Enhanced Biodegradation of Petroleum Hydrocarbons in Polluted Soil Augmented with Nitrogen-Fixing Bacteria

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Abstract: Biodegradation of crude oil-polluted soil augmented with nitrogen-fixing bacteria (NFB) and amended with inorganic fertilizer (NPK) was examined *ex situ*. Three setups were designed and they include: T_1 (un-amended control- 700g of soil + 70 ml of crude oil); T_2 (700g soil, 70 ml crude oil, 70 ml hydrocarbon utilizing bacteria (HUB) and 70 g NPK) and T_3 (700g soil, 70 ml crude oil, 70 ml HUB, 70 g NPK and 70 ml Nitrogen Fixing Bacteria-NFB). In all treatment setups, increase in total heterotrophic bacterial (THB) counts, HUB and NFB counts were obtained. The HUB isolated include; *Corynebacterium; Staphylococcus; Pseudomonas; Achromobacter; Klebsiella; Serratia; Bacillus; Micrococcus; Clostridium; Acinetobacter; Flavobacterium; Citrobacter* and *Alcaligenes* whereas, the isolated NFB used for augmentation of the polluted soil include; *Nitrobacter, Nitrosomonas*, *Archromobacter, Burkholderia, Azotobacter, Arthrobacter* and *Alcanivorax*. After 28 days of treatment, reduction in total petroleum hydrocarbon (TPH) content of soil was 78.5 % in T_3, 75.8 % in T_2 and 10.8 % in T_1. The % TPH removal in the polluted soil was in the order: $T_3 > T_2 > T_1$. Gas chromatograms of soil samples indicated considerable attenuation of peaks of various carbon fractions in T_2 and T_3 when compared to the control (T_1) which suggests the enhanced biodegradation of the petroleum hydrocarbons in the polluted soil especially in T_3 augmented with NFB. This study thus, highlights the biotechnological potential of employing NFB as an agent for nitrogen content elevation in polluted soils. Their application in bioremediation protocols could enhance the fixation of nitrogen in a form that is readily utilizable by HUB *in situ* without adverse effects on the environment.
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Key words: Crude oil, *ex situ* biodegradation, nitrogen-fixing bacteria, nitrogenous fertilizer

1 Introduction

The haulage of petroleum and allied products across the world is frequent and the amounts of petroleum stocks in Organization of Petroleum Exporting Countries (OPEC) are enormous. Consequently, the potential for oil spillage is significant and research on the removal of petroleum hydrocarbons in soil environment is important to ameliorate its attendant consequences on soil ecosystem.

Crude oil pollution caused by spillages from the oil industry located primarily in the Niger Delta region has caused massive destruction to natural resources including farmlands (UNEP, 2006; Mbakwem-Aniebo *et al*., 2014).

Upon discharge into the soil, crude oil undergoes physical, chemical and biological weathering and modification (Bordenave and Goni-Urriza, 2004; Edlund and Jasson, 2004), thereby polluting environmental media. Several reports have it that oil polluted soil can be bioremediated by microbes or by organic nutrients (Bragg *et al*., 1994; Okpokwasili, 1994, 2006).

Biodegradation involves microbial transformation and detoxification of organic contaminants. It is a unique natural weathering process in which organic contaminants are broken down, over time, by microbes that then produce simple substances such as CO_2 and H_2O, thereby growing in the process producing biomass.

The bioremediation process is enhanced when sufficient amendments and ambient conditions are provided, including the manipulation of a number of physical, biological and chemical parameters in the site to remove the chemical compound or alter its original state. According to Okpokwasili (2006), bioremediation can be defined as assisted, augmented, accelerated or enhanced biodegradation.

The rate of biodegradation is limited by several factors such as oxygen, nutrients, salinity, temperature, pH, bioavailability of contaminants, moisture content, soil type, molecular weight of oil compounds, frequency of oil exposure and seasonal effects (Nakles and Loehr, 2002; Prince and McMillen, 2002; Rowland *et al*., 2002). However, the most common limiting factors are the nutrients:

phosphorus and nitrogen (Rosenberg *et al.*, 2002; Liebeg and Cutright, 1999). Therefore, amendment of polluted media with nitrogenous-based fertilizers containing nitrogen and phosphorus could increase the carbon, nitrogen and phosphorus ratio in media for resident hydrocarbon-degraders to use.

Nitrifying bacteria belonging to the genera, *Archromobacter, Burkholderia, Azotobacter, Arthrobacter* and *Alcanivorax* are found in the soil and contain the enzyme, nitrogenase, which is responsible for nitrogen fixation. Thus, the *in situ* seeding of hydrocarbon polluted soil with adapted microbes such as nitrogen-fixing bacteria which aids in co-metabolism and nitrogen fixation to the autochthonous bacteria may also help in providing the needed nitrogen for enhanced degradation of these hydrocarbons in the soil.

Their numbers in the soil can be increased through bioaugmentation in order to speed up the rate of biodegradation. Enhanced bioremediation of crude oil polluted soil using a microbial consortium has been reported elsewhere (Hammer, 1993; Agarry and Ogunleye, 2012; Nwogu *et al.,* 2015).

Hence, in this study, the biodegradation of petroleum hydrocarbons in polluted soil augmented with nitrogen fixing bacteria was investigated to determine their bioremediation enhancement potentials.

2 Materials and Methods

2.1 Sample collection

The soil samples used in this study were obtained from Imiringi in Ogbia Local Government Area of Bayelsa State, Nigeria. Composite soil samples were collected witha soil augerinto a clean container, bulked and thereafter, transported to the laboratory within five hours for analyses.

Nitrogenous fertilizer (NPK 15:15:15) was collected from a fertilizer Company in Port Harcourt while Bonny light crude was obtained from a Petroleum Refining Companyin Nigeria.

2.2 Soil treatment design

Enhancement of petroleum hydrocarbon biodegradation in soil through amendment with a microbial inoculant (nitrogen fixing bacteria) was determined using three different treatment set ups designated as T_1, T_2 and T_3. The various treatments are as described in Table 1.

During the treatment, the microbiological and physicochemical parameters were measured every 7 day-interval for a period of 28 days.

Table 1: Laboratory scale bioremediation protocol

Treatment cell	Cell content
$T_{1\ (Control)}$	soil (700 g)+ Crude oil (70 ml) (no amendment)
T_2	Soil (700 g) + Crude oil (70 ml) + HUB (70 ml) + NPK (7 g)
T_3	Soil (700 g) + Crude oil (70 ml) + HUB (70 ml) + NPK (7 g) + NFB (70ml)

HUB: hydrocarbon utilizing bacteria, **NFB:** nitrogen fixing bacteria, **NPK:** nitrogen, phosphorus and potassium

2.3 Enumeration of Microorganisms

Soil slurry was prepared and used for 10-fold serial dilution by mixing 1g of wet soil with 9ml of sterile physiological saline suspension in a test tube. Subsequently serial dilution from that test tube was performed to obtain 10^{-7} dilution. Total culturable heterotrophic bacteria (TCHB) in soil were enumerated on nutrient agar which comprised the following: meat extract 1g, yeast extract 2g; peptone 5g, $NaCl_2$ 5g, agar No. 2 powder 15g, and distilled water 1litre.

The final pH was 7.4±0.2. Nitrogen-fixing bacteria (NFB) were isolated from the soil samples using the method employed by Okpokwasili and Odokuma (1996a, b) and Colwell and Zambruski (1972). Enumeration of NFB in soil was carried out using the mannitol Ashby nitrogen-free agar (Sigma-Aldrich, USA). This medium was composed of 0.1 g K_2HPO_4, 2.5 g $CaCO_3$, 10.0 g $NaCl_2$, 0.05 g K_2SO_4, 0.1 g $MgSO_4.7H_2O$, 10 g agar No. 2 powder and 1 liter of distilled water. After inoculation, plates were incubated at 28±2^{0}C for 24 h.

Hydrocarbon utilizing bacteria (HUB) in soil samples were enumerated using a modified mineral salt medium of Mills *et al.* (1978). It contained: $MgSO_4.7H_2O$ 0.40g; KCl, 0.28g; KH_2PO_4 0.80g; Na_2HPO_4 1.20g; NH_4NO_3 0.40g; NaCl 15g and agar No. 2 powder 20g, all in 1 liter of de-ionized water. The pH of the medium was adjusted to 7.1 and subsequently sterilized at 121^{0}C for 15 min. Crude oil was introduced to the medium through vapour phase transfer by soaking a 9cm Whatman No. 1 filter paper with 10 ml of fresh Bonny light crude oil.

The flooded filter paper was then placed on the lid of the agar plate and incubated for 7 days at 25±8 ^{0}C in an inverted position following the method of Abu and Ogiji (1996). The filter papers served as a source of energy and carbon and supplied the hydrocarbons by vapour-phase transfer to inverted inoculums. Determinations of counts of various physiological groups of bacteria were carried out in triplicates and counts obtained expressed as colony-forming units per gram of soil analyzed.

2.4 Identification of Bacterial Isolates

Colonies of nitrogen-fixing bacteria and hydrocarbon utilizing bacteria were picked randomly using a sterile inoculating wire loop and purified by sub-culturing on nutrient agar plates. The plates were incubated at 28±2^{0}C for 24 h to obtain pure colonies.

Gram reaction, cell arrangement, colonial morphology and biochemical characteristics of purified colonies were examined. Gram-negative, grayish, mucoid and flat colonies with a pear-shaped suggestive of *Nitrobacter* were picked and identified with reference to Bergey's Manual of Systematic Bacteriology (Holt *et al.*1994).

2.5 Physicochemical Analyses

The physicochemical parameters were determined using standard methods adopted from APHA (1998). The pH was determined using a pH meter (Jenway 3015) whereas the total organic carbon (TOC), moisture content, phosphate, sulphate and nitrate contents were determined based on standard methods (APHA, 1998).

For gas chromatographic analysis, the residual total petroleum hydrocarbon content in soil was determined using a modified EPA 8015 technique. All analyses were carried out in triplicates and the results obtained expressed as mean ± standard deviation.

3 Results and Discussion

Data obtained on the changes in population of various physiological groups of bacteria in soil of various treatments during the 28 day study are as presented in Figs. 1–3.

In T_1, the $\log_{10}$counts of TCHB increased from 5.38CFU/gat onset of experiment to 6.46CFU/g by day 28 (Fig. 1). Similarly, the $\log_{10}$counts of TCHB in T_2 and T_3increased from 5.81CFU/g on day 0 to 8.91 CFU/g by day 28 and from 6.84CFU/g on first day of treatment to 8.79CFU/g by day 28 respectively. The TCHB increased significantly (p<0.05) with time in all the three treatments and the highest count was obtained in T_3 (8.79$\log_{10}$CFU/g) after day 28 of treatment (Fig. 1).

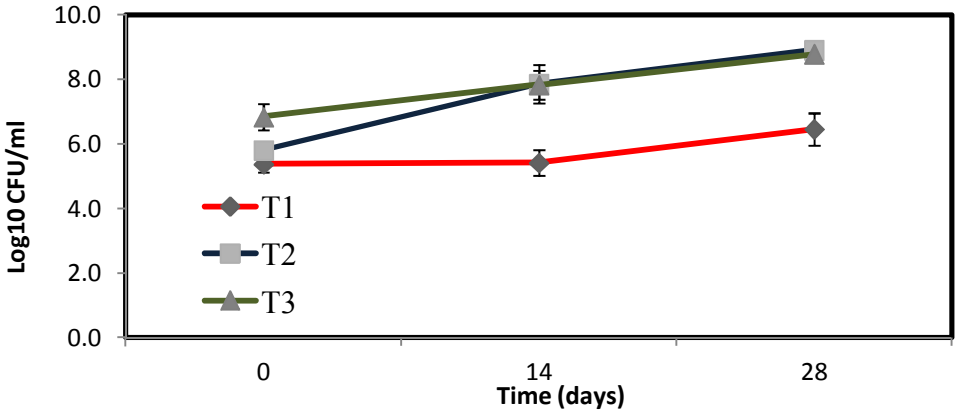

Fig. 1. Changes in total culturable heterotrophic bacterial (THB) counts of crude oil polluted soil in TI, T2 and T3 treatment setups during the study period.

The changes in population of HUBs in various treatment set ups are as presented in Fig. 2. In T_1, the change in population of the HUBs was not significant (p<0.05) as increase in $\log_{10}$count was from 4.36 CFU/g to 4.49 CFU/g. However, in T_2, and T_3, there were significant increases (p<0.05) in the HUB population from $\log_{10}$ 4.38 CFU/g on day 0 to $\log_{10}$ 6.46 CFU/g by the 28th day and from $\log_{10}$ 4.39 CFU/g at onset of treatment to $\log_{10}$8.54 CFU/g by end of treatment, respectively. The highest HUB count of 3.5x10^8 CFU/g was obtained in T_3 at day 28 of the study period (Fig. 2).

The rapid proliferation of HUBs in T_2 and T_3 may be attributable to the addition of NPK fertilizer to the soil in those treatment set ups. These nutrients (nitrogen, phosphorus and potassium) probably stimulated microbial growth and allowed microbes to synthesize the necessary enzymes needed to break down the petroleum hydrocarbon contaminants since they are the basic building blocks of life (Vidali, 2001). Albeit, the HUBs were present in contaminated soil, their numbers may not have been sufficient to initiate effective remediation of contaminated soil. Hence, the growth and activities of the HUBs must be stimulated through the provision of nitrogen, phosphorous, and carbon as building blocks which are utilized by these degrading microorganisms for their active growth and metabolic performance (Van Hamme *et al.*, 2003). Previous studies have demonstrated that nitrogen is essential for cellular protein and cell wall configuration, while phosphorus is needed for nucleic acids, cell membrane and ATP formation (Swindell *et al.*, 1988).

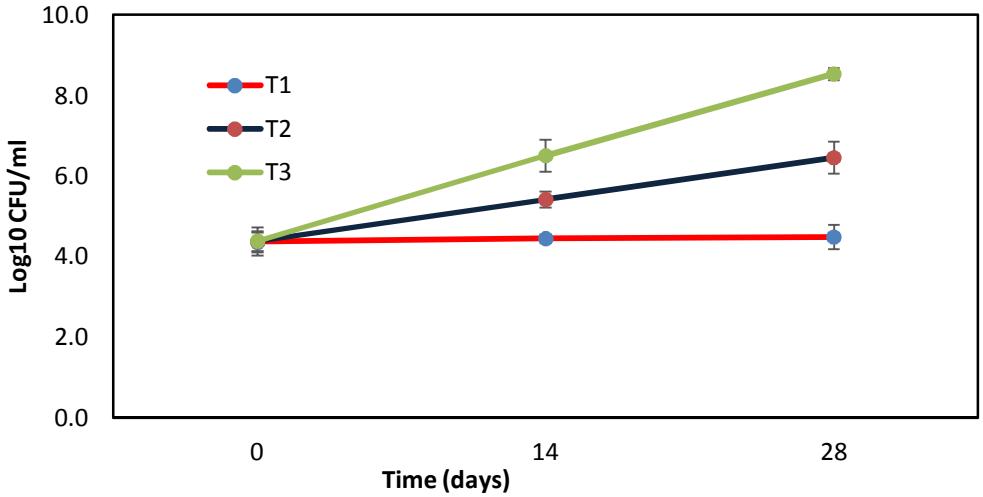

Fig. 2. Changes in hydrocarbon utilizing bacterial (HUB) counts of crude oil polluted soil in TI, T2 and T3 treatment setups during the study period.

The NFB population in T_1showed a marginal increase from $\log_{10}$3.32 CFU/g on day 0 to 3.51 CFU/g by the 28th day whereas in T_2, increase in $\log_{10}$ NFB was from 2.20 CFU/g at onset of treatment to 3.26 CFU/g by day 28. The population and proliferation of nitrogen fixing bacterial (NFB) population in $T_2$was not significant (p>0.05) and this could be attributable to the toxic effect of hydrocarbon fractions in the crude on the microorganisms. Although the nitrifying bacteria have similar cell wall morphology as Gram-negative rods (Holt *et al.*, 1994), they responded differently to hydrocarbon components in T_2. The difference in response of these bacteria to crude oil components in soil environment may be due to genetic differences (Greenwood *et al.*, 1996). However, in T_3amended with the microbial inoculant (NFB), there was a significant proliferation (p<0.05) of the NFB from $\log_{10}$ 4.41 CFU/g on day 0 to $\log_{10}$8.42 on day 28 and this could be attributed to the augmentation of the NFB population in T_3at the onset of treatment. The highest population of NFB (2.6 x 10^8CFU/g) was obtained in T_3at the end of the treatment (Fig. 3).

The hydrocarbon utilizing bacterial (HUB) isolates obtained belonged to the following genera: *Corynebacterium; Staphylococcus; Pseudomonas; Achromobacter; Klebsiella; Serratia; Bacillus; Micrococcus; Clostridium; Acinetobacter; Flavobacterium; Citrobacter* and *Alcaligenes.* Some of these isolated bacterial genera have been reported to contain special enzyme system including dehydrogenase and oxygenase that help degrade crude oil contaminants (Atlas, 1981; Delille and Coulon, 2008; Agarry and Ogunleye, 2012).

The nitrogen fixing bacteria (NFB) isolated in this study were mainly members of the following genera: *Nitrobacter, Nitrosomonas*, *Archromobacter, Burkholderia, Azotobacter, Arthrobacter* and *Alcanivorax.*

Previously, Ibiene and Okpokwasili (2011) had reported that autotrophic adaptation and transformation by nitrifying bacteria may be hindered in an ecosystem polluted with crude oil, as nitrification processes will be reduced in the environment. In that report, the crude oil had negative effects on both nitrite oxidations by *Nitrobacter*sp. and ammonia oxidation by *Nitrosomonas* sp. in the crude oil polluted soil as were indicated by their reduced number and this was attributed to the toxicity of the crude oil components. Other researchers also corroborate this observation (Bitton, 1983; Watanabe, 2001).

Changes in nitrogen content, phosphate content, total organic content and pH of soil in T_1, T_2 and T_3treatment cells during the study are as presented in Fig. 4. In the T_1 cell (Figs. 4–7). Fluctuations in soil nitrogen, phosphorus and TOC in all treatment cells were devoid of a trend and insignificant (p<0.05) though there was a slight decrease in TOC in T_3. The breakdown of hydrocarbons without a concomitant mineralization of the biodegradation metabolites may not result in a decline in TOC of soil which may explain the reason for TPH decline in T_2 and T_3treatment cells without a considerable TOC removal.

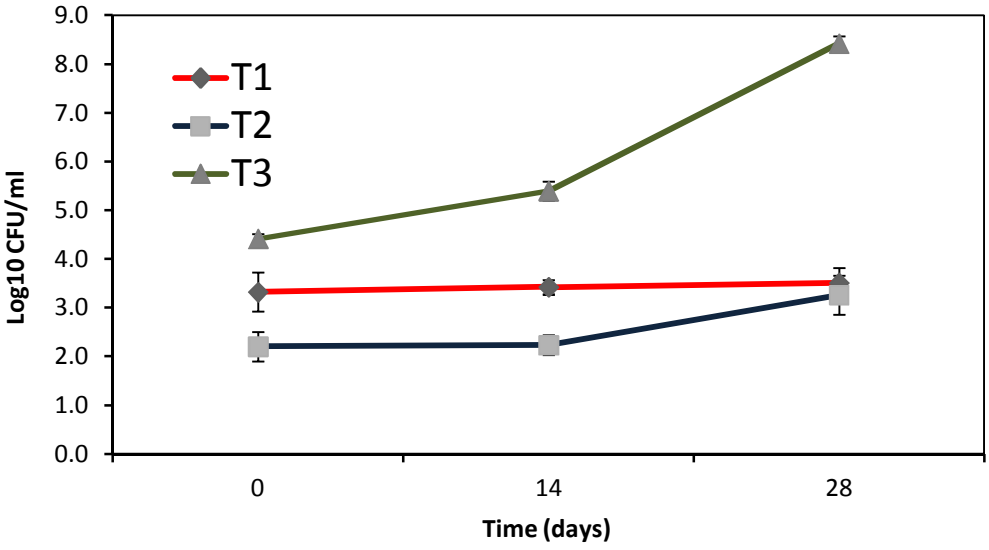

Fig. 3. Changes in nitrogen fixing bacterial (NFB) counts of crude oil polluted soil in TI, T2 and T3 treatment setups during the study period.

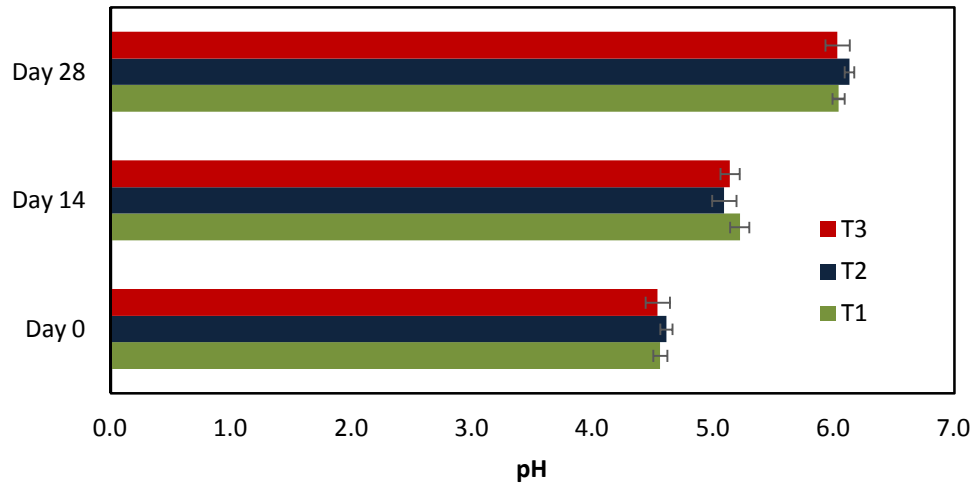

Fig. 4. Changes in pH in various treatment setups during the 28 day study period

Soil pH in all three treatment cells tended towards alkalinity (Fig. 4) suggesting the production of alkaline metabolites from the degradation of the petroleum hydrocarbons by the HUBs. The shift in pH towards neutrality during the study period may have facilitated the extinction of the petroleum hydrocarbons in the polluted soil since neutral pH is optimal for bacterial growth and metabolism. Previous reports have attributed optimum biodegradation of petroleum hydrocarbons in soil to shift in pH from acidic range to neutral range Atlas, 1981; Hussemann, 1993).

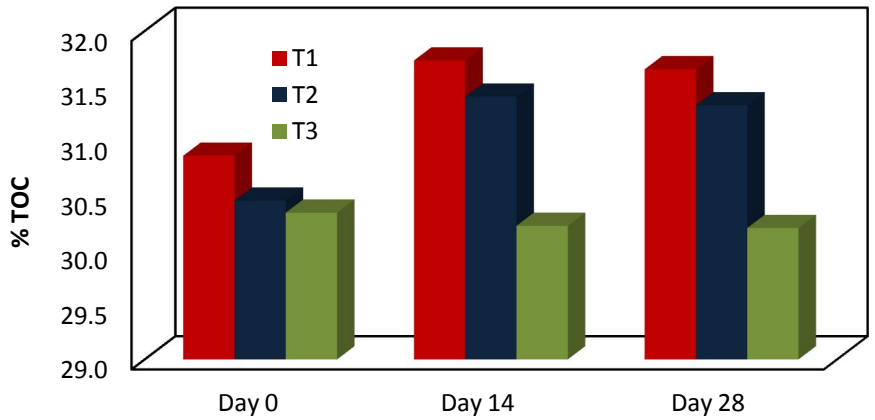

Fig. 5. Changes in % total organic carbon in various treatment setups during the 28 day study period

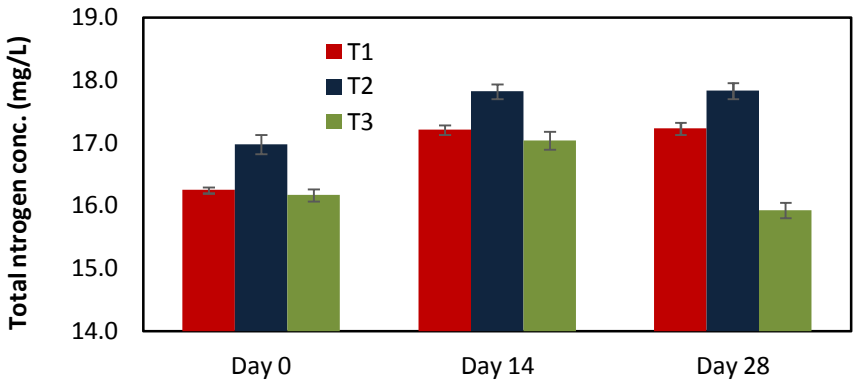

Fig. 6. Changes in total nitrogen concentration (mg/L) in various treatment setups during the 28 day study period

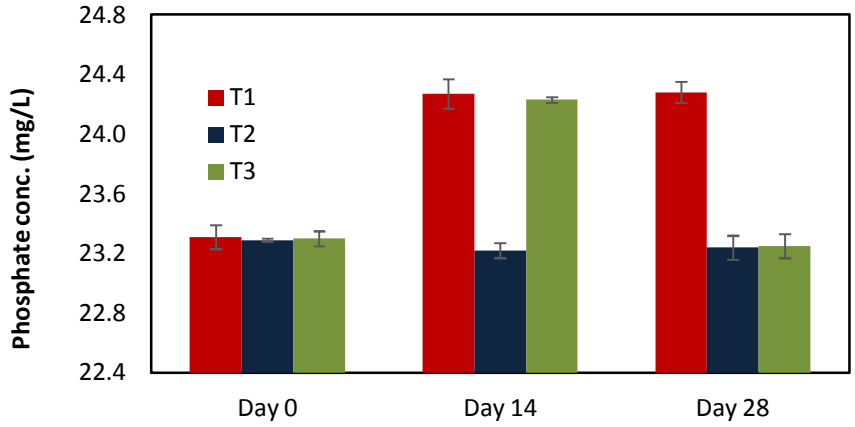

Fig. 7. Changes in phosphate concentration (mg/L) in various treatment setups during the 28 day study period

The changes in TPH of soil in T_1, T_2 and T_3 during the 28 day treatment period are as depicted in Figs. 8–9. Reduction in TPH in the control setup (T_1) was just 10.8 % after 28 days. However, in T_2 amended with NPK, decline in TPH was 75.8% whereas in T_3 amended with NPK and NFB, a decrease of 78.5 % was obtained in 28 days (Fig. 9). The order of TPH extinction rate in the three bioremediation treatment cell (setups) during the 28 day study period was T_3> T_2> T_1. The NPK amendments in T_2 and T_3 were responsible for the enhanced bioremediation of the soil in those treatments when compared to the course of events in T_1 treatment cell (non-amended polluted soil). Nitrogen and phosphorus in the appropriate ratio favour the proliferation of microorganisms in the soil and allow microbes to synthesize the necessary enzymes needed to break down the petroleum hydrocarbon contaminants (Vidali, 2001). The activities of these microbes may have resulted in decrease in TPH concentration obtained. Furthermore, the introduction of NFB in $T_3$was probably the reason for the enhanced bioremediation process (78.5%) when compared to T_2 (75.8%) since NFB can fix nitrogen gas from the atmosphere in the soil thus, increasing the nitrogen content of the soil and ameliorating the imbalanced carbon: nitrogen ratio occasioned by the crude oil spike in the soil. Other researchers have reported that the toxicity arising from accumulation of excess nitrogen and its compounds from mineral and chemical fertilizers can be greatly reduced by *in situ* seeding of these microorganisms (Bitton, 1983; Delille and Coulon, 2008; Solomon *et al.,* 2016).

Albeit, HUB are present in soil, their numbers might not be adequate to initiate effective remediation of contaminated sites. Hence, the growth and activities of these hydrocarbonoclastic bacteria must be stimulated through the extraneous provision of nitrogen and phosphorous which they require as building blocks. Previous studies have demonstrated that nitrogen is essential for cellular protein and cell wall configuration, while phosphorus is needed for nucleic acids, cell membrane and ATP formation (Swindell *et al.*, 1988). Thus, bioremediation of petroleum contaminated soil requires an adequate supply of these elements, which in turn are utilized by HUB for their active growth and metabolic performance (Van Hamme *et al.*, 2003).

This result is in accordance with a similar trend reported by other researchers (Odokuma and Inor, 2002; Solomon *et al.*, 2015). Their report showed 80% loss of crude oil when 5g slurries of microorganisms including *Azotobacter* and inorganic nutrient were used as amendment to enhance bioremediation of a crude oil polluted soil *ex situ*.

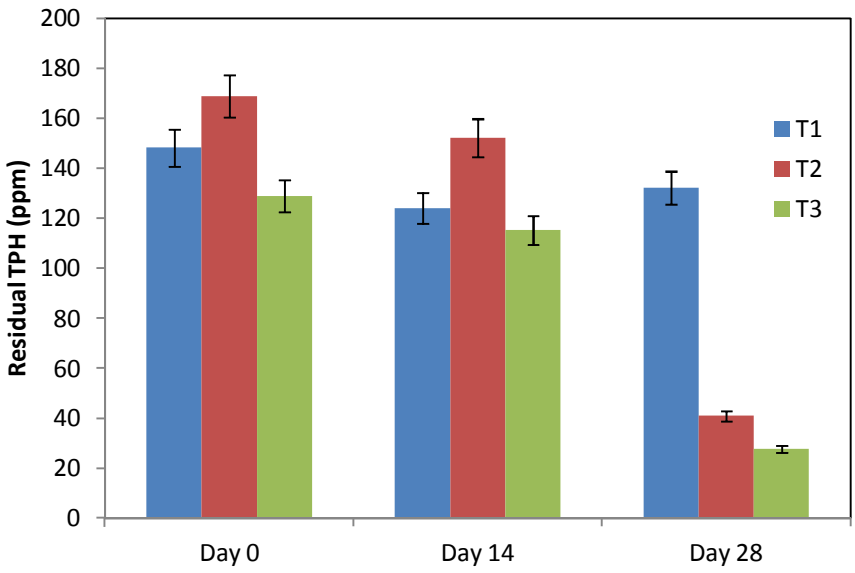

Fig. 8. Residual TPH in soil of various treatment setups (T1, T2 and T3) during the 28-day study period

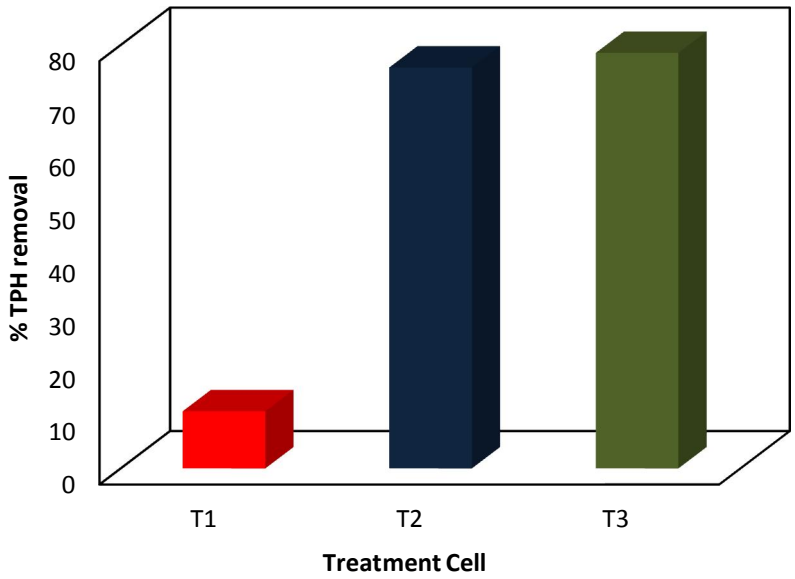

Fig. 9. Percentage TPH removal in various treatment cells due to the actiivities of HUB

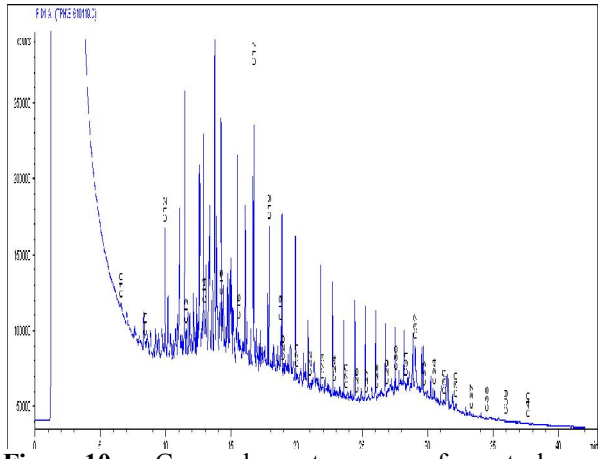

Fig. 10. Gas chromatogram of petroleum contaminated soil in T_1 subjected to 28 days of treatment.

Gas chromatograms of soil samples from the three treatment set ups (T_1, T_2 and T_3) indicating peak attenuation of various carbon fractions in the crude oil after the 28–day study period are as presented in Figs. 10—12. Peak extinction of the various carbon fractions after 28 days was more evident in soil from T_3 treatment cell when compared to T_2 and T_1. In the non-amended soil (T_1), many of the peaks of the various TPH fractions were not attenuated (Fig. 10) thus, suggesting the post-spike persistence of these fractions even after 28 days. However, the attenuation of the fractions in the amended soil especially in T_3 (Fig. 12) when compared to T_1 was an indication of petroleum hydrocarbon degradation by the soil microbe facilitated by NFB seeding and NPK amendment. Hydrocarbon utilizing microorganisms mineralize TPH into harmless carbon dioxide via Rubredoxin: NADH oxidoreductase reactions during catabolic reactions for energy generation. Carbon fractions in the range of C_2-C_8 chain length were not detected by the GC analysis since carbon chain lengths< C_8 are volatile and may have escaped from the soil into atmosphere via volatilization soon after spiking.

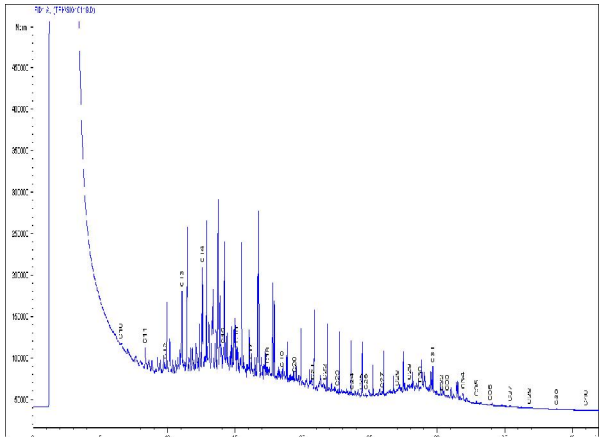

Fig. 11. Gas chromatogram of petroleum contaminated soil in T_2 subjected to 28 days of treatment.

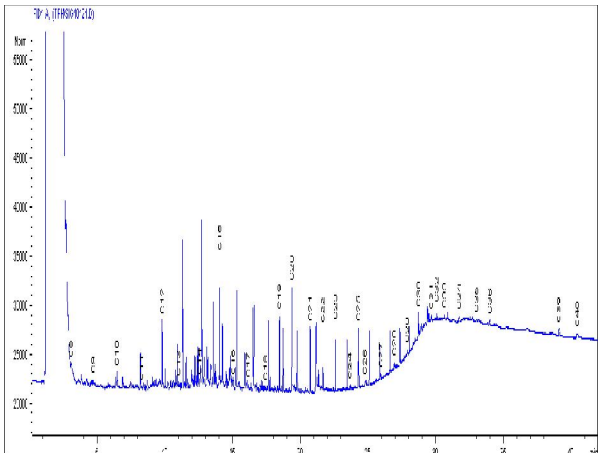

Fig. 12. Gas chromatogram of petroleum contaminated soil in T_3 subjected to 28 days of treatment.

4 Conclusion

The data from this study espouse the stimulation of biodegradation activities of HUB by NFB seeded to petroleum contaminated soil after 28 days. The NFB in conjunction with NPK amendment enhanced the bioremediation of a petroleum hydrocarbon polluted soil thus suggesting its biotechnological potential as a veritable agent for nitrogen content elevation in such polluted soils.

The seeding of nitrifying bacteria which play a vital role in nitrogen cycle and thus soil fertility to crude oil contaminated soil could help in preventing the accumulation of nitrogen (caused by excessive NPK application) by enhancing its fixation in a form that is readily utilizable by microbial cells *in situ* without adverse effects on the environment. This will stabilize soil nitrogen, organic nutrients and improve HUB metabolic activities, thereby enhancing biodegradation rates under conditions of nitrogen deprivation.

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