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Full Length Research Paper

Bacterial diversity in a tropical crude oil-polluted soil undergoing bioremediation

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The bacterial diversity in a tropical soil experimentally polluted with crude oil during a 57 days bioremediation was investigated in five 1 m^2 plots using total culturable hydrocarbon utilizing bacteria, heterotrophic bacteria and gas chromatographic analyses. Four out of the five experimental plots received each 4 L of Bonny light crude oil while three treatment plots received 3 kg of NPK, urea fertilizers or poultry droppings with periodic tilling. Two plots, oil-contaminated and pristine served as controls. Bacterial counts increased 200 fold and 2 fold in the NPK treated and poultry-dropping-treated plots respectively, by day 31 post-inoculation. Detectable hydrocarbons in the treatment plots decreased by 84 - 95% and 96 - 99%, 31 and 57 days post-inoculation, respectively, compared with the petroleum contaminated control. Bacterial strains isolated included *Rhodococcus* sp., *Nocardia* sp., *Arthrobacter* sp., *Gordonia* sp., *Mycobacterium* sp., *Corynebacterium* sp., *Bacillus* sp., *Micrococcus* sp., *Flavobacterium* sp., *Pseudomonas* sp. and *Alcaligenes* sp. The overall data suggest an important contribution of *Actinobacteria* during bioremediation of crude oil-polluted soil.

Key words: Niger Delta, Nigeria, crude oil pollution, bonny light, bioremediation, *Actinobacteria*, fertilizers.

INTRODUCTION

Exploration and production of crude oil in Nigeria is carried out in the oil-rich Niger Delta region. Over 80% of the country's oil comes from this ecological zone and its surrounding offshore areas (Okpokwasili and Amanchukwu, 1988; Okpokwasili and Odokuma, 1994; Odokuma and Dickson, 2003). Within the Delta, the numerous oil fields, tank farms, flow stations, pipelines, tankers and loading jetties constantly provide potential sources of oil pollution (Ijah and Antai, 2003; Chikere and Chijioke-Osuji, 2006).

Large scale pollutions of both the terrestrial and aquatic environment in the area, consequent on activities of the oil industry have been documented (Odokuma and Ibor, 2002; Abu and Chikere, 2006; Okpokwasili, 2006). The economic and environmental impacts of oil pollution on the soil are enormous causing serious damage to vegetation, soil fertility (Nwachukwu and Ugorji, 1995) and soil-borne micro organisms.

Bioremediation has long been applied as a treatment technology that is cost-effective, ecologically friendly and efficient for the decontamination of hydrocarbon polluted soils (Leahy and Colwell, 1990; Rosenberg and Ron, 1996; Balba et al., 1998; Bouchez-Naitali et al., 1999; Margesin et al., 2003; Nweke and Okpokwasili, 2004; Kaplan and Kitts 2004; Quatrini et al., 2008). Bioremediation techniques for removing petroleum hydrocarbons in the soil are developed around strategies for delivering moisture, aeration and nutrients in order to optimize microbial activity and degradation of the pollutants (Macnaughton et al., 1999; Kaplan and Kitts, 2004; Ayotamuno et al., 2006; Stroud et al., 2007).

The ability to degrade utilize hydrocarbon substrates is exhibited by a wide variety of bacterial genera (Leahy and Colwell, 1990; Dally et al., 1997; Bogan et al., 2003) that are widely distributed in oil-polluted as well as pris-

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tine soils (Smitts et al., 1999; Bogan et al., 2003; Van Beilen and Funhoff, 2005; Cappello et al., 2007; Hamamura et al., 2006; Van Beilen and Funhoff, 2007). Using culture dependent and independent isolation techniques, different bacterial genera have been characterized from hydrocarbon polluted soils in different geographical and ecological contexts (Van Hamme et al., 2003; Maila et al., 2004; Maila and Cloete 2005; Maila et al., 2006, Hamamura et al., 2006).

Although experimental and climatic conditions differed considerably in each study, some general trends have indicated that Gram negative *Proteobacteria* and *Cytophaga-Flavobacterium-Bacteroides* group dominate during bioremediation and the diversity shift with time to this group (Macnaughton et al., 1999; Kaplan and Kitts, 2004). These groups were usually associated with the fast degradation phase and their abundance was positively correlated to hydrocarbon attenuation. Gram positive bacteria if detected are never diverse and dominant during bioremediation (Kaplan and Kitts, 2004). However, recent reports have shown that Gram positive bacteria mainly the *Actinobacteria* can actually dominate during bioremediation of petroleum hydrocarbon owing to their metabolic versatility and their widespread occurrences both in pristine and hydrocarbon polluted soil (Bell et al., 1998; Bouchez-Naitali et al., 1999; Bogan et al., 2003; Margesin et al., 2003; Larkin et al., 2005; Hamamura et al., 2006; Quatrini et al., 2008).

In this study, the bacterial diversity in an oil-polluted tropical soil that was undergoing bioremediation by nutrient supplementation and periodic tilling was investigated using culture-dependent technique over a 57 day period.

MATERIALS AND METHODS

Experimental design

The soil used for the bioremediation field study was located behind the Faculty of Science complex, Abuja campus University of Port Harcourt, Nigeria. The pristine soil is of sandy clay texture with specific gravity of 2.57. The baseline microbiological, chromatographic and physicochemical analyses of the soil before the experimen-tal oil contamination as determined using the methods in APHA (1998) are presented in Table 1. Five composite soil samples were augered from 15 cm depth, bulked to get a representative sample and taken to the laboratory for analyses.

The bioremediation protocol consisted of five experimental plots measuring 1 m^2 and respectively designated:

(i) PN - Plot fertilized with N.P.K (20:10:10) fertilizer.
(ii) PN - Plot fertilized urea fertilizer.
(iii) PP - Plot fertilized with poultry droppings.
(iv) OC - Plot that served as oiled control (represented natural attenuation plot and received no treatment).
(v) PC - Pristine control plot.

Each oil-contaminated plot was experimentally polluted with 4 L of Bonny Light crude oil with a sprinkler. The polluted plots were left undisturbed for 3 days after which the different treatment options commenced. The treatment plots (PN, PU and PP) received 3 kg each of N.P.K. 20:10:10 fertilizer, Urea fertilizer and poultry droppings respectively. They were tilled to 15 cm depth every other day till day 57 to mix nutrient with polluted soil properly.

Sampling

Sampling was done on days zero, that is, 3 days post-contamination/pollution, 7, 34 and 57 respectively. Five composite samples were removed with soil auger from 15 cm depth of each plot, bulked and taken to the laboratory for microbiological, chromatographic and physicochemical analyses.

Bacterial counts and isolation

The total heterotrophic bacterial (THB) count was determined using the spread plate method on nutrient agar (Sigma-Aldrich) according to APHA (1998). Soil suspensions were prepared by 10 fold serial dilutions with 1 g of soil and then 10^{-5} dilution was spread on the plates in triplicates. The CFU of heterotrophs were counted after incubation at 28°C for 18 h. Hydrocarbon utilizing bacteria (HUB) were enumerated as adopted from Hamamura et al. (2006) using mineral salts medium with crude oil supplied by the vapour phase transfer. Isolated colonies were further purified by subculturing and identified using biochemical tests and microscopy.

Determination of physico chemical parameters of soil

Parameters such as pH, moisture content, nitrate, phosphate, total organic carbon (TOC) and potassium were determined using the methods from APHA (1998).

Chromatographic analysis

Residual total petroleum hydrocarbons (TPH) and polycyclic aromatic hydrocarbons (PAHs) were extracted from the soil samples and quantified using gas chromatograph - flame ionization detector (GC-FID) according to the methods of ASTDM 3921 and USEPA 8270B (TPI, 2007).

Statistical analysis

Two-way ANOVA test was used to test whether the different nutrient amendments given to the crude oil-contaminated plots were statistically significant.

RESULTS AND DISCUSSION

The bacterial diversity of an experimentally oil-contaminated soil in Abuja Campus, University of Port Harcourt, Nigeria was investigated. The concentration of petroleum hydrocarbons in the contaminated soil prior to treatment, that is, day zero was 3,666 mg/kg for TPH and 33.07 mg/kg for PAHs at 15 cm depth respectively. The total heterotrophic bacterial count in the polluted soil was 1.20×10^6 CFU g^{-1} while the hydrocarbon utilizing bacterial count was 2.51×10^2 CFU g^{-1}. The physico-

Table 1. Baseline properties of soil prior to oil contamination.

Parameter	Measurement
THB	1.83×10^6
HUB	3.5×10^3
TPH (mg/kg)	147.17
PAH (mg/kg)	1.66
pH	6.50
Moisture (%)	13.42
Nitrate (mg/kg)	17.65
Phosphate (mg/kg)	24.09
TOC (%)	3.46
Potassium (mg/kg)	9.60

[a]THB: total heterotrophic bacteria; [b]HUB hydrocarbon utilizing bacteria; [c]TPH: total petroleum hydrocarbons; [d]PAHs: polycyclic aromatic hydrocarbons; [e]TOC: total organic carbon.

chemical parameters as determined for the polluted soil were as follows pH: 6.39; moisture content: 13.60%; nitrate 10.85 mg/kg; phosphate 15.28 mg/kg; total organic carbon: 10.48% and potassium: 8.60 mg/kg.

It was observed that the THB and HUB increased during the study resulting in corresponding attenuation of the hydrocarbons with time (Tables 2 and 3) in the nutrient-amended plots when compared with the oil-contaminated control. Different bacterial genera were isolated from each of the treatment plots. The plot that was fertilized with NPK 20:10:10 (PN) had the highest bacterial counts and was dominated by the Actinobacteria throughout the study. The isolates identified belonged to genera *Gordonia*, *Rhodococccus*, *Nocardia*, *Corynebacterium*, *Arthrobacter*, *Mycobacterium*, *Pseudomonas*, *Bacillus* and *Flavobacterium*.

However, the Actinobacteria appeared from the day zero to the day 57 with *Rhodococcus*, *Gordonia Arthrobacter* and *Nocardia* being the dominant genera in the hydrocarbon degraders isolated. PU also had the Actinobacteria as the dominant bacterial group but was less diverse than PN. The genera identified were *Gordonia*, *Rhodococcus* and *Arthrobacter*. *Pseudomonas* and *Alcaligenes* were identified from the 2nd week of the study. PP had *Pseudomonas*, *Corynebacterium* and *Rhodococcus* spp. as the dominant bacterial group from day zero to 57. However, *Salmonella* sp. and *Escherichia coli* were isolated from this plot during the first week of the study. This is expected as these bacteria are commensals found in poultry.

The OC plot had the Actinobacteria mainly *Rhodococcus*, *Nocardia*, *Gordonia* and *Arthrobacter* dominating from the day zero to day 57 of the study. *Pseudomonas* spp. increased appreciably from day 31 but was not dominant. Other bacterial genera isolated from the oiled control were *Bacillus* and *Alcaligenes*. The pristine control during the course of the study showed less population shift as compared with the treatment plots. However, the Gram positive Actinobacteria developed more confluent spreading growth on media discouraging the growth of other bacteria. These groups of bacteria are well known for antibiotic production (Boudjella et al., 2007) and this may be the explanation for the suppression of other bacterial colonies. The population of culturable hydrocarbon degraders from the different treatment plots investigated showed that majority of the bacteria were Gram positive belonging to the Actino-bacteria group.

Although some studies have shown that, oil-polluted soils are dominated by Gram negative bacteria (Macnaughton et al., 1999; Kaplan and Kitts, 2004), the dominant culturable hydrocarbon utilizing bacteria from the experimental plots were made up of Gram positive Actinobacteria of the genera *Gordonia*, *Rhodococcus*, *Corynebacterium*, *Nocardia*, *Mycobacterium* and *Arthrobacter*. This corroborates the findings of Quatrini et al. (2008) who isolated 2 *Rhodococcus*, 2 *Gordonia* and 1 *Nocardia* strains as the dominant hydrocarbon degraders from a hydrocarbon contaminated Mediterranean shoreline.

Earlier studies have also demonstrated the prevalence of Actinobacteria in hydrocarbon polluted soils from different geographical locations (Bouchez-Naitali et al., 1999; Margesin et al., 2003; Bogan et al., 2003; Chaillan et al., 2004; Hamamura et al., 2006) and the presence of multiple hydrocarbon catabolic genes in this group of bacteria (Smitts et al., 1999; Van Beilen et al., 2002; Larkin et al., 2005; Van Beilen et al., 2005; Kloos et al., 2006). The Actinobacteria isolated from the treatment plots were also isolated in the pristine control showing that they are well adapted to harsh environmental conditioning such as oil pollution.

This observation is consistent with the results of Hamamura et al. (2006) and Quatrini et al. (2008). Gram positive hydrocarbon degraders have also been detected in pristine soils (Margesin et al., 2003; Kloos et al., 2006). It has been hypothesized that Actinobacteria generally adapt to nutrient limited conditions and consequently do not fluctuate in response to hydrocarbon enrichment (Quatrini et al., 2008) which normally causes nutrient deficiencies (Stroud et al., 2007). The bacterial population dynamics and counts observed during crude oil degradation could also be associated with the degradation patterns of the total petroleum hydrocarbons and polycyclic aromatic hydrocarbons in the different treatment plots when compared with the oiled control.

The polluted soil had 3,666 mg/kg of TPH and 33.07 mg/kg of PAHs as at day zero. This concentration of total petroleum hydrocarbon in PN, PP and PU plots decreased appreciably by 95, 89.7 and 84%, respectively. The PAHs level decreased by 97, 96 and 95% respectively, by day

Table 2. Bacterial Counts (10^6 $cfug^{-1}$) in the experimental plots.

Bacteria	Day 7	Day 31	Day 57
[c]PN [a]THB	13.7	289	450
[b]HUB	0.042	1.28	38
[d]PU THB	5.6	1.32	21.3
HUB	0.007	0.026	0.051
[e]PP THB	1.17	21.9	19.7
HUB	0.021	0.11	0.257
[f]OC THB	0.31	1.63	1.14
HUB	0.001	0.02	0.02
[g]PC THB	4.7	0.39	0.18
HUB	0.003	0.003	0.027

[a]THB total heterotrophic bacterial counts; [b]HUB-hydrocarbon utilizing bacteria; [c]PN: NPK plot; [d]PU: urea plot; [e]PP: poultry plot; [f]OC: oiled control; [g]PC: pristine control.

Table 3. Residual petroleum hydrocarbons (mg/kg) during 57 day bioremediation study.

Hydrocarbon	Day 0	Day 7	Day 31	Day 57
PN [a]TPH	3,666	918	171.30	89.68
[b]PAHs	33.07	3.01	0.99	BDL*
PU TPH	3,666	1,990	584.85	162
PAHs	33.07	6.31	1.67	BDL*
PP THP	3,666	2,961	377.94	135.01
PAHs	33.07	5.56	1.29	BDL
OC THP	3,666	3328	1122	900.1
PAHs	33.07	7.53	2.47	0.01

*BDL below detectable level; [a]TPH: total petroleum hydrocarbons; [b]PAHs: polycyclic aromatic hydrocarbons.

31 where as more than 96% of the TPH were lost by the 57 day in all the treatment plots. The different treatments given to the plots PN, PP and PU showed that the results for the TPH degradation are significant $P < 0.1$ but for the PAHs the results were not significant when compared with the oil-contaminated control. Day 31 (4^{th} week) of the study corresponded with high bacterial counts recorded for PN which increased 200 fold from day 7.

This was followed by PP (2 fold) and PU which decreased 4 fold when compared with day 7. Different trends have been reported for biostimulation with fertilizers, for instance, Ayotamuno et al. (2006) found that the addition of NPK fertilizer to a polluted agricultural soil in Nigeria significantly enhanced the biodegradation of TPH in the test cells which was also observed in the PN plot in this study. The PN plot had the highest bacterial counts (both for THB and HUB) and degradation of the petroleum hydrocarbons when compared with PU, PP and the oil-contaminated control.

However, urea fertilizer used for PU reduced the THB counts 4 fold by day 31. This finding is consistent with the report of Sarka et al. (2005) which stated that urea fertilizer caused either NH_3 overdosing or increased pH that negatively affected bacteria. In the same vein, Chaillan et al. (2006) found out that urea fertilizer has a fungicidal effect and caused a toxic concentration of NH_3 gas when applied to the soil which in turn limited the rate of hydrocarbon degradation. However PU plot recorded significant attenuation of the petroleum hydrocarbons despite the observed deleterious effect of the fertilizer on soil bacteria.

The PP plot showed the second highest bacterial counts and hydrocarbon degradation when compared with PU and oil-contaminated control. This finding is consistent with the work done by Williams et al. (1999) who used poultry litter to enhance the degradation of petroleum hydrocarbons in the soil. The result showed that a significant first order rate of TPH biodegradation was measured for all treatment units containing the poultry litter.

The results of the present study revealed that Nigerian soil may habour hydrocarbon degraders that have been exposed to hydrocarbons as a result of the increased multifarious activities of the oil industry especially in the Niger Delta region (Chikere and Okpokwasili, 2003, 2004; Ayotamuno et al., 2006; Okpokwasili, 2006). It was observed that the Actinobacteria played a significant role in petroleum hydrocarbon degradation having shown dominance both in the treatment plots and pristine soil.

Studies have shown that the Actinobacteria can habour multiple aliphatic and aromatic hydrocarbon degradative genes with overlapping hydrocarbon substrate ranges (Van Beilen and Funhoff, 2007) and it could be that the genera isolated in this study may have these catabolic capabilities as shown by the disappearance of the hydrocarbons even in the oil-contaminated control without any amendment.

It is well documented that Actinobacteria like *Rhodococcus*, *Gordonia*, *Nocardia* and *Mycobacterium* which are known hydrocarbon degraders could be cosmopolitan (Margesin et al., 2003; Hamamura et al., 2003; Van Beilen and Funhoff, 2007). The Actinobacteria are of considerable environmental and biotechnological importance because of their wide catabolic abilities, their resilience in harsh environment and biosurfactant production (Arenskotter et al., 2004; Larkin et al., 2005; Van Beilen and Funhoff, 2005; Hamamura et al., 2006; Van Beilen and Funhoff, 2007). Further molecular studies are needed to decipher the catabolic genes resident in these tropical isolates that were isolated from hydro-carbon polluted soils and their hydrocarbon specificities. This will invariably assist in developing cost effective and efficient bioremediation protocol for Nigerian oil polluted soil.

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