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NITROGEN FIXING BACTERIA ENHANCED BIOREMEDIATION OF A CRUDE OIL POLLUTED SOIL

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ABSTRACT

The use of nitrogen fixing bacteria to enhance bioremediation of a crude oil polluted soil was investigated in a 56-day study period. Soil pH ranged between 5.3 - 6.8. Soil moisture content ranged between 10-30%. Counts of heterotrophic bacteria ranged between 4.5 x 10^6 - 6.0 x IO^7Cfu/g. Counts of hydrocarbon-utilizing bacteria ranged between 2.5 x 10^6 - 2.0 x IO^7 Cfu/g. Levels of total organic carbon, nitrogen, phosphorus and hydrocarbons decreased with time during the study period except in the kill control cell. The highest percentage loss of crude oil (84%) was recorded in cells, which contained seeds *of Phaseolus vulgaris* (White beans) and 5g slurry of *Bacillus polymyxa.* Cells in which 5g slurry of *Anacystis (Chroococcus)* sp., 5g slurries each of *Azotobacter* sp., *Bacillus polymyxa* and *Anacystis (Chroococcus)* sp. were applied, recorded 56% and 80% losses of crude oil respectively. Cells in which 5g slurry of *Azotobacter* sp., *5g* slurries each of *Azotobacter* sp. and *Bacillus polymyxa* were applied, recorded 80% loss of crude oil. The fertilizer (NPK 15:15:15) treated cell showed a 64% loss of crude. The cell containing fertilizer (NPK 15:15:15) with seeds of *Phaseolus vulgaris,* and the cell containing fertilizer NPK 15:15:15 with 5g slurry of *Anacystis (Chroococcus)* sp showed 64% and 72% losses of crude oil respectively. The cell in which all treatment options were applied showed 56% loss of crude oil. The cell in which tilling alone was employed showed 72% loss of crude oil. The kill control cell recorded the least percentage loss of crude oil (24%) at the end of the study period. Results indicate that at 0.05 (95%) level of significance, there is a significant difference between nutrient level arising from treatment option and hydrocarbon level in soil samples. Thus the % losses of crude oil recorded in treatment cells were due to treatment options applied (at 95% level of significance) The results indicated a higher bioremediation rate when nitrogen-fixing bacteria were used instead of when inorganic nutrient (fertilizer) were applied.

Key words: - Nitrogen fixing bacteria, Bioremediation, Crude oil, Polluted soil, Fertilizer.

INTRODUCTION

In the Niger Delta terrestrial and aquatic systems are generally the main recipients of crude oil spillages, sometimes resulting in large-scale contamination of these environments. Terrestrial and aquatic (surface water and groundwater) contamination in this area by crude oil is gaining more prominence as a result of increased upstream and downstream activities of the petroleum industry. Because of the sensitive nature of the ecology of this area, the issue is gaining more publicity (Ifeadi and Nwankwo, 1989, Okpokwasili and Odokuma, 1990).

The soil is a complex portion of the biosphere it consists of 21% of the global surface (Alexander, 1997). In Nigeria land agriculture practices and products account for about 10% of her foreign exchange (Odeyemi and Ogunseitan, 1985).

Although attempts have been made at careful handling and containment of the large amounts of petroleum and its products produced on land every year in this area, there is still the possibility that some may enter the soil environment. The effects of any oil spill situation in the environment will depend on the quantity, type and mobility of spilled oil and other environmental factors. The variability of these and other factors and their interactions can lead to a wide range of ecological, economic and physical effects, ranging from the barely tolerable to the utterly disastrous.

Lolomary (1979) summed up the effect of oil spillage on soil as one that decreases its porosity. Improvement of soil biochemical processes, eg., organic matter decomposition, ammonification, nitrification, symbiotic and non-symbiotic nitrogen fixation and geochemical cycling of elements tend to occur. Odu and Isinguzo (1979) and Odeyemi and Ogunseitan, (1985) have reported that slight contamination in the order of I % of crude oil of soil improves crop growth. This improvement is attributed to nitrogen fixation in soil and also enrichment of soil nutrient from oil killed

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microorganisms and the soil itself.

Oil spills in the last two decades have given rise to increased scientific knowledge of the behaviour of hydrocarbons and have led to the development of new intervention methods (Ladousse and Tramier, 1991). Of the many remediation methods currently in use, Bioremediation is viewed as one of the most promising technologies. Bioremediation involves the use of biological processes to return a polluted environment to its original state. The actual mechanism involved is biodegradation. This is mediated by about 200 microbial species representing approximately 30 genera of bacteria, yeast, and even algae.

Oil biodegradation is a slow but natural process limited mainly by scarcity of nitrogen and phosphorous in the environment (Ladousse amid Tramier, 1991). Many authors have shown that oil biodegradation can be accelerated by the addition of nitrogen and phosphorus-containing fertilizer, both in aqueous environments and sediments (Dibble and Bartha, 1979, Stevens, 1991, Ladousse and Tramier, 1991, Holf, 1992). Formulation application envisaged and reported by some investigators (Raymond *et al.*, 1976) were overcome during this study.

This study was therefore carried out to exploit the nitrogenase enzyme system of nitrogen fixing bacteria in enhancing bioremediation of crude oil polluted soil. This was in addition to comparing nitrogen fixing bacteria enhancement of bioremediation of crude oil polluted soils with enhancement of bioremediation by inorganic fertilizer formulations.

LOCATION AND CHARACTERISTICS OF EXPERIMENTAL SITE

The experimental site was located south west of the multipurpose (Ofirima hall, Unipark, University of Port Harcourt. The area was characterized by the typical grassland vegetation, reasonably flat with slopes not exceeding 2°. The land type was dry land with natural drainage. The subsoil consisted of moderately, firm, light brown, silty clay becoming dense with depth and covering brown medium sand. The climate was tropical with relative humidity ranging between 80-100% throughout the study period (July-September). The study period had high rainfall exceeding 25mm, which however did not affect work because the soil was dry with natural drainage.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

The method employed was adopted from Raymond *et al.* (1976). The site was 4m x 3m in area, thus was divided into 12 test cells of 1m x 1m, each using wood plank 1ft x 30cm. The wood planks were cut to specification and buried 25cm deep into the soil to expose about 5cm above soil level. Period of testing was 56 days, and 12 separate treatment options were employed to assess the nitrogen fixing bacteria enhancement of bioremediation of crude oil polluted soils. In each treatment, parameters, such as pH, % fresh moisture content, total hydrocarbon-utilizing bacterial count, total heterotrophic bacterial count, total organic carbon, nitrogen, phosphorus, hydrocarbon level were determined. Application of treatment options was preceded by pre-treatment analysis of test plot soil using parameters outlined below.

STATISTICAL ANALYSIS

Results were subjected to statistical analysis employing the student t-test at 95% probability level and the correlation coefficient test. (Finney, 1978).

ISOLATION, PRE-ADAPTATION AND SCALE UP OF TEST MICROORGANISMS

Three different organisms, *Azotobacter* sp., *Bacillus polymyxa* and a *Chroococcus* (cyanobacterium) were used in the experiment. These were obtained by plating aliquot (0.1ml) of 10^2 dilution of 1g of soil obtained from the experimental plot on synthetic media of Abd-el Malek and Ishac medium, (1968), Nutrient agar, Difco agar medium, respectively. The organisms were purified amid stored on agar slants as stock cultures. Pure and characterized isolates (*Bergey's Manual of Determinative Bacteriology,* 1993) of test microorganisms were streaked on solid mineral salt medium (Mills *et al.*, 1978) using the vapour phase transfer method (Amanchukwu *et al.*, 1989). Plates were enumerated after incubation at room temperature (28 ± 2°C) for 7 days. A wire loopful each of the pure cultures of test microorganisms was inoculated into sterile 10ml of their corresponding selective media.

These were incubated appropriately until growth was dense enough. These were subsequently transferred to successive larger volumes of broth media such that the volume inoculated constituted at least 3% of that into which it was inoculated. There was an accompanying incubation with aeration at each stage until 5g/4 liters slurries of each organism were obtained for each treatment application. Slurries were obtained by centrifugation at 5000 revolutions for 30 mins.

TREATMENT APPLICATION AND SAMPLE COLLECTION

Five hundred milliliters (500ml) of crude oil (1.25ml/hectare) was applied in each test cell only on day 0 of experiment with accompanying application of treatment options once a week for 1 month as outlined below.

The traditional planting of seeds of *Phaseolus vulgaris* was done once (Day 0). Mercury sulphate ($HgSO_4$) (toxicant) was applied after a preliminary determination of the concentration for the toxicity. Test cells except those containing *Phaseolus vulgaris* were tilled and harrowed thrice per week. Sample collection, which was done bi-weekly involved duplicate topsoil samples (0- 15cm). These were collected into appropriately labeled sterile plastic bags and were processed within six hours of collection in the laboratory.

SOURCE OF MATERIALS

All chemical reagents employed were of analytical grade and were purchased from May & Baker, England.

The crude oil type "Bonny light " was obtained from Shell Petroleum Development Company, Port Harcourt, Nigeria.

The fertilizer type (NPK15:15:15) was obtained from National Fertilizer Company of Nigeria (NAFCON) Port Harcourt, Nigeria. Wood planks "Obeche" were purchased from the Timber Market Marine Base, Port Harcourt, Nigeria.

MICROBIOLOGICAL ANALYSIS

The total heterotrophic bacterial count of soil sample was, performed in duplicates on Nutrient agar plates using the spread plate method by Pelczer *et al.* (1983). Plates were enumerated after 48 hours of incubation.

The total hydrocarbon-utilizing bacterial count of soil samples was performed in duplicate on

TABLE 1: APPLICATION OF TREATMENT OPTIONS.

TEST CELL IDENTITY	DESCRIPTION OF TREATMENT OPTION
TCA	5g slurry of *Chroococcus* (cyanobacterium)
TCB	40g of NPK 15:15:15: + 5g slurry of *Chroococcus* (cyanobacterium).
TCC	Traditional planting of seeds of *Phaseolus vulgaris*
TCD	40g of NPK 15:15:15.
TCE	40g of NPK 15:15:15 + Traditional planting of seeds of *Phaseolus vulgaris*
TCF	5g slurry each of *Azotobacter* sp, *Bacillus polymyxa* and *Chroococcus* (cyanobacterium).
TCG	5g slurry of *Bacillus polymyxa*
TCH	5g slurry of *Azotobacter* sp
TCI	5g slurry of *Azotobacter* sp and *Bacillus polymyxa*
TCJ	40g of NPK 15:15:15: + 5g slurry each *Azotobacter* sp, *Bacillus polymyxa* cyanobacterium, + Traditional planting of seeds of *phaseolus vulgaris*
TCK	500ml of crude oil type.
TCL (control)	100g of $HgSO_4$ (Toxicant) + 500ml of crude oil type

Keys: TCA = Test cell A.
TCB = Test cell B.
TCC = Test cell C.
TCD = Test cell D.
TCE = Test cell E.
TCF = Test cell F.
TCG = Test cell G.
TCH = Test cell H.
TCI = Test cell I.
TCJ = Test cell J.
TCK = Test cell K.
TCL = Test cell L. (control).

The test plot outlay is as represented in the figure below:

TCJ	TCI	TCD	TCC
Oil + NPK 15:15:15 + *Bacillus polymyxa* + *Azotobacter* sp + *Chroococcus* + Seeds of *Phaseolus vulgaris*	Oil + *Azotobacter* sp + *Bacillus polymyxa*	Oil + NPK 15:15:15	Oil + Seeds of *Phaseolus vulgaris.*
TCK	TCH	TCE	TCB
Oil	Oil + *Azotobacter* sp	Oil + NPK 15:15:15 + Seeds of *Phaseolus vulgaris*	Oil + NPK 15:15:15 + *Chroococcus*
TCL	TCG	TCF	TCA
Oil + $HgSO_4$	Oil + *Bacillus polymyxa*	Oil + *Azotobacter* sp + *Bacillus polymyxa* + *Cyanobacterium*	Oil + *Chroococcus*

Fig 1: Test plot outlay
Key: TC= Test cell identity.

modified mineral salt agar of Mills *et al* (1978) using the spread plate technique. The vapour phase transfer method (Amanchukwu *et al.*, 1984) was used in estimating the population of hydrocarbon utilizing microbes. Plates were enumerated after incubation at room temperature for 5 days.

BIOCHEMICAL CHARACTERISATION AND IDENTIFICATION OF HYDROCARBON - UTILIZING ISOLATES.

Pure stock cultures of' crude oil utilizing bacteria isolates were examined for their colonial appearance and then used to carry out the following test methods: Gram staining, catalase test, oxidase test, citrate utilization test, motility test, urease test, methyl red test, Voges-Proskauer test and Kligler iron agar test. These were based on the criteria of Allen and Lechevalier (1973), *Bergey's Manual of Determinative Bacteriology* (1993), key for identification of freshwater algae common in water supplies and polluted waters are APHA, AWWA and APCF (1985) and the methods of Cruickshank *et al.* (1980), Collins *et al.* (1984)

Table 2a: Characteristics of *Bacillus polymyxa*

TEST	OBSERVATIONS
1.Description of colony	Cream colour, round with scalloped margin.
2.Gram reaction	Gram positive or Gram negative
3.spore test	Positive; spores centrally located; star-shaped spores; sores swell the sporangium.
4. Motility	Positive.
5. Catalase	Positive
6. Voges-Proskauer	Positive
7. Gas production (fermentation of carbohydrates)	Positive
8. Acid production (Glucose, Mannitol, Mannose, Xylose)	Positive
9. Reduction of NO_3 to NO_2	Positive

TABLE 2b: Characteristics of *Azotobacter* sp

TEST	OBSERVATIONS
1.Description of colony	Grey colour, round.
2.Gram reaction	Gram negative cocci
3. Motility	Positive.
4. Catalase	Positive
5.spore test	Negative
6. Fermentation	
a. Rhamnose	Negative
b. Caproate	Positive
c. Caprylate	Negative
d. Meso-inositol	Negative
e. Mannitol	Positive
f. Malonate	Positive

TABLE 2c: Characteristics of *cyanobacterium- Anacystis (Chroococcus)* sp.

TEST	OBSERVATIONS
1.Description of colony	Translucent, round.
2. Microscopic observation	
a. Appearance of cells	Spherical
b. Distribution of cells	Spherical
c. Colour pf cells	Blue green
d. Plastids	Absent
e. Heterocysts	Absent
3.Gram reaction	Negative cocci
4. Motility	Negative

and Carpenter (1977).

DETERMINATION OF PHYSICOCHEMICAL PARAMETERS

The following parameters were determined: pH, %moisture content, total organic carbon, nitrogen and hydrocarbon level.
Apart from total organic phosphorus determined using APHA AWWA and APCF (1985) and hydrocarbon level done using the photometric method adapted from the Shell manual.

RESULTS

In Table 2a-2c are recorded observations made during the isolation of the three test microorganisms employed in this study, viz: *Bacillus__pollymxa*, *Azotobacter* sp and a cyanobacterium. The cyanobacterium isolated was identified as *Anacystis (Chroococus)* sp.
Mineral salt agar plates for each test microorganisms, showed very minute forms lot of growth of organisms along streak lines. Even with prolonged period of incubation (14 days), organisms did not show any difference in growth from initial observations; an indication that organisms merely tolerated hydrocarbons.
The results of toxicity determination of $HgSO_4$ to soil microorganisms are presented in Table 3. One Gram of HgSO4 was completely toxic to soil microorganisms. 0. lg showed slight toxicity. Other concentrations; 0.01g, 0.001g, and 0.001g respectively showed decreasing levels of toxicity.
In Table 4 the results of soil pH during this study period is presented. Generally pH values showed acidic range with relatively marked acidity in TCL (kill control). In TCB, TCD, TCE, and TCJ where NPK 15:15:15 was applied either singly or in combination with other treatment options, the alkaline range however reverted to the acidic range subsequently
(Day 42-56).
The results of determination of percentage fresh moisture content of soil samples are represented in Table 5. Percentage fresh moisture content of soil samples did not vary considerably in test cells during study period in relation to pre-treatment values. There was however marked increase in moisture content in TCB, TCD, TCE, and TCJ.
In figures 2 and 3 changes in the plate counts of heterotrophic and hydrocarbon-utilizing bacteria are shown respectively, during the study period. There was a noticeable, corresponding increase in both heterotrophic and hydrocarbon-utilizing

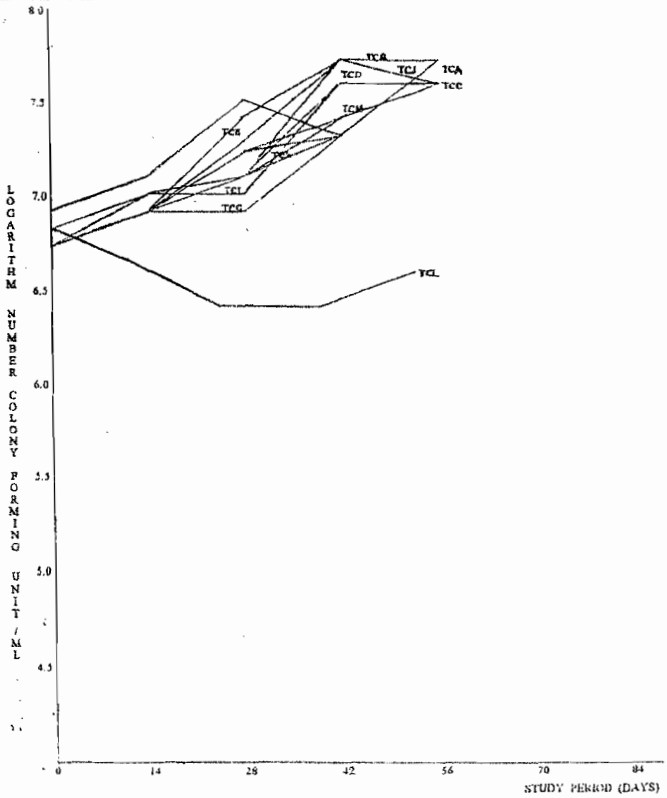

Fig. 2 : Changes in the plate counts of heterotrophic bacterial population during study period

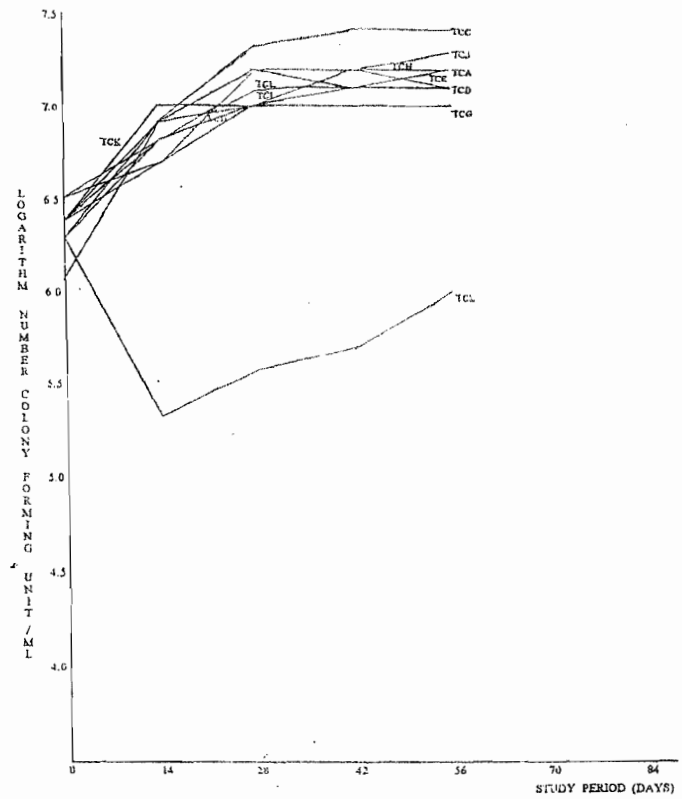

Fig. 3 : Changes in the plate counts of hydrocarbon-utilizing bacterial population during study period.

TABLE 3 Toxicity of Mercuric Sulphate ($HgSO_4$) to Soil Microorganisms

CONCENTRATION (GRAMME) OF TOXICANT ($HgSO_4$)	OBSERVATION MADE ON NUTRIENT AGAR PLATES
1.0	-
0.1	+
0.01	++
0.001	+++
0.0001	++++

– = No growth

+ = Slight growth

++ = Fairly profuse growth

+++ = very profuse growth

++++ = Confluent growth

TABLE 4: pH of soil samples

DAYS	TREATMENT CELL IDENTITY											
	TCA	TCB	TCC	TCD	TCE	TCF	TCG	TCH	TCI	TCJ	TCK	TCL
PRE-	5.9	6.6	6.4	6.5	6.4	6.2	6.5	5.9	6.8	6.7	6.2	6.6
TREATMENT	±0.005	±0.00	±0.02	±0.00	±0.00	±0.02	±0.00	±0.02	±0.00	±0.00	±0.02	±0.08
0	6.5	7.6	6.6	7.6	7.5	6.0	6.0	6.2	6.3	7.4	5.8	5.9
	±0.00	±0.08	±0.00	±0.00	±0.02	±0.02	±0.00	±0.02	±0.02	±0.00	±0.08	±0.00
14	6.2	7.4	6.6	7.4	7.6	6.5	6.5	6.7	6.7	7.1	6.4	5.3
	±0.02	±0.02	±0.00	±0.08	±0.08	±0.00	±0.02	±0.02	±0.08	±0.02	±0.08	±0.00
28	6.5	7.6	6.0	7.4	7.5	6.4	6.2	6.0	6.8	7.4	6.7	5.3
	±0.00	±0.00	±0.02	±0.02	±0.00	±0.08	±0.08	±0.00	±0.08	±0.02	±0.02	±0.00
42	6.2	6.4	6.8	6.8	6.6	6.5	6.4	6.7	6.8	6.2	6.4	5.9
	±0.02	±0.00	±0.08	±0.00	±0.00	±0.02	±0.08	±0.00	±0.00	±0.08	±0.00	±0.02
56	6.0	6.2	6.0	6.5	6.2	6.8	6.6	6.8	6.0	6.4	6.8	5.7
	±0.00	±0.00	±0.02	±0.00	±0.02	±0.02	±0.00	±0.02	±0.02	±0.02	±0.08	±0.02

Results represent means ± standard deviation of duplicate reading.

bacterial counts within the study period. This increase was more in the heterotrophic population than the hydrocarbon utilizing population.

In Table 6-8 are presented results of the physiochemical parameters, carbon, phosphorus and Nitrogen. Organic carbon level increased by an average of 29.96% on Day 0. This trend was reverted between Day 28-56 as an average increase of 2.04% was observed in Test cells. The phosphorus level appreciated between Day 0-28. This was accompanied by a period of gradual decrease in phosphorus level (Day 42-56). Nitrogen level appreciated between Day 0-42 with an accompanying period of slight decrease in level (Day 56). % Nitrogen contribution was predominant in TCC (kill control), their was no noticeable variation in values of carbon, phosphorus and Nitrogen along the course of experiment as in other Test cells, from the values obtained on Day 0.

The carbon-Nitrogen (C/N) ratio (Table 9) was slightly narrowed down on Day 0 in relation to pre-treatment values. This tendency remained in all test cells with study period.

In TCL however, the ratio slightly appreciated and remained so throughout the study period.

In Table 10, there was an accompanying decrease in hydrocarbon level with time of experiment varying according to treatment option applied. There was more decrease in TCC and TCE. In TCL, there was only a slight decrease in hydrocarbon level.

In figure 4 % losses of crude oil varied in test cells during the study period. The tabular representation using Total /Net % losses is presented in Table 11.TCC, TCF and TCE recorded the highest % losses of crude oil (84%) and TCL (kill control) recorded the least % loss of 24%.

DISCUSSION

Bioremediation as an effective and economic tool of post oil spill clean up is yet to be fully exploited in Nigeria. Even when it was not so, there still lingers the limitation problem in ecosystems of nitrogen and phosphorus, essential nutrients that

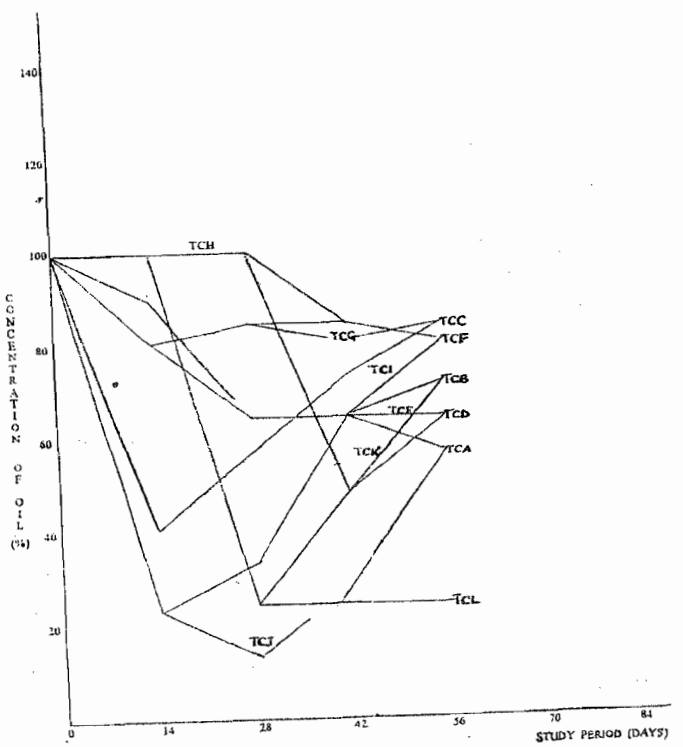

Fig. 4 : Percentage loss in crude oil with study period.

TABLE 5: % Fresh moisture content of soil samples

DAYS	TREATMENT CELL IDENTITY											
	TCA	TCB	TCC	TCD	TCE	TCF	TCG	TCH	TCI	TCJ	TCK	TCL
PRE-	8.0	10.0	12.0	10.0	8.0	10.0	8.5	12.0	10.0	8.5	10.0	10.0
TREATMENT	±2.0	±2.0	±0.0	±2.0	±0.0	±2.0	±0.0	±2.0	±2.0	±0.05	±0.0	±0.0
0	10.0	15.0	14.0	15.0	15.0	14.5	13.0	13.0	13.5	16.0	12.0	12.0
	±2.0	±8.0	±0.02	±0.005	±2.0	±0.0	±2.0	±2.0	±2.0	±8.0	±0.008	±0.05
14	12.0	20.0	12.0	24.0	22.0	16.0	15.0	15.0	16.0	20.0	18.0	14.0
	±8.0	±2.0	±1.0	±0.02	±8.0	±8.0	±8.0	±8.0	±8.0	±8.0	±5.0	±0.02
28	11.0	28.0	20.0	30.0	27.0	15.0	10.0	18.0	20.0	24.0	22.0	10.0
	±8.0	±2.0	±2.0	±2.0	±2.0	±2.0	±8.0	±2.0	±2.0	±2.0	±8.0	±0.08
42	12.0	30.0	18.0	35.0	30.0	12.0	18.0	20.0	26.0	28.0	26.0	12.0
	±8.0	±2.0	±2.0	±2.0	±2.0	±8.0	±2.0	±2.0	±8.0	±8.0	±2.0	±2.0
56	10.0	24.0	18.0	28.0	27.0	14.0	12.0	16.0	18.0	24.0	20.0	14.0
	±2.0	±2.0	±8.0	±8.0	±8.0	±2.0	±2.0	±2.0	±2.0	±2.0	±2.0	±2.0

Results represent means ± standard deviation of duplicate soil samples.

TABLE6: Organic Carbon Level (ppm) of soil samples

DAYS	TREATMENT CELL IDENTITY											
	TCA	TCB	TCC	TCD	TCE	TCF	TCG	TCH	TCI	TCJ	TCK	TCL
PRE-	19680	19680	19680	19680	19680	19680	19680	19680	19680	19680	19680	19680
TREATMENT	±0.00	±0.00	±0.00	±0.00	±0.02	±0.08	±0.00	±0.00	±0.00	±0.02	±0.00	±0.02
0	28100	28100	28100	28100	28100	28100	28100	28100	28100	28100	28100	28100
	±0.00	±0.02	±0.02	±0.02	±0.08	±0.05	±0.02	±0.00	±0.08	±0.05	±0.02	±0.02
14	27600	26880	24480	25200	25440	24480	28100	25200	26880	26880	26880	28100
	±0.08	±0.005	±0.08	±0.02	±0.00	±0.05	±0.08	±0.02	±0.02	±0.02	±0.02	±0.00
28	26880	22440	22200	22080	22560	22800	22200	22480	22560	25200	25200	28100
	±0.00	±0.08	±0.005	±0.02	±0.05	±0.02	±0.02	±0.08	±0.08	±0.05	±0.08	±0.00
42	25440	24120	21000	21600	22320	21840	22080	22080	22200	25200	24480	28100
	±0.005	±0.02	±0.05	±0.02	±0.08	±0.02	±0.00	±0.00	±0.02	±0.02	±0.08	±0.02
56	24720	21600	20160	20400	20640	20880	20400	20640	20520	24240	22080	27600
	±0.02	±0.08	±0.02	±0.02	±0.08	±0.08	±0.05	±0.02	±0.02	±0.08	±0.08	±0.02

Results represent means ± standard deviation of duplicate soil samples.

enhance the success of bioremediation techniques.

Although the counts of hydrocarbon utilizers were low comparable to the total heterotrophic bacterial count, their presence at all, in pre treatment soil samples (data not shown) confirms the assertion by Okpokwasili and Odokuma (1990) of their enduring presence in Niger Delta ecosystems, following increased oil exploration and production activities. Increases in counts of hydrocarbon utilizing bacteria were accompanied by experimental increases in counts of heterotrophic bacteria (fig .2 and 3). Amanchukwu *et al* (1989) have reported corresponding increases in counts of heterotrophic and hydrocarbon utilizing bacteria during hydrocarbon degradation. This may have been due to factors of acclimatization of hitherto intolerant species and, or, presence of bacteria to treatment options in TCA, TCB, TCC, TCF, TCJ, TCI and TCK throughout the study period shows the bioremediation potentials of the treatment options. Counts of both heterotrophic bacteria and hydrocarbon utilizing bacteria were drastically low in TCL. The counts recorded at all may have been due to the leaching effect of rainfall experienced during study period. There products of mineralization from degradative activities of the hydrocarbon utilizing population. The observation of increased counts of hydrocarbon utilizing bacteria with progress of experiment (fig.4) indicates enrichment for hydrocarbon utilizing population by the crude oil applied and other treatment options. Put together the increase in counts of hydrocarbon utilizing

TABLE 7: Organic phosphorus level (ppm)

DAYS	TREATMENT CELL IDENTITY											
	TCA	TCB	TCC	TCD	TCE	TCF	TCG	TCH	TCI	TCJ	TCK	TCL
PRE-	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
TREATMENT	± 0.02	± 0.05	± 0.00	± 0.00	± 0.00	± 0.00	± 0.02	± 0.02	± 0.02	± 0.02	± 0.00	± 0.00
0	11.6	11.6	11.6	11.6	11.6	11.6	11.6	11.6	11.6	11.6	11.6	11.6
	±0.02	±0.02	±0.08	±0.08	±0.08	±0.05	±0.05	±0.02	±0.08	±0.02	±0.02	±0.02
14	14.4	10.8	16.4	33.4	21.2	32.4	32.4	25.2	28.8	28.8	20.2	9.0
	±0.00	±0.02	±0.00	±0.005	±0.08	±0.005	±0.08	±0.08	±0.00	±0.00	±0.08	±0.02
28	33.4	25.2	25.2	25.2	25.2	8.00	20.2	9.0	25.3	33.4	25.2	9.0
	±0.05	±0.05	±0.08	±0.02	±0.08	±0.00	±0.02	±0.08	±0.02	±0.08	±0.08	±0.02
42	25.2	10.0	25.2	9.0	9.2	8.00	11.6	7.2	8.4	8.0	9.0	9.0
	±0.08	±0.00	±0.00	±0.02	±0.02	±0.08	±0.08	±0.005	± 0.00	± 0.00	±0.08	±0.00
56	17.2	9.0	10.8	4.4	6.0	9.0	10.8	5.2	4.4	4.6	9.2	9.0
	±0.08	±0.08	±0.02	±0.08	±0.02	±0.02	±0.02	±0.08	±0.00	±0.08	±0.02	±0.00

Results represent means ± standard deviation of duplicate soil samples

TABLE 8: Organic Nitrogen level (ppm) of soil samples

DAYS	TREATMENT CELL IDENTITY											
	TCA	TCB	TCC	TCD	TCE	TCF	TCE	TCH	TCI	TCJ	TCK	TCL
PRE-	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
TREATMENT	±0.00	±0.00	±0.00	±0.02	±0.01	±0.05	±0.00	±0.05	±0.05	±0.00	±0.00	±0.02
0	151	154	151	154	154	151	154	154	154	154	120	100
	±0.02	±0.00	±0.02	±0.00	±0.08	±0.005	±0.00	±0.00	±0.02	±0.02	±0.005	±0.00
14	155	158	158	158	158	165	175	165	165	158	154	100
	±0.08	±0.08	±0.08	±0.08	±0.08	±0.02	±0.005	±0.02	±0.02	±0.08	±0.02	±0.00
28	160	158	175	160	165	170	175	170	165	155	154	100
	±0.02	±0.00	±0.02	±0.08	±0.08	±0.00	±0.02	±0.08	±0.005	±0.08	±0.02	±0.01
42	160	155	190	160	160	165	185	170	165	155	150	105
	±0.02	±0.02	±0.08	±0.02	±0.02	±0.00	±0.08	±0.08	±0.05	±0.00	±0.02	±0.00
56	160	155	190	155	160	165	180	165	160	150	150	100
	±0.08	±0.00	±0.02	±0.01	±0.005	±0.00	±0.08	±0.00	±0.08	±0.08	±0.08	±0.005

Results represent means ± standard deviation of duplicate soil samples

was no growth of seeds of *Phaseolus vulgaris* (white beans) planted in TCE and TCJ comparable with TCC where a luxuriant growth was observed. The planting of the seeds at once with fertilizer application may have accounted for the no growth situation. This may well be stated as expected since traditionally fertilizers are applied to farmlands weeks and months before planting. Otherwise they are applied after crops would have shown appreciable growth. Thus the release rate of the NPK 15:15:15 applied may have stepped up beyond a tolerable limit, moisture content of the soil suitable for growth of seeds. Although toxicity particularly of the saturated components of crude oil to agricultural crops has been reported (Kinako, 1990) the seeds still flourished in TCC (fig.1). This may be a factor of the quantity of crude oil applied, which may not have been as much as such that could cause asphyxiation of the seeds and clogging of soil air pores so as to prevent growth.

The slightly acidic (pH 5.9-6.8) nature of the soil recorded at the onset of the experiment remained generally constant throughout the study period with only slight variations in certain treatment cells (Table 6). Only in TCB, TCD. TCE and TCJ where NPK 15:15:15 was applied simply or in combination with other options was a noticeable slight shift towards alkalinity.

The comparatively high value of 72 % total loss of crude oil recorded in TCK (where tilling alone was done) agrees with the assertion by Ekundayo and Omokaro (1987) and Odu and Isinguzo (1987) that natural biodegradation is the major and ultimate mechanism for elimination of oil especially in aquatic environment, which may also apply to soil environment. The highest total % loss of crude oil was recorded in TCC and TCG (84 % respectively). This value recorded in TCC may be accounted for by adequate aeration in soil by roots of the plant. The effect of nitrogen fixing plants will ordinarily be minimal in relation to the non-symbiotic effect in TCE. The result suggests the possibly of ploughing back nitrogen plants into the soil for a meaningful contribution of nutrient enhancement of bioremediation. Although several investigations (Olivieri *et al*, 1976, Westlake *et al*, 1978) have indicated the effectiveness of fertilizers to enhance biodegradation, the comparatively low values of total % losses noticeable in TCD, TCE and TCJ where NPK 15:15:15 alone, or in combination with other treatment options was applied may be due to

TABLE 9: Carbon - Nitrogen (C/N) ratios of soil samples

DAYS	TREATMENT CELL IDENTITY											
	TCA	TCB	TCC	TCD	TCE	TCF	TCE	TCH	TCI	TCJ	TCK	TCL
PRE-TREATMENT	246	246	246	246	246	246	246	246	246	246	246	246
0	186.09	182.47	186.09	182.47	182.47	186.09	182.47	182.47	182.47	182.47	234.17	281
14	178.06	170.13	154.94	159049	161.01	148.36	139.89	170.30	152.72	170.13	174.55	281
28	168	161.01	126.01	138	136.73	134.12	120	132.23	136.72	162.58	163.64	281
42	159	155.61	110.53	135	135.27	132.36	119.35	129.88	134.55	162.58	163.20	267.61
56	154.50	139.35	106.11	131.61	129	126.55	113.33	125.09	128.25	161.60	147.20	276

TABLE 10: Hydrocarbon (oil and grease) level (ppm) of soil samples

DAYS	TREATMENT CELL IDENTITY											
	TCA	TCB	TCC	TCD	TCE	TCF	TCG	TCH	TCI	TCJ	TCK	TCL
PRE-	200	200	200	200	200	200	200	200	200	200	200	200
TREATMENT	±0.00	±0.00	±0.02	±0.005	±0.00	±0.005	±0.005	±0.08	±0.02	±0.02	±0.02	±0.00
0	12000	12000	12000	12000	12000	12000	12000	1200	12000	1200	12000	1200
	±0.00	±0.00	±0.02	±0.00	±0.02	±0.005	±0.00	±0.00	±0.005	±0.00	±0.005	±0.02
14	1420	9120	7200	9120	9120	2400	6240	200	2400	9120	7200	12000
	±2.0	±0.08	±0.005	±0.10	±80	±2.0	±8.0	±8.0	±0.02	±0.02	±8.0	±0.02
28	4320	8160	12960	8640	8160	1920	1920	200	4320	10560	200	9120
	±8.0	±0.005	±0.02	±0.02	±2.0	±2.0	±2.0	±2.0	±0.08	±0.08	±2.0	±0.005
42	4320	5280	3360	6240	5280	1920	2400	1920	4320	8640	6240	9120
	±2.0	±0.08	±0.10	0.10	±0.10	±8.0	±8.0	±0.02	±0.08	±2.0	±0.08	±0.005
56	5280	3360	1920	4320	4320	2400	1920	2400	2400	5280	3360	9120
	±8.0	±0.02	±0.10	±0.02	±2.0	±2.0	±18.0	±0.08	±0.02	±2.0	±0.08	±0.00

Results represent, means ± standard deviation of duplicate soil sample

TABLE 11: Total/Net % losses of crude oil at the end of study period

TREATMENT CELL IDENTITY/ TREATMENT OPTION	TOTAL % LOSS	NET % LOSS
PRE - TREATMENT	N/A	N/A
TCA	56	32
TCB	72	48
TCC	84	60
TCD	64	40
TCE	64	40
TCF	80	56
TCG	84	60
TCH	80	56
TCI	80	56
TCJ	56	32
TCK	72	48
TCL	24	N/A

N/A = Not applicable

fertilizer. These workers used fertilizers with a higher nitrogen or phosphate ratio (5:1,3:5:1,10:1,7:3:2) to achieve a higher removal of hydrocarbons. The ratio of the readily available and cost effective fertilizer used in this study (NPK 15:15:15 with a ratio of 1:1:1) may have been minimal to contest with novel fertilizers employed in some bioremediation experiment (Lee, and Levy 1987,1989, Lee *et al.*, 1993).
Results obtained indicated that at 0.05 (95%) level of significance, there is a significant difference between nutrient level arising from treatment options and hydrocarbon level in soil samples.

CONCLUSION

This study shows that greater percentage losses of crude oil were recorded in cells in which nitrogen-fixing bacteria were applied than cells in which inorganic nutrients (fertilizer) alone or combination was applied. The use of nitrogen fixing bacteria as a substitute for novel nutrient formulation, in the enhancement of bioremediation of crude oil polluted soils in the Niger Delta is advisable. It holds good promise in combating oil pollution and offers itself for testing in other environment where oil is explored and produced.

REFERENCES

Abd – el Malek, T. Ishac, Y.Z., 1968. Evaluation of methods used in counting *Azotobacter*. Journal of Applied Bacteriology. 31:267-275.

Alexander, M., 1977. An introduction to soil Microbiology (2nd ed) John Wiley and Sons, New York. pp 1-546.

Allen, L.L., and Lechevalier H.A. 1973. CRC Handbook of Microbiology (vol.1) pp 71-88

Amanchukwu, S.G., Obafemi, A and Okpokwasili, G.C., 1989. Hydrocarbon degradation and utilisation of a palm- wine yeast isolate. FEMS Microbiol. Letters 57:151-154.

American Petroleum Institute 1980. RP 45: Recommended practice for analysis of Oil field waters pp 3-6.

A.P.H.A., A.W.W A., A.P.C.F. 1985. Standard methods for water and waste water Examination 15th Edition, Washington D.C.

Bergey's Manual of Determinative Bacteriology 1993. Williams and Wilkins ed A Waverly Company USA.

Bossert, I. and Bartha, R. 1984: The fate of petroleum in soil ecosystems. In Petroleum Microbiology Atlas R.M. Ed. Macmillan, New York p. 435-473.

Brown, K.W., Donely, K.C. and Dewel,-L.E. 1983: Effects of mineral nutrients sludge applications, and application frequency on biodegradation of two oily sludges. *Micro. Eco6*. 9:363.

Collins, P., M. Legne, and A.G.J. Proto. 1984: Microbiological method vol. 5 ed. Butterworths. pp 253-406.

Carpenter, P.L., 1977. Microbiological method vol. 5 ed. Butterworths. pp 253-406.

Cruickshank, R., Duguid, J.P. Marmoin, B.P. and Swain, Dibble, J.T. and Bartha, R. 1979: Effects of environment parameters on the biodegradation of oil spillage. Applied Environmental microbiology. 37: 729-739.

Ekundayo, J.A. and Omokaro, R. 1978. Use of indigenous microorganism In ridding the environment of spilled oil. The petroleum industry and the Nigerian Environment proceedings of 1987.International Seminar NNPC. pp 139-148.

Finney, D.J., 1978. Statistical Methods in Biological Assay 3rd Editor Charles Criffin London pp1-252.

Hoff, R. 1992. Bioremediation: A counter measure for oil spill Technology Newsletter. 17:1-14.

Ifeadi, C.N. and Nwankwo, J.N. 1989. Oil spill incident in Nigeria petroleum Industry: A critical analysis. *Napector*. 8:11-45.

Jones, M.A. and Greenfield. J.H. 1991: In sites comparism of bioremediation Methods For No.6 residual oil spill in country, Florida. Proceedings of the 1991 oil/spill conference. American Petroleum Institute. Washington D.C.

Kinako, P.D.S. 1990: Ecology and human welfare. Academic Digest 1 (1): 3-8.

Ladousse, A. and Tramier. B., 1991. Results of 12years of research in spilled oil Bioremediation. Inipol EA 22. Proceedings of the 1991 oil spill conference American petroleum Institute. Washington D.C.

Lee, K. and Levy E.M., 1987. Enhanced biodegradation 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 E.M., 1987. Biodegradation of Petroleum in the marine environment and its enhancement in aquatic Toxicity Water Management

Nriagu J.A. ed. John Wiley and sons, New York pp 217-224

Lee, K. Tremblay GH. and Levy E.M., 1993. Bioremediation Application of slow release fertilizers on low energy shorelines. Proceedings of the 1993 Oil Spill Conference. American Petroleum Institute Washington D.C. pp 449-453.

Lolomari, D., 1979. Factors in crop productivity. Soil biotechnology. Blackwell scientific, Oxford. 249p.

Mills, A. L., Brevil, C. and Colwel, R.R., 1978. Enumeration of petroleum degrading marine and estuarine microorganisms by the most probable number method. Canadian Journal Of Microbiology. 24:552-557.

Odeyemi O. It's pollution potential in Nigeria. Oil and petrochemical pollution 2: 223-229.

Odu C.T.I and Isinguzo, O.S.N., 1979. Oil spillage and the use of infant organisms for enhanced biodegradation. The petroleum industry and the Nigerian Environment proceedings of 1987 International seminar, NNPC. pp 133-138.

Okpokwasili, G.C. and Odokuma, L.O., 1990. Effects of salinity on biodegradation of oil spill dispersants. Waste management. 10, 141-146.

Olivieri R. Bacchin P., Robertullo, A., Oddo N., Deger, L. and Tonolo, A.1976 microbial degradation of oil spills enhanced by a slow release fertilizer. Journal of Applied microbiology 31 (6): 624-634.

Pelczar, M. J. Jr., Reid R.D. and Chan E.C.S. 1983 Microbiology. Tata McGraw Hill Publishing Company Ltd. New Delhi. pp 1-845.

Raymond R.L, Hudson, J.O., Jamison, V.W. (1976). Oil degradation in soil. Applied Environmental Microbiology 31: 522-535.

Stevens, S., 1991. Selection of nutrients to enhance biodegradation for the remediation of oil spilled on beaches. Proceedings of the 1991 oil spill conference American petroleum Institute Washington DC.

Westlake, D.W.S., Jobson A.M. and Cook F.D. 1978 In situ biodegradation of oil in a soil of Boreal region of the Northwest territories. Canadian Journal of Microbiology 24:254-260.