture, pH, organic carbon, total nitrogen, carbon/nitrogen (C/N) ratio, phosphorous and total hydrocarbon) before clean-up, after physical clean-up and during remediation are presented in Tables 1-6. The soil moisture content of the various options dropped from 19% to 14% after physical clean-up (Table 1) and dropped further to 4% (in option A) prior to remediation (Table 2) and increased later to 13 % in some options after remediation treatment (Tables 3-6). The initial drop is expected because in heavily polluted soils, water droplets adhere to the hydrophobic layer formed, and this prevents wetting of the inner parts of the soil aggregates. (Rowell,1977)

Prior to remediation (Table 2), there was a further drop in moisture due to soil drying and evaporation of water. As the soil samples were remediated through the introduction of microbes, fertilizers continuous watering and tilling, moisture content increased (Table 3-6). There was a correlation (r = +0.011) between soil moisture content and remediation period

Table 1: Some Physicochemical Characteristics And Heavy Metal Concentrations Before And After Clean-Up

SAMPLE STATN	SAND	PERCENT(%) SILT	CLAY	MOISTUE	pH 1:1	THC mg/kg	ORGANIC C	PERCENT TOTAL N	C/N RATIO	AVAIL P mg/kg	HEAVY METALS mg/kg Fe	Mn	Pb	Zn	Ni
BC,	75.8±0.5	6.4±0.2	17.18±0.5	18±1	6.34±0.1	12,749±100	4.906±0.05	0.023±0.001	213±1	19± 0.1	896±10	80.80±0.5	0.66±0.1	70.0±0.1	0.40±0.1
AC,	74.8±0.5	7.2±0.1	18.0±0.4	14±1	5.75±0.2	6,643±150	4.900±0.05	0.023±0.001	213±1	1.3±0.1	874±15	42.00±0.5	0.58±0.1	61.80±0.1	0.40±0.1

BC = Before clean-up

AC = After clean-up

Results represent mean and standard deviations of treatment cells

TABLE 2: SOME PHYSICOCHEMICAL CHARACTERISTICS OF THE CELLS PRIOR TO REMEDIATION

REMEDIATION TREATMENT TYPE	% MOISTURE	pH 1:1	THC mg/kg	ORGANIC C	PERCENT TOTAL N	C/N RATIO	AVAIL P mg/kg
O	8± 1	5.42± 0.20	11,671± 20	4.862± 0.05	0.021± 0.001	232± 5	2.20± 0.1
A	4± 2	5.19± 0.15	6,123± 100	4.685± 0.15	0.022± 0.001	213± 3	1.66± 0.2
B	6± 1	5.34± 0.30	4,471± 200	4.590± 0.12	0.022± 0.001	209± 2	1.35± 0.09
C	5± 1	5.37± 0.25	8,586± 200	4.607± 0.10	0.021± 0.001	219± 5	1.52± 0.09
D[1]	7± 1	5.40± 0.20	7,613± 250	4.620± 0.10	0.022± 0.001	210± 3	1.09± 0.07
D	5± 1	5.31± 0.25	11,528± 300	4.860± 0.20	0.022± 0.001	221± 2	1.90± 0.05

Results represent means and standard deviations of three Replicates

as 1.56% (Tables 6) in some options during remediation treatment. There was a correlation (r = -0.576) at 1% probability level between organic carbon and remediation period (Table 7). This suggest that organic carbon reduced with time.

Unlike organic (C), total nitrogen (N) of various options increased with increase in the period of remediation (0.021% to 0.081%). The relationship between nitrogen and total hydrocarbon content (THC) reduction was significant (r = +0.684 at 1% probability level) implying that nitrogenous nutrients supplied, provided a favourable environment for THC degradation.

At the end of the study, the C/N ratio narrowed from 213 after clean-up (Table 1) to 24 in option A, 21 in option B and 14 in option C (Table 6). The C/N ratio of the control however increased to 255 at the termination of the study. The narrowed C/N ratios implied that remediation in the various options except control was successful. The increased C/N ratio of the control is due to lack of metabolic feedstock like aeration and inorganic nutrient sources for the microbial population. This resulted in partial degradation of hydrocarbons and such degradation products were added to the organic C pools whereas the little nitrogen in the soil was used up by the microbes carrying out the degradation process. There was a significant correlation between C/N ratio and total hydrocarbon content (r = -890) at 10% probability levels meaning that wide C/N ratios led to reduced THC degradation and vice-versa. The correlation analysis between C/N ratio and remediation period (Table 7) also showed a relationship (r = -0.404) at 10% probability level.

The available phosphorous (P) levels after clean-up (1.3 mg/kg) and during remediation in some options(3.65mg/kg) were very low. This is due to the immobility (reduced availability) of P. It may not have been sufficiently dissolin the soil to make it available, while the little that was dissolved is rapidly utilized by existing soil microbial populations.

There was a marked decrease in the percentage THC of all the options except the control. After nine weeks of remediation, the percentage THC reduction for all the options were 2%, 73%,

TABLE 5: Some Physicochemical Characteristic Of The Cells Seven Weeks After Remediation

[illegible]	MOISTURE	1:1	mg/kg	ORGANIC C	PERCENT TOTAL N	C/N RATIO	AVAIL P mg/kg
O	6± 0.5	5.07± 0.05	11,669± 205	5.07± 0.01	0.021± 0.001	241± 5	3.85± 0.15
A	11± 0.5	4.83± 0.02	1,110± 111	2.86± 0.5	0.070± 0.005	41± 10	5.10± 0.25
B	12± 1	5.02± 0.05	1,504± 250	3.02± 0.03	0.069± 0.010	44± 15	4.31± 0.2
C	12± 1.5	4.94± 0.1	1,053± 102	2.99± 0.3	0.078± 0.020	38± 10	4.83± 0.10
D[1]	13± 0.5	4.97± 0.06	1,078± 150	3.00± 0.01	0.076± 0.030	39± 5	3.50± 0.3
D	12± 0.5	4.88± 0.1	2,098± 30	3.16± 0.05	0.78± 0.10	41± 15	4.20± 0.20

Results represent means and standard deviations of three replicates

TABLE 6: Some Physicochemical Characteristic Of The Cells Nine Weeks After Remediation

REMEDIATION TREATMENT	% MOISTURE	pH 1:1	THC mg/kg	ORGANIC C	PERCENT TOTAL N	C/N RATIO	AVAIL P mg/kg
O	6± 0.3	5.04± 0.05	11,394± 105	5.10± 0.5	0.020± 0.001	255± 5	3.94± 0.10
A	10± 1	4.99± 0.10	955± 30	1.98± 0.2	0.081± 0.005	24± 2.0	5.37± 0.15
B	11± 1	5.25± 0.07	1,215± 60	2.94± 0.03	0.143± 0.001	21± 3.0	4.60± 0.25
C	12± 1.5	5.02± 0.5	908± 25	1.556± 0.2	0.109± 0.002	14± 2.5	4.92± 0.3
D[1]	12± 1	5.05± 0.05	896± 50	2.861± 0.3	0.156± 0.003	18± 1.5	3.96± 0.20
D	11± 1	4.92± 0.01	2,068± 12	3.039± 0.02	0.174± 0.003	18± 20	4.52± 0.23

Results represent means of three replicates

Table 7 The relationship Between time and some measured Characteristics during Remediation as Expressed By Correlation coefficient(γ)values and regressions

CORRELATION REGRESSION FACTION	γ	SIGNIFICANCE LEVEL	EQUATION
Time Vs Moisture	+ 0.011	ns	Y = 11.08 + 0.01X
Time Vs pH	+ 0.291	ns	Y = 4.60 + 0.0X
Time Vs THC	+ 0.257	ns	Y = 0.58 - 0.04X
Time Vs THC reduction	-.0. 414	*	Y = 31.28 - 4.70X
Time THC Organic C	- 0.576	**	Y = 4.73 - 021X
Time Vs Total N	+ 0. 694	**	Y = 0.004 + 0.11X
Time Vs C/N ratio	- 0.404	*	Y = 93.58 - 11.41X
Time Vs P	+ 0.626	**	Y =3.20 + 0.153x

'ns ⇒ not significant, * ⇒ significant at 5%, ** ⇒ significant at 1%

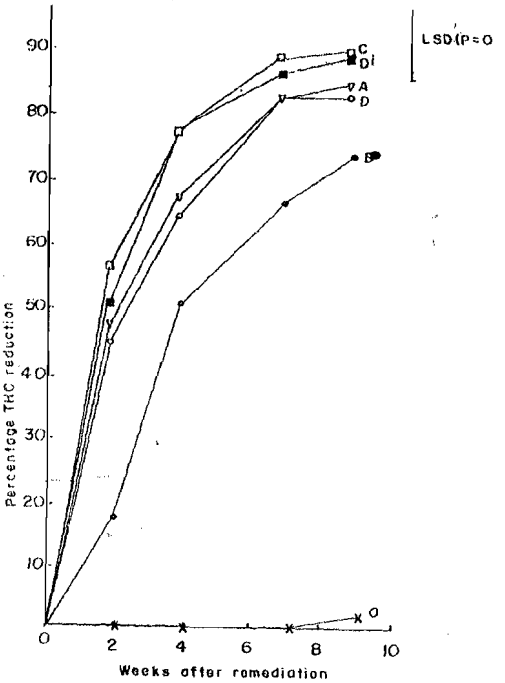

Figure2 : Rate of hydrocarbon loss.

TABLE 8: Total Heterotrophic Bacterial Count

CELL CODE	SAMPLING PERIOD (weeks) 0	2	4	7	9
			($\times 10^5$Cfu/g)		
O	70	90	360	2,700	3,200
A	420	3,700	61,000	290.000	350,000
B	310	5,500	92,000	300,000	330,000
C	700	2,400	41,000	240,000	390,000
D^1	690	7,200	31,000	290,000	310.000
D	990	4,400	43,000	270,000	330.000

82%,84%,88% and 89% for option O, B, D, A, D1 and C respectively (Figure 2)

In Table 8, 9 and 10, the results of THB, HUB and the percentage HUB population are presented. There was an increase in the THB (Table 8), HUB (Table9) and %HUB (Table10) in all the options. This increase is however least in control and in week 0 for all other options. The low values obtained in the control throughout the study period and in week 0 for other options, may have resulted from toxicity of the crude oil to soil microbes brought about by the high concentration of the crude oil before remediation treatment. Similar observations have been made by Atlas, (1981), Wang,

(1984), Bauda and Block (1985) and Pettibone and Cooney (1988). These microbiological results corroborates the results in Figure 2. The introduction of non-indigenous genetically engineered bacteria (GEM), did not make any appreciable difference on the HUB count in the soil. This observation was corroborated by the results of hydrocarbon loss recorded in option D and option C. The GEM is a strain of *Pseudomonas*. This organism probably required longer period to adapt to the foreign soil. It is also possible that the new environment was not suitable for the growth of the organism, since 9weeks period of remediation was not long enough for the organism to adapt and exhibit it's full potentials.

CONCLUSION

Looking at the performance of the individual treatment options treatment A (hot water washing followed by additions of fertilizer, indigenous HUB and tilling) recorded one of the highest THC losses. This development indicated that hot water washing of the polluted soil to remove water soluble fractions facilitated

Table 9: Hydrocarbon Utilizing Bacterial Count

CELL CODE	SAMPLING PERIOD (weeks) 0	2	4	7	9
			($\times 10^3$Cfu/g) %		
O	6.5	6.9	4.8	42	50
A	4.2	6.1	520	3,000	6,100
B	3.1	89	360	4,000	58,000
C	3.3	84	200	2,500	55,000
D^1	3.1	47	380	3,700	58,000
D	3.3	70	150	3,300	63.000

Table 10: Percentage Hydrocarbon Utilizing Bacterial Population

CELL CODE	SAMPLING PERIOD (weeks) 0	2	4	7	9
			(%)		
O	0.09	0.08	0.13	0.2	0.02
A	0.01	0.02	0.01	0.01	0.22
B	0.01	0.02	0.004	0.01	0.20
C	0.005	0.04	0.01	0.01	0.14
D^1	0.004	0.01	0.01	0.01	0.20
D	0.005	0.02	0.004	1.01	0.19

hydrocarbon degradation and loss the soil. But previous reports by Foster *et al(1990)* indicated that hot water washing affect soil structure and texture and also destroy some of the soil biota.

The mixing of uncontaminated soil with contaminated soil and the subsequent addition of fertilizer, indigenous hydrocarbon utilizing bacteria and tilling (treatment B) gave a relatively lower rate of hydrocarbon loss as compared to other treatment options apart from the control. This may be due to the slow rate of acclimatization to the new soil by the indigenous soil bacterial population. In this study, the uncontaminated soil was obtained from a totally different location from the contaminated soil. The result revealed that mixing uncontaminated soil with contaminated soil delayed hydrocarbon degradation rate.

Addition of indigenous bacteria followed by fertilizer application and tilling (treatment C) recorded the highest rate of hydrocarbon loss . The introduction of non-indigenous genetically engineered bacteria (treatment D) did not record the anticipated increase in hydrocarbon loss. The genetically engineered strain of bacteria probably require a longer length of time to adapt to the new environment it was introduced to or it was not suitable for this environment. It was therefore concluded that the introduction of this strain of genetically engineered bacteria into hydrocarbon contaminated soils in the Niger Delta areas may not present any advantage over the use of those indigenous to the spill site.

Application of fertilizer alone plus tilling (treatment D) produced a similar level of hydrocarbon degradation and loss as the use of indigenous HUB plus fertilizer application and tilling (treatment C). This development revealed that nutrient enhanced bioremediation (application of fertilizer alone) could achieve the desired level of hydrocarbon loss, as the introduction of indigenous HUB populations plus fertilizer. This is because HUB are present in almost all types of soils in this area and would multiply in their numbers where the right types and quantities of metabolic feed stock are provided. There is therefore no need for culturing indigenous HUB populations and introducing same to the contaminated soil. All that is needed is the application of the right types and quantities of fertilizers and also providing optimum moisture content, pH, aeration level and other environmental conditions for microbial growth.

REFERENCES

American Public Health Association, 1985. Standard Methods for water and Wastewater Analyses, 13ed Washington D.C

Atlas, R.M., 1981. Microbial degradation of petroleum hydrocarbon Hydrocarbons An environmental perspective. Microbiol. Rev. 45: 180-541

Atlas, R. M. A. Horowitz M. Krichevsky and Bej A. K., 1991. Response of microbial population to environmental disturbance. Ecol. 22: 249-256

Atlas, R. M. and Bartha R., 1973. Stimulated bioremediation of oil slick using oleophilie fertilizers. Environs Sc. Technol. 1: 538-541.

Bauda, P. and Block, J. C., 1985. Cadmium biosorption and toxicity to laboratory grown bacteria. Environs. Technol. Let 6: 445-454

Black, C. A., Evans, D. D., Whils J. L., Ensminger, L. E. and Clerk, F. E., 1965. Methods of Chemical analysis of ecological materials. American society of Agronomy. Inc Publisher Madson, Winscon USA.

Button, D.K., Robertson, B. R., Macintosh, D. and Juttner, F., 1992. Interactions between marine bacterial and dissolved-phase and beached hydrocarbons after the Exxon valdex oil spill. Appl Environs Microbiol 58: 43 - 251.

Dalyan, U., Harder, H. and Hopner, T. H., 1990. Hydrocarbon biodegradation in sediments and soils. A systematic examination of physical and chemical conditions- part 11. pH values. Wissenschaft and Technik Science and Technology 43:337-342

Finney, D. J., 1978. Statistical Methods in Biological Assay 3rd Edition, Charles Griffin London.

Lee, K. and Levy, E. M., 1981. Bioremediation; Waxy Crude oils standard on low-energy shorelines. Proceedings of the 1991 oil spill Conference. America Petroleum Institute, Washington DC. pp. 541-547.

Lee, K. and Levy, E. M., 1987. Enhanced bioremediation of a light crude oil sandy beaches. Proceedings of the 1987 oil spill Conference, American Petroleum Institute, Washington DC pp. 411-416.

Lee, K. and Levy, E. M., 1989. Bioremediation of Petroleum in the Marine environment and it's enhancement. In: Aquatic Toxicology Manager. J. A. Nriagu ed. John Wiley and Sons, New York. Pp.217-24.

Lee, K., Tremblay, G. H. and Levy, E. M., 1993. Bioremediation application of slow release fertilizers on low energy shorelines. Proceedings of the 1991 oil spill Conference. America Petroleum Institute, Washington DC. pp. 449 –453.

Mills, A., Brenil L. C. and Colwell R. R, 1978. Enumeration of Petroleum Degrading Marine and Estuarine micro organisms by the probable number method. Can. J., Microbiol 24: 552 – 557.

Nadeau, R. R., Singhri, J., Ryabik, Y., Lin, I. and Syslo, J., 1993. Monitoring Bioremediation for bioremediation efficacy; the marrow marsh experience. Proceedings of the 1991 oil spill Conference. America Petroleum Institute, Washington DC. pp. 477 - 485.

Okpokwasili, G. C., Sommerville, C. C., Sullivan, M., Grimes, D. J. and Colwell R. R., 1986. Plasmid – mediated degradation of hydrocarbons in estuarine bacteria. Oil Chem. Pollut. 3: 177-129.

Pettibone, G. W. and Cooney, J. J., 1988. Toxicity of methyltins to microbial populations in estuarine sediments. J. Indust. Microbiol. 2 373-378

Rowell, M.J., 1977. The effect of crude oil Spills on Soil. A review of Literatures. In The Reclamation of Agricultural Soil after oil spills Part 1: Research J.A Toogood (ed.) API Pub 1. 27p

Tisdale, S. and Nelson. W., 1975. *Soil Fertility and Fertilizer*. Third edition Macmillan Pub. Co. Inc. New York 964p

Wang, W., 1984. Time response of *Nitrobacter* to toxicity. Environ Inter. 10:21 26