Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 2 (2): 149-155, 2007
ISSN 1816-4935

Characterization of Hydrocarbon-Degrading Microorganisms Isolated from Crude Oil Contaminated Soil and Remediation of the Soil by Enhanced Natural Attenuation

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Abstract: Remediation by Enhanced Natural Attenuation (RENA) was employed to remediate an oil contaminated site in the Gokana Local Government Area of Rivers State, Nigeria between January and September 2006. Mineral salt medium to which crude oil had been added was used as a sole source of carbon and energy to isolate hydrocarbon utilizers from the sample collected from different plots of the contaminated site. Two fungi, *Articulosporium inflata* and *Zoopage mitospora*; as well as five genera of bacteria, *Lactobacter*, *Arthrobacter*, *Bacillus*, *Pseudomonas* and *Micrococcus* were isolated and identified. The microbial and physicochemical properties of the soil samples varied with the different plots and at different periods of remediation.

Key words: Bioremediation, hydrocarbon-utilizers, oil-spillage, bacteria, fungi

INTRODUCTION

Petroleum is at present Nigeria's and indeed, the world's most important derived energy source ((Moffat and Linden, 2005; Nigerian Environmental Study Action Team, 2006). However, the growth and activities of petroleum and petroleum associated industries in Nigeria and in other parts of the world has led to increased oil pollution in our environment. Petroleum in its natural state is called crude oil (Ukoli, 2003). Crude oil, because of its characteristics, is one of the most significant pollutants in the environment as it is capable of causing serious damages to humans and the ecosystem (Okpokwasili, 1996). In this context pollution is defined as the addition to any segment of the environment, any material which has detrimental effect on the ecosystem (Aboribo, 2001).

The greatest single environmental problem connected with crude oil exploration in Nigeria is oil spillage both on-shore and off-shore. The rate of oil spillage reported in the country has been rising with a corresponding increase in petroleum production. Only a single spill was reported in 1970, whereas in 2001, the number shot up to 14. In 2003, 105 spills were reported, 154 in 2004 and 216 in 2005 (Nigerian Environmental Study Action Team, 2006).

It is known that greater degradation of oil pollutants is carried out in situ by a consortium of microorganisms (Okpokwasili, 1996, 2003) and more than 200 species of bacteria, fungi and even algae can biodegrade hydrocarbons. The various genera that have been reported to contain hydrocarbon-degrading species include *Pseudomonas*, *Vibrio*, *Corynebacterium*, *Arthrobacter*, *Brevibacterium*, *Flavobacterium*, *Sporobolomyces*, *Achromobacte*, *Bacillus*, *Aeromonas*, *Thiobacillus*, *Lactobacter*, *Staphylococcus*, *Penicillium* and *Articulosporium*. These organisms have been isolated in large numbers from many oil polluted waters and soils, but are found in less numbers in uncontaminated environments (Walworth and Reynolds, 1995; Okoh, 2003).

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The principle by which a mixture of microorganisms act together to bring about oxidation of complex compounds is known as co-metabolism. This principle is employed by oil companies in Nigeria to remediate oil polluted sites in a process known as Remediation by Enhanced Natural Attenuation (RENA). This is because the microbial degradative mechanisms appear to be the natural processes which eliminate the bulk of oil pollutants after initial physical and chemical breakdown has occurred (Floodgate 1984; Atlas, 1995).

Many studies have been conducted to isolate and characterize hydrocarbon degraders from oil spill sites but little have been done to determine the changes in soil mineral nutrients and total petroleum hydrocarbon as bioremediation of the spill site progresses. This study will provide information on the effectiveness of microorganisms in eliminating oil pollutants from the oil polluted areas of the Niger delta region in Nigeria. Specifically, the experiment was designed to; isolate and characterize hydrocarbon utilizing bacteria and fungi from crude oil impacted soil samples and also determine the extent to which the spilled crude oil has been degraded.

MATERIALS AND METHODS

Collection of Soil Samples

Soil samples used were obtained from an oil spilled area in the Gokana Local Government Area of Rivers State, Nigeria, between January and June 2006. The samples were aseptically collected using soil sampler to a depth of 20 cm, stored in sterile aluminum foils and transported to the laboratory within 48 h of collection. The spill site was cleared and divided into 3 portions which were labeled A, B and C, respectively. The spill site was cleared so as to allow for easy access to the site, for easy determination of the extent of spill and more importantly, for the construction of windrows. Samples were collected from the different parts of each portion and bulked for homogeneity. Thereafter, windrows were constructed and leveled at intervals of two weeks so as to enhance the vaporization of the volatile components of the spilled crude oil, to ensure an even distribution of the spilled crude and to expose the hydrocarbon degraders, which are basically aerobes, to oxygen (Woods, 2005).

Enumeration of total heterotrophic bacteria and fungi in the soil samples 1 g of each sample was serially diluted (10^{-1} to 10^{-7}). One milliliter aliquots from dilutions of 10^{-2} and 10^{-3} were plated in duplicate on sterile sabouraud dextrose agar plates while dilutions of 10^{-5}, 10^{-6} and 10^{-7} were plated in duplicate on sterile nutrient agar plates, using the pour plate method. Incubation was carried out at 28±2°C for 7 days for the sabouraud dextrose agar plates and 37°C for 24 h for the nutrient agar plates. Colonies on the plates were afterwards enumerated according to the American Public Health Association (1995).

Enumeration of Hydrocarbon-Utilizing Bacteria and Fungi

One gram of each sample was diluted serially (10^{-1} to 10^{-7}).One milliliter from dilutions of 10^{-2}, 10^{-3}, 10^{-5}, 10^{-6} and 10^{-7} were plated in duplicate on pre dried mineral salt agar using the spread plate technique. For the 10^{-2} and 10^{-3} dilutions, 0.5 mL of streptomycin was added to the mineral salt agar to suppress bacterial growth. A filter paper saturated with sterile crude oil was aseptically placed on the inside of the inverted Petri dishes and the culture plates were incubated for 7 days at 28±2°C for fungi and 4 days at 37°C for bacteria. Plates yielding 30 to 300 colonies were afterwards enumerated for bacterial isolates. Plates with fungal colonies were also enumerated.

Isolation and Characterization of Hydrocarbon-Utilizing Bacteria and Fungi

Colonies of different hydrocarbon utilizing bacteria and fungi were picked randomly using a sterile inoculating wire loop and subcultured to purify, by streaking on nutrient agar plates and sabouraud dextrose agar plates respectively. The plates were incubated at 30°C for 24 h and at room

temperature for 3 days, respectively to obtain pure colonies. All the isolates were characterized using the techniques of Carpenter (1977), Gerhardt *et al*. (1981) and Bargey's (1994).

Determination of Total Petroleum Hydrocarbon

Fourier Transform Infrared Spectrophotometer (FTIR, Genesis Series) was used for the test. Prior to analysis the soil samples were extracted with carbon tetrachloride and treated with 2% deactivated silica gel. The equipment was calibrated with isooctane/octane in carbon tetrachloride. TPH concentrations in the samples were determined by using the stored calibration graph in the software of the equipment as a reference.

Moisture Content Determination

A weighed amount of the soil sample was placed in a weighed crucible and dried at 105°C in the oven until a constant weight was reached. From the difference in weight, the percentage moisture content was calculated.

Phosphate Content Determination

Samples were extracted with 25% acetic acid and the extract run on the Unicam UV/visible spectrophotometer at a wavelength of 700 nm. A spike sample was analyzed in every batch of analysis. A standard was analyzed after every batch of samples and the first value of the standard was used to plot the means control chart.

Nitrate and pH Determination

Soil samples were extracted with sodium acetate in the presence of sulphuric acid and measured at a wavelength of 470 nm using Unicam UV/visible spectrophotometer. To assess the pH, electrodes of a multimeter were dipped into a mixture of soil sample and deionized water. The pH values of the samples were subsequently read on the multimeter.

Total Organic Matter Content Determination

Five hundred milligram of soil sample was mixed with 25 mL chromic acid in a 250 mL conical flask. The mixture was boiled for 1 h, allowed to cool and diluted with 100 mL of water. This was followed by the addition of 5 mL of indicator solution and titration of dichromate with ferrous ammonium sulphate solution. 2.5 mL of dichromate mixture was further added when the first colour change occurred and the titration was completed drop wise. A blank determination was carried out and subtracted as shown below. For example: If T mL of ferrous Ammonium sulphate is used in the titration, then: percentage organic matter was expressed as;

$$(\%)\ 0 = \frac{(2.7 - T)\text{mL} \times 0.12}{\text{Sample weight or sample volume}}$$

Analyses of physicochemical properties of the soil samples were performed according to the American Society for Test and Materials-ASTM (1998).

Statistical Analysis of Data

All the data obtained were subjected to statistical analysis of variance (ANOVA) using computer-aided SPSS statistical program. All the means were separated and compared using Duncan Multiple Range Test at 5% level of significance.

RESULTS

Microbial Counts and Identification

The hydrocarbon utilizing microbial isolates encountered were two fungi, *Articulosporium inflata* and *Zoopage mitospora*; as well as five genera of bacteria, *Lactobacter*, *Arthrobacter*, *Bacillus*, *Pseudomonas* and *Micrococcus* were isolated and identified. *Bacillus* spp and *Zoopage mitospora* were more frequently isolated among the bacteria and fungi respectively.

The counts of the total heterotrophic bacteria (THB) in crude oil polluted soil ranged from 4.10×10^4 to 2.73×10^7 cfu g^{-1}, while in the crude oil free soil (control), the counts ranged from 3.00×10^6 to 2.99×10^7 cfu g^{-1} of soil. Generally, the THB counts were higher in crude oil free soil than in crude oil polluted soil. However, statistical analysis revealed that the difference in counts between the two sites was not significant ($p\leq0.05$). There were higher counts of hydrocarbon utilizing bacteria (HUB) in crude oil polluted soils (3.60×10^3 to 7.60×10^4 cfu g^{-1} of soil) than crude oil free soil (1.33×10^4 to 1.66×10^4 cfu $g^{-1}$of soil) (Table 1).

The counts of total heterotrophic fungi (THF) ranged from 2.10×10^2 to 1.06×10^3 sfu g^{-1} of soil in crude oil polluted soil and from 2.67×10^2 to 3.02×10^2 sfu g^{-1} of soil in crude oil free soil. On the other hand, the counts of hydrocarbon utilizing fungi (HUF) ranged from 2.0×10^1 to 2.31×10^2 sfu g^{-1} of soil in crude oil polluted soil and 1.1×10^1 to 2.0×10^1 sfu g^{-1} of soil in crude oil free soil (Table 2). The difference in counts between the 2 sites for THF was not significant ($p\leq0.05$), but there were significant differences in the total counts between the 2 sites for HUF (Table 2).

Physicochemical Properties

The pH of crude oil polluted soil ranged from 4.00-4.54 while that of crude oil free soil ranged from 4.52-4.85. The pH values of crude oil polluted soil were significantly lower compared to those of crude oil free soil. The TOC of crude oil polluted soil (1.49-3.13%) was higher than that of crude oil free soil (1.26-1.90%). The nitrogen level of the crude oil polluted soil ranged from (3.00-6.00%), while that of crude oil free soil ranged from (1.51-2.86%), indicating the availability of more nitrogen in the polluted soil than the free soil. The values were significantly different, ($p\leq0.05$).

Table 1: Total heterotrophic and hydrocarbon utilizing bacterial counts (cfu/ml) in Samples 1-4

	Sample 1			Sample 2			Sample 3			Sample 4		
Plot	THB	HUB	% of HUB to THB	THB	HUB	% of HUB to THB	THB	HUB	% of HUB to THB	THB	HUB	% of HUB to THB
A	2.73×10^7	5.00×10^3	0.018315	4.55×10^4	1.95×10^4	42.85714	4.10×10^4	3.22×10^4	78.53659	4.90×10^3	7.60×10^4	15.5102
B	3.00×10^4	1.02×10^4	0.34	5.10×10^3	2.06×10^4	4.039216	4.27×10^4	2.97×10^4	69.55504	9.40×10^3	6.20×10^4	6.595745
C	1.20×10^7	3.60×10^3	0.03	5.00×10^3	1.50×10^4	3	2.91×10^7	3.81×10^4	13.09278	1.79×10^7	5.30×10^4	0.296089
Ct1	2.95×10^7	1.62×10^4	0.054915	2.730×10^7	1.37×10^4	0.050183	2.91×10^7	1.53×10^4	0.052577	3.00×10^4	1.44×10^4	0.48
Ct2	2.76×10^7	1.41×10^4	0.051087	2.81×10^7	1.33×10^4	0.047331	2.99×10^7	1.66×10^4	0.055518	3.17×10^4	1.39×10^4	0.438486

THB: Total Heterotrophic Bacteria, Sample 1: Soil samples collected prior to remediation, HUB: Hydrocarbon Utilizing Bacteria, Sample 2: Soil samples collected 6 weeks into remediation, CFU: Colony Forming Unit, Sample 3: Soil samples collected 12 weeks into remediation, Ct 1 and Ct 2: Control 1 and Control 2, Sample 4: Soil samples collected 18 weeks into remediation

Table 2: Total counts (cfu/ml) of heterotrophic and hydrocarbon utilizing fungi in samples 1-4

	Sample 1			Sample 2			Sample 3			Sample 4		
Plot	THF	HUF	% of HUF to THF	THF	HUF	% of HUF to THF	THF	HUF	% of HUF to THF	THF	HUF	% of HUF to THF
A	4.90×10^2	5.0×10^1	10.20408	4.30×10^2	1.07×10^2	24.88372	3.77×10^2	1.60×10^2	42.44032	5.30×10^2	2.31×10^2	43.58491
B	2.50×10^2	4.06×10^1	16.24	2.43×10^2	1.08×10^2	44.44444	2.10×10^2	1.33×10^2	63.33333	5.09×10^2	1.90×10^2	37.32809
C	4.06×10^2	2.0×10^1	4.926108	2.11×10^2	1.10×10^2	52.1327	5.00×10^2	2.30×10^2	46	1.06×10^3	1.10×10^2	10.37736
Ct1	2.80×10^2	1.6×10^1	5.714286	2.67×10^2	1.9×10^1	7.116105	3.02×10^2	1.8×10^1	5.960265	2.75×10^2	2.0×10^1	7.272727
Ct2	2.90×10^2	1.1×10^1	3.793103	2.79×10^2	1.1×10^1	3.942652	2.81×10^2	1.3×10^1	4.626335	2.70×10^2	1.5×10^1	5.555556

THF: Total Heterotrophic Fungi, Sample 1: Soil samples collected prior to remediation, HUF: Hydrocarbon Utilizing Fungi, Sample 2: Soil samples collected 6 weeks into remediation, SFU: Spore Forming Unit, Sample 3: Soil samples collected 12 weeks into remediation, Ct 1 and Ct 2: Control 1 and Control 2, Sample 4: Soil samples collected 18 weeks into remediation

Table 3: Physicochemical properties of soil samples collected prior to remediation

Parameters	Method	Plot A	Plot B	Plot C	Control 1	Control 2
TPH	GC (FID)	291.3	379.7	353.2	54.46	142.7
TOC	Titrimetry (%)	2.57	2.77	2.18	1.86	1.56
NO_3	UV/Spectrophotometer (%)	3.55	3.00	3.26	1.51	2.00
PO_4	UV/Spectrophotometer	0.67	0.41	0.69	0.19	0.23
pH	Electrometry	4.33	4.24	4.00	4.52	4.52
MC	(%)	6.00	6.00	5.80	10.44	11.00

Table 4: Physicochemical properties of soil samples collected 6 weeks into remediation

Parameters	Method	Plot A	Plot B	Plot C	Control 1	Control 2
TPH	GC (FID)	207.5	302.2	276.2	45.38	57.48
TOC	Titrimetry (%)	1.956	1.752	1.494	1.26	1.40
NO_3	UV/Spectrophotometer (%)	4.62	3.97	4.11	2.17	2.61
PO_4	UV/Spectrophotometer	0.16	0.35	0.24	0.10	0.17
pH	Electrometry	4.36	4.32	4.20	4.67	4.61
MC	(%)	6.56	5.60	6.20	12.62	13.51

Table 5: Physicochemical properties of samples collected 12 weeks into remediation

Parameters	Method	Plot A	Plot B	Plot C	Control 1	Control 2
TPH	GC (FID)	103.6	186.5	157.2	35.76	53.44
TOC	Titrimetry (%)	2.46	2.81	2.61	1.87	1.70
NO_3	UV/Spectrophotometer (%)	6.00	5.55	4.98	2.77	2.86
PO_4	UV/Spectrophotometer	0.46	0.39	0.39	0.25	0.13
pH	Electrometry	4.41	4.41	4.35	4.80	4.73
MC	(%)	7.11	7.50	7.00	12.59	12.97

Table 6: Physicochemical properties of samples collected 18 weeks into remediation

Parameters	Method	Plot A	Plot B	Plot C	Control 1	Control 2
TPH	GC (FID)	86.10	68.79	54.72	25.97	37.30
TOC	Titrimetry (%)	2.97	3.13	3.00	1.90	1.79
NO_3	UV/Spectrophotometer (%)	5.37	5.20	4.50	2.02	1.90
PO_4	UV/Spectrophotometer	0.55	0.49	0.66	2.29	0.20
pH	Electrometry	4.50	4.45	4.54	4.85	4.82
MC	(%)	8.00	7.60	8.20	13.71	12.62

TPH: Total petroleum hydrocarbon, PO_4: Phosphate content, pH: Soil acidity/alkalinity, TOC: Total crganic matter content, C: Moisture content, NO_3: Nitrate content

The available phosphorus level of crude oil polluted soil (0.16-0.69) was higher than that of crude oil free soil (0.10- 0.29) and the differences were also significant, ($p \leq 0.05$). However, the MC of crude oil polluted soil (5.60-8.20%) was lower than that of crude oil free soil (10.44-13.71%) and statistical analysis showed a significant difference between them (Table 3-6). In the crude oil polluted soil, the TPH decreased sharply through the investigation period while the decrease in the crude oil free soil was gradual and statistically, the difference in the TPH level between both soils was significant ($p \leq 0.05$).

DISCUSSION

The results of the study indicated that the total heterotrophic bacteria (THB) counts varied over the period of 18 weeks. The difference in counts may be due to changes in the physicochemical properties of the soil. However, the difference in counts of THB and THF between the crude oil polluted soil and the crude oil free soil was not significant, probably due to rapid biodegradation of the crude oil in the soil. The counts of hydrocarbon utilizing bacteria (HUB) in crude oil polluted soil were higher than those of the crude oil free soil. Statistical analysis revealed that the difference was not significant ($p \leq 0.05$). The reason for higher counts in crude oil polluted soil may be due to the presence of residual crude oil in the polluted soil which boosts the carbon supply in the soil, hence favor the growth of the hydrocarbon utilizing bacteria as compared to crude oil free soil (Ijah and Abioye, 2003; Ijah and Antai, 2003).

The bacterial counts in both polluted and free soil where higher than the fungal counts in both soils and the HUF count in the polluted soil was higher than that of the crude oil free soil. The higher counts of bacteria compared to fungi may be as a result of the nutrient status of the soil (Jobson *et al*., 1979) and the presence of some toxic components which do not favor fungal growth (Colwell and Walker, 1977).

The pH values of crude oil polluted soil were lower as compared to those of crude oil free soil, a finding, which is in line with the report of Ijah and Abioye (2003). The decrease in pH value may be due to increased degradation of crude oil by microorganisms in the soil, resulting in accumulation of acidic metabolites. However, the moisture content of crude oil polluted soil was lower than that of crude oil free soil. This may be due to the fact that crude oil can coat the soil and consequently prevent the penetration of water compared to kerosene that was used in their study.

Both nitrogen and phosphorus levels were higher in crude oil polluted soil than crud oil free soil. This agrees with the finding of (Odu, 1972), who reported increase in nitrogen and phosphorus contents of a crude oil polluted soil. The reason could be due to higher organic matter content of the polluted soil.

The rate of crude oil biodegradation in the soil seems to be rapid. This may be due to the fact that the microorganisms in the soil have efficient ability in utilizing the residual crude oil as a source of carbon and energy (Ijah and Antai, 2003). Crude oil contains hydrocarbon and does not resist attack by microorganisms (Atlas, 1995). The hydrocarbon utilizing microorganisms isolated from the soil were species of *Bacillus*, *Lactobacter*, *Arthrobacter*, *Pseudomonas*, *Micrococcus*, *Zoopage* and *Articulosporium*. *Bacillus* species predominated, especially in the crude oil polluted soil. This may be due to the ability of the organisms to produce spores, which may shield them from the toxic effects of the hydrocarbons.

In conclusion, the study shows that there was residual crude oil in the soil after 18 weeks of investigation. The study also shows that the physiochemical properties of the soil were significantly affected ($p \leq 0.05$). Finally, the study has shown that Remediation by Enhanced Natural Attenuation is effective in the clean up of polluted sites in the Niger Delta.

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