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Laboratory Scale Bioremediation of Petroleum Hydrocarbon – Polluted Mangrove Swamps in the Niger Delta Using Cow Dung

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ABSTRACT

Aim: The aim of the study was to carry-out Laboratory–scale bioremediation of Petroleum hydrocarbon polluted Mangrove swamps using Cow dung as source of limiting of nutrients.
Methodology and Results: In a 70 days study, the cow dung treated polluted soil had its total culturable hydrocarbon utilising bacterial/fungi, heterotrophic bacterial and fungal counts increased progressively from the 28th day to the 70th day. The control set- up showed very slight increment in its microbial growth. Alkaline pH was observed in all the treatments and control during the study period. The conductivity values of cow dung decreased progressively. In the cow dung treatment option, the nitrate concentration decreased from 35.44 mg/kg to 14.28 mg/kg. Phosphate concentration of cow dung option decreased from 25.41 mg/kg to 9.31mg/kg. The control had the nitrate decreased from 8.42 mg/kg to 6.98 mg/kg. Percentage total organic carbon (% TOC) in the cow dung option decreased from 4.06 % to 0.96 % Control experiment had the % TOC decreased from 3.32 % to 2.99 %. Studies using Gas chromatographic analyses showed that 0 %, 49.88 %, and 69.85 % of Total petroleum hydrocarbon (TPH) were lost at zero hour, 28th day and 70th day respectively in the cow dung option. In addition, in the control experimental set-up, 0 %, 7.14 % and 13.42 % of TPH were lost at zero hour, 28th day and 70th day respectively.
Conclusion significance and impact of study: The use of organic nutrient sources such as cow dung has shown good promises in bioremediation of crude oil impacted Mangrove Swamps in the Niger Delta. The next line of action is to transfer the technology to pilot scale study.

Keywords: Petroleum Hydrocarbon, Pollution, Mangrove Swamps, Bioremediation, Niger Delta, Nigeria

INTRODUCTION

The Niger Delta Development Commission (NDDC) estimates the size of the Niger Delta at 112,000 square kilometres, inhabited by more than 3,000 long-settled communities. The population in the oil and gas producing regions of the Niger Delta is constantly rising. In 1991 there were approximately 20.5 million people there. Today there are about 30 million (SPDC, 2011) and that figure will rise to 46 million by 2020 according to a recent report compiled by the United Nations Development Programme (UNEP, 2011). Subsistence farming/fishing is the mainstay of the people (UNEP, 2011).

The Niger Delta ecosystem is particularly sensitive to changes in water quality, such as salinity or pollution. The Niger Delta is a wetland containing a number of ecological zones: sandy coastal ridge barriers, mangroves, freshwater permanent and seasonal swamp forests, and lowland rain forests. The Niger Delta Mangrove Swamps provide grounds for commercial fishing, timber production, biotechnologically important microorganisms. However, pollution caused by petroleum and its by-products has greatly impacted negatively on the mangrove swamps leading to reduction in seafood output, increased food security challenges, reduction in biodiversity of mangrove biota, youth restiveness and violence in this region.

Bioremediation technology which gives much hope on the restoration of polluted mangrove swamps is being utilized for the degradation of crude oil in soil matrix by using microorganisms, to transform the petroleum hydrocarbons into less toxic compounds (Davies & Westlake, 1979).This is achieved by the help of bacteria, fungi, algae that produce enzymes capable of degrading harmful organic compounds (Davies & Westlake, 1979). In the presence of abundant hydrocarbon in the environment after a spill, microorganisms cannot effectively attack and utilise the hydrocarbon unless limiting nutrients such as nitrate, phosphate, even micro-elements are incorporated into the polluted medium (Van Hamme *et al*, 2003).

Different methods have been employed in order to deliver limiting nutrients and restore petroleum polluted media. Some of these include the use of oil degrading microorganisms, inorganic fertilizers, chicken droppings,

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periwinkle shell, liming and tilling (Leahy and Colwell 1990; Ijah *and Antai* 2003). Biostimulation using inorganic fertilizer has been extensively employed worldwide in reclaiming oil polluted soil (Dibble and Bartha 1979). The use of inorganic fertilizers as source of limiting nutrients has been extensively carried out. However, the use of inorganic fertilizers is still challenged by the large cost of bioremediation and likely chance of eutrophication/agal bloom especially in aquatic environments. It is worthy to state that a good remediation method must be environmentally friendly and affordable. The use of organic nutrients such as chicken droppings, periwinkle shells, cow dung for the bioremediation of crude Oil polluted environments other than mangrove swamps have been previously reported in Nigeria (Ijah and Antai, 2003; Obire *et al.*, 2008).

The objectives of this study were; to determine the effectiveness of cow dung as source of limiting nutrients in bioremediation of crude oil polluted mangrove, determine the possibilities of managing the underutilised cow dung waste through bioremediation. This study reports the use of cow dung for provision of limiting nutrients for microbial remediation (laboratory-scale) of crude Oil polluted mangrove swamps in the Niger Delta.

MATERIALS AND METHODS

Study area

The hydrocarbon polluted soil was obtained from mangrove swamp at Elibrada, Emuoha Local Government Area of Rivers State. The mangrove swamp is accessible through Elibrada junction. The mangrove swamp links Emuoha and Kalabari. This site was selected due to high level of pollution as a result of oil spillage form a pipeline owned by an upstream industry in Nigeria. The predominant mangrove plant in this area is *Rhizophora racemosa* as identified by Dr. Godfrey Akani of the Department of Applied and Environmental Biology, Rivers State University of Science and Technology, Port Harcourt, Nigeria. The major occupation of Elibrada people is fishing in the mangrove and agricultural/ land farming.

Sample collection

The soil sample was collected with a spade into a plastic pail which was cleaned with cotton wool soaked in 70 % alcohol (Eziuzor and Okpokwasili, 2009). Four sampling points were sampled and the soils from the sampling points were mixed together after excavation. The excavated soil was transported to the Environmental Microbiology laboratory of the University of Port Harcourt, Nigeria for bioremediation study. Co-ordinates of the sampling points were determined using Global Positioning System (GPS). The co-ordinates of the sampling points were: 453'53.990"N; 650'43.745"E and 4'5353.874"N; 650'43.564"E for soil sampling points 1 and 2(SS 01 and SS 02) respectively. The sampling Co-ordinates for soil sampling points 3 and 4 (SS 03 and SS 04) were 454'59.990"N; 620'43.745"E and 454'56.886"N 657'43.775"E respectively.

Soil contamination

The Elibrada mangrove has been contaminated by petroleum hydrocarbon about two years as a result of pipeline vandalization. Fifty millilitres of Bonny light crude oil was poured in each treatment option containing 500 g of the mangrove soil (including the controls). The aim of this further contamination was to simulate condition of a major spill. The polluted soil was at this point sampled for baseline studies. Bonny light crude oil used in this study was obtained from Shell Petroleum Development Company of Nigeria (Rumuobiakani, Port Harcourt, Nigeria

Preparation of cow dung

Cow dung of about 3 kg were obtained from cow Slaughter house in Choba cattle market and transported to the microbiology laboratory. The cow dung was sun dried for 5 days until moisture was driven off completely. The cow dung was stored for usage.

Experimental design

This is a laboratory scale experiment carried out in plastics pots.

Table 1: Bioremediation Design of the study

Experimental Group	Test experiment
Set A	500 g of polluted soil + 50 g of sterile cow dung
Set C (Control)	500 g of polluted soil + no nutrient

Each Experimental Group is established in three replicates

Bioremediation study

The polluted soils in plastic pots were amended with 50 g of cow dung. The control was never amended with nutrient. Sampling for laboratory studies started immediately after the soils were amended with nutrients (for the treatments). This is called zero day or zero hour study. The Cow dung treated plot and control were regularly turned using different hand trowels. Samples were taken for laboratory analyses at 2 weeks intervals on the 14^{th}, 28^{th}, 42^{nd}, 56^{th} and 70^{th} day. The bioremediation of petroleum hydrocarbons in the different experimental designs/set-up was studied/monitored using parameters such as total culturable heterotrophic bacteria and fungi counts, total culturable hydrocarbon utilising bacteria and fungi count, total hydrocarbon content (THC), total petroleum hydrocarbon (TPH), percentage loss in percentage total organic Carbon (%TOC), nitrate level, phosphate level, conductivity, pH.

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Enumeration of total culturable heterotrophic bacteria (TCHB)

Total culturable heterotrophic bacterial count present in the 3 different groups was determined at zero hour, 14th, 28th, 42nd and 56th day and 70th day of the experiment. The spread plate method on nutrient agar (Antech Laboratories LTD) was used. Swamp soil suspensions were prepared by 10 fold serial dilutions with 1 g of soil, using normal saline as diluents. 0.1 mL aliquots of appropriate dilutions were spread on triplicates of sterile nutrient agar. The plates were incubated for a period of 18-48 h in the incubator at 28 °C. Colonies that formed during this incubation period were counted using this formula;

$$\frac{\text{No of colonies x dilution factor}}{\text{Amount used}}$$

Values were expressed as colony forming units per g (Cfu/g). Enumeration of total heterotrophic bacteria was carried out using the stated procedures have been previously reported by some authors/workers (Chikere *et al.,* 2009; Nwachukwu *et al*., 2010).

Enumeration of total culturable hydrocarbon utilizing bacteria (TCHUB)

The enumeration of total culturable hydrocarbon utilizing bacteria was done by using the vapour phase method. Appropriate dilutions of the samples withdrawn from the three different conditions of 0 h, 14, 28, 42, 52 and 70 days of analyses were inoculated into modified mineral salt medium. The medium components were 0.42 g of $MgS0_4.7H_2O$, 0.297 g of KCl, 0.85 g of KH_2PO_4, 0.424 g of $NaNO_3$, 1.27 g of K_2HPO_4, 20.12 g NaCl, 250 mg of Amphotericin B (sold as Fungizone), and 20 g of Agar powder (Oxoid, Basingstoke, Hants, United Kingdom). These were weighed out and hydrated in 1000 mL of sterile distilled water in an Erlenmeyer flask. The medium was sterilized by autoclaving at 121 °C, 15 Psi for 15 min before dispensing into sterile petri dishes. The gelled mineral salt agar (MSA) was inoculated with appropriate dilutions of the swamp soil sample. Filter paper (Whatman No 1) was saturated with bonny light crude oil and the crude oil impregnated papers were aseptically placed onto the covers and Petri dishes inverted. The hydrocarbon saturated filter papers supply hydrocarbon by vapour-phase transfer to the inoculums (Amanchukwu *et al.,* 1989a; Amanchukwu *et al*., 1989b Atuanya and Ibeh, 2004; Abu and Chikere, 2006). The plates were incubated at 28 °C ± 2 °C for 7 days and colonies were counted from triplicates and mean values were recorded in colony forming units per g (Cfu/g).

Enumeration of Total culturable Hydrocarbon utilizing fungi count

Enumeration of total culturable hydrocarbon utilizing fungi was done using mineral salt agar (MSA) containing 10 g of NaCl, 0.45 g of $MgSO_4$ $7H_20$, 0.42 g KCl, 0.29 g of KH_2PO_4, 0.86 g of Na_2HPO_4, H_2O, 0.43 g of $NaNO_3$ and 25.0 g of Agar powder in 1000 mL. The medium was sterilized by autoclaving at 121 °C for 15 min, 10 Psi. Crude oil (Bonny light) served as both the sole carbon and energy sources. Three replicate plates inoculated by spread plating, were inverted over sterile filter papers moistened with sterile crude oil, which were placed on the lid of Petri dish covers. The plates were inverted over the dish covers containing the crude oil impregnated filter papers (Chukwura *et al.,* 2005). The crude oil used was bonny light, and the compounded medium for the hydrocarbon utilizing fungal count was amended with 250 mg of chloramphenicol and tetracycline (Chikere and Chijioke-Osuji, 2006; Obire *et al.,* 2008). Incubation was within 7 – 10 days at 28 °C ± 2 °C (Okpokwasili and Amanchukwu, 1988).

Enumeration of total culturable heterotrophic fungi (TCHF)

The medium of choice was the potato dextrose agar, (PDA) with 10 % tartaric acid using the spread plate method. The medium was prepared according to the manufacture's (Oxoid LTD) instruction and sterilized at 121 °C, 15 psi and 15 min before dispensing into sterile Petri plates. A 0.1 mL aliquot of appropriate dilutions (Normal saline diluent) of sample was inoculated unto the media. The plates were incubated in for 5-7 days at room temperature and colonies formed were counted and expressed as Cfu/g

Identification of bacterial isolates and fungal species

The identification of hydrocarbon utilising bacterial was based on biochemical characterisation such as sugar and alcohol sugars fermentation tests, citrate, catalase, indole, methylred, voges-prauskauer, starch hydrolyses, oxidase etc. The identification of hydrocarbon utilising fungal isolates was based on colonial appearances, wet mount preparation and the use of different staining technique such as Methylene cotton-blue, Indian Ink/Nigrosin Ink (Chukwura *et al*., 2004).

Physicochemical Analyses

pH and conductivity measurement (pH/conductivity meter electrometric method): The pH of the samples withdrawn at baseline, zero hour, 14, 28, 42, 56 and 70th day of the study was determined using a digital pH/conductivity meter (Jenway 3015, United Kingdom). At each point, three values were obtained and the mean of the values was used.

Measurement of Nitrate (NO_3^-) level of Polluted soil and composite analyses of cow dung: The brucine method was used. One millilitre of soil filtrate was measured into a clean test tube and 1 mL of distilled water was measured into a clean test tube and 1 mL of distilled water was measured into another test tube as blank

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solution. Half millilitre of brucine reagent was gently introduced into both test tubes. Two millilitre of concentrated sulphuric acid was then added and shaken to homogenize. The resulting solution was allowed to cool to room temperature. The solution turned yellow and was measured at 470 nm on spectrophotometer (UNEP, 2004).

Phosphate content of polluted mangrove soil and composite analyses of cow dung: The method used was the colourimetric method as described in United Nations Environmental Pro gme (UNEP, 2004). One-tenth of 2.5 % glacial acetic acid was prepared was prepared and used for the extraction of phosphate in 250 mL capacity conical flask. The mixture was stirred for 10 min. Fifty millilitre sample extract was pipetted into a clean conical flask. This aliquot was autoclaved with $K_2S_2O_8$ and H_2SO_4 for 30 min at 121 °C, Five millilitre of ammonium molybdbate was added to the autoclaved mixture to form heteropoly molybdophosphoric acid and was reduced with stannous chloride in an aqueous sulphuric acid medium, at 30 °C, to form a molybdenum blue complex. The resulting blue colour was measured spectro-photometrically at 660 nm and compared to identically prepared standard (water). The detection limit of this method is 0.001 mg/L (UNEP, 2004).

Total organic carbon (%TOC) determination: Total organic carbon is an alternative analytical method for measuring petroleum hydrocarbons using the wet oxidation technique as previously reported by Nelson and Sommers (1975). One gram of the sample was transferred into a clean pyrex conical flask. Five millilitre potassium chromate solution and 7.5 mL concentrated sulphuric acid were added. The mixture was heated on an electrothermal heater for 15 min to reflux. The sample was cooled to room temperature and diluted to 100 mL with distilled water. Twenty five millilitre of the sample solution was titrated with 0.2 M ferrous ammonium sulphate using Ferrion as indicator. A blank containing oxidant (potassium chromate) and sulphuric acid was titrated as in the sample and the titre value was recorded. Calculation was as follows:

$$\% \ TOC \ = \ \frac{Titre\ value\ of\ blank - sample\ titre}{sample\ weight} \ \times \ 0.003 \times 100$$

Chemical Analyses

Total Hydrocarbon Content (THC): Five grams of soil sample was weighed into a beaker and 10 mL of xylen was added under cork cover for 30 min. Aliquot of the extract was placed in the infrared spectrophotometer analyzer. The total hydrocarbon (THC) value was determined by comparism to a calibration curve constructed from dilutions of a stock solution of a 1:1 bonny light crude, and bonny medium. The spectrophotometric measurement was at 420 nm (UNEP, 2004; Osuji and Ezebuiro, 2010).

Total petroleum hydrocarbon (TPH): The extraction of petroleum hydrocarbon was done with dichloromethane (DCM) using cold extraction method with ASTM D-3694 heavy machine for 1 h. Procedurally, 20 g of dried soil samples was weighed into 100 mL conical flask. Twenty grams of activated anhydrous sodium sulphate and 20 mL of DCM were gently added into the barrier containing the test soil sample. This was allowed to stand for 1 hour and then filtered into 50 mL conical flask using filtration plugged/packed with cotton wool. Procedure was repeated on the residual soil until a colourless solution was obtained. The extracts were analyzed by gas chromatography, using Hp Agilent 6890 gas chromatography (Agilent technologies, 610 Wharfdale Road, Wokingham, Berkshire, United Kingdom) equipped with a FID detector, an Agilent 7673 auto sampler and 5 capillary column (15m x 0.25mm) with a nominal film thickness of 0.25 µm, splitless injection method (all in batch). Injection volume was 1 µL and injection temperature was 330 °C. Helium was used as a carrier gas (2 mL/min). The column was held at 35 °C for 1.50 min. Real values of TPH were calculated as product of raw data on FID table or graph and dilution factor used for each sample (Saari *et al.,* 2007).

Calibration Check and Instrument precision: Calibration was done using Bonny light crude oil, acetone, and mixture of Bonny light crude and acetone. The gas chromatography machine and peak sum method can only detect ≥ C_{10} as was saved in the sequence.

Calculation of Percentage loss in THC /TPH: This was calculated using the formula below:

% Loss in TPH/THC =

$$\frac{\text{Concentration at a point — Conc. at time zero}}{\text{Concentration at time zero}} \ \text{X } 100$$

Statistical Analysis

Statistical analyses were carried out using statistical package for social sciences (SPSS, Version 17.0). Analysis of variance (ANOVA), P – values, tests of significance, was carried out at 95 % level of confidence using statistical package for social sciences. P – Values were used to determine the significance levels between various treatments and data obtained during the experimental study.

RESULTS AND DISCUSSION

Microbiological studies

The initial/baseline physico-chemical and microbiological characteristics of the crude-oil polluted mangrove soil was documented in **Table 2**. The pH, conductivity, nitrate, phosphate, and percentage total organic carbon were 7.5 ± 0.04, 2380 ± 3.12, 10.52 ± 0.286, 8.11 ± 0.021, 3.30 % respectively. The total petroleum hydrocarbon and total hydrocarbon content were 12934.75 ± 00 and 14102.22 ±

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0.04 respectively. The total culturable heterotrophic bacterial count, total culturable heterotrophic fungal count, total culturable hydrocarbon utilizing bacterial count, total culturable hydrocarbon utilizing fungal count were 6.1 x 10^5 ± 0.004 Cfu/g, 4.8 x 10^5 ± 0.111 Cfu/g, 3.5 x10^4 ± 0.025 Cfu/g, 2.6 x 10^4 ± 0.025 Cfu/g respectively. The baseline data results showed that the hydrocarbon utilizing microbes in the mangrove soil is relatively adequate for bioremediation (Ebuehi *et al*., 2005). This is not surprising as the mangrove soil has history of crude oil pollution due to pipeline vandalization. The populations of heterotrophic and hydrocarbon utilizing bacteria and fungi are presented in **Figures 2-3**. In addition, during the study period, the total culturable heterotrophic bacterial population of the cow dung (CD) amended option increased from 6.03 x 10^5 Cfu/g at zero hour to 2.82 x 10^7 Cfu/ g at the 70th day (**Figure 2**). The total heterotrophic bacterial count in the control experiment ranged between 5.6 x 10^5 Cfu/g to 6.20 x 10^5 Cfu/g. In both cases the growth of the heterotrophic bacterial organisms was lowest at zero hour and highest on the 70th day. There was no significant difference at p<0.05 level for cow dung and control experiments (F [2, 15] = 2.277).

The total culturable heterotrophic fungal count at baseline was 4.8 x 10^5 Cfu/g. This increased in cow dung amended polluted soil from 4.87 x 10^5 Cfu/g at zero hour to 2.45 x 10^7 Cfu/ g at the 70th day, (**Figure 1**). The control option which was not amended showed very slight increase in the total culturable heterotrophic fungal count. Statistical analyses have shown that there was significant difference at p<0.05 level for the two conditions (cow dung and control).

The hydrocarbon utilizing bacterial organisms responded to the nutrient amendment with cow dung. The population of hydrocarbon utilizing bacteria count ranged between 3.4 x 10^4 Cfu/g to 2.8 x 10^7Cfu/g from zero hour to 70th day of study (**Figure 2**). The total culturable hydrocarbon utilizing bacterial counts in the control experiment ranged from 2.5 x 10^4Cfu/g at zero hour to 1.68 x 10^5Cfu/g at 70th day of the study (**Figure 2**). Post HOC comparisons using the tukey HSD test indicated that the mean score for control (m = 4.7259, SD = 0.48758) differed significantly from the mean scores of cow dung treated option. In the cow dung experiment, the total culturable hydrocarbon utilizing fungal count ranged from 2.7 x 10^4 Cfu/g at zero hour to 1.6 x 10^7 Cfu/g at 70th day. The control had its total culturable hydrocarbon utilising fungi increased from 2.6 x 10^4 to 1.81 x 10^5Cfu/g. (**Figure 2**) Statistical analyses have also shown that there was significant difference at the p<0.05 level for the two conditions [f (2, 15) = 8.969, p<0.003). The response of indigenous hydrocarbon utilizing bacteria and fungi to the bioremediation treatment was generally positive with higher population occurring progressively as time elapsed. The bacterial and fungal species exhibited ability to either degrade or utilize the different petroleum hydrocarbon components as sole carbon sources. Okolo *et al.,* 2005 in studies on bioremediation of crude oil polluted sandy-loamy soil, reported that poultry dropping was able to significantly sustain increase in the population of fungal organisms utilizing crude oil.

Table 2: Baseline Physico-chemical and Microbiological properties of crude oil-impacted mangrove soil in the Niger Delta

Parameters	Values S.D
pH	7.5 ± 0.04
Conductivity (µs/cm)	2380 ± 3.12
Nitrate (NO_3^-) (mg/kg)	10.52 ± 0.286
Phosphate (PO_4^-)(mg/kg)	8.11 ± 0.021
Total organic carbon (%)	3.30 ± 0.00
Total petroleum hydrocarbon (mg/kg)	12934.75±0.00
Total hydrocarbon (mg/kg)	14102.22 ± 0.127
Total culturable heterotrophic bacterial count (Cfu/g)	6.1x 10^5 ± 0.004
Total culturable heterotrophic fungal count (Cfu/g)	4.8 x 10^5 ± 0.111
Total culturable hydrocarbon utilizing bacterial count (Cfu/g)	3.5 x 10^4 ±0.025
Total culturable hydrocarbon utilizing fungal count (Cfu/g)	2.6 x 10^4 ± 0.024

Cfu/g; colony forming unit per g, µs/cm; microsiemmens per centimetre

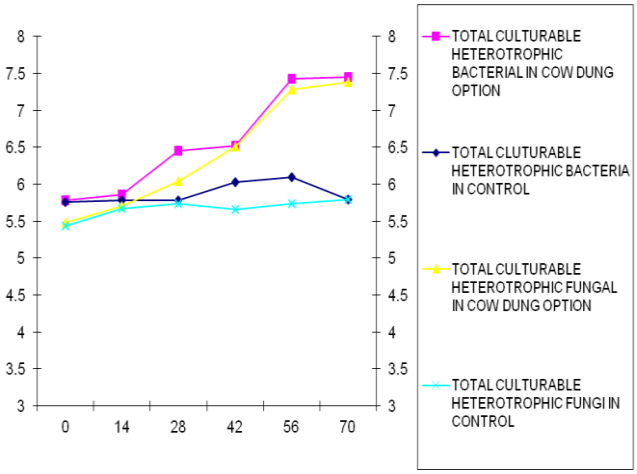

Figure 1: Changes in total culturable heterotrophic bacterial/ fungal counts during the 70 days study

The hydrocarbon utilizing bacterial isolates from this study included *pseudomonas* species, *Bacillus* species, *Citrobacter* species, *Micrococcus* species, *Vibrio* species, *Flavobacterium* species, and *Corynebacterium* species (**Table 3**). In addition the hydrocarbon utilizing fungal isolates include *Rhizopus* species, *Aspergillus* species, *Fusarium* species, *Penicillium* species, *Sacharomyces* species, and *Mucor* species (**Table 4**). Eziuzor and Okpokwasili (2009) studied bioremediation of crude oil polluted mangrove soil in Port Harcourt. They used NPK

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(Nitrogen phosphorus potassium) fertilizer as source of limiting nutrient and the hydrocarbon utilizing bacteria isolated include: *Acinetobacter*, *Arthrobacter, Bacillus, Citrobacter, Alcaligenes, Flavobacteria*, *Pseudomonas*, *Vibrio* and *Corynebacterium*. In Nigeria, the fungi reported as oil degraders in aquatic environments of petroleum producing areas by Obire (1988), are *Aspergillus, Aureobasidium, Candida, Rhodosporodium, Cepthalosporium*, *Rhodotorula, Saccharomyces*, *Aspergillus niger, Aspergillus terreus, Blastomyces* spp., *Botryodioplodia*, *Fusarium* spp., *Nigrospora* spp., *Penicillium* and *Trichoderma harianum*.

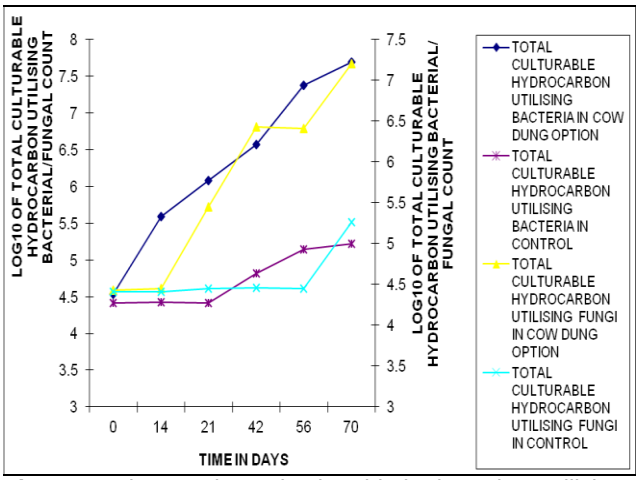

Figure 2: Changes in total culturable hydrocarbon utilising bacteria and fungi counts

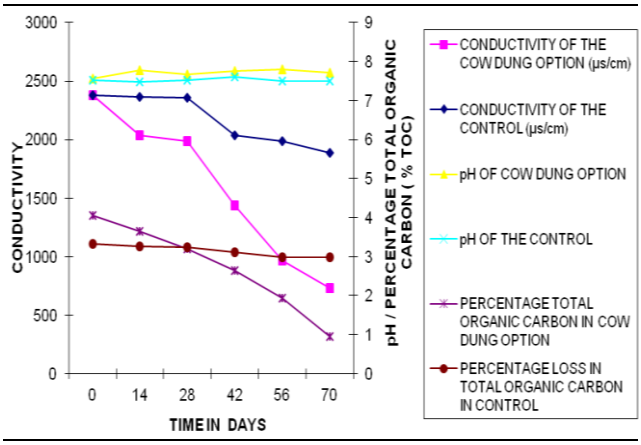

Figure 3: Changes in Conductivity (µS/cm), pH, and Percentage Total Organic Crabon (% TOC).

Physico-chemical studies

The pH of the polluted mangrove soil was slightly alkaline. In the cow dung experimental set-up, the pH at 0 hour, 14th day, 28th day, 42nd day, 56th day and 70th day were 7.59, 7.78, 7.68, 7.77, 7.91, 7.71 respectively. In the control (without nutrient), the pH for the zero hour to 70th day were 7.59, 7.47, 7.68, 7.75, 7.79 and 7.71 respectively (**Figure 3**). Statistical analyses showed that there was statistical significance at the p<0.05 for the two conditions [f (2, 25) = 8.969, p<0.003]. Post HOC comparisons test indicated that the mean score for the control (M = 7.5250, SD = 0.04637) differed significantly from the mean score for cow dung (m = 7.7167, S. D = 0.074) at 95 % confidence interval. The pH value of the cow dung from the composite study was 8.20 ± 0.820 (**Table 5**).

Table 3: Biochemical identities of the hydrocarbon utilising bacterial species

S/No	Isolate No	Morpholo-Gy & Gram Reaction	Identity Of Bacterial Species	Sucrose	Mannitol	Lactose	Glucose	Oxidase	H_2S Production	Starch Hydrolsis	Voges Pros-kauer	Methyl Red	Indole	Catalase	Citrate	Motility
1.	HUB_1	Rods -	*Pseudomonas* species	+	+	+	+G	+	-	-	-	-	-	+	+	+
2.	HUB_2	Rods -	*Pseudomonas* species	+	+	+	+G	+	-	-	-	-	-	+	+	+
3.	HUB_3	Rods +	*Bacillus* species	-	+	+	+	-	-	+	+	+	-	+	+	+
4.	HUB_4	Rods +	*Bacillus* species	-	+	+	+	-	-	+	+	+	-	+	+	+
5.	HUB_5	Rods +	*Bacillus* species	-	+	+	+	-	-	-	+	+	-	+	+	+
6.	HUB_6	Rods -	*Citrobacter* species	+	+	+	+G	-	+	+	-	+	-	+	+	+
7.	HUB_7	Cocci+	*Micrococcus* species	+	+	+	+	+	-	-	-	+	-	-	-	-
8.	HUB_8	Rods -	*Vibrio* species	+	+	-	+	+	-	+	+	+	+	+	+	+
9.	HUB_9	Rods -	*Vibrio* species	+	-	-	+	+	-	-	+	+	+	+	+	+
10.	HUB_{10}	Cocci +	*Micrococcus* species	+	+	+	+	+	-	-	-	+	-	+	-	+
11.	HUB_{11}	Rods -	*Flavobacterium* species	+	-	-	+	+	+	-	-	+	-	+	+	-
12.	HUB_{13}	Rods -	*Flavobacterium* species	+	-	-	+	+	+	-	-	+	-	+	+	-
13.	HUB_{14}	Cocci +	*Micrococcus* species	+	+	+	+	+	-	-	-	+	-	+	-	-
14.	HUB_{15}	Rods +	*Corynebacterium* species	+	-	-	-	-	-	-	+	-	-	+	-	-
15.	HUB_{16}	Cocci +	*Micrococcus* species	+	+	+	+	+	-	-	+	+	-	+	+	+

+G-Positive with gas production positive, -; negative, and +; positive

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Table 4: Identities of the hydrocarbon utilizing fungal isolates

Isolate	Culture characteristics	Microscopic characteristics	Probable genera
HUF_1	Pure white, thick and abundant cottony mycelium. Reverse was white	Non-septate sporangiophores, rhizoid, spongisphore and black sporangium containing hemispherical collumela	*Rhizopus* spp.
HUF_2	Powdery, dark brown, flatty spread on the surface of the solid medium and brown reverse.	Septate and branched hyphae with conidia in chains	*Aspergillus* spp.
HUF_3	Whitish and cottony mycelium with pink at the centre, and brown reverse	Multi segmented canoe-shaped spores , and branched Conidiophores.	*Fusarium* spp.
HUF_4	Powdery, dark brown, flaty spread on the surface of the solid medium with brownish reverse	Septate and branched hyphae and conida in chains	*Aspergillus* spp.
HUF_5	Grey colonies that were large With white border. Reverse was white	Long conidiophores consisting of broom like conida in chains	*Penicillium* spp.
HUF_6	Whitish and cottony	Segmented canoe-like spores, with branched and segmented conidiophores	*Fusarium* spp.
HUF_7	Yellow green dense mycelia. Powdery, and light yellow reverse	Long conidiophores consisting of broom like conidia in Chains	*Penicillium* spp.
HUF_8	White colony mycelium which developed within 4 days with extensive sub-surface	Coarse hyphae that segment into rectangular arthrospores varying in sizes	*Geotrichium* spp.
HUF_9	White and cottony mycelium	Multi-segmented canoe-like spores with branched and segmented conidiophores	*Fusarium* Spp.
HUF_{10}	Creamy ovoid colonies which were easily picked	Spherical cells in clusters with buds	*Saccharomyces* Sp.
HUF_{12}	Grey to black and thick abundant cottony mycelium, and white reverse	Non-septate hyphae with sporangium containing black, sporangiospores, columella separated by septum, and without rhizoids	*Mucor* spp.

In the cow dung amended polluted mangrove soil, conductivity decreased from 2380 µs/cm to 713 µs/cm. In addition, control experimental set-up showed a slight decrease in conductivity from 2381 µs/cm - 1884µs/cm (**Figure 3**). The conductivity value of the cow dung was 60µs/cm **±** 0.01 (**Table 5**).There was significant difference in the (2) conditions [f (2, 15) = 0.289, p = 0.753) at 95 % confidence interval. It is worthy to mention that the pH values recorded in the experiment did not follow a consistent pattern/trend. This pH fluctuation may be as a result of production of metabolites at different stages/period of the bioremediation. Conductivity studies in bioremediation experiments both in-situ and ex-situ is related to salinity but it is often used than salinity as a result of ease of measurement (Zhu *et al.,* 2001). In the baseline study of the polluted mangrove soil, the conductivity level was 2380 µs/cm. This high value shows that the environment is salt water mangrove or brackish water mangrove. But observation at the environment shows fluctuation of the salty taste of mangrove water within two tides of the day. This fluctuation is an indication that the mangrove of study is a brackish water mangrove. Abu and Akomah (2008) also reported a decrease in the conductivity of a treated wetland undergoing bioremediation under laboratory simulation. They cited the uptake of exchange ions by microorganisms as the major reason for such decrease. Furthermore, amendment of the soil with cow dung increased the percentage total organic carbon (%TOC) to 4.06 %. The %TOC decreased from 4.06 % to 0.96%. The control experiment showed little decrease from 3.32 % to 2.99 % within the 70 days period of study (**Figure 3**). There is a co-relation between the changes in the Conductivity and Total Organic Carbon of the treated polluted soil. The parameters decreased downwards in their different values as the experiment progressed to the 70^{th} day.

The nitrate concentration in the cow dung was observed to be 25.06 mg/kg (**Table 5**). In addition, in the cow dung amended polluted soil, the nitrate level at zero hour

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(immediately after amendment) increased to 35.44 mg/kg. This later decreased to 14.28 mg/kg (**Figure 4**). In the control experiment, there was slight decrease in nitrate concentration from 10.48 mg/kg to 8.44 mg/kg (**Figure 4**). There was significant difference for the two experimental conditions [f (2, 14) = 5.654, p < 0.015].

Table 5: Chemical composition of cow dung (organic fertilizer) used in the study

Parameters	Cow dung ± S.D
pH	8.20 ± 0.820
Conductivity (µs/cm)	60.00 ± 0.001
Nitrate (Mg/kg)	25.06 ± 0.052
Phosphate (Mg/kg)	19.32 ± 0.056

Real values are mean of triplicate analyses. S.D-Standard Deviation

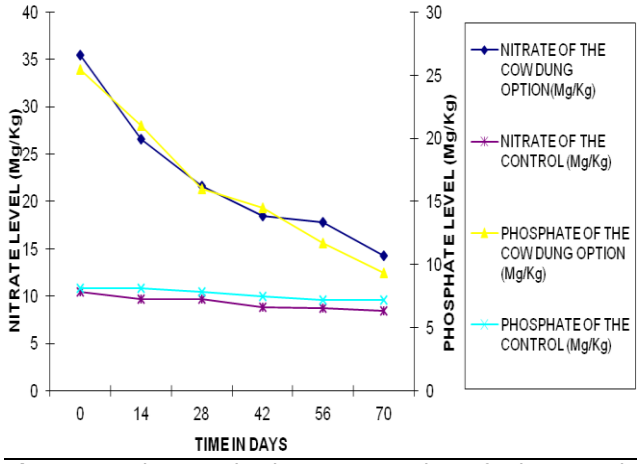

Figure 4: Changes in the concentration of Nitrate and phosphate levels in the cow dung treated soil and Control

The concentration of phosphate in the cow dung was 19.32 mg/kg (**Table 5**). The concentration of phosphate immediately after amendment increased to 25.41 mg/kg in the cow dung amended option (**Figure 4**). The amount of phosphate in cow dung amended option decreased from 25.41 mg/kg to 9.31 mg/kg during the 70 days study. The control experiment showed slight decrease at 8.10 mg/kg at zero hours to 7.21 mg/kg at the 70th day. There was significant difference of phosphate concentration at p<0.05 level for the two conditions [f (2, 15) = 4.922, p<0.023]. The phosphate concentration in the treated option decreased downward from the 28th day to the 70th day. This is an indication that the Phosphate was used by micro organisms during the bioremediation study. It has been well established that the availability of nitrogen and phosphorus limits the microbial degradation of hydrocarbon (Abu and Ogiji, 1996; Zhu *et al.,* 2001). The decrease in both nitrate and phosphate as the experiment progresses is due to the fact as they were used in metabolism of organisms in building biomass. There is a positive correlation in the utilization of both nitrate and phosphate. This indicates their importance in cell metabolism.

However, the slight decrease in the phosphate and nitrate levels at the control experiment (without amendment) indicated that there was no active bioremediation in the control since it was not amended with nutrient. There is an inverse relationship between the microbial counts and the concentrations of the limiting nutrients. The microbial counts increased progressively from the 28th day to 70th day whereas the nitrate and phosphate decreased downwards from the 28th day to the 70th day. The amount of Total hydrocarbon content (THC) at zero hour in cow dung amended option was 14103.02 mg/kg. This decreased to 5222.99 mg/kg at 70th day of the study (**Figure 5**)

In the control experiment, the total hydrocarbon content (THC) decreased at zero hour from 14146.19 mg/kg to 10114.29 mg/kg at the 70th day of the study (**Figure 5**). There was significant difference at the p<0.05 level for the two conditions [f (2, 15) = 7.933, p>0.007]. Post HOC comparisms using tukey HSD test indicated that the mean score for the control (m = 2645.50, SD = 1161.100) differed significantly from both the mean scores Cow dung option (m = 7775.20, SD = 2258.238), with p<0.098 and cow dung (m = 6928.60, SD = 2273.688). The percentage loss of THC for the cow dung treated polluted mangrove soil were 0 %, 23.23 %, 44.23 %, 55.01 %, 60.08 %, 62.08% for the zero day, 14th, 28th, 42nd, 56th and 70th day respectively. In addition, 0 %, 1.25 %, 11.36 %,12.42 %, 23 %, and 28 % for the zero, 14th, 28th, 42nd, 52nd and 70th day respectively for the control option (**Figure 5**). Changes in the concentration the petroleum hydrocarbon (TPH) was monitored at the zero hour/day, 28th day and 70th day of the study for the treatment and control. In the cow dung amended option, it decreased from 12419.89 mg/kg to 3743.979 mg/kg (**Figure 6**). In the control experiment, the total petroleum hydrocarbon decreased at zero hour from 12329.677 mg/kg to 10674.506 mg/kg (Figure 7). There was significant differences at the p<0.05 level for two conditions (cow dung, and control). It was observed that as the pollutants/chemical of concerns (COC's i.e. TPH) decreased during the 70 days study period, the counts/loads of the hydrocarbon utilising bacterial and fungal isolates increased progressively.

In a crude oil contaminated agricultural soil at Federal University of Technology, Owerri -Nigeria, the amendment of 100 g of contaminated soil with 30 g of organic nutrient (poultry droppings) led to the loss of 40 % total petroleum hydrocarbon (Ibekwe *et al.,* 2006). The percentage loss in TPH as recorded in the cow dung option were 0 %, 49.88 %, and 69.853 % for zero day, 28th day, and 70th day respectively (**Figure 6**). In a tropical crude oil polluted soil undergoing bioremediation, Chikere *et al.,* 2009, observed and reported that the use of NPK fertilizer, urea fertilizer and poultry droppings effectively stimulated bacterial organisms into utilization of crude oil. NPK 20:10:10 fertilizer option reduced TPH from 3666.0 mg/kg to 89.68 mg/kg for 57 days where as urea fertilizer option reduced TPH from 3666 mg/kg to 162 mg/kg for 57 days. In the poultry droppings option, the TPH was reduced from

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3666.0 mg/kg of soil to 135.01 mg/kg of soil (Chikere *et al.,* 2009).

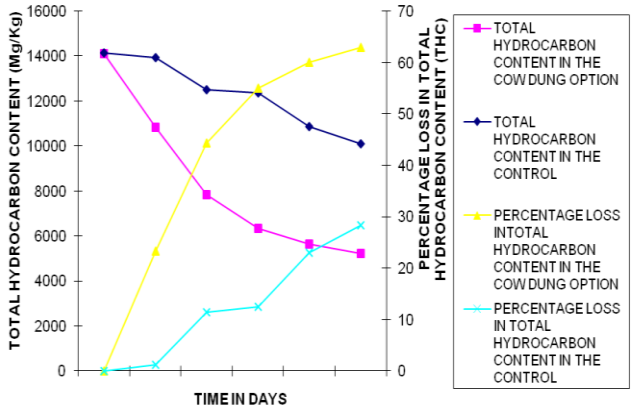

Figure 5: Changes in the concentration of Total Hydrocarbon Content (THC) and percentage loss in Total Hydrocarbon Content in the cow dung treated soil and Control

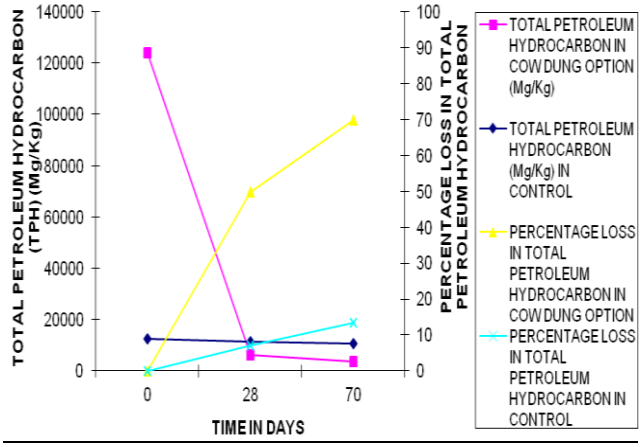

Figure 6: Changes in the concentration of Total Petroleum Hydrocarbon (TPH), and Percentage loss in TPH in the Cow dung treated and control options within the study period

CONCLUSION

The result of this research study has shown the cheap fertilizers such as cow dungs are effective in the supply of limiting nutrients necessary for bioremediation of crude oil impacted media such as mangrove soils. This cost effective organic fertilizers can be harnessed into preserved forms and be used for bioremediation. The long term aim of bioremediation design is to develop a cost effective and environmentally friendly approach. Cow dung apart from being cost effective is also environmentally friendly. This environmental friendly remedial action (Use of cow dung for bioremediation) in polluted mangrove swamp soils are geared towards sustainable development in the Niger Delta. The use of the cow dung for bioremediation is in line with international convention on waste utilisation (Awodun, 2008). Further research attention is on the contributions of biosurfactants in the microbial utilization of petroleum hydrocarbon in cow dung – amended polluted mangrove environment. There is also need for larger scale studies or pilot-scale studies on the use of Cow dung to bio-remediate petroleum-hydrocarbon impacted mangrove soils. As a result of incessant crude oil pollution in the Niger Delta, this is the right time for Nigerian Government to establish agencies for conservation of Mangrove Forests. These agencies are expected to work collaboratively with Environmental regulatory agencies to conserve the threatened Mangroves in the Niger Delta.

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REFERENCES

Abu, G. O and Ogiji, P.A. (1996). Initial test of a bioremediation scheme for the cleanup of an oil-polluted water body in a rural community in Nigeria. *Bioresource Technology* **58: 7-12.**

Abu, G.O. and Chikere, B.O. (2006). Cell surface properties of hydrocarbon-utilizing bacterial isolate from Port Harcourt marine environment. *Nigerian Journal of Microbiology* **20 (1): 809-816.**

Abu, G.O., and Akomah, O.N. (2008). A laboratory assessment of anaerobic biodegradation of petroleum hydrocarbons in a typical Niger Delta Wetland. *Global Journal of Pure Applied Sciences* ***14 (1)*****: 97-102.**

Amanchukwu, S.C., Obafemi, A. and Okpokwasili, G.C. (1989a). Hydrocarbon degradation and utilization by a palmwine yeast isolate. *FEMS Microbiology Letters,* ***57*****:151-154.**

Amanchukwu, S.C., Obafemi, A. And Okpokwasili, G.C. (1989b). Single cell protein production by *Schizosaccharomyces pombe* isolated from palmwine using hydrocarbon feedstock. *Folia Microbiologia* **34: 112-119.**

Atuanya, E.I. and Ibeh, I.N. (2004). Bioremediation of crude oil contaminated loamy-sand and clay soils. *Nigerian Journal of Microbiology* **18(1-2): 6373-6386.**

Awodun, M.A. (2008). Effect of nitrogen release from rumen digesta and cow dung on soil and leaf nutrient content of Gboma (*Solanum macrocarpon* L.). *Journal of Applied Biosciences* ***7: 202-206.***

Chikere, B.O and Chijioke-Osuji, C.C. (2006). Microbial diversity and physiochemical properties of a crude oil-polluted soil. *Nigerian Journal of Microbiology* **20 (2):**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

1039-1046.

Chikere, C.B., G.C., Okpokwasili and B.O Chikere, (2009). Bacterial diversity in a tropical crude oil-polluted soil undergoing bioremediation. *African Journal of Biotechnology **8*** **(11): 2535-2540.**

Chukwura, E.I., Nwokolo, C.I. and Nwachukwu, S.C.U. (2005). Bioremediation of crude oil polluted Escravos River using *Candida utilis. Nigerian Journal of Microbiology* **19(1-2): 623-630.**

Davies, J.S. and Westlake, D.W.S. (1979). Crude oil utilization by fungi. *Canadian Journal of Microbiology* **25: 146-156.**

Dibble, J.T. and Bartha, R. (1979). Effects of environmental parameters on biodegradation of oil sludge. *Applied Environmental Microbiology* **37: 729-739.**

Ebuehi, O.A.T., Abibo, I.B., Shekwolo, P.D., Sigismund, K.I., Adoki, A. and Okoro, I.C. (2005). Remediation of Crude oil contaminated soil by Enhanced natural attenuation technique. *Journal of Applied Environmental Management* **9(1): 103-106.**

Eziuzor, C.S. and Okpokwasili, G.C. (2009). Bioremediation of hydrocarbon contaminated mangrove soil in a bioreactor. *Nigerian Journal of Microbiology* **23 (1)*: 1777-1791.***

Ijah,U.J.J.; and Antai, S.P. (2003). The potential use of chicken-drop micro-organisms for oil spill remediation. *The Environmentalist* **23: 89-95.**

Ibekwe, V.I., K.C. Ubochi and E.U. Ezeji (2006). Effects of organic nutrient on microbial utilization of hydrocarbon on crude oil contaminated soil. *African Journal of Biotechnology* **5 (10)*: 983-986.***

Leahy, J.C.; and Colwell, R.R. (1990). Microbial degradation of hydrocarbons in the environment. *Microbiological Review* **54: 305-15.**

Nwachukwu, N.C., Orji, F.A., Iheukwumere, I. and Ekeleme, U.G (2010). Antibiotic resistant environmental isolates of *Listeria monocytogenes* from anthropogenic lakes in Lokpa –Ukwu, Abia State of Nigeria. *Australian Journal of Basic and Applied Sciences* **4(7): 1571-1576.**

Obire, O. (1988). Studies on the biodegradation potentials of some microorganisms isolated from water systems of two petroleum producing areas in Nigeria. *Nigerian Journal of Botany* **1: 81-90.**

Obire, O., Anyanwu, E.E., and Okigbo, R.N. (2008). Saprophytic and crude oil degrading fungi from cow dung and poultry droppings as bioremediating agents. *Journal of Agricultural Technology* **4 (92): 81-89.**

Okolo, J.C., E.N. Amadi and C.T.I. Odu (2005). Effects of soil treatments containing poultry manure in crude oil degradation in a sandy loam soil. *Applied Ecology and Environment* **3 (1): 47-53.**

Okoro, C.C. (2010). Enhanced Bioremediation of hydrocarbon contaminated mangrove swamp in the Nigerian oil rich Niger Delta using sea water microbial inoculate amended with crude oil biosurfactants and macronutrients. *Nature Science* **8 (8): 195-206.**

Okpokwasili, G.C. and Amanchukwu, S.C. (1988). Petroleum hydrocarbon degradation by *Candida* species. *Environment International* **14: 243-247.**

Osuji, L.C., and Ezebuiro, P.C. (2006). Hydrocarbon contamination of typical mangrove floor in Niger Delta, Nigeria. *International Journal of Environmental Science Technology* **3(3):313-320.**

Saari, E., Perämaki, P. and Jalonen, J. (2007). A comparative study of solvent extraction of total petroleum hydrocarbons in soil. *Microchimica Acta* **158: 261-268.**

SPDC (2011). Shell in Nigeria, the Operating Environment. Online Technical Sheet. Available at http//www.shellnigeria/operatingenvironment%sheel.com (Accessed 4th December, 2011).

Singh, D. and Fulekar, M.H. (2007). Bioremediation of phenol using microbial consortium in bioreactor. *Innovation Rommanian Food Biotechnology* **1: 31-36.**

Nelson, D. W., and Sommers, L.E. (1975). A rapid and accurate method for estimating organic carbon in soil. *Proceedings of the Indiana Academy of Science.* **84: 456-462.**

UNEP (2004). United Nations Environmental Programmes, *Analytical Methods for water quality*. Burllinton publishing house, Ontario, Canada, pp 160. Available at http://www.gemswater.org (Accessed 9th June, 2009).

United Nations Environmental Programmes (UNEP) (2011). UNEP Environmental Assessment of Ogoniland. **Pp262**.

Van Hamme, J.D., Singh, A., and Ward, O.N.W. (2003). Recent Advances in Petroleum Microbiology. *Microbiological and Molecular Biology Review* **67(4): 503-549.**

Zhu, X., Venosa, A.D., Suidan, M.T., and Lee, K. (2001). Guidelines for the bioremediation of marine shorelines and freshwater wetlands. Report under a contract with office of Research and Development, US Environmental Protection Agency. **Pp 201**.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538