

Bioremediation of Petroleum Hydrocarbon-Polluted Mangrove Swamps Using Nutrient Formula Produced From Water Hyacinth (*Eichhornia crassipes*)

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ABSTRACT

Laboratory-scale studies were carried out using a nutrient formula produced from *Eichhornia crassipes* plant to achieve bioremediation of crude oil impacted mangrove soil. In a 70 day study, the culturable heterotrophic bacterial population of the *Eichhornia crassipes* recipe increased from 6.26×10^5 Cfug to 2.69×10^7 Cfug. The control set-up had its total culturable bacterial count increased from 5.76×10^5 Cfug to 1.24×10^6 Cfug. Statistical analyses showed significant difference for the two conditions ($p \geq 0.05$). The total culturable heterotrophic fungal count in the *Eichhornia crassipes* recipe treatment increased from 5.36×10^5 Cfug to 2.50×10^7 Cfug respectively. The total culturable hydrocarbon utilising bacteria in *Eichhornia crassipes* treated polluted mangrove soil increased from 2.52×10^4 Cfug to 3.81×10^7 Cfug. Statistical analyses showed significant difference at $p \geq 0.05$ level for the two conditions (*Eichhornia crassipes* nutrient treated soil and control). The total culturable hydrocarbon utilising fungal counts increased significantly for both the control and *Eichhornia crassipes* treatment. There was no regular trend in pH changes in all the conditions. The conductivity value of the *Eichhornia crassipes* recipe treated soil decreased progressively. Phosphate, nitrate, %total organic carbon, Total Hydrocarbon Content (THC). Studies using Gas chromatographic analyses showed that in the *Eichhornia crassipes* recipe treated polluted mangrove soil, 0, 58.92 and 75.36% were lost at zero hour, 28th day and 70th day respectively. 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. There was no significant difference between the control experiment and *Eichhornia crassipes* ($p = 0.054$). The use of organic nutrient sources such *Eichhornia crassipes* recipe/nutrient powder is of good use as source of limiting nutrient needed for bioremediation of crude oil impacted medium.

Keywords: Bioremediation, Nutrient Formula/Powder From Water Hyacinth, Petroleum Hydrocarbon Pollution, Biotechnology, Mangrove Swamps, Niger Delta

1. INTRODUCTION

Spillage of petroleum hydrocarbons either by accident or vandalization causes significant environmental pollution (Mukherjee and Bordoloi, 2010). Crude petroleum oil and its derivatives

released in the environment either accidentally or deliberately pose problems of increasing magnitude throughout the world (Okoh and Trejo-Hernandez, 2006). Furthermore, these problems are more aggravated because of the expensive disposal methods (Rahman *et al.*, 2003; Das and Mukherjee, 2007). The

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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/derivatives has greatly impacted negatively on the Mangrove swamps leading reduction in seafood output, increased food security challenges, reduction in biodiversity of mangrove biota, youth restiveness and violence in this region. The majority of the population depend largely on food products (Periwinkles, oysters, in the mangroves in the Niger Delta. The mangroves provide ecological protection against tsunamis, erosion. It provides herbs with pharmacological benefits. Today, mangrove forests and swamps are among the most threatened habitats in the Niger Delta. The mangrove Environment is disappearing at an accelerated rate, yet with little public notice and research attention. Lenticels in the exposed portions of the mangrove roots are highly susceptible to clogging by crude oil and other pollutants, attacks by parasites and prolonged flooding from artificial dykes or causeways. Petroleum hydrocarbon pollution of mangrove swamps has greatly endangered the Niger Delta mangrove ecosystem. This has led to increased toxicity and bioaccumulation of Petroleum hydrocarbon in the seafoods from the mangroves (Oudot *et al.*, 1981; Vandermeulen and Gilfillan, 1985; Odokuma and Dickson, 2003). Among the various strategies adopted to clean petroleum oil-contaminated soil, bioremediation is recognized as one of the effective and inexpensive technologies (Das and Mukherjee, 2007). This approach involves supplying hydrocarbon-degrading efficient microorganisms to degrade target compounds, along with appropriate nutrients, to the subsurface (petroleum oil-contaminated sites). It should be noted that site remediation technology must be simple and cost-effective for both industrialized as well as for developing countries to be able to sort out and resolve this menace. Bioremediation of complex hydrocarbons usually requires the co-operation of more than a single species because the individual microorganism can metabolize only a limited range of hydrocarbon substrates/fractions (Mukherjee and Bordoloi, 2010). Bioremediation is an effective process to remediate petroleum oil contaminated mangrove ecosystem (Eziuzor and Okpokwasili, 2009).

However, the use of NPK fertilizer as source of limiting nutrients for bioremediation poses great environmental challenges including eutrophication, toxicity to marine lives including fishes. Above all, it not cost effective when compared with the use of waste

materials or organic fertilizers. In response to these existing challenges encountered with the use of NPK fertilizers during remediation of polluted aquatic environment, this study used nutrient formula/recipe for the bioremediation of Crude-oil polluted Mangrove swamps in the Niger Delta. The objectives of the study were; to determine the possibility of developing a fertilizer/nutrient formula/recipe for bioremediation from Water hyacinth (*Eicchornia crassipes*), an invasive/problematic species that has become a nuisance in water ways of the Niger Delta and other parts of the world and to determine the effectiveness of the produced water hyacinth recipe/powder in the bioremediation of crude oil polluted mangrove in the Niger Delta, Federal Republic of Nigeria.

2. MATERIALS AND METHODS

2.1. Study Area

The hydrocarbon polluted soil was excavated from mangrove swamp at Elibrada, Emuoha Local Government Area of Rivers State, Nigeria. 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 from a pipeline over two decades. 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.

2.2. 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/lumped together after excavation. The excavated soil was transported to the Environmental Microbiology laboratory of the University of Port Harcourt for bioremediation study. Co-ordinates of the sampling points were also 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.

2.3. Soil Contamination

The Elibrada mangrove has been contaminated by petroleum hydrocarbon before the time of the study. However, 50 mL sec of Bonny light crude oil was poured in each treatment cell/pot containing 500 g of the mangrove soil (including the controls). The aim of this further contamination was to simulate condition of a major spill (Odokuma and Dickson, 2003). 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).

2.4. Preparation of the Water Hyacinth Recipe Nutrient Formula/Fertilizer/Recipe

The water hyacinth used for this study was obtained from Choba River, accessible through Choba cattle Market, Port Harcourt. They were identified as Water hyacinth (*Eichhornia crassipes*) by Dr. Godfrey Akani of the Department of Applied Environmental Biology, Rivers State University of Science and Technology, Port Harcourt. Large quantity of the water hyacinth was harvested and transported to the Environmental Microbiology Laboratory, University of Port Harcourt. The water hyacinth recipe was chopped into pieces and air-dried. The dried water hyacinth was grinded with local mortar and pestle and the organic fertilizer in powdery form was stored for use during the bioremediation experiment.

2.5. Experimental Design

This is a laboratory scale experiment carried out in plastics pots as described in **Table 1**. The decision to use 50grams of the water hyacinth nutrient for 500grams of the polluted soil was based on findings from preliminary investigation.

2.6. Bioremediation Study

The polluted soils in plastic pots were amended with 50 g of recipe/powder produced from water hyacinth. The control was never amended with nutrient. Sampling for laboratory studies started immediately after the soils were amended with nutrients. This is called zero (0) day or zero hour study. The water hyacinth nutrient powder treated polluted soil and control were regularly turned using different hand trowels to avoid cross-contamination of Petroleum hydrocarbon fractions in both sets (1 and 2).

Table 1. Bioremediation experimental design of the study

Experimental group	Test experiment
Set 1	500 g of polluted soil + 50 g of sterile water hyacinth recipe/powder
Set 2 (Control)	500 g of polluted soil + no nutrient

Each Experimental group had three (3) replicates

Samples were taken for laboratory analyses in two (2) weeks intervals at 14th day, 28th day, 42nd day, 56th day and 70th day.

2.7. Monitoring of Bioremediation/ Biodegradation Studies

The bioremediation of petroleum hydrocarbons in the different experimental designs/set-up was studied/monitored by withdrawing samples before initiation of the experiment i.e., baseline and at time zero and subsequently at the 14th, 28th, 42nd, 56th and 70th days of the experiment for the following analyses.

2.8. Microbiological Analysis

2.8.1. 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, 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 1g 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 period of 18-48 h in the incubator at 28°C. Colonies that formed during this incubation period were counted using this formula:

$$\frac{\text{No of Colonies} \times \text{Dilution Factor}}{\text{Amount used}}$$

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

2.9. 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.

2.10. Enumeration of Total Culturable Hydrocarbon Utilizing Bacteria (TCHUB)

The enumeration of Total Culturable Hydrocarbon Utilizing Bacteria (TCHUB) was done by using the vapour phase method (Atuanya and Ibeh, 2004). Appropriate dilutions of the samples withdrawn from the two different conditions of 0 h., 14, 28, 42, 52 and 70 days of analyses were inoculated into modified Mineral Salt Medium (MSM). The culture medium components (MSM) were 0.42 g of $MgSO_4 \cdot 7H_2O$, 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 milligrams 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 of Petri dishes and inverted. The hydrocarbon saturated filter papers supply hydrocarbon by vapour-phase transfer to the inoculums (Amanchukwu *et al.*, 1989a; 1989b; Chikere and Chijioke-Osuji, 2006). The plates were incubated at 28°C±2°C for seven (7) days and colonies were counted from triplicates and mean values were recorded in colony forming units per gram (Cfu/g).

2.11. Enumeration of Total Culturable Hydrocarbon Utilizing Fungi Count (TCHUF)

Enumeration of total culturable hydrocarbon utilizing fungi was done using Mineral Salt Agar (MSA) containing 10 g of NaCl, 0.45 g of $MgSO_4 \cdot 7H_2O$, 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 (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 milligrams of Chloramphenicol and tetracycline (Chikere and Chijioke-Osuji, 2006). Incubation was within 5-7days at 28±2°C (Okpokwasili and Amanchukwu, 1988).

2.12. Characterisation and Identification of Hydrocarbon Utilising Bacterial 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 utilisation, methyl red, Voges-prauskauer, starch hydrolyses, oxidase reaction. The macroscopic colonial appearances of fungal growth in plates were observed and recorded. The macroscopic examinations were based on texture of the colony, colony size, pigmentation, time of growth, color on the reverse side of plate and margin of colony (Chukwura *et al.*, 2005). A drop of lactophenol cotton blue was placed on a grease free, scratch-free glass slide (Mbakwem-Aniebo and Ezekwem, 2010). A small portion of the fungal growth was picked with a wire loop and teased out using a mounting needle. The preparation was covered with a cover slip (Chukwura *et al.*, 2005). The slide was observed under ×10 and ×40 objective lenses. Observed characteristics were recorded and compared with the established identification keys of (Barnett and Hunter, 1972).

2.13. Physico-Chemical Analyses

2.13.1. pH Measurement (pH 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 meter (Jenway 3015, United Kingdom). At each point, three values were obtained and the mean of the values was used.

2.14. Conductivity Measurement

Conductivity of withdrawn samples was measured with a conductivity meter (Jenway 4010, United Kingdom) during the period of the experiment. The conductivity values were measured in $\mu s/cm$ and it gives a surrogate value of level of salinity and Total Dissolved Solids (TDS).

2.15. Measurement of Nitrate (NO₃⁻) Level of Amended Polluted Soil

The brucine method was used. One (1 mL) of soil filtrate was measured into a clean test tube and 1 mL of distilled water was measured into a clean test tube and one (1 mL) of distilled water was measured into another test tube as blank solution. About 0.5 mL of brucine reagent was gently introduced into both test tubes. Two (mL) 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).

2.16. Determination of Phosphate Content of the Amended Polluted Soil

The method used was the colourimetric method as described in United Nations Environmental Programme (UNEP, 2004). One-tenth of 2.5% glacial acetic acid was prepared and used for the extraction of phosphate in 250 mL capacity conical flask. The mixture was stirred for 10 min. Fifty (mL) sample extract was pipetted into a clean conical flask. This aliquot is autoclaved with K₂S₂O₈ and H₂SO₄ for 30 min at 121°C, (5 mLs) of ammonium molybdate was added to the autoclaved mixture to form heteropoly molybdophosphoric acid and is reduced with stannous chloride in an aqueous sulphuric acid medium, at 30°C, to form a molybdenum blue complex. The resulting blue colour is measured spectrophotometrically at 660 nm and compared to identically prepared standard (water). The detection limit of this method is 0.001 mg L⁻¹ (UNEP, 2004).

2.17. Proximate Analyses of the Water Hyacinth Nutrient Powder

The produced Water hyacinth nutrient powder was analysed for pH, conductivity. Nitrate and Phosphate concentrations were also studied to determine the amount of the two limiting nutrients required for microbial remediation of polluted sites. The methods used for determination of these four parameters are still the procedures for the determination of pH, conductivity, Nitrate and Phosphate concentrations in the amended polluted soil under-going remediation.

2.18. Percentage 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 milliliters (mLs) potassium chromate solution and milliliters (7.5 mLs) of 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 mLs with distilled water. Twenty five mLs of the sample solution was titrated with 0.2 molar 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:

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

2.19. Total Hydrocarbon Content (THC)

Five grams of soil sample was weighed into a beaker and 10 mLs of xylene 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 comparison to a calibration curve constructed from dilutions of a stock solution of a 1:1 bonny light crude and bonny medium. The Ultraviolet light spectrophotometric measurement was at 420 nm (UNEP, 2004; Osuji and Ezebuio, 2006).

2.20. Total Petroleum Hydrocarbon (TPH)

2.20.1. Extraction

Five grams portion of soil sample was weighed out and 0.1 mg was weighed out and transferred into extraction vessels. Fifty milliliters of acetone was added and the vessel was closed. The vessel was heated under 200psi, temperature of 150°C, time of 15 min. The vessels were at this point allowed to cool to room temperature. The vessels were opened at room temperature and five grams of sodium sulfate was added. The mixture was gently swirled. The extract mixture filtered through ashless filter paper rinsing and washing the sediment with acetone into a bigger container (Saari *et al.*, 2007).

2.21. Preparation of the Extracts for the Analysis

Before GC-FID measurement, all extracts volume was adjusted to (20 mLs) with (20 mLs) hexane solution to prevent possible change in concentration of the

extracted solutions because of evaporation losses of solvent mixtures during an extraction stage. Polar compounds, such as vegetable oils, animal fats were then removed by solid phase extraction under silica gel (florisil) (Saari *et al.*, 2007).

2.22. Calibration Checks 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 $\geq C_{10}$.

2.23. Gas Chromatography-Flame Ionization Detection System

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 (15 m \times 0.25 mm) with a nominal film thickness of 0.25 μ m, splitless injection method (all in batch). Injection volume was 1 μ L and injection temperature was 330°C. Helium was used as a carrier gas (2 mL min⁻¹). The column was held at 35°C for 1.50 min. The temperature was increased from 15°C min⁻¹, to 310°C min⁻¹ and held for 10 min. This enabled complete run within 27 min. The amount of Total Petroleum Hydrocarbon (TPH) was then determined as a sum total of resolved and unresolved components eluted from the GC capillary column between retention times of 5 to 35 min. This method called peak sum calculates TPH by summing up all components of crude oil from C10 and upwards. 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).

2.24. Calculation of Percentage Loss in THC/TPH

Percentage loss in THC and TPH was calculated using the formula below:

$$\% \text{ Loss in TPH / THC} = \frac{\text{Concentration at a point} - \text{Conc. at time zero}}{\text{Concentration at time zero}} \times 100$$

2.25. Statistical Analysis

Statistical analyses were carried out using Statistical Package For Social Sciences (SPSS, Version 17.0). Analysis Of Variance (ANOVA), P-values and tests of significance were carried out at 95% level of confidence using statistical package for social sciences. P-Values

were used to determine the significance levels between treatment and control.

3. RESULTS

3.1. Baseline Studies

The initial physico-chemical and microbiological characteristics of the crude-oil polluted mangrove soil are documented in **Table 2**. The changes in the populations of heterotrophic and hydrocarbon utilizing bacteria and fungi are presented in **Fig. 1-4**.

4. DISCUSSION

4.1. Baseline Studies

The initial physico-chemical and microbiological characteristics of the crude-oil polluted mangrove soil are documented in **Table 2**. The pH, conductivity, nitrate, phosphate and percentage total organic carbon were 7.5 \pm 0.04, 2380 \pm 3.12, 10.52 \pm 0.286, 8.11 \pm 0.021, 3.30% respectively. The Total Petroleum hydrocarbon and Total hydrocarbon content were 12934.75 \pm 00 and 14102.22 \pm 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 \times 10⁵ \pm 0.004 Cf/g, 4.8 \times 10⁵ \pm 0.111 Cf/g, 3.5 \times 10⁴ \pm 0.025 Cf/g, 2.6 \times 10⁴ \pm 0.025 Cf/g respectively (**Table 2**).

4.2. Microbial Dynamics

The total culturable heterotrophic bacterial counts in the water hyacinth powder treated polluted mangrove soil were 5.8 \times 10⁵ Cf/g, 7.5 \times 10⁵ Cf/g, 2.09 \times 10⁶ Cf/g, 2.90 \times 10⁷ Cf/g, 2.43 \times 10⁷ Cf/g, 2.43 \times 10⁷ Cf/g, 2.69 \times 10⁷ Cf/g for the zero hour, 14th, 28th, 48th, 52nd and 70th day respectively (**Fig. 1**). The total heterotrophic bacterial count in the control experiment ranged between 5.6 \times 10⁵ Cf/g to 6.20 \times 10⁵ Cf/g. In both cases the growth of the heterotrophic bacterial organisms was lowest at zero hour and highest on the 70th day. There was significant difference at p<0.05 level for water hyacinth powder treated polluted soil and the un-amended control (F [2, 15] = 2.277).

In addition, the total culturable heterotrophic fungal count at baseline was 4.8 \times 10⁵ Cf/g. This increased from 5.3 \times 10⁵ Cf/g to 2.5 \times 10⁷ Cf/g and 4.3 \times 10⁵ Cf/g to 6.2 \times 10⁵ Cf/g for water hyacinth recipe/nutrient powder option and control experiments respectively (**Fig. 2**).

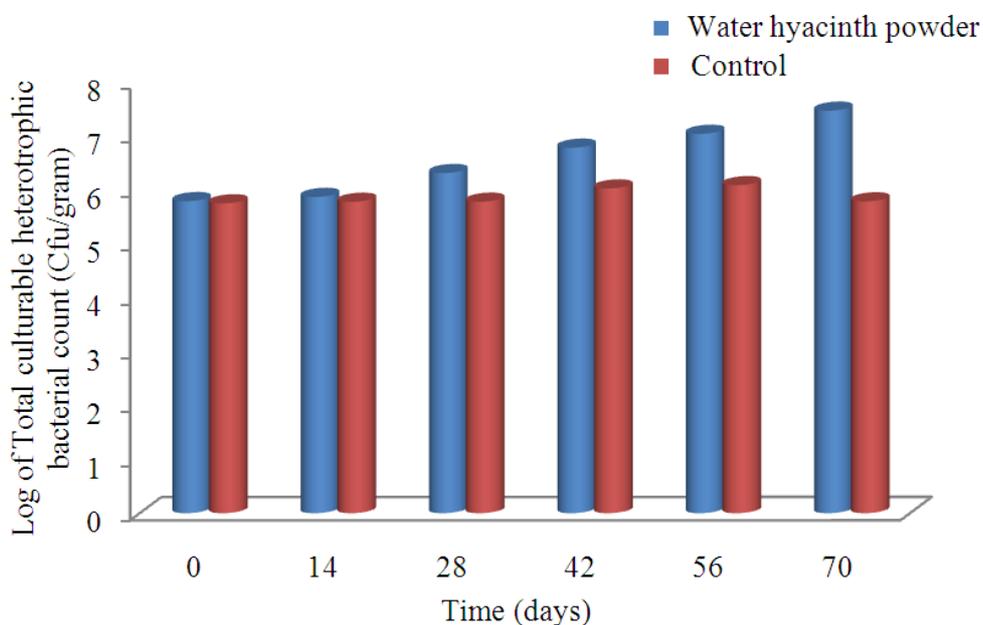


Fig. 1. Changes in total culturable heterotrophic bacterial counts in the water hyacinth powder treated polluted soil and control during the 70th day study

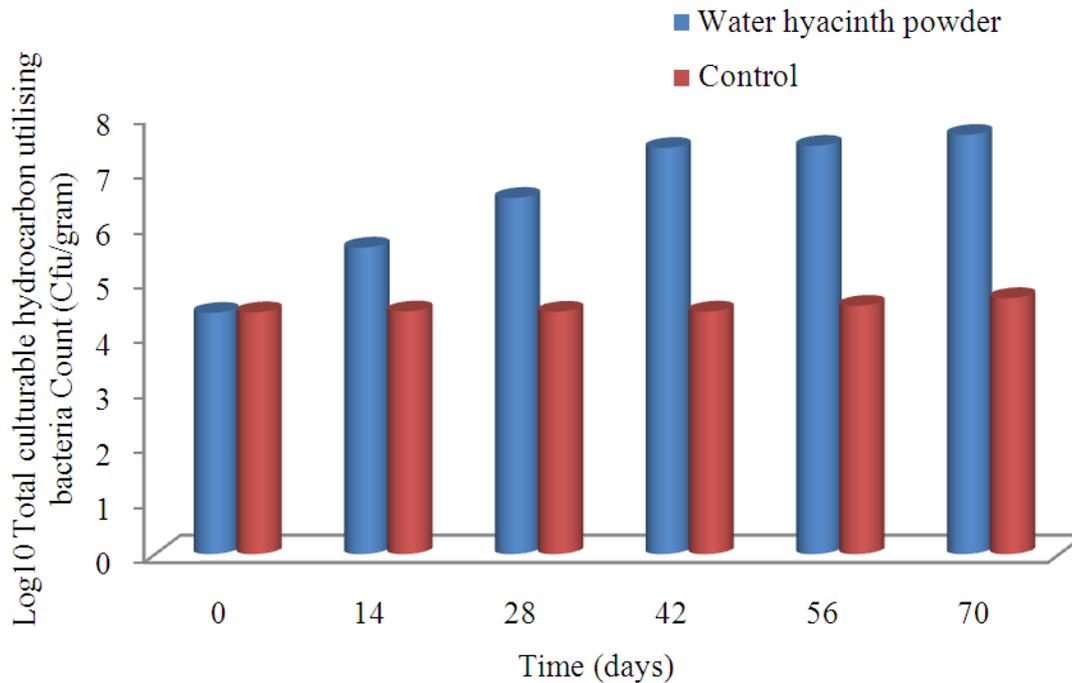


Fig. 2. Changes in total culturable heterotrophic fungal counts in the water hyacinth powder treated polluted soil and control during the 70th day study

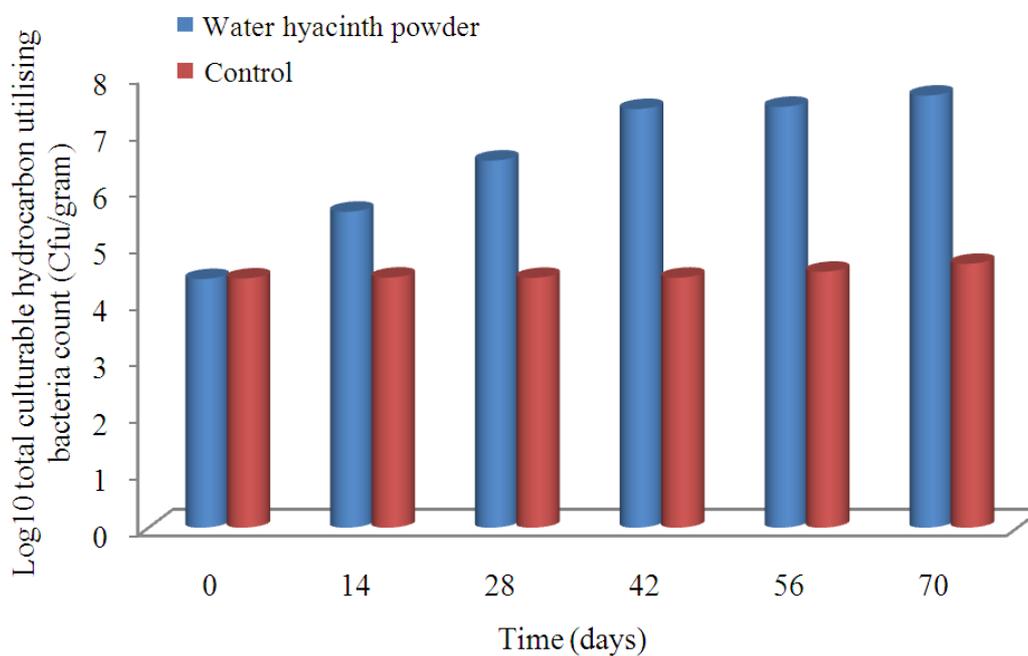


Fig. 3. Changes in total culturable hydrocarbon utilizing bacterial counts in the water hyacinth powder treated polluted soil and control

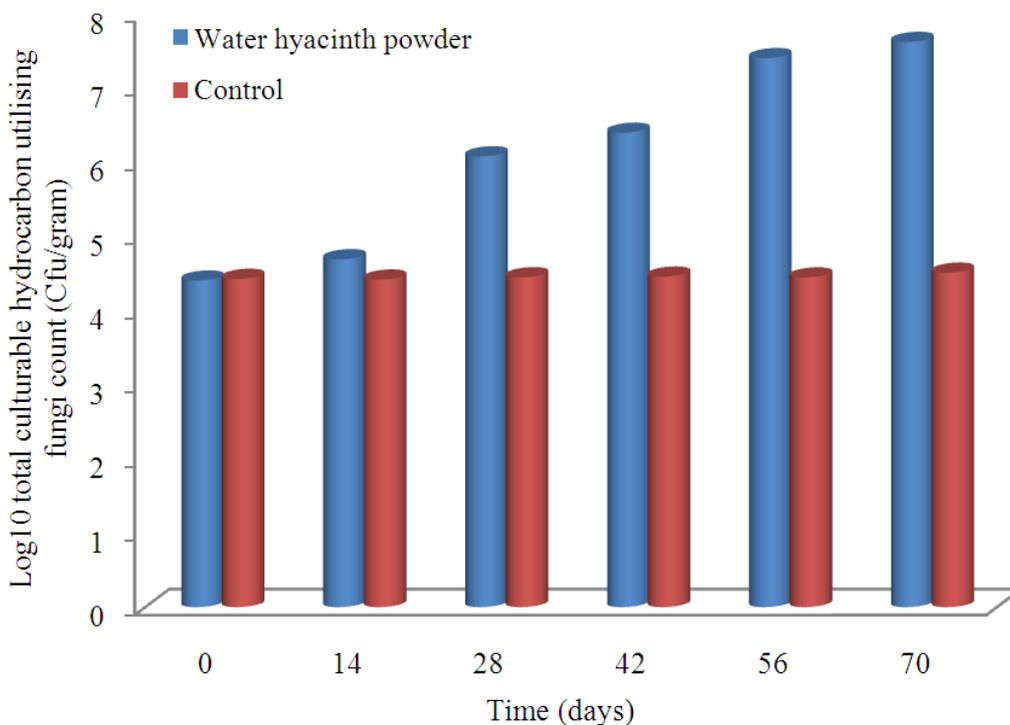


Fig. 4. Changes in the total culturable hydrocarbon utilizing fungi count of the Water hyacinth powder treated option and control

Statistical analyses have shown that there was significant difference at $p < 0.05$ level for the two conditions (water hyacinth and control).

The hydrocarbon utilizing bacterial species responded to the nutrient amendment with water hyacinth recipe. The total culturable hydrocarbon utilizing bacterial count in water hyacinth expect increased at zero hour from 2.5×10^4 Cfug to 3.7×10^7 Cfug during the period of study that lasted for seventy (70) days. The total culturable hydrocarbon utilizing bacterial counts in the control experiment ranged from 2.5×10^4 Cfug at zero hour to 1.68×10^5 Cfug at 70th day of the study (Fig. 3). Post HOC comparisons using the tukey HSD test indicated that the mean score for control ($m = 4.7259$, $SD = 0.48758$) differed significantly from both the mean scores of water hyacinth recipe and control experimental groups.

In the Water hyacinth nutrient powder amended option experiment, the total culturable hydrocarbon

utilizing fungal counts ranged were 2.5×10^4 Cfug, 2.03×10^3 Cfug, 1.20×10^6 Cfug, 2.49×10^6 Cfug, 2.68×10^7 Cfug at zero hour, 14th 28th 48th, 56th and 70th day of the experiment respectively.

The un-amended control experiment had its culturable hydrocarbon utilising fungi count increased from 2.6×10^4 to 1.81×10^5 Cfug (Fig. 4). The amounts of limiting nutrients such as nitrogen and phosphorus present in this polluted soil were very low. This called for the amendment of the polluted mangrove soil with water hyacinth nutrient powder. Managing water hyacinth which is a problematic plant species in the Nigerian water ways by putting it into effective use such as in bioremediation will definitely be a welcome development in Sub-Saharan Africa and other parts of the world where the plant posses' ecological problems and cheaper remediation technologies are regularly sought for.

Table 2. Baseline physico-chemical and microbiological properties of petroleum hydrocarbon-impacted mangrove soil in the Niger Delta

Parameters	Values \pm S.D
pH	7.5 \pm 0.04
Conductivity (μ s/cm)	2380 \pm 3.12
Nitrate (NO ₃ ⁻) (mg/kg)	10.52 \pm 0.286
Phosphate (PO ₄ ⁻) (mg/kg)	8.11 \pm 0.021
Total organic carbon (%)	3.30 \pm 0.00
Total petroleum hydrocarbon (mg/kg)	12934.75 \pm 0.00
Total hydrocarbon (mg/kg)	14102.22 \pm 0.127
Total culturable heterotrophic bacterial count (Cfu/g)	6.1 $\times 10^5 \pm 0.004$
Total culturable heterotrophic fungal count (Cfu/g)	4.8 $\times 10^5 \pm 0.111$
Total culturable hydrocarbon utilizing bacterial count (Cfu/g)	3.5 $\times 10^4 \pm 0.025$
Total culturable hydrocarbon utilizing fungal count (Cfu/g)	2.6 $\times 0^4 \pm 0.024$

Cfu/g; colony forming unit per gram, μ s/cm; microsiemens per centimetre, S.D; Standard deviation

Table 3. Identities of the hydrocarbon utilising bacterial isolates

Isolate no	Morphology and Gram\		Biochemical tests											Identities of the bacterial isolates	
	Reaction	Motility	Citrate	Catalase	Indole	Red	Methyl Voges Proskauer	Starch Hydrolysis	H ₂ S Production	Oxidase	Glucose	Lactose	Mannitol		Sucrose
HUB ₁	Rods -	+	+	+	-	-	-	-	-	+	+	+	+	+	<i>Pseudomonas</i> species
HUB ₂	Rods -	+	+	+	-	+	-	+	+	-	+G	+	+	+	<i>Citrobacter</i> species
HUB ₃	Rods +	+	+	+	-	+	+	+	-	-	+	+	+	-	<i>Bacillus</i> species
HUB ₄	Rods +	+	+	+	-	+	+	+	-	-	+	+	+	-	<i>Bacillus</i> species
HUB ₆	Rods -	+	+	+	-	+	-	+	+	-	+G	+	+	+	<i>Citrobacter</i> species
HUB ₇	Cocci+	-	-	-	-	+	-	-	-	+	+	+	+	+	<i>Micrococcus</i> species
HUB ₈	Rods -	+	+	+	+	+	+	+	-	+	+	-	+	+	<i>Vibrio</i> species
HUB ₉	Rods +	-	-	+	-	-	+	-	-	-	-	-	-	+	<i>Corynebacterium</i> species
HUB ₁₀	Cocci+	+	-	+	-	+	-	-	-	+	+	+	+	+	<i>Micrococcus</i> species
HUB ₁₁	Rods -	-	+	+	-	+	-	-	+	+	+	-	-	+	<i>Flavobacterium</i> species
HUB ₁₃	Rods -	-	+	+	-	+	-	-	+	+	+	-	-	+	<i>Flavobacterium</i> species
HUB ₁₄	Cocci+	-	-	+	-	+	-	-	-	+	+	+	+	+	<i>Micrococcus</i> species
HUB ₁₅	Rods +	-	-	+	-	-	+	-	-	-	-	-	-	+	<i>Corynebacterium</i> species

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

The slight increase observed in the heterotrophic/hydrocarbon utilising bacterial, fungi counts in the control experiment from the zero day to the 70th day is as a result of the fact that it is not amended with nutrient. Again, the little nutrients (nitrate and phosphate) available and present are likely not available to the microorganisms. The response of indigenous hydrocarbon utilizing bacteria 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. Similarly, (Odokuma and Dickson, 2003) in a

bioremediation study in a mangrove swamp in new Calabar River, Rivers State was able to scale-up the hydrocarbon-utilizing bacteria using NPK fertilizer. The NPK fertilizer increased the bioload of the polluted mangrove soil from 1.5×10^3 to 1.5×10^9 Cfug. In addition, in another study in a contaminated sandy-loamy soil in the Port Harcourt, Okolo *et al.* (2005) documented the use of poultry manure to increase the hydrocarbon utilizing bacteria count significantly. The hydrocarbon utilizing bacterial isolates obtained from this study included *Pseudomonas* species, *Bacillus* species, *Citrobacter* species, *Micrococcus* species, *Vibrio* species, *Flavobacterium* species and *Corynebacterium* (Table 3).

Table 4. Identities of the hydrocarbon utilizing fungal isolates in the water hyacinth nutrient powder treated polluted soil

Isolate	Culture characteristics	Microscopic characteristics	Fungal identity
HUF ₁	Pure white, with abundant cottony mycelium and white reverse	sporangioophores, rhizoid, Spongisphore Non-septate and black sporangium containing	<i>Rhizopus</i> spp. hemispherical
HUF ₂	Powdery, dark brown, flatty spread on the surface of the solid medium with brown reverse.	Septate and branched hyphae with conidia in chains	<i>Aspergillus</i> spp.
HUF ₃	Whitish and cottony mycelium with pinkish pigments at the centre, and reverse side was brown	Segmented canoe-shaped spores and branched Conidiophores.	<i>Fusarium</i> spp.
HUF ₄	Surface was first greyish white and woolly,	Dark septate mycelium with short conidiophores. Conidia were large with longitudinal septations	<i>Alternaria</i> spp.
HUF ₅	Powdery, dark brown, flaty spread on the surface of the solid medium with brownish reverse	Septate and branched hyphae and conida in chains	<i>Aspergillus</i> spp.
HUF ₆	Grey colonies that were large With white border. Reverse was white	Long conidiophores consisting of broom like conida in chains	<i>Penicillium</i> spp.
HUF ₇	Yeast-like colony, soft and smooth. Pinkish in colour and turned red as it was old	Oval shaped budding cells with few rudimentary pseudohyphae. No ascospores present.	<i>Rhodotorula</i> spp.
HUF ₈	Whitish and cottony	Segmented canoe-like spores, with branched and segmented conidiophores	<i>Fusarium</i> spp.
HUF ₉	Yellow green dense mycelia. Powdery and light yellow reverse	Long conidiophores consisting of broom like conidia in Chains	<i>Penicillium</i> spp.
HUF ₁₀	White colony mycelium which developed within 4 days with extensive sub-surface	Coarse hyphae that segment into rectangular arthrospores varying in sizes	<i>Geotrichium</i> spp.
HUF ₁₁	White and cottony mycelium	Multi-segmented canoe-like spores with branched and segmented conidiophores	<i>Fusarium</i> spp.
HUF ₁₂	Creamy ovoid colonies which were easily picked	Spherical cells in clusters with buds	<i>Saccharomyces</i> Sp.

Eziuzor and Okpokwasili (2009) studied bioremediation of crude oil polluted mangrove soil in Port Harcourt. They used NPK as source of limiting nutrient and reported the isolation of hydrocarbon utilizing bacteria such as *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Citrobacter*, *Alcaligenes*, *Flavobacteria*, *Pseudomonas*, *Vibrio* and *Corynebacterium*. The hydrocarbon utilizing fungal organisms isolated from this study include the following: *Penicillium*, *Mucor*, *Fusarium*, *Saccharomyces*, *Rhodotorula*, *Alternaria*, *Rhizopus*, *Geotrichium* and *Aspergillus* (Table 4). In Nigeria, the fungi reported as oil degraders in aquatic environments in the petroleum producing areas by (Obire, 1988) were *Aspergillus*, *Aureobasidium*, *Candida*, *Rhodospiridium*, *Cephalosporium*, *Rhodotorula*, *Saccharomyces*, *Aspergillus niger*, *Aspergillus terreus*, *Blastomyces* spp., *Botryodiplodia*, *Fusarium* spp., *Nigrospora* spp., *Penicillium* and *Trichoderma harianum*. The use of white-rot fungus, *Lentinus subdunus* for mycoremediation of engine oil polluted soil in western Nigeria has been reported (Adenipekun and Fasidi, 2005).

4.3. Physico-Chemical Studies

The pH of the polluted mangrove soil was slightly alkaline soil. In the water hyacinth powder amended soil, the pH for the zero hour and 70th day were 7.55, 7.54, 7.68, 7.69 and 7.49 respectively (Fig. 5). The pH of the control experiment was alkaline within the 70 days study, but in a static pattern (Fig. 5). 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 the Water hyacinth treated option ($m = 7.7167$, $S. D = 0.074$) at 95% confidence interval. In addition, from composite analyses, the pH value of water hyacinth organic fertilizer/powder was 9.91 ± 0.027 respectively (Table 5). It is worthy to mention that the pH values recorded in the water hyacinth powder 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.

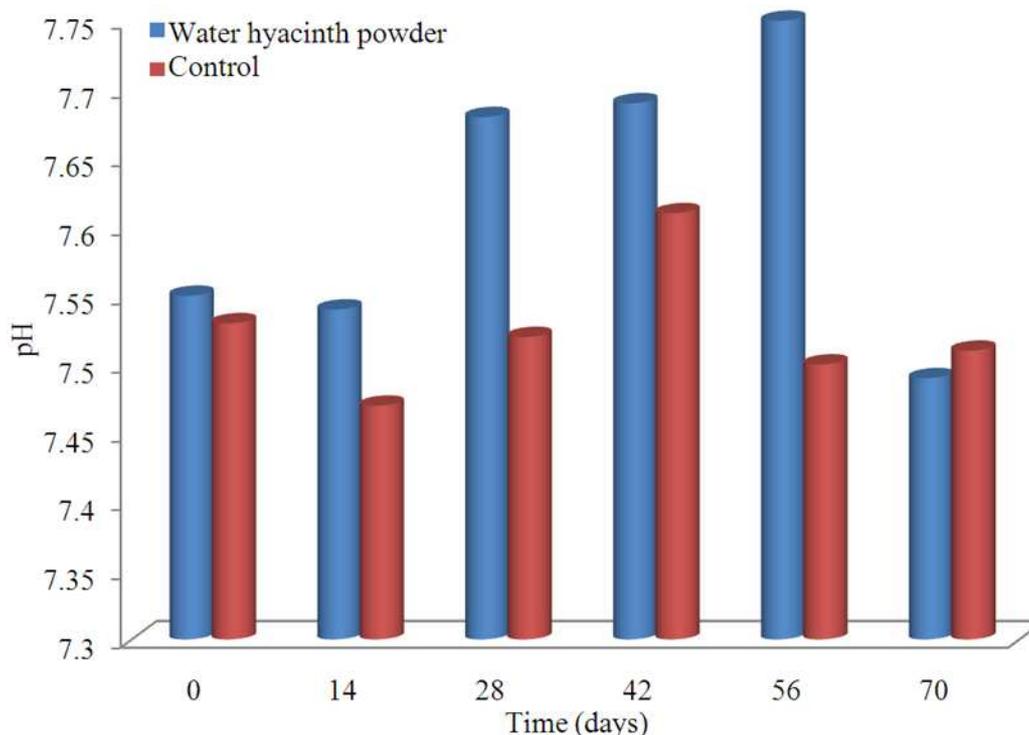


Fig. 5. Changes in the pH of the Water hyacinth powder treated option and control during the 70day study

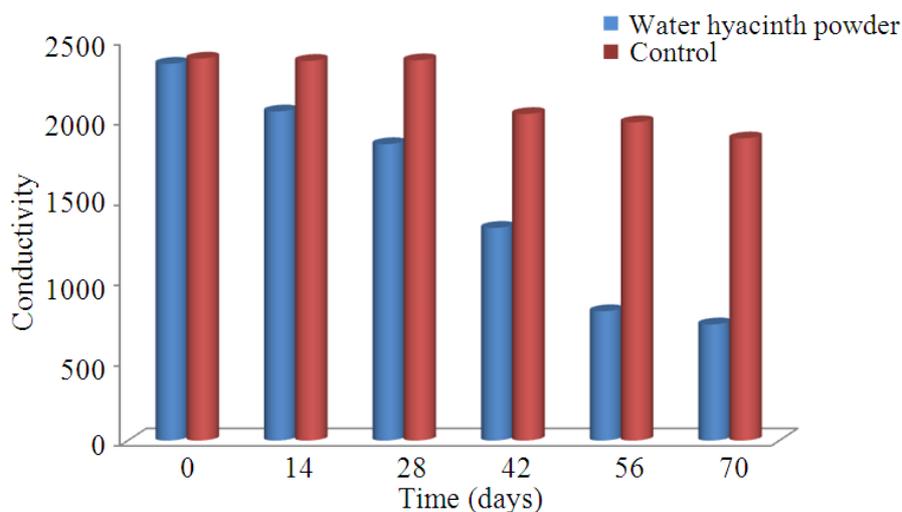


Fig. 6. Changes in the conductivity (µS/cm) of the water hyacinth powder treated option and control during the 70day study

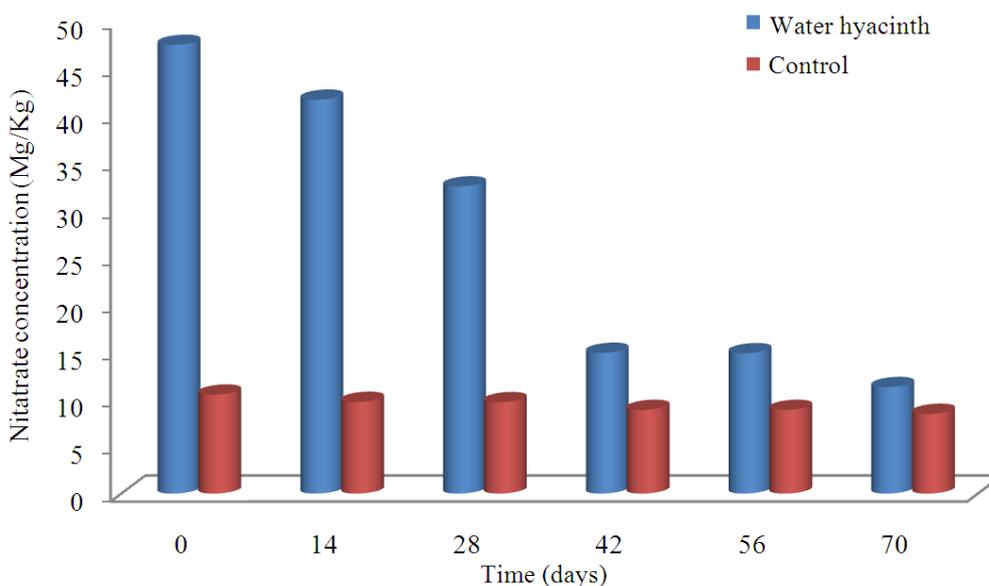


Fig. 7. Changes in the concentration of nitrate (Mg/Kg) of the Water hyacinth powder treated option and control during the 70day

In the baseline study of the polluted mangrove soil, the conductivity level was $2380 \mu\text{S cm}^{-1}$. 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. In the water hyacinth recipe amended polluted soil, conductivity decreased from 2399

$\mu\text{S cm}^{-1}$ to $724 \mu\text{S cm}^{-1}$ during the 70 days study. In addition, control experimental set-up showed very slight decrease in conductivity from $2381\text{-}1884 \mu\text{S cm}^{-1}$ (Fig. 6). There was significant difference in the two (2) conditions [$f(2, 15) = 0.289, p = 0.753$] at 95% confidence interval. The conductivity value of the water hyacinth recipe from composite analyses was $89.47\mu\text{S/cm} \pm 5.110$ (Table 5). This high conductivity value is because the life water hyacinth was plucked from marine environment.

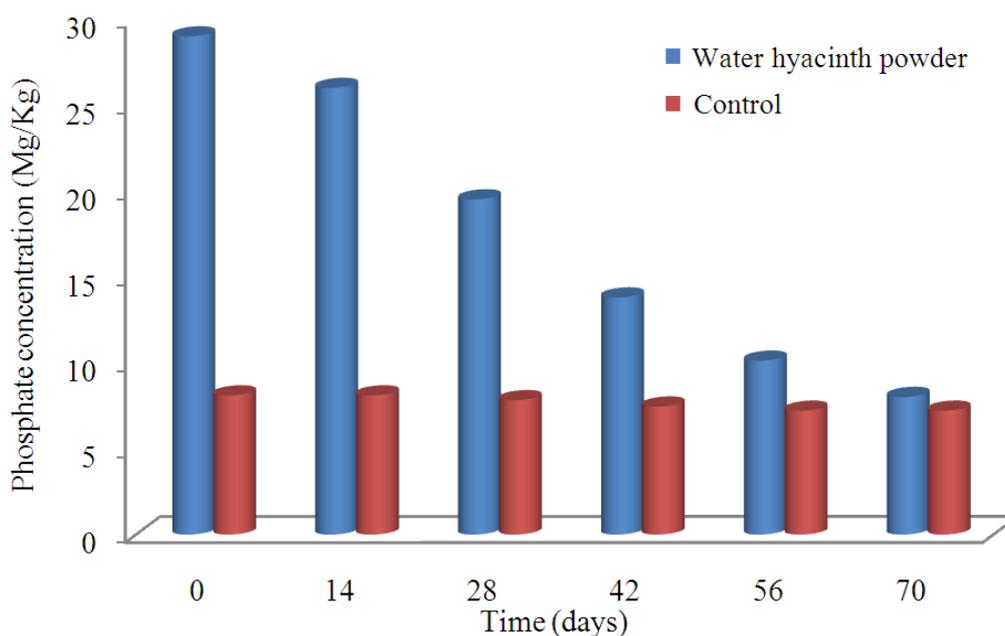


Fig. 8. Changes in the concentration of phosphate (Mg/Kg) in the polluted soil amended with water hyacinth powder and control during the 70day study

Table 5. Chemical composition of the water hyacinth recipe organic fertilizer used in the study

Parameters	Values±S.D
pH	9.91±0.027
Conductivity ($\mu\text{s cm}^{-1}$)	89.47±5.100
Nitrate (Mg kg^{-1})	38.48±0.050
Phosphate (Mg kg^{-1})	27.89±0.018

Real values are mean of triplicate analyses, S.D: Standard deviation

Conductivity study 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). This observation of decrease in conductivity could be as a result of the facts that the salts present in the polluted mangrove soil may have been utilised as micro-nutrients by microorganisms. Invariably, it means that the exchange ions in the polluted mangrove soils were used by the hydrocarbon utilizers for increase in biomass. As the salts are used as micronutrients, this eventually lead to reduction of conductivity.

Abu and Akomah (2008) similarly reported a decrease in the conductivity of a treated wetland undergoing bioremediation under laboratory simulation. They cited uptake of exchange ions by microorganisms as the major reason for such decrease.

There was a decrease in the level of nitrate in water hyacinth recipe amended polluted soil as it decreased from 47.52 mg kg^{-1} to 11.28 within the 70 days study period (Fig. 7). In the control experiment, there was slight decrease in nitrate concentration from 10.48 mg kg^{-1} to 8.44 mg kg^{-1} (Fig. 7). There was significant difference for the two experimental conditions [$f(2, 14) = 5.654$, $p < 0.015$]. The nitrate concentration in the water hyacinth fertilizer was 38.48 mg kg^{-1} (Table 5). This showed that the powder from water hyacinth is nitrate rich.

The concentration of phosphate in the water hyacinth fertilizer was 27.89 mg kg^{-1} (Table 5). This also showed that the water hyacinth powder is rich in phosphate. The concentration of phosphate immediately after amendment increased to 29.01 mg kg^{-1} in the water hyacinth amended option (Fig. 8). The amount of phosphate decreased in the water hyacinth recipe amended option from 29.01 mg kg^{-1} to 8.01 mg kg^{-1} during the 70 days study. The control experiment showed slight decrease at 8.10 mg kg^{-1} at zero hours to 7.21 mg kg^{-1} . There was significant difference at $p < 0.05$ level for the two conditions [$f(2, 15) = 4.922$, $p < 0.023$]. The phosphate and nitrate concentrations in the treated option decreased downwards in specific pattern from the 28th day to the 70th day. This is an indication that the limiting nutrients (Phosphate and nitrate) were used by microorganisms during the duration of study.

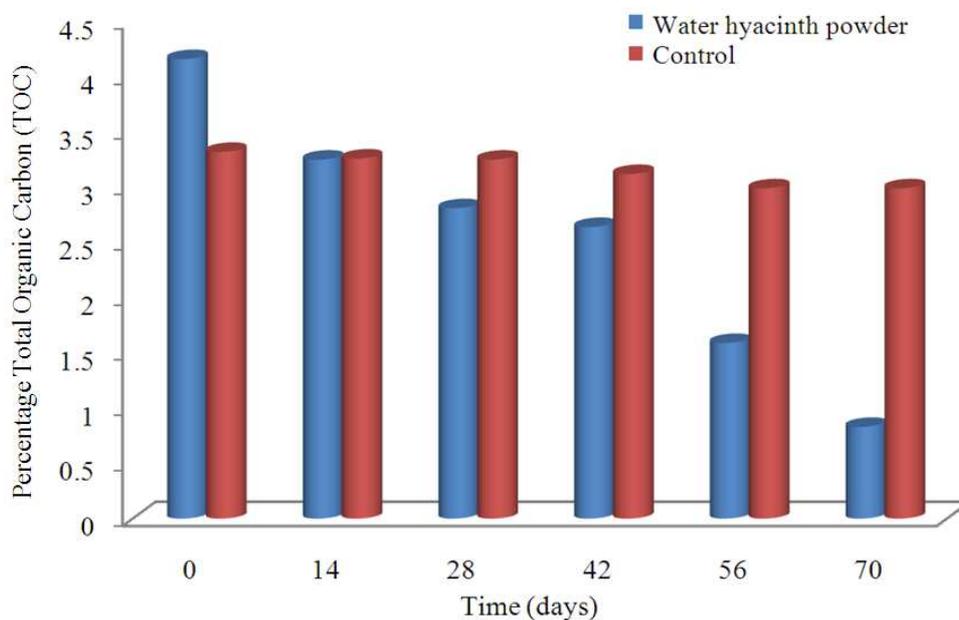


Fig. 9. Changes in the percentage Total Organic Carbon (%TOC) in the polluted soil amended with water hyacinth powder and control during the 70day bioremediation study

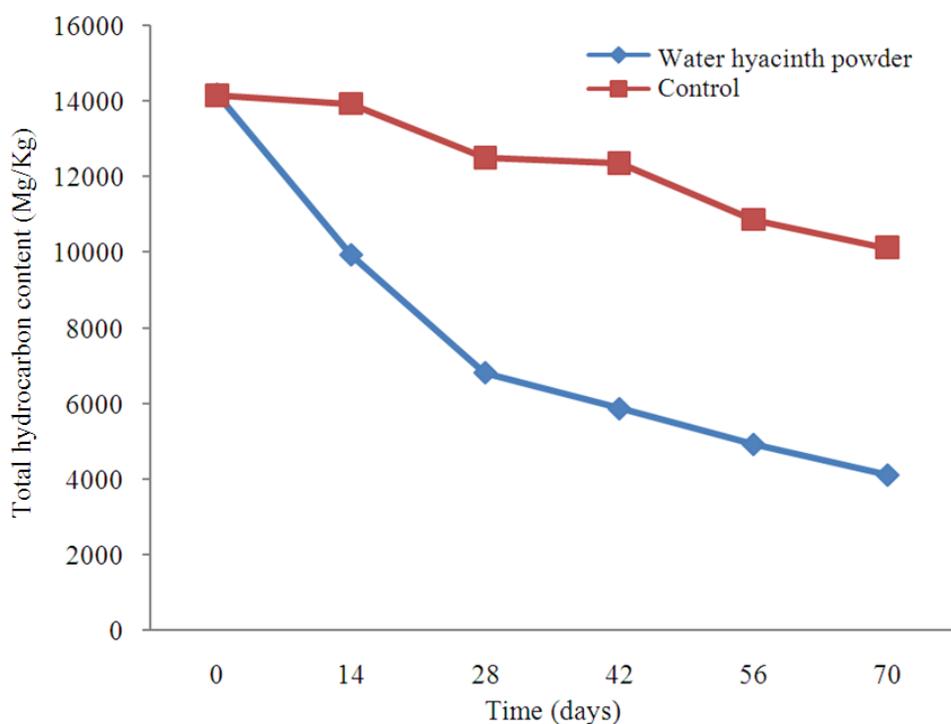


Fig. 10. Changes in the Total Hydrocarbon Content (Mg/Kg) in the polluted soil amended with Water hyacinth powder and control during the 70 day bioremediation study

Adjustment of carbon/nitrogen/phosphorus ratios by the addition of nitrogen and phosphorus in the form of oleophilic fertilizers including paraffinized urea have been shown to stimulate microbial organisms into utilization of crude oil in different ecosystems, (Atlas and Bartha, 1972).

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). However, the slight decrease in the control experiment (without amendment) indicated that there was no active bioremediation in the control since it was not amended with nutrients.

Furthermore, amendment of the polluted mangrove soil with water hyacinth fertilizer increased the percentage Total Organic Carbon (%TOC) at zero hour to 4.16%. The %TOC decreased during the 70day study decreased from 4.16 to 0.83% in water hyacinth amended option. The control experiment showed little decrease from 3.32 to 2.99% within the 70 days period of study (Fig. 9). There was no significant different observed within the two conditions (treatment and control) [F (2, 15) = 0.631, p = 0.545].

The Total Hydrocarbon (THC) in the water hyacinth recipe amended crude-oil polluted mangrove soil, decreased from 14187.03 mg kg⁻¹ to 4119.52 mg kg⁻¹ (Fig. 10). In the control experiment, the Total Hydrocarbon Content (THC) decreased at zero hour from 14146.19 mg kg⁻¹ to 10114.29 mg kg⁻¹ at the 70th day of the study (Fig. 10). 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 water hyacinth (m = 7775.20, SD = 2258.238), with p<0.098.

The changes in the concentration the Petroleum Hydrocarbon (TPH) was monitored at the zero hour/day, 28th day and 70th day of the study for each of treatments. In the water hyacinth recipe amended soil, Total Petroleum Hydrocarbon (TPH) reduced at zero hour from 12517.89 mg kg⁻¹ to 3083.408 mg kg⁻¹ at the 70th day (Fig. 11). In the control experiment, the total petroleum hydrocarbon decreased at zero hour from 12329.677 mg kg⁻¹ to 10674.506 mg kg⁻¹ (Fig. 11).

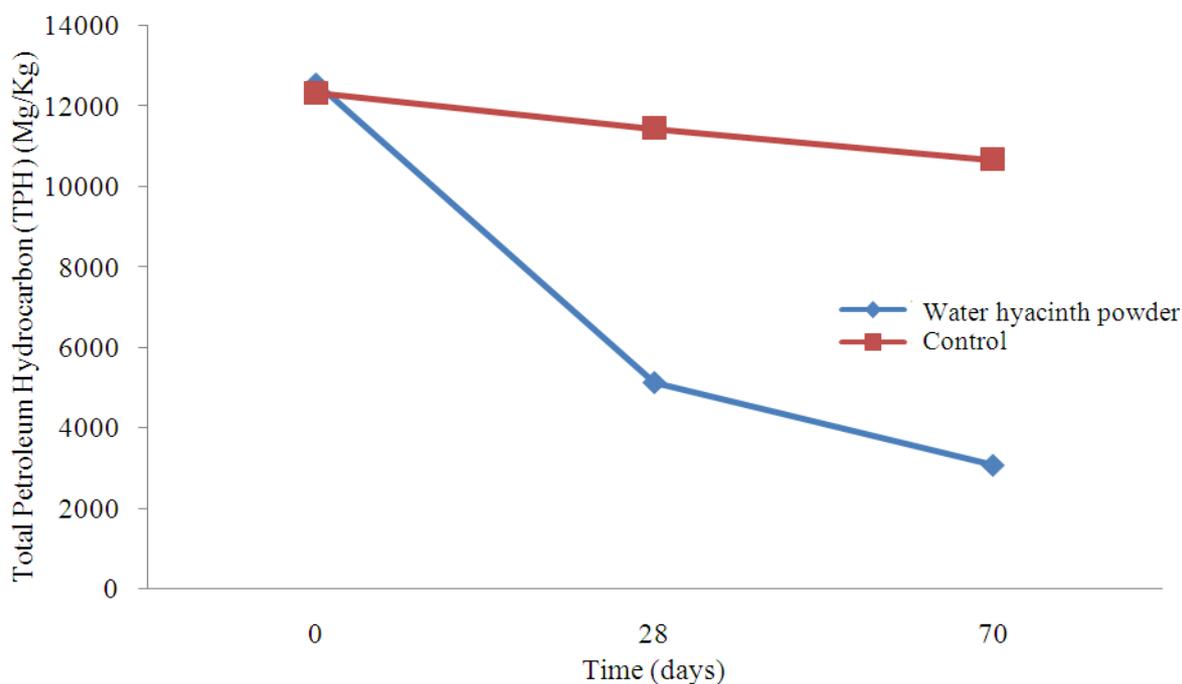


Fig. 11. Changes in the Total Petroleum Hydrocarbon (Mg/Kg) in the polluted soil amended with Water hyacinth powder and control during the 70 day bioremediation study

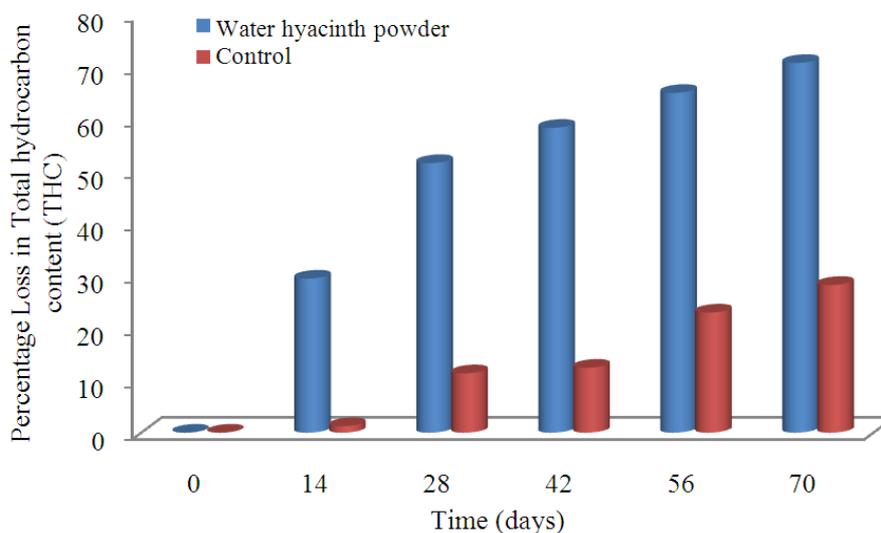


Fig. 12. Changes in the percentage loss of Total Petroleum Hydrocarbon (Mg/Kg) in the polluted soil amended with Water hyacinth powder and control during the 70 day bioremediation study

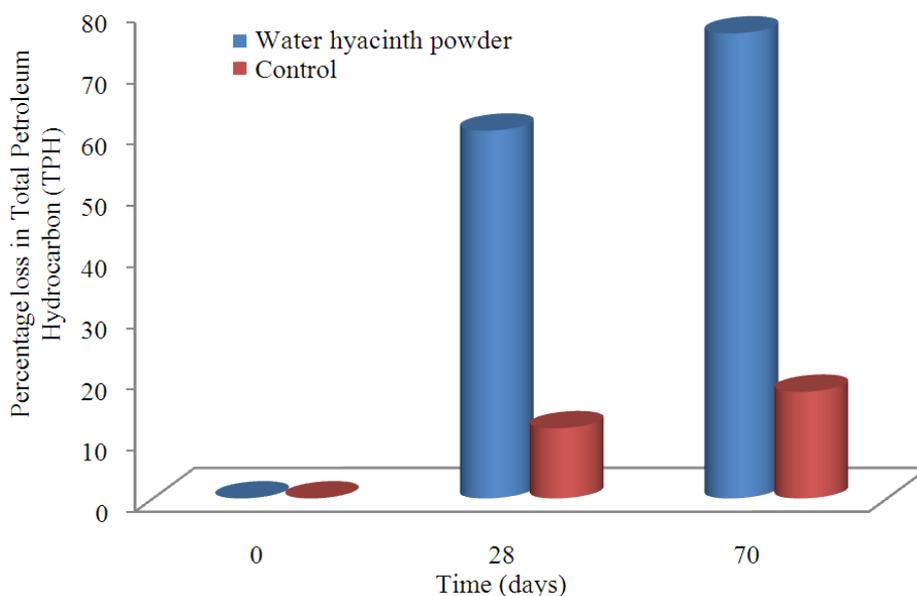


Fig. 13. Changes in the Percentage loss in Total Petroleum Hydrocarbon (Mg/Kg) in the polluted soil amended with Water hyacinth powder and control during the 70day bioremediation study

Post HOC comparisons using the tukey HSD test indicated that the mean scores of Total Petroleum Hydrocarbon (TPH) for the control ($m = 1.2315 E4$, $SD = 1608, 14004$) differed significantly from the mean score for water hyacinth ($m = 7.6455E3$, 3786.93565) with $p < 0.050$ at the 95% confidence level.

The percentage loss in Total Hydrocarbon Content (THC) on the zero day/hour was 0% for the two conditions. However, for Water hyacinth fertilizer experimental set-up, percentage Total Hydrocarbon Content (THC) lost were 29.50 to 70.78% between the 28th day to 70th day respectively (**Fig. 12**). In the control

experimental set-up, 1.25 to 28.28% of THC were lost between the 28th day and 70th day of the study respectively (**Fig. 12**). There was significant difference at $p < 0.05$ level for the two conditions. Post Hoc comparisons using the Tukey HSD test indicated that the mean score for control at each point other than zero hour/day differed significantly from the means of water hyacinth ($p < 0.008$). The percentage loss of Total Petroleum Hydrocarbon (TPH) on the zero hour or day was 0% for all the two conditions (water hyacinth treatment option and the control). Furthermore on the 28th day of the bioremediation study, 58.92 and 7.14% of TPH were lost in water hyacinth amended polluted soil and control experiment respectively (**Fig. 13**). On the 70th day, 75.36 and 13.42% of TPH were lost in the water hyacinth recipe amended soil and control experiments (**Fig. 13**). There was significant difference between the two conditions ($p = 0.50$). Abu and Akomah (2008) studied laboratory assessment of anaerobic biodegradation in another Niger Delta wetland at Ebubu, Rivers State Nigeria. In a biostimulation laboratory model by (Abu and Akomah, 2008) using NPK fertilizer the total hydrocarbon at baseline (1000 mg kg^{-1}) reduced to 600 mg kg^{-1} , 586 mg kg^{-1} , 560 mg kg^{-1} and $387.21 \text{ mg kg}^{-1}$ for zero hour, 14th day, 28th day, 42nd day and 56th day respectively. Abu and Akomah (2008) also reported that the Total Hydrocarbon Content (THC) lost in autoclaved control was not greater than 120 mg kg^{-1} . This little amount of THC lost can be accounted for by natural attenuation process such as photo-oxidation, volatilization and evaporation. It is not surprising that at zero hour in all the experimental groups, there was no loss of either TPH or THC. This is because at the zero hour, microbial metabolism has not actually begun.

In Guanabara Bay, Marciel-Souza *et al.* (2006) reported a positive contribution of natural attenuation in reduction of total petroleum hydrocarbon, Polycyclic Aromatic Hydrocarbons (PAH's) in such mangrove sediments. Okoro (2010) studied bioremediation in Escravos mangrove swamp polluted by crude oil using NPK fertilizer. The scholar reported a decrease in the mean total petroleum hydrocarbon from 5360 ppm to 2360 ppm at zero hour to 5th week of study respectively. It was reported that inclusion of biosurfactant established a significant biodegradation after 7 days of application of the biosurfactant and NPK oleophilic fertilizer. The use of water hyacinth recipe to make provision for limiting nutrient for the purpose of bioremediation has never been published by any known scholar. This means that this study is a pioneer and frontier bioremediation study

using water hyacinth recipe/nutrient powder. In a related development, water hyacinth recipe produced using different approach from our method, was been successfully used in Pakistan for fish ponds fertilization in order to supply nutrients which will promote the growth of grasses for fish grazing in the pond (Widjajant *et al.*, 2002). This study in Pakistan buttresses the fact that water hyacinth is nutrient rich (Widjajant *et al.*, 2002). In Rwanda, water hyacinth recipe/fertilizer produced in a different procedure with our own recipe, was used to increase soil fertility and maize (*Zea mays*) performance/output. In a 90 days study, it was observed that there was a significant increase in output maize in the water hyacinth recipe treated plot and no significance was statistically observed in the control which was not treated with water hyacinth recipe (Gashamura, 2009).

5. CONCLUSION

The results of this research study have shown that water hyacinth recipe/nutrient formula are effective in the supply of limiting nutrients necessary for bioremediation of crude oil impacted media such as mangrove soils. This cost effective fertilizer can be harnessed into preserved powdery forms and be used for bioremediation by Oil and Gas Companies. This study is a pioneer research on the use of water hyacinth powder for bioremediation calls for more research attention especially in field scales where factors such as tidal influence, emulsification cannot be eliminated and are better understood. The expanded scope will include enzymes profile studies during the remediation process. The long term aim of bioremediation design is to develop a cost effective and environmentally friendly approach. Water hyacinth powder apart from being cost effective is also environmentally friendly. These environmental friendly remedial actions are geared towards sustainable development in the Niger Delta.

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6.1. Dedication

The study is dedicated to a family friend of the lead author; late Dr. Nnaukwu Ukoha, who died in July 2007, barely six weeks after his appointment as Secretary to Abia State Government of Nigeria.

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