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Victoria Ginika Awari
1. Microbiology Department, Faculty of Natural and Applied Sciences, Tansian University Umunya, Anambra State, Nigeria
2. Microbiology Department, Faculty of Science, Rivers State University, Nkpolu-Oroworukwo, Port Harcourt, Rivers State, Nigeria

David Nwagozie Ogbonna
Microbiology Department, Faculty of Science, Rivers State University, Nkpolu-Oroworukwo, Port Harcourt, Rivers State, Nigeria

Akani Nnedie Patience
Microbiology Department, Faculty of Science, Rivers State University, Nkpolu-Oroworukwo, Port Harcourt, Rivers State, Nigeria

Douglas Salome I
Microbiology Department, Faculty of Science, Rivers State University, Nkpolu-Oroworukwo, Port Harcourt, Rivers State, Nigeria

Correspondence
Victoria Ginika Awari
3. Microbiology Department, Faculty of Natural and Applied Sciences, Tansian University Umunya, Anambra State, Nigeria
4. Microbiology Department, Faculty of Science, Rivers State University, Nkpolu-Oroworukwo, Port Harcourt, Rivers State, Nigeria

Hydrocarbonoclastic bacteria and organic nutrients mixture potential for biodegradation of hydrocarbon contaminated soils in Niger Delta

Victoria Ginika Awari, David Nwagozie Ogbonna, Akani Nnedie Patience and Douglas Salome I

Abstract
Biodegradation, the use of hydrocarbon degrading microbes also known as hydrocarbonoclastic microbes and/or the use of nutrient organics to clean up crude oil hydrocarbon contaminated soils by assessment of the potentials of the bacteria; *Comamonas testosteroni* and nutrient organics; goat manure (GM), fish waste (FW) were investigated. The aim is to adopt it as treatment approach in biodegradation of crude oil contaminated soils in Niger Delta regions. A total of ten experimental setups of 5000g soil in each, where; a group contained 5% crude oil (5% HCS) and another group contained 10% crude oil (10%HCS) were laid out in an experimental plot for a period of 3 weeks. Applications of treatments (CM, GF and FS) were carried out. Baseline characteristics of soils were determined. Soil samples were collected at seven days interval to monitor microbiological parameters including; total heterotrophic bacteria and fungi, hydrocarbon utilizing bacteria and fungi and physiochemical parameters including; nitrate, sulphate, potassium, magnesium, pH, temperature and total petroleum hydrocarbon. The values of the bioremediation efficiency were determined by the differences in the percentage of TPH concentrations on initial day and on the final day of experiment. The results of the experiments were statistically analyzed. The TPH concentration value on day 1 recorded; 10328.03mg/kg. This reduced in the different treatments employed as degradation progressed and revealed percentage of TPH concentrations in increasing order accordingly: Ctrl (125.71 mg/kg; 1.21%) < CM (2261.01 mg/kg; 21.89%) < GM+CM (7313.47 mg/kg; 70.81%) < FW+CM (7614.74 mg/kg; 1.21%) < GM+FW+CM (8100.59 mg/kg; 78.43%). 5% CS followed similar progression. This result showed that the nutrient organics enhanced the biodegradiation capability of the hydrocarbon degrading bacteria. Although, biodegradation using combination treatment of the nutrient organics added to the bacteria proved more effective and achieved a greater percentage of TPH biodegraded. It is therefore recommended as effective biodegradation option in cleaning up of hydrocarbon contaminated soils in Niger Delta.

Keywords: Bacteria, biodegradiation, hydrocarbon contaminated soil, Nutrient organics, TPH concentration.

Introduction
Petroleum hydrocarbon spill usually pose serious threat to the environment in Niger Delta due to the frequent crude oil spillage and accidental discharge during oil exploration and exploitation [1]. Annually, millions of tons of crude petroleum oil enter the marine environment from either natural or anthropogenic sources. This has over the years impacted the water bodies and farmlands negatively and causing environmental pollution in affected areas [1]. More so, despite its negative effect on the environment, it has greatly affected ecological diversity and agriculture, leaving a large mass of land areas infertile thereby depreciating the local economy of these affected areas [2]. Crude oil is a complex mixture of hydrocarbons. Hydrocarbon is a family of organic compounds or a class of organic chemicals composed entirely of carbon and hydrogen atoms, which bond together as structure of the compound. It is considered to be an organic compound of simplest composition and may be thought to be the parent substance from which other compounds are derived [3]. Annually, million tons of crude petroleum oil seeps into the environment from either natural or man-made sources.
Basically, hydrocarbons is categorized into four groups namely: saturates, aromatics, asphaltenes like; phenols, fatty acids, ketones, esters and porphyrins and the resins like; pyridines, quinolines, carbazoles, sulfoxides and amides. Hydrocarbon compounds bind to soil components and they are difficult to be degraded and eliminated [4].

The various types of hydrocarbons are differentiated by their susceptibility capability to microbial attack. Generally, this susceptibility capability of hydrocarbons to microbial attack in the degradation process can be categorized in decreasing order as follows: cyclic alkanes < small aromatics < branched alkanes < linear alkanes [4]. Although, some compounds like, the high molecular weight polycyclic aromatic hydrocarbons (PAHs) are usually not easily degraded [3]. Petroleum hydrocarbons, which are naturally occurring chemicals, are known to be a major constituent of crude oil and petroleum products. They are used for various anthropogenic activities; such as fueling of vehicles, operations of various machineries and domestic usage [4]. Petroleum hydrocarbons are also very important energy resources used in the various industries and in our daily life. Its constant day to day production in high volumes contributes to constant pollution of the environments and when their presence becomes above regulatory consent-limits in the environments, it automatically indicates environmental pollution [1]. To control such risk, biodegradation constitutes a naturally and environmentally friendly alternative technology that has been established, adopted and applied in order to reduce the menace caused by crude oil spill as a result of exploitation and exploration [2].

Petroleum hydrocarbon can spill into the environment during the refining, during transportation, in storage of petroleum and other petroleum products causing hydrocarbon spill pollution. Petroleum hydrocarbon spill pollution refers to the negative effects of pollution of the environment that hydrocarbon spills have on the environments and living organisms including humans, animals and plants due to the deliberate discharge of petroleum hydrocarbon and other various organic compounds that are contained in petroleum and other distillate products. This can be recycled or eliminated to a great degree as possible, but in some cases, it is usually difficult to recover the spilled materials because, its remains in the environments results in contaminating the affected areas, thereby, posing persistent risks to the environments and the health of humans, animals and plants [4].

Hydrocarbon pollution of the environments and soil usually occur in so many ways. It can occur in the areas where petroleum is found through seepage of petroleum hydrocarbons from shallow reservoirs into the environments. It can also occur by accidental spillage of crude oil on the ground which spills to the surroundings polluting the environments and soil. Regardless of the source of hydrocarbon contamination, once these hydrocarbons come into contact with the soil, first, they contaminate the environments and soil and alter the physical and chemical properties of soil, thereby deteriorating the soil quality. Usually, the degree of this alteration depends on the soil type, the specific compositions of the hydrocarbon constituents spilled, the quantity spilled and also the present environmental condition as at the time of spilling. In occasions where only a small quantity of volatile hydrocarbon spills onto the dry sand, the hydrocarbons will evaporate very quickly, causing little or no chemical and physical damages to the soil and in other situations where a heavy crude oil spill occur onto the clay soil type, the chemicals can remain within the soil for several years, altering the permeability property of the soil [4]. This can cause toxicity in soil and lowering or destroying of the quality of the soil, thereafter. In such conditions, the soil will become a source of pollution itself and these will eventually cause reduction in agricultural produce resulting to famine and depreciation of the economy at large [4]. In such circumstance, soil which acts as reservoir of residual pollution, will then release contaminants into groundwater or air over extended periods of time, usually, even after the original source of pollution has been eliminated [5].

Hydrocarbon spills causes imbalance in the physicochemical properties of soil. It alters the carbon-nitrogen contents of soil once a spill occur from the site. This is because some of the essential components of crude oil include mixture of carbon and nitrogen [6]. After an oil spill occurs, it causes a nitrogen deficiency in an oil soaked soil [6]. Other nutrients such as: phosphorus and sulphate also play a more critical role in limiting the capacity of biodegradation rate of petroleum and it products in soil [6].

Over the years, the technology commonly used for soil remediation includes methods like; mechanical, burying, evaporation, dispersion and washing [5]. However, these technologies are expensive, they take a very long time to be actualized and it can result in incomplete decomposition of contaminants present in the petroleum. In recent times, the process of bioremediation which constitutes the primary mechanism for the elimination of hydrocarbons from contaminated soils or sites by natural existing consortium of hydrocarbon degrading microorganisms [4]. The concept of bioremediation functions basically on biodegradation. Biodegradation involves mineralization of harmful and toxic organic contaminants into less harmful and less toxic compounds like; cell protein, carbon dioxide, water and other inorganic compounds. Biodegradation can also be described as the transformation of complex organic contaminants into simpler organic compounds by means of biological agents like microorganisms found in the environments [4]. According to [7], it can be defined as the usage of microorganisms to decontaminate, detoxify, or eliminate pollutants from polluted soil or site. Considering the diverse metabolic capabilities of the associated hydrocarbon degraders, it is an evolving and innovating method for the removal and degradation of many environmental pollutants from the environment including the products of petroleum industy [7]. In addition, bioremediation technology is believed to be a non-invasive and a relatively cost effective method of removal of environmental pollutants from the environments [7] and alternative treatment strategy that is considered effective, minimally hazardous, economical, versatile and environment-friendly in the cleaning-up of pollutants from the environment [8].

[7] stated clearly that basically, there are two main approaches to hydrocarbon spill bioremediation / biodegradation that has gained increased interest and application over the past decades. These are (a) bioaugmentation, in which known hydrocarbon degrading bacteria are added to supplement the existing indigenous microbial population, and (b) bio stimulation, in which the growth of the indigenous hydrocarbon degraders are stimulated by the addition of nutrients or other growth-limiting co-substrates.

Bioremediation methods are currently receiving favorable publicity as promising environmental friendly treatment technologies for the remediation of hydrocarbons [9]. Many of the components of crude oil products such as benzene,

toluene, ethyl benzene and xylene and compounds that are readily biodegradable are frequent ground water contaminants and are generally utilized as growth substrates or utilized as sole source of carbon for growth and energy by many Genera of bacteria [10, 11].

Recommendations have been advocated for an environmentally friendly approach that can be cheap and at the same time readily available for the remediation of oil spills. Several researchers including: [12, 13, 14, 15, 16, 17, 18] have investigated on the applications of nutrients formulations for bio stimulation of hydrocarbon contaminated soil which were very effective as the use of bioaugumentation with indigenous and exogenous hydrocarbon degrading bacteria. Hence, this study was designed to evaluate the efficacy of hydrocarbon degrading bacterium in combination with organic nutrients: goat manure and fish wastes with the aim to adopt it as an effective treatment approach to enhance the biodegradation rate of crude oil contaminated soils or sites in the Niger Delta regions, Nigeria in the quickest possible period of time in order to restore the environment to its initial status and for sustainability that can enhance the economy of the oil producing regions that were previously affected by crude oil spills.

Materials and Methods

A. Study Area / Sample Collection

The choice of agricultural training and developmental farming centre of Rivers State University, Port Harcourt was considered based on factors including; the farm has recorded no history of crude oil spill over the years, availability of water, easy accessibility and sufficient space with relatively flat topography. The yearly rainfall is bimodal. This begins from middle March and ends in late November observing their peaks in June and September with short periods of lower rainfall in August, which is usually referred to as the August Break. The only dry months are observed in January and February. While, the annual temperature ranges between 21 ^{o}C and 31 ^{o}C. The well-secured area serves acts as a centre for farming, training, research, demonstration, production of crops including yam, cassava, cocoyam, sweet potato, maize, rice, beans, plantains, vegetables and fruits as well as development for sustainable agricultural practices. The farm land area used for the present study is a pristine patch of land within coordinates 4.80474 Lat.N4^{0}48'1707804'' and 6.97579 Lon.E6^{0}58'33.15828'' measuring 525cm x 525cm with a total area of 275,625cm^{2}. This area was cleared and partitioned equally for the various experimental setups.

B. Collection of Soil Samples

A total of five (5) sampling points were considered for the study for the sample collection. Soil samples were collected from the different sampling points from agricultural training and developmental farming centre of Rivers State University, Port Harcourt, Rivers State. Global Positioning System (GPS) machine was used for measurement of the position and location of the sampling site. Thereafter, top soil samples were collected using standard procedures as adopted and stated in food and agricultural organization (FAO) [19]. This was carried out by tilling of the soil with clean manual soil auger from a depth of 0-15cm. The soil samples collected were of sandy-loamy texture with specific gravity of 2.61, were bulked after collection to obtain and 400kg quantity of the representative composite soil sample were weighed. The soil samples were then packed into freshly bought moderate size black poly-ethylene bags **[15, 20]** and transported immediately to the experimental plot.

C. Collection of Crude Oil Sample

Crude oil was aseptically collected in twenty litres large sterile plastic jerry cans from an oil company located at Nembe Creek, Bayelsa State. The crude oil collected was of Bonny light and was filtered to minimize contamination from point of collection.

D. Preparation of Soil for Contamination

The soil sample was prepared for bioremediation experiments in line with the experimental unit design (Figure 2.1). Five (5KG) of composite soil were weighed separately into the freshly bought unused black poly-ethylene bags. The bags were perforated with spatulas to allow for aeration and orderly laid out in the experimental plot. The pristine soil was separated to serve as control (CTRL 1) for standard reference. The experimental set up about were then allowed to fallow for a period of six days. After which, contamination began on the following day [15, 20, 21]. The sample treatment codes for the bioremediation procedures as applied on the experimental units (SU) used in the study was made up of five units in duplicates and these represented two batches of contamination levels (5% and 10% contaminated sample setups containing 250mls and 500mls of crude oil (CO) in each setup respectively with control setup samples (CTRL 1 and 2). All the setup soil samples were thoroughly mixed with separate spatulas and left for a period of three weeks for proper contamination so as to allow even distribution and mixture of soil and oil in order to mimic a typical crude hydrocarbon spill site according to [15, 20].

E. Source of Nutrients for Biostimulation

Two nutrient amendments were used in this study for biostimulation. These included; goat manure which was aseptically obtained from the abattoir located at Rukpokwu District in Port Harcourt, Rivers State. Whereas, fish wastes were obtained from fish market located at Rumokoro District in Port Harcourt, Rivers State.

F. Source of Bacteria for Bioaugumentation

The bacteria used in this study; *Comamonas testosteroni*. This specie of bacteria was isolated using standard microbiological methods from the top layer of the soil down to a depth of 5cm. The soil sample collected from the study site was homogenously mixed. The required culture media as freshly prepared and poured into different Petri-dishes, thereafter distinct colonies with varying cultural characteristics, suspected to be *Comamonas* species were picked and sub-cultured onto freshly prepared culture media plates. Plates were incubated at 37 ^{o}C for 24 hours and observed for viable colonies.

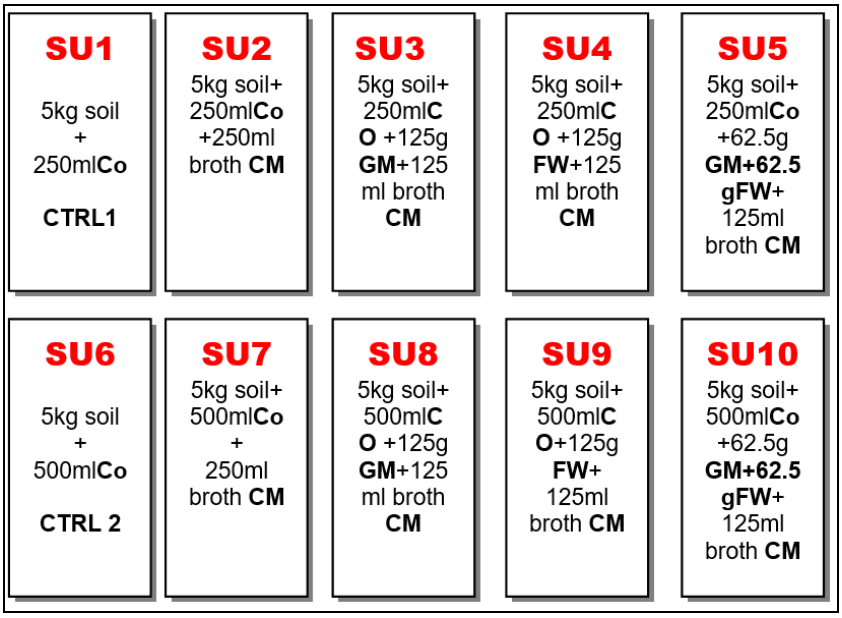


Fig 1: Experimental setup layout

G. Microbiological Analyses

1- Serial dilution

One (1) gram of soil sample from each of the bags was added separately to nine (9) mls of sterile normal saline in separate test tubes which was used as diluents. The test tubes were stirred thoroughly and ten- fold (volume/volume) serial dilutions were made by transferring 1ml of the original solution using pipette into freshly prepared sterile normal saline diluents for up to 10^{-6} dilution [22].

2- Inoculation and incubation

Separate sterile pipettes were used to collect 0.1 ml aliquots from the different dilutions and were inoculated onto the surfaces of the dried appropriate medium in triplicates and spread uniformly using sterilized glass spreaders. Inoculated plates were then incubated for 24 hours at 37º C and for 72 hours at room temperature for the bacteria and the fungi plates respectively [22].

3- Enumeration of Total Heterotrophic Bacteria and Fungi Count

An aliquot (0.1mls) of 10^{-7} dilution was transferred aseptically unto properly dried freshly prepared nutrient agar plates containing fungusol (antifungal) and tetracycline (antibiotic) in order to suppress the growth and multiplication of fungi and bacteria respectively, in triplicates. Plates were spread uniformly using sterilized glass rods and incubated for up to 24 to 48 hours at 37ºC and. for up to 3 days at 28ºC, for the enumeration of total heterotrophic bacterial and fungal counts respectively. Appropriate dilution was transferred aseptically unto properly dried Nutrient Agar and Sabouraud Dextrose Agar plates accordingly [22].

At the end of the incubation period, the viable bacterial and fungal colonies observed on the plates were counted and sub-cultured unto freshly prepared appropriate plates accordingly. Counts for each sample were then enumerated and extrapolated, using the formula as shown in equation (1).

$$\text{THBC/FC (CFU/g)} = \frac{\text{Number of Colonies}}{(\text{Dilution} \times \text{Volume plated})}$$ [22]. (1)

4- Enumeration of Hydrocarbon Utilizing Bacteria and Fungi Count

Aliquots (0.1mls) from the dilutions of 10^{-2} were plated separately in triplicates using separate pipettes on freshly sterilized and prepared Mineral Salt Agar (MSA) plates using the spread plate technique. The antifungal; fungusol was added to the mineral salt agar plates for the enumeration of hydrocarbon utilizing bacteria in order to suppress the growth of fungi, while, the antibiotics; tetracycline was added to the mineral salt agar plates for the enumeration of hydrocarbon utilizing fungi in order to suppress any bacterial growth. Filter papers saturated with sterile crude oil was aseptically placed on the inside of the cover plates and then inverted. Inoculated plates were incubated for 5 days at 28°C. Plates were observed at the end of the incubation period. Plates that yielded growth between 30 to 300 colonies were enumerated afterwards for bacterial isolates. Also, plates with fungal colonies were enumerated accordingly [23].

5- Purification of pure microbial isolates

Distinct representative bacterial colonies were picked aseptically and repeatedly transferred by the streak-plate method for purification onto sterilized culture plates of freshly prepared nutrient agar plates. Inoculated plates were allowed to grow for 24 hours at 37ºC. Discrete colonies as observed on the nutrient agar plates were transferred aseptically into 10% (volume/ volume) bijour bottles of glycerol suspension, well labeled and stored at temperature of -4ºC as stock cultures of pure microbial isolates. The isolates were preserved for further investigations.

H. Identification of Bacterial Isolates for the Biodegradation Procedure

Isolated colonies of the different hydrocarbon degrading bacteria were identified and characterized based on their colonial morphology and microscopic examination as described by [22], while identification to species was done using molecular identification method according to [24].

I. Physiochemical Parameters

The physiochemical parameter analyzed in this study were

carried out according to standards methods as adopted by APHA standard methods [25].

J. Determination of the Total Petroleum Hydrocarbon Content

The Total Petroleum Hydrocarbon (TPH) contents of each set up of the experimental plot was determined using Gas Chromatography (GC) analytical method (1440 GC-FID: California, USA) according to [25]. Samples were analysed at seven days interval, on day 7, 14, 21, 28, 35, 42, 47 and on day 56 accordingly, for up to eight samplings.

K. Bioremediation Experiment Procedures

The bioremediation experiment in this study was carried out in five stages accordingly; Nutrients were prepared, bacteria were screened for their hydrocarbon degradation capabilities, bacteria inoculum were prepared, treatment procedures were applied and experimental setups were monitored.

1- Preparation of nutrients

The nutrients amendments to be used including; goat manure and fish wastes were spread on wooden pallets and allowed in the open air in order to be sun dried for about 20 days. Thereafter, each nutrients was ground to powder using clean washed laboratory grinder, after which, the particles recovered was sieved separately through a 2mm particle size sieve in order to obtain smooth texture fine particles. Thereafter, 125g and 250g of each nutrient was carefully weighed separately and wrapped in clean foil papers, ready for use, for the bioremediation work [21, 23].

2- Screening of bacteria for hydrocarbon degradability

The bacteria were screened for hydrocarbon degradation using the turbidometric method. A UV Spectrophotometer (S-HP10012414-50, 72ID-UK) programmed at a wavelength of 600nm. A volume of 50ml Bushnell Hass broth containing (1%, volume/ volume) of crude oil containing 0.05% Tween 80 added to the mixture was dispensed into 100ml contents Erlenmeyer's flasks. The Erlenmeyer's flasks were covered with clean cotton wool, sealed with foil paper and then sterilized in an autoclave for 15 minutes at 121^{o}C (15 psi). After the sterilization of the consortium, the broth was cooled and thereafter, inoculated with standardized bacterial inoculum (5%, volume/ volume). The inoculated flasks were then, incubated in a rotary shaker incubator for 14 days at 28^{o}C to 30^{o}C and at 150 rpm rotation speed/rate. The turbidity of all samples were determined at the end of the incubation period [26].

3- Preparation of bacteria inoculum

The test organisms to be used for the biodegradation experiment were sub-cultured onto sterilized and freshly prepared Bushnell Hass Agar plates. Inoculated plates were incubated for 96 hours at 30^{o}C [26]. At the end of the incubation period, the plates were observed and examined for any viable and distinct colonies on the surfaces of the culture media. Thereafter, pure cultures were obtained separately, for each of the representative isolate and were inoculated separately into Bushnell Hass broth in 1000ml Erlenmeyer flasks which were plugged with clean and sterilized cotton wool, sealed with foil paper and allowed to stand in order to observe for the growth of the augmenting test organisms. Broth cultures were also standardized using 0.5 McFarland solutions and used for the biodegradation process [26, 27].

4- Application of treatment procedures

The different treatments was applied accordingly into the different setups as designed (Figure 1) in order to evaluate the efficiency of crude oil degradation capacities in the contaminated soils using the untreated soil setups as controls for comparism. The treatment procedures were as follows; (a) 250mls broth of *Comamonas testosteroni* (CM) (b) 125mls broth of *Comamonas testosteroni* (CM) and 125g of goat feaces (GF) (c) 125mls broth of *Comamonas testosteroni* (CM) and 125g of fish waste (FW) (d) 125mls broth of *Comamonas testosteroni* (CM), 62.5g of goat feaces (GF) and 62.5g of fish waste (FW) was added to the required setup soil samples, according to the experimental setup layout and mixed [28, 29, 10]. Tilling and watering were carried out in all the setup sample treatments with separate spatulas every three days to allow for sufficient aeration according to [30].

5- Monitoring of the bioremediation process

Samples were collected and monitored at seven days interval for up to fifty (56) days, after the application of the different treatments on the different setup samples (at day 7, 14, 21, 28, 35, 42, 49 and 56) accordingly. Soil samples from each of the fourteen bag setups were collected after mixing thorougly using separate soil spatulas to obtain homogenous mixture according to the methods adopted by [16]. Microbiological, physicochemical as well as chromatographic analyses were determined. Composite soils were obtained from each sample setup by homogenizing 5g of soil collected from five different points from the setup and taken immediately to the laboratories for analyses. The percentage bioremediation was calculated by the expression as shown in equation (2) [16, 29, 31].

$$\% \text{ B.R} = \left(\frac{TPHCi - TPHCf}{TPHCi}\right) x \left(\frac{100}{1}\right) \quad (2)$$

Where;

TPHCi is the initial concentration of petroleum hydrocarbons (TPH) of the experiment on day 7, TPHCf is the final concentration of petroleum hydrocarbons (TPH) of the experiment on day 56% B.R is the percentage bioremediation of the total petroleum hydrocarbon content in the soil [16, 29, 31].

L. Statistical Analyses of Data

All experiments analyzed were statistically compared using one-way ANOVA followed by Duncan's multiple comparism test in order to compare and ascertain for the significant differences in the various. Statistical significance was expressed as the *P*-values of less than 0.05 (< 0.05) at 95% confidence interval. Chromas-Lite was used to analyse the sequence data generated while phylogenetic trees were extrapolated from Neighbour-Joining method from the Gene Bank.

Results and Discussion

A. Results of microbial counts

The microbial counts revealed that, the total heterotrophic bacterial count (THBC) for goat manure ($1.41\pm0.05x10^{9}$cfu/g) was significantly higher than the fish

wastes ($7.0\pm0.35x10^8$cfu/g) with a P-value of 0.0012. However, the fungal count (FC) for goat manure ($2.0\pm0.42x10^5$cfu/g) was also higher than that of fish wastes ($1.0\pm0.28x10^5$cfu/g) although the difference between the mean count was not significant and recorded a P-value of 0.1091 (Table 1). The high microbial count results as recorded in the stimulants in the present experiment could be as a result of the high nutrient contents in both stimulants. This concur with suggestions in research works by [15]. The nutritional composition of fish wastes carried out by [32] showed that, iron and crude protein were their highest composition. Iron is necessary for microbial growth due to its role in the synthesis of vital enzyme in the electron transport chain, while, protein is also necessary for the structural and functioning of a living organism. Goat manure on the other hand has also been reported to contain high level of proteins and iron due to the nature of feed the goats are given [33].

The result of the microbial counts of the crude oil contaminated and uncontaminated soil samples enumerated, all the microbiological parameters analyzed showed significant difference ($p<0.05$) between the two samples except the total heterotrophic bacterial count which had higher counts ($2.58\pm0.07x10^8$) in the uncontaminated soil than the crude oil contaminated soil ($2.10\pm0.50x10^8$). Whereas, the fungal counts (FC) in the uncontaminated soil sample (2.0 ± 0.08 x10^5cfu/g) were significantly higher than the crude oil contaminated soil sample ($1.6\pm0.05x10^5$cfu/g) (Table 2). Our results further showed that, the hydrocarbon degrading bacterial (HDB) counts in hydrocarbon contaminated soil sample ($5\pm0.50x10^4$cfu/g) was significantly higher than the uncontaminated soil sample ($8\pm0.50x10^3$cfu/g). Also, the hydrocarbon utilizing fungal (HUF) counts in crude oil contaminated soil sample ($7\pm0.50x10^4$cfu/g) was significantly higher than the uncontaminated soil sample ($9\pm0.50x10^3$cfu/g) (Table 2). The results of this present study, obtained from the higher microbial counts in normal soil than in the crude oil contaminated soils showed that, bacteria and fungi were higher in normal soil than in the crude oil contaminated soil, thereby, implying that, these microorganisms grow better in nutrient sufficient conditions [34]. Suggested that the presence of hydrocarbon in the crude oil contaminated soil depletes the nutrient level in soil. This result is similar to the study performed by [35] who in their studies, recorded higher counts in the total heterotrophic bacteria and total fungi present in unpolluted soil than polluted soil and recorded higher counts of HUB and HUF in the soil collected from a hydrocarbon contaminated site than the unpolluted site.

The microorganisms isolated from the stimulants showed that the goat manure had more bacteria and fungi diversity than the fish wastes (Table 3). This may be as a result of the higher microbial counts obtained from the goat manure than the counts obtained from the fish wastes.

Similar bacteria including: *Comamonas* sp., *Staphylococcus* sp., *Citrobacter* sp., *Pseudomonas* specie and *Bacillus* specie were isolated from both soil samples and in addition, the uncontaminated soil sample only had *Proteus* specie. Also, similar fungi (*Mucor* sp., *Rhizopus* specie, *Penicillium* specie and *Fusarium* specie) were isolated from the uncontaminated and the contaminated soil samples (Table 3). These results therefore, demonstrate the presence of more microbes and diversity of bacterial population available in the soil not contaminated than in the soil that has been contaminated with crude oil.

The result indicated that, crude oil contamination shifts the dynamics of microbial population towards crude oil degrading microbes [20]. This result concurs with research by [24] who reported significant difference between the heterotrophic microorganisms and oil-degrading microorganisms of pristine soil and the crude oil polluted soil

In Environmental Microbiology, it is important to accurately identify organisms that are responsible for degradation of specific materials such as crude oil in a contaminated environment. However, molecular Identification revealed the hydrocarbonoclastic bacteria; *Comamonas testosteroni MN273753* and some other individual organisms identified to their respective strain levels by Polymerase chain reaction (PCR) method as; and *MA1 | Comamonas testosteroni MN273753 MA2 | Staphylococcus saprophyticus MN273754 MA3 | Chryseobacterium cucumeris MN273755, MA4 | Pseudomonas aeruginosa MN273756MA5 | Bacillus amyloliquiefaciens MN273757*, as shown in Figure 2.

Table 1: Microbial Counts of Fish Wastes and Goat Manure

Microbiology / Parameter	Fish Wastes (CFU/g)	Goat Manure (CFU/g)	P-value
Total Heterotrophic Bacterial Count (THBC)	$7.0\pm0.35x10^8$	$1.41\pm0.05x10^9$	0.0012
Fungal Count (FC)	$1.0\pm0.28x10^5$	$2.0\pm0.42x10^5$	0.1091

Statistical significance was considered at P-value of < 0.05

Table 2: Microbial Counts of Soil Samples (Uncontaminated and Crude oil Contaminated)

Microbiology Parameter	Soil (Uncontaminated)	Soil (Crude oil Contaminated)	P-value
THBC (cfu/g)	$2.58\pm0.07x10^8$	$2.10\pm0.50x10^8$	0.1375
FC (cfu/g)	$2.0\pm0.08x10^5$	$1.6\pm0.50x10^5$	< 0.0001
HUBC (cfu/g)	$8\pm0.50x10^3$	$5\pm0.50x10^4$	0.0045
HUFC (cfu/g)	$9\pm0.50x10^3$	$7\pm0.50x10^4$	0.0086

Key: THBC=Total heterotrophic bacterial count; FC=Fungal count; HUBC=Hydrocarbon utilizing bacterial count; HUFC=Hydrocarbon utilizing fungal count. Statistical significance was considered at P-value of < 0.05.

Table 3: Microorganisms Isolated From Goat Manure and Fish Wastes

Microorganisms	Fish wastes	Goat manure	Uncontaminated soil	Crude oil contaminated soil
Bacteria	Escherichia coli Bacillus specie Proteus pecie Staphylococcuspecie Acinetobacter specie	Klebsiella pecie Staphylococcus specie specie Bacillus specie Sarratia specie Pseudomonas specie Shigella specie Escherichia coli	Comamonas specie Staphylococcus specie Citrobacter specie Pseudomonas specie Bacillus specie Proteus specie	Comamonas specie Staphylococcus specie Citrobacter specie Pseudomonasspecie Bacillus specie
Fungi	Candida specie Mucor specie Penicillium specie	Mucor specie Penicillium specie Candida specie Yeast	Mucor specie Rhizopus specie Penicillium specie Fusarium specie	Mucor specie Rhizopus specie Penicillium specie Fusarium specie

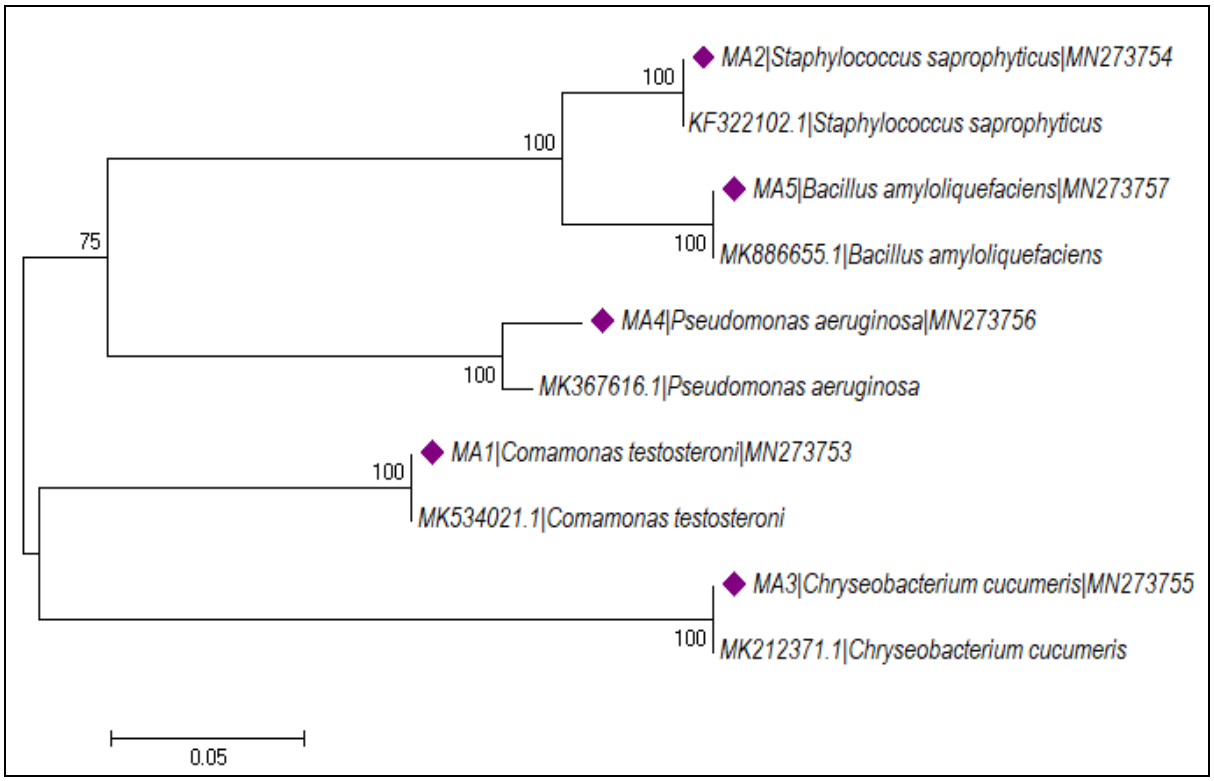
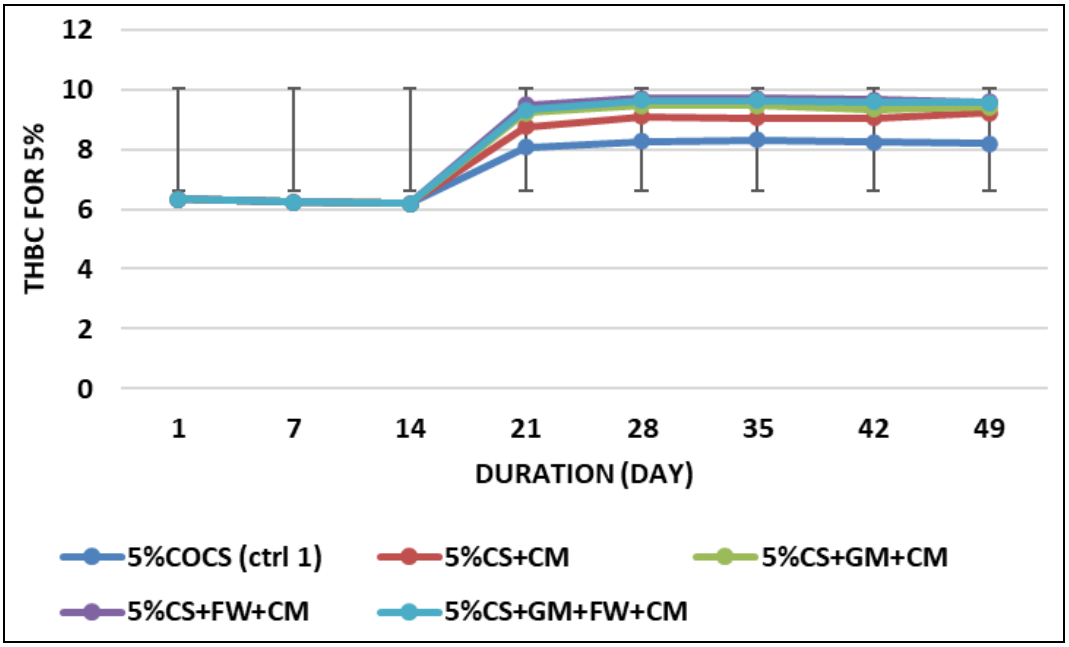


Fig 2: Phylogenetic Tree of the Hydrocarbon utilizing bacteria (*Comamonas testosteroni*) and other Bacterial Isolates with their Accession Numbers

The microbial count obtained within the sampling period for the various setup samples from day 7 to 56 during the study are graphically presented in Figures 3i, 4i, 5i and 6i. Also, Figures 3ii, 4ii, 5ii and 6ii below for the total heterotrophic bacterial (THB), total heterotrophic fungal (TF), hydrocarbon utilizing bacterial (HUB) and hydrocarbon utilizing fungal (HUF) counts for 5 and 10 percent crude oil contaminated soil samples respectively. The Hydrocarbon utilizing bacterial counts (HUBC) ranged from $7.94x10^3$cfu/g (control sample) to $2.4x10^4$cfu/g (treated samples).The Hydrocarbon utilizing fungal counts (HUFC) ranged from $5x10^3$cfu/g (control sample) to $1.9x10^4$cfu/g (treated samples). The total heterotrophic bacteria counts (THBC) ranged from $1.55x10^6$ cfu/g (control sample) to $5.5x10^9$ cfu/g (treated samples). The total fungal counts (TFC) ranged from $9x10^3$cfu/g (control sample) to $7x10^4$cfu/g (treated samples).

The results show that the microbial counts were observed to be lower in all the control samples than in the supplemented soil sample setups. This result is in accordance with work done by [21], who reported lower counts in the control samples than in the treated soil samples. Similarly, the low microbial counts in the control samples corroborate work done by [31]. Futhermore, microbial counts increased with time as degradation progressed and thereafter, maintained negligible changes across the trend throughout the experiment.



Key: ctrl: control, cocs/cs: contaminated soil, CM: *Comamonas* specie, GM: goat manure, FW: fish waste.

Fig 3i: Total Heterotrophic Bacterial Counts (THBC) of 5% crude oil contaminated soil samples

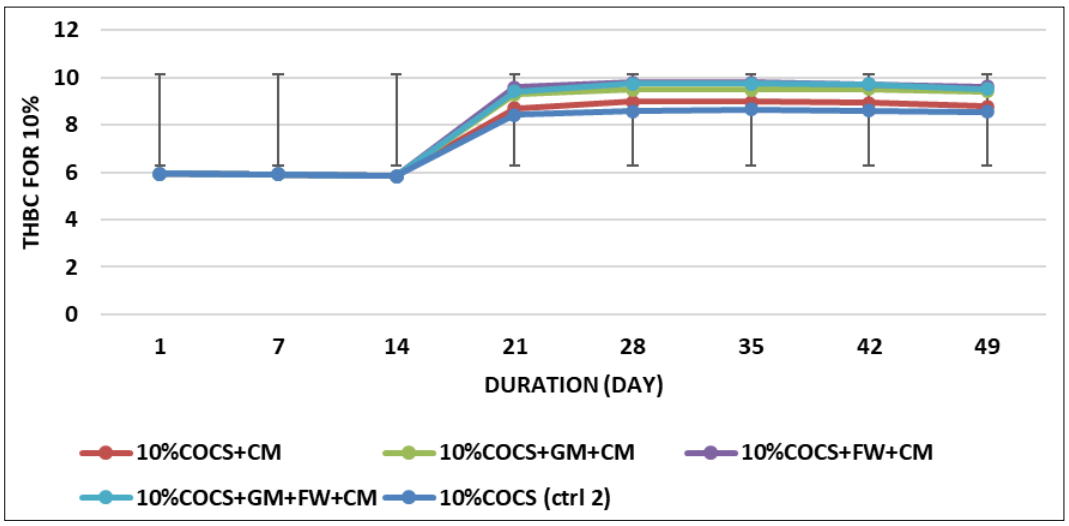


Fig 3ii: Total Heterotrophic Bacterial Counts (THBC) of 5% crude oil contaminated soil samples

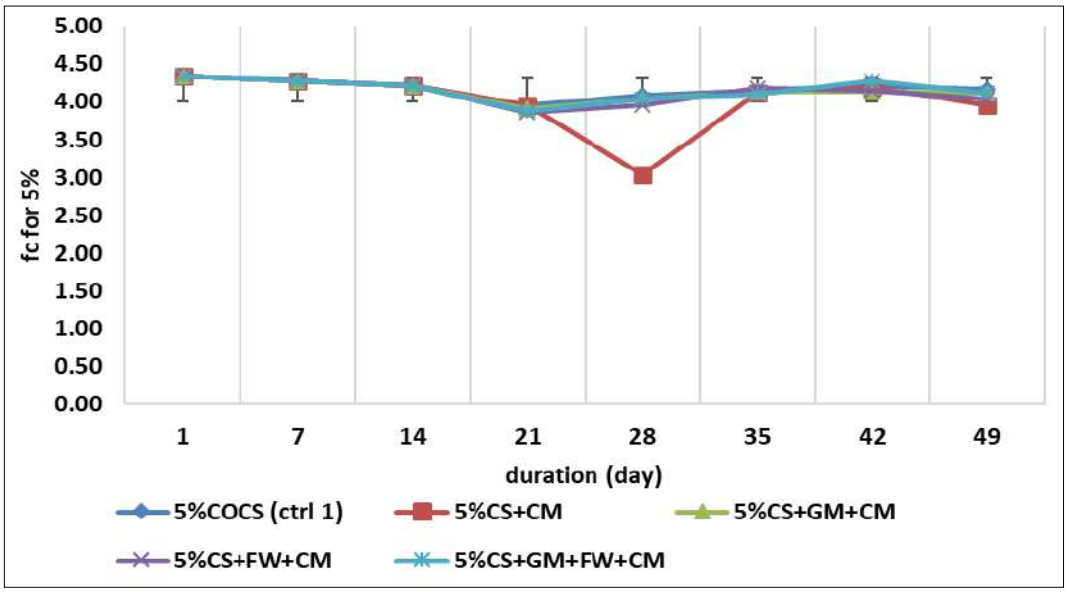


Fig 4i: Total Fungal Counts (TFC) of 5% crude oil contaminated soil samples

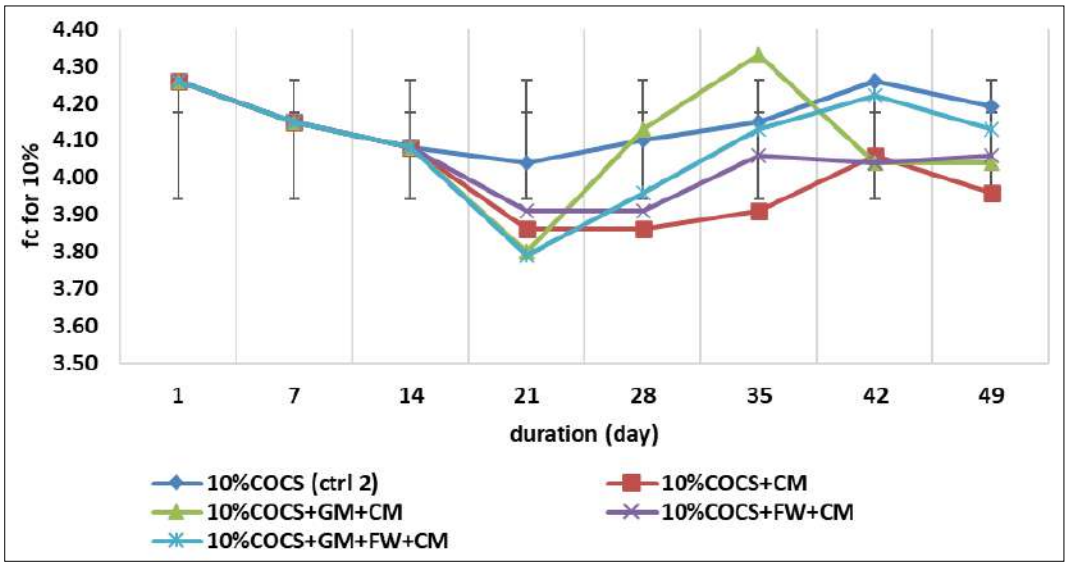


Fig 4ii: Total Fungal Counts (TFC) of 10% crude oil contaminated soil samples

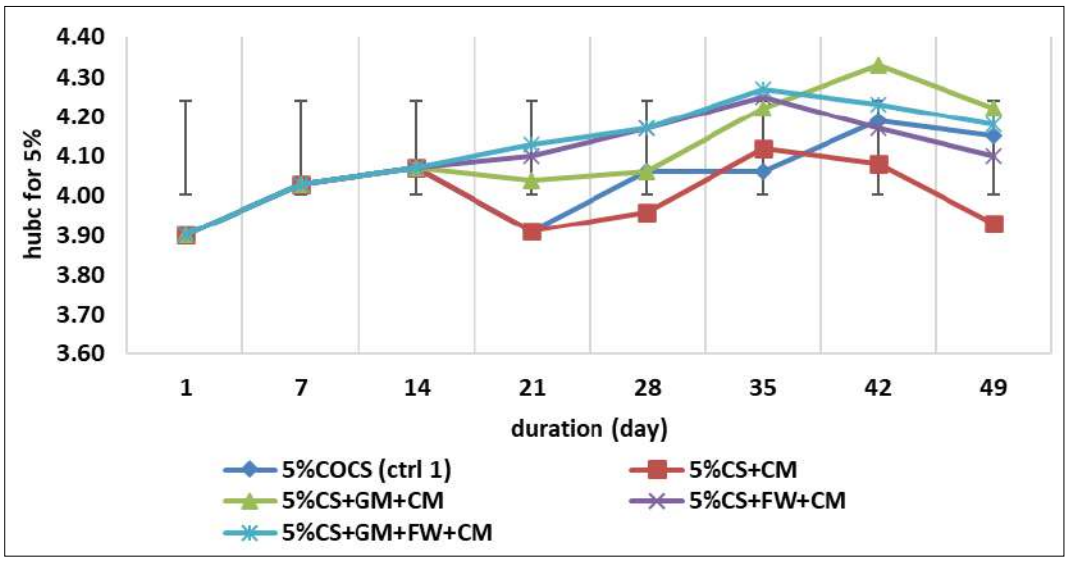


Fig 5i: Hydrocarbon Utilizing Bacterial Count(HUBC) 5% crude oil contaminated soil

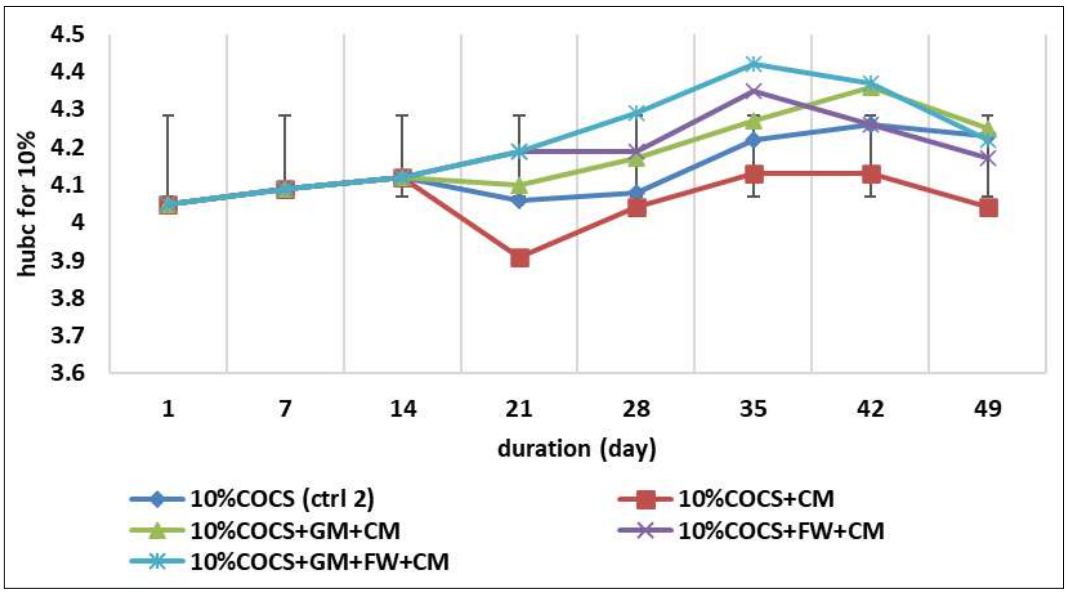


Fig 5ii: Hydrocarbon Utilizing Bacterial Count (HUBC) 10% crude oil contaminated soil

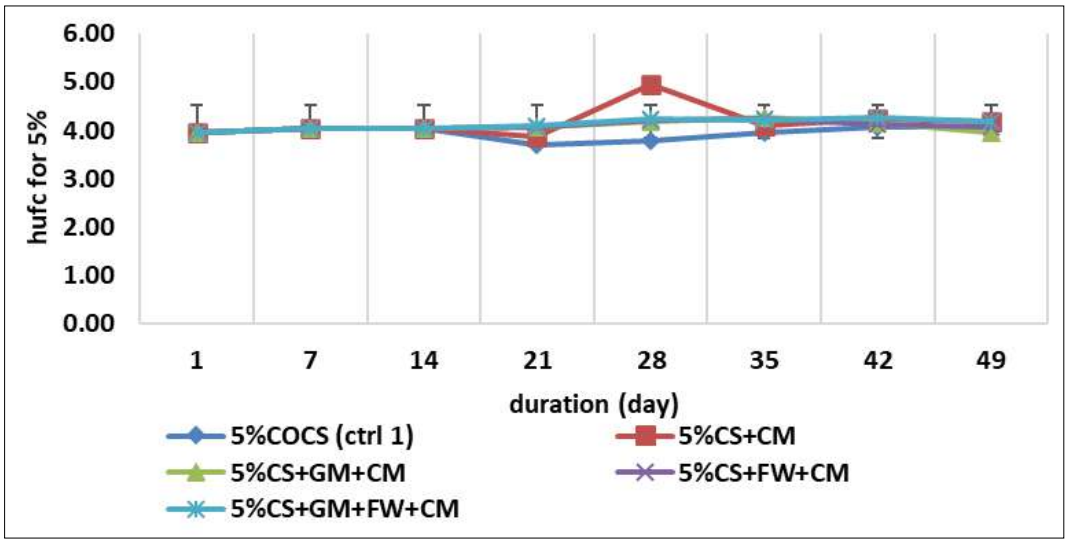


Fig 6i: Hydrocarbon Utilizing Bacterial Count (HUBC) 5% crude oil contaminated soil

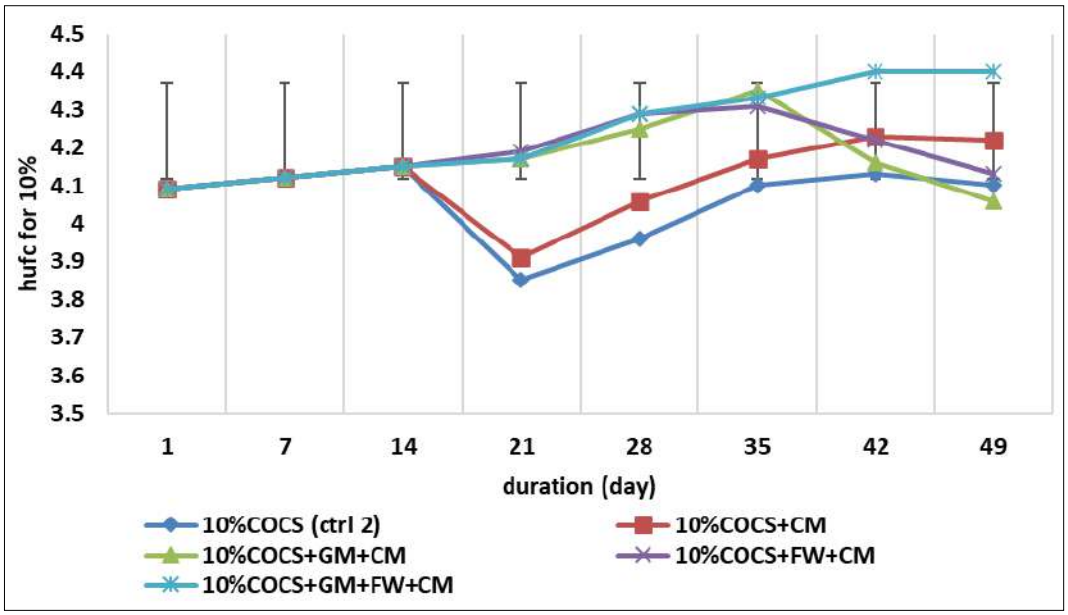


Fig 6ii: Hydrocarbon Utilizing Bacterial Count (HUBC) 10% crude oil contaminated soil

B. Results of physiochemical parameters

Physicochemical parameters of soil are greatly affected by crude oil contamination [24]. All the parameters analyzed revealed significant variation between the contaminated soil (CS) and uncontaminated soil (UCS) and recorded: Nitrate(mg/kg) (811.5±0.70: UCS) and (791.5±0.65:CS), (P = < 0.0001), Sulphate (mg/kg) (734.1±20.1:UCS) and (735.9 ±15.5:CS, Phosphate (mg/kg) (15.78±8.0:UCS) and (15.9±63.84:CS) (P=0.0057), magnesium (mg/kg) (3.3±0.07638 UCS) and (3.1±0.07638 CS) (P = 0.0327). (P = 0.0003), THC (mg/kg) (2.02±0.02: UCS) and (6546±5.74: CS), (*p*=< 0.0001) as shown in Table 4. The difference in the level of hydrocarbon contents of the CS and UCS samples confirms the availability of crude oil in the CS which was significantly higher than the UCS. The results also showed that nitrate and magnesium were significantly higher in the UCS than in the CS. This could be due to the fact that the higher number of the HUB and HUF as reported in the microbial counts of the contaminated soil could be have using up some of the essential nutrients available in the contaminated soil. Although on the contrary, the concentration of the phosphate and sulphate contents were significantly higher in the CS than the UCS sample. This report concurs with [15] who reported a higher concentration of nitrate and magnesium in their soil samples collected from an unpolluted site than in the soil samples collected from a crude oil polluted site.

Table 4: Physicochemical Analyses of Uncontaminated and Crude Oil Contaminated Soil Samples

Physicochemical Parameter	Soil (Uncontaminated)	Soil Crude Oil Contaminated	P-Value
Nitrate (mg/kg)	811.50±0.70	791.50±0.65	< 0.0001
Nitrogen(mg/kg)	1036.20±0.77	993.70±0.68	< 0.0001
Phosphate (mg/kg)	15.782±8.09	15.982±8.46	0.0057
Phosphorous(mg/kg)	23.21±8.59	24.30±8.63	0.0087
Sulphate (mg/kg)	734.13±12.10	735.94±15.59	0.0003
pH	6.85±0.31	5.95±0.26	0.9895
Magnesium (mg/kg)	3.38±0.07	3.18±0.07	0.0327
Potassium (mg/kg)	1.09±0.02	1.11±0.03	0.3305
Temperature (°C)	27.33±0.47	28.10±0.36	0.0892
Total Hydrocarbon Content(mg/kg)	6546±5.74	2.02±0.02	< 0.0001

The mean physicochemical characteristics including parameters like: nitrate, sulphate, potassium, magnesium, pH and temperature, were analyzed at seven days interval during the monitoring process as shown in Table 5. This individual physicochemical parameter was used as criteria to check for the effects of control samples and the supplemented soil samples for 5%CS and 10%CS samples and also, to check for any significant difference in the physicochemical parameters monitored for all the setup treatments. Generally, there were no significant differences in most of the parameters monitored. However, there were significant differences in nitrate concentration for some of the treatment setups for 5%CS samples and in potassium level for most of the 10% treatments. Our result recorded a decline in the nitrate level which increased and dropped again along the trend. Our finding is in line with [36], who observed a decline in nitrate level at the beginning of their study on the bioremediation approach using animal waste and also observed notable fluctuations of the physico-chemical parameter varied along the trend as the degradation rate progressed.

During the monitoring of the sulphate level, an insignificant stability pattern was observed in all the percentages of contaminated soil as there were a drastic increase in the quantity of sulphate level and a decline afterwards for most of the treatment setups. In this study, there was a stable pattern of flow observed in the potassium and magnesium level from the initial day of the experiment to day 21 for 5%CS and 10%CS samples, after which, there were drastic increase in the level of potassium and magnesium followed by a decline in concentrations for most of the treatments. The same fluctuation scenario was observed with 5%CS and 10%CS samples, although, the 10%CS samples experienced a more irregular pattern of flow during the study period. Our findings are in agreement with works done by [15, 36] who observed changes in the level of magnesium, potassium, sulphate and nitrate concentrations as bioremediation progressed with time. There were reductions in the pH level as degradation progressed. Our results on the decrease in the pH level as degradation progressed corroborates work done by [37] who stated clearly that, the reason for the reductions in the pH level could be attributed to the utilization of nutrients by microorganisms and the release of metabolites which in turn acidifies soil samples as degradation progressed thereby, reducing the pH of the medium. Decline in the temperature was observed with negligible changes along the trend during the study period. This agrees with studies carried out by [31] who reported insignificant differences in the temperature during their bioremediation studies.

Table 5: Means of the Physicochemical Characteristics of 5% and 10% Crude Oil Contaminated Soil during Bioremediation

Setup	Treatments	Nitrate (mgKkg)	Sulphate (mg/Kg)	Potassium (mg/Kg)	Magnesium (mg/Kg)	pH	Temperature (ºC)
SU1	5%CS (control 1)	778.7±26.53	724.0±20.63	1.04 ±0.72	1.94 ±1.12	6.44 ±0.3116	27.78±0.17
SU2	5%CS+CM	798.6±3.625^{b}	735.2±9.12	1.03 ±0.23	1.94 ±1.01	6.57 ±0.5748	27.63±0.34
SU3	5%CS+GM+CM	791.7±7.370	746.8±15.71	2.05 ±1.22	2.06 ±0.92	6.79 ±0.4198	27.64±0.28
SU4	5%CS+FW+CM	797.3±5.618^{b}	751.2±21.39	2.14 ±1.29	2.07 ±0.91	6.54 ±0.4897	27.54±0.42
SU5	5%CS+GM+FW+CM	797.2±7.925^{b}	756.3±19.95^{a}	2.22 ±1.34	2.10 ±0.89	6.47 ±0.5290	27.46±0.42
SU6	10%CS (control 2)	792.4±50.63	759.4±52.13	1.58 ±0.89	1.51 ±1.03	6.61 ±0.09	28.53±0.33
SU7	10%CS+CM	813.2±26.18	770.1±36.44	1.20 ±0.50	1.54 ±0.97	6.84 ±0.30	27.84±0.70
SU8	10%CS+GM+CM	804.6±33.24	791.5±37.47^{a}	2.07 ±0.57^{a}	1.63 ±0.91	6.92 ±0.36	28.24±0.51
SU9	10%CS+FW+CM	809.1±29.41	797.3±45.67^{a}	2.11 ±0.57^{a}	1.65 ±0.90	6.72 ±0.29	28.21±0.45
SU10	10%CS+GM+FW+CM	808.8±29.65	803.5±46.23^{a}	2.21 ±0.62^{a}	1.67 ±0.88	6.68 ±0.18	28.19±0.43
5%	F Value	2.799	3.945	2.137	0.06654	0.4111	0.8316
	P Value	0.0019	0.0353	0.0182	>0.9999	0.9627	0.6260
10%	F Value	0.4214	5.067	4.744	0.3298	1.389	1.267
10%	P Value	0.9588	0.0282	< 0.0001	0.9857	0.1781	0.2458

Key: SU: Setup, CS: Crude oil contaminated soil, CM: *Comamonas testosterone*, FW: Fish wastes, GM: Goat manure
P-value: significant when compared to 5% and 10% CS (ctrl 1) – b

C. Results of TPH Contents

The effect of the TPH content on the different treatments used in this study for 5%CS and 10%CS were estimated. The control samples (without treatment) recorded TPH values of 6548.06mg/kg and 10328.03mg/kg on day 1 and this was used as a representative value for the initial TPH concentrations for the various treatment set up prior to the addition of the various treatments in each set up while the TPH contents on the last day of the experiment was 5984.54mg/kg and 10202.32mg/kg for 5%CS and 10%CS

setup samples respectively. The difference in the TPH concentrations were extrapolated by subtracting TPH concentration on the last day of the experiment from the TPH concentration on the first day of the experiment in order to ascertain the amount of TPH left, which is usually referred to as the 'TPH remediated' and then, the percentage bioremediation (% BR) for the various treatments were thus, estimated as represented in the pie charts as shown in Figures 7 and 8.

From our overall results, in the group of 5% crude oil contamination levels; the setup treated with *Comamonas testosteroni* (CM) only, recorded the highest TPH concentration (4707.48 mg/kg) on the last day of the experiment. Where's, the setup treated with the hydrocarbonoclastic bacterial isolate*; Comamonas testosteroni* and both nutrients (GM+FW+CM) revealed the lowest TPH concentration (1265.31 mg/kg) on the final day of the experiment. The group of 10% crude oil contamination level followed similar trend.

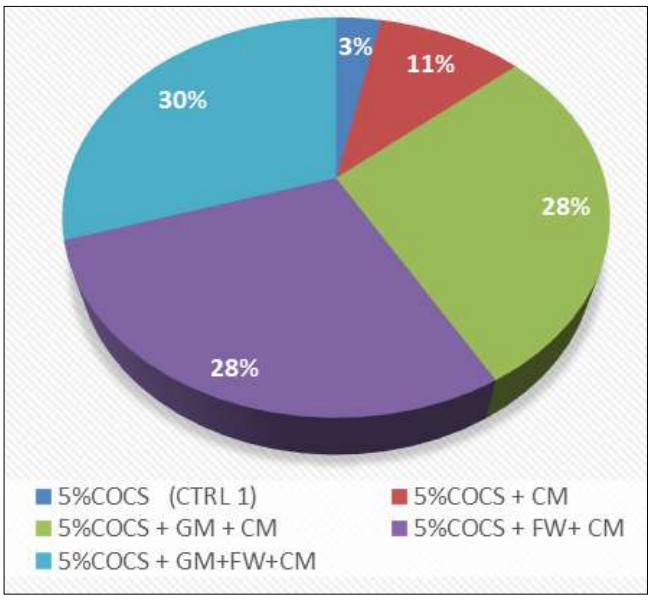


Fig 7: Percentage bioremediation of TPH for 5% hydrocarbon contaminated soils

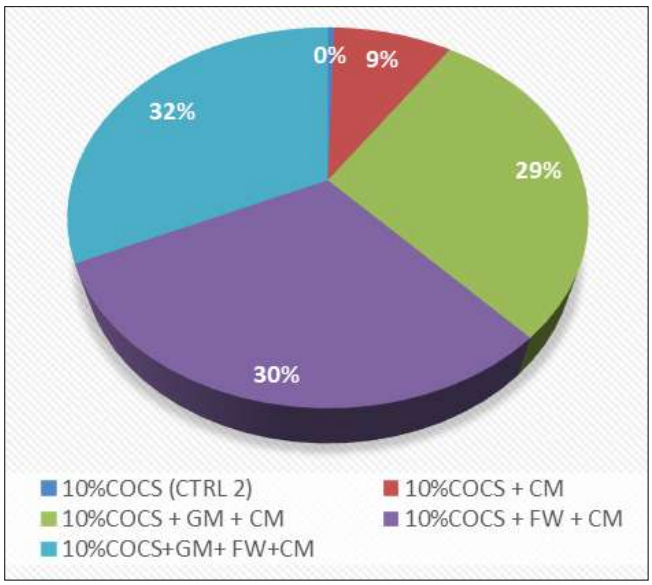


Fig 8: Percentage bioremediation of TPH for 10% hydrocarbon contaminated soils

This results, therefore revealed that the setup with combination of treatments using: *Comamonas testosteroni* and both nutrients had more capabilities to degrade the TPH contents in the soil samples than the setup with combination of treatments using: *Comamonas testosteroni* and the organic nutrients singly. Our results are in line with works done by [15, 38], in their bioremediation study carried out using *Pseudomonas aeruginosa*, *Comamonas testosteroni* and *Bacillus subtilis* as amendments, who also reported increased biodegradation potentials of combination treatments of microbial population with organic nutrients used in bioremediating contaminated soils. Futhermore [15], stated clearly, that the introduction of organic nutrients could support the degradation process and ensure a faster and better effective remedy for cleanup of crude oil contaminated soils or sites. However [39], suggested that bacterial population is necessary for complete biodegradation of oil pollutants as the enzymes required for the degradation of petroleum are usually better found in such organisms. Overall, the combination treatments using *Comamonas testosteroni*, combined with both nutrients recorded the highest degradation potentials.

The present study also concurs with some other studies carried out on bioremediation like; [10], who reported a significant decrease in the total hydrocarbon contents (THC) with percentage THC reductions of 73%, 82%, 84%, 88% for four treatment options used except for the control sample that had insignificant reduction in THC with percentage THC reductions of 2%. Various environmental conditions as well as activities of the microbial populations usually play a role in the degradation process of the hydrocarbon contaminants. The increased hydrocarbon utilizing bacteria and fungi count in the contaminated soil samples indicates that these microorganisms are capable to degrade compounds including petroleum hydrocarbons [30, 31, 32, 33]. Our result is in accordance with work done according to [40], who reported more microbial populations observed with less TPH content and organic matter content analyzed in the aged crude oil polluted sites as compared with the results obtained from the fresh spilled sites.

D. TPH degradation modeling pattern using best treatment option

Total petroleum hydrocarbon (TPH) contents in the various treatments applied were monitored for 56 days at 7 days intervals within a period of 56 days. Figures 9 and 10 presents the mathematical modelling of TPH degradation through linear regression method for the best treatment option for 5% and 10% crude oil contaminated soil samples. Mathematical modelling of TPH degradation through linear regression method revealed that TPH content degradation followed a linear pattern for the best treatment option (GM + FW + CM). The coefficient of determination R^2 (also known as the Goodness of Fit) is a measure of how well our derived model fits the experimental data and can be used to predict TPH degradation with time for the best treatment option. The R^2 value can be interpreted as the proportion of the variance in hydrocarbon (TPH) content attributable to the variance in time. This report concurred with report by [27, 41], who carried out researches on biodegradation of total petroleum hydrocarbon using a consortium of the bacteria; *Cyanobacteria* and other bacterial isolates and observed that, the TPH degradation modelling using the hydrocarbon degraders in the present studies, showed that, TPH degraded

with corresponding exponential growth and the coefficient of determination values recorded 0.7597 throughout the period of the study. They stated clearly, that, it is an indication that if the experiment is repeated employing procedures as same, then there are possibilities of obtaining result as same or very similar result at the confidence level of 75.97%.

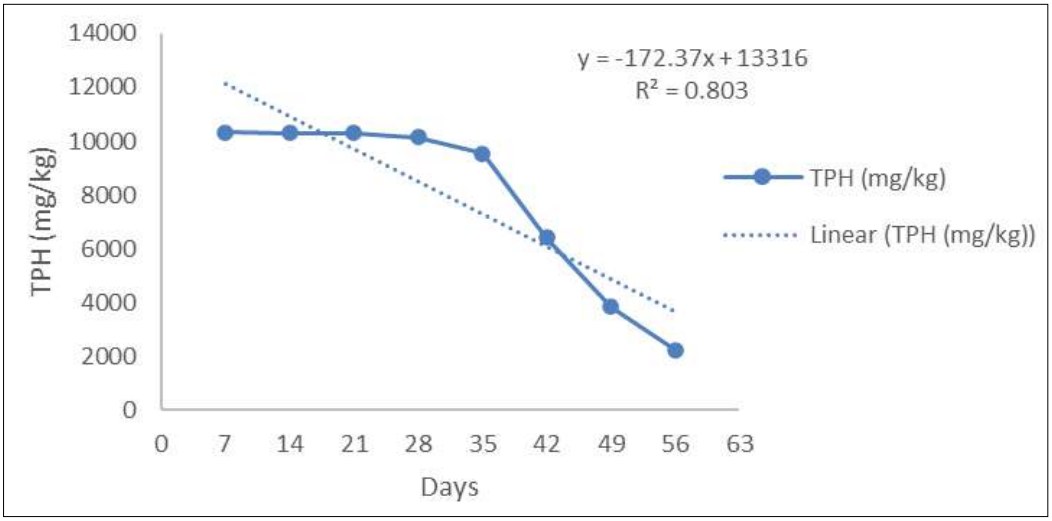


Fig 9: TPH degradation modeling pattern using best treatment option for 5% crude oil contaminated soils

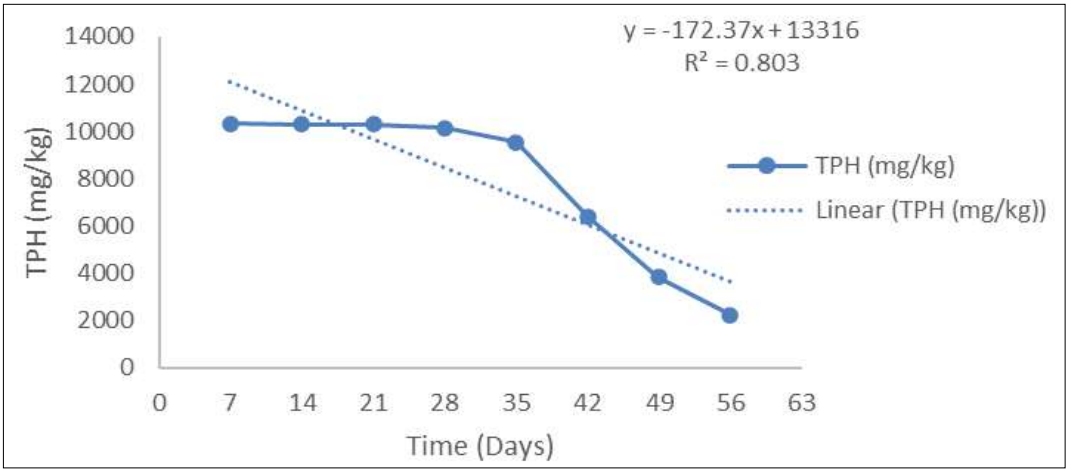


Fig 10: TPH degradation modeling pattern using best treatment option for 10% crude oil contaminated soils

Conclusion and Recommendation

The use of nutrient organics as biostimulants singly and the bacterial isolate in the present study has proved to enhance the biodegradation rate of hydrocarbon contaminated soil. Although a mixture of the treatments using the stimulants and the bioaugumenting organism showed greater and faster potentials in the biodegradation of crude oil contaminated soil. However, a combination approach in employing a mixture of the nutrient organics with bacterial isolate; *Comamonas testosteroni* in the biodegradation procedure of hydrocarbon contaminated soil resulted in faster and more effective biodegradation process as a greater reduction in petroleum hydrocarbon was achieved.

It is therefore recommended that environments situated around companies and refineries whose day to day activities contaminate the soils and surrounding environments with petroleum and other petroleum products are to be encouraged to simply consider the application of this treatment combination strategy as a low-cost and environmental friendly option that is readily available in order to enhance remediation capability to decontaminate any hydrocarbon polluted soils or sites. The results obtained in this study will be useful for the development of effective biodegradation/ bioremediation programme in the oil producing regions such as the Niger Delta states in Nigeria that are specifically noted for crude oil pollution.

Conflict of Interest

Not available

Financial Support

Not available

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