Remediation of Oily Waste using Soil Organic Nutrient Stimulant

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Authors' contributions

This work was carried out in collaboration among all authors. Author OUMJ designed and performed the experiments, analyzed and interpreted the data and wrote the manuscript. Authors SIE, VON and RAO assisted in the literature and writing of the manuscript. All authors read and approved the submitted final manuscript.

Original Research Article

ABSTRACT

Aims: This present study aim at assessing the efficacy of soil-organic nutrient stimulant in the remediation of oily waste.
Study design: Preparation of Soil-goat dung mix was used as stimulant for the remediation of oily waste.
Place and duration of study: The study was carried out at the Department of Microbiology University of Uyo, Uyo, Akwa Ibom state, Nigeria in the dry season months of January - March
Methodology: Remediation of oily waste using soil - organic (goat dung) nutrient stimulant was assessed for 12 weeks using standard culture-dependent microbiological, chemical and enzyme activity assay procedures.
Results: The results indicate increased counts of hydrocarbon utilizing bacteria, fungi and actinomycetes with remediation time. Microorganisms belonging to the genera *Bacillus, Pseudomonas, Acinetobacter, Alcaligenes, Serratia, Penicillium, Aspergillus, Cladosporium, Rhodococcus, Nocardia* and *Streptomyces* were recovered from the remediated waste. The pH of

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the soil-goat dung treatment ranged from 6.5 ± 0.02 to 7.1 ± 0.05. Enzyme activity by dehydrogenase and urease were higher than phenol oxidase with time. PAHs were below detectable limits (< 0.01) and reduction in total petroleum hydrocarbon was 99.3% for the remediated waste.

Conclusion: Overall, enhanced microbial activities correlated positively with reduction in Total Petroleum Hydrocarbon and PAH composition which resulted in ecofriendly waste product. Soil-goat dung stimulant can therefore serve as a cheap alternative in the management of oily waste.

1. INTRODUCTION

Crude oil exploration and production activities have led to economic boom in oil producing countries [1]. However, these activities are often of great concern because of accidental, deliberate or indiscriminate discharge of wastes into the ecosystem which cause ecological problems [2,3,4,5]. Bioremediation, a cost-effective process which functions basically on biodegradation leading to complete mineralization of contaminants by biological agents is a means of mitigating the problem of environmental pollution [6,7]. It can be achieved *in situ* or *ex situ* through biostimulation, bioaugmentation achieved by land filling, land farming, use of bioreactor, composting or vermiculture [4,7] The cost of treating waste generated from industrial activities is often high, thus most industrial waste are not treated before disposal into the environment. Bioremediation is an efficient, economic and alternative to physicochemical treatment of waste generated from the oil industry [8]. Microorganisms are able to use the total petroleum hydrocarbons of the crude oil as source of carbon and energy and break them down to simpler non-toxic compounds such as carbon (iv) oxide and water [7]. Composting, a remediation process which involves the careful control and addition of nutrients, suitable microorganisms and bulking agents to improve the remediation of contaminated environments indicated 85 – 90% reduction of petroleum hydrocarbons (including PAHs concentrations), enhanced soil quality and facilitate the re-use of the restored environment. The bioremediation of hydrocarbon contaminated Nigerian soils using organic nutrient amendment has been reported [1,9]. Bioremediation is a promising technique to restore hydrocarbon contaminated soils, manage waste associated with crude oil exploration and production activities in Nigeria. The increased concern on restoring the oil polluted soils in the Niger Delta region of Nigeria and ensure environment friendly disposal of oily waste at a reduced cost necessitated this study.

2. MATERIALS AND METHODS

2.1 Remediation Study

This was carried out based on the principles of biostimulation**,** bioaugmentation and composting [8,10,11]. The soil-goat dung nutrient stimulant treatment and control were set up in triplicates using transparent, sterile small sized buckets with perforated lids. Precisely 3 kg of oily waste was mixed with 150 g of soil (5 % of oily waste weight) obtained from Unyenge coastal wetland, 300 g of goat dung (10 % of oily waste weight) and 15 g of bulking agent (wood chips) (0.5% of oily waste weight). The control was also set up without the soil and goat dung.The different treatment cells were covered with nets and perforated lids and incubated for twelve weeks at ambient temperature. The treatment and control cells were assessed immediately after set up (i.e. 0 week) and also monitored weekly for changes in Total Viable Counts (TVC) of hydrocarbonoclastic organisms, pH, Total Petroleum Hydrocarbon (TPH) reduction and enzyme activities.

2.2 Isolation and Enumeration of Microorganisms

Ten-fold serial dilutions of oily waste samples were made according to the method described by [12] using Tween 80 as diluent. The first ten-fold dilution was made using 10g of the oily waste sample in 90ml of diluent. The dilution were shaken and further serial ten-fold dilutions made up to 10^{-8}. The isolation and enumeration of hydrocarbon utilizing microorganisms were done by vapour-phase transfer method as described by [13,14,15], Aliquots (0.1ml) of appropriate dilutions (10^{-2} to 10^{-6}) of oily waste samples were inoculated onto mineral salt medium (MSM) using the surface spreading technique. The medium used for the isolation of

oil-degrading bacteria was supplemented with 50μml[-1] Nystatin to inhibit interfering yeast and mould and pH adjusted to 7.6. The medium for the enumeration of oil degrading mould was supplemented with 50 μml[-1] of penicillin G and streptomycin to inhibit interfering bacteria and pH adjusted to 5.6 while that for the enumeration of oil-degrading Actinomycetes was supplemented with cyclohexamide to prevent fungal growth and pH adjusted to 5.5 to arrest the growth of non-filamentous bacteria. Sterile filter papers (Whatman1) soaked with filter sterile crude oil (Nigerian light crude) were aseptically placed inside the lid of each Petri-dish and inverted over the inoculated plates. These filter papers supplied the hydrocarbon by vapour phase transfer to the inocula. Control plates were also prepared without crude oil and incubations made at $28 \pm 2^{o}C$ for 5 – 7 days. Colony forming Units (cfu/g) were enumerated and due number of hydrocarbon-utilizing microorganisms (i.e. Bacteria, Fungi and Actinomycetes) calculated by subtracting the number of colony forming units in control from those in test cultures. The cultural characteristics of emerging colonies were observed after the incubation periods. Different colonies which appeared after the incubation periods were carefully sub-cultured on appropriate media originally used for their isolation. On further sub-culturing, the resulting pure cultures were preserved in the refrigerator for further use.

2.3 Characterization and Identification of Microbial Isolates

Characterization and identification of bacterial isolates and actinomycetes was based on the examination of cultural colonial morphology on plates, microscopy after staining techniques were applied and biochemical tests carried out .The bacteria and actinomycetes were characterized and identified by comparing to known taxa using Bergey's Manual of Determinative Bacteriology [16].Characterization and identification of fungal isolates were based mainly on their cultural and microscopic morphology and with the presence or absence of special reproductive structures [17,18].

2.4 Physicochemical Analysis of Samples

Chemical characteristics of oily waste and organic manure samples were determined according to techniques described by [19,20]. pH was determined by electrometric method using the pH meter. Total nitrogen in the samples were determined by macrokjeldahl digestion and distillation method. Phosphorus was extracted from the samples by the Bray P-1 method and determined by Murphey Riley Method. The total organic carbon content of the samples were determined using the method of Walkley and Black. Heavy metals were determined by Atomic Adsorption Spectrophotometer after acid digestion.

The Total Petroleum Hydrocarbon (TPH) content of the samples was assessed using Toluene extraction method as described by [21]. Five gram of the oily waste sample was measured into a beaker and 10 mL of toluene (Analar grade) was added to it. After shaking vigorously for 5 min, it was allowed to stand for 20 min. After which, two layers were formed and the supernatant (toluene-residual oil extract) was put into fresh test tubes (curvette). The hydrocarbon content (oil) extracted was determined spectrophotometrically at 420 nm using spectronic-20 Spectrophotometer. The absorbance reading was recorded after reading from a standard curve of the absorbance of different known concentrations of hydrocarbon extractant (toluene). Hydrocarbon concentrations were calculated by multiplying with the appropriate dilution factor and the results expressed as milligrams per kilogram (mg kg^{-1}).

Enzyme (Dehydrogenase, urease and phenol oxidase) activities of samples were determined as described by [22]. The Triphenyltetrazolium Chloride (TTC) method based on the estimation of TTC reduction rate to Triphenylformazan (TPF) in samples after incubation was employed to determine dehydrogenase activity of the treated waste. 5g of sample was weighed into test tubes and mixed with 5ml of Triphenyltetrazolium Chloride (TTC) solution. The tubes were sealed with rubber stoppers and incubated for 24hours at $30^{o}C$. The control containing only 5ml of Tris-HCl buffer (i.e. Hydroxy-methyl-aminomethane in distilled water + HCl) without TTC was also prepared. After incubation, 40ml acetone was added to each tube and shaken thoroughly and further incubated at room temperature for 2 hours in the dark, shaking the tubes at intervals. The suspension was then filtered and the optical density of the clear supernatant measured against the blank at 546nm (red colour). Urease activity was determined by the non-buffered method based on the determination of ammonium ions after incubation of sample with urea. 2.5ml of urea was mixed with 5g of sample

and incubated at 37^0C for 2 hours. After incubation, 50ml of KCl solution was added and shaken for 30 minutes, then filtered and the filtrate analyzed for ammonium content using Salicylate/NaOH solution and Sodium dichloroisocyanide at room temperature before measuring the optical density at 690nm. To determine Phenol Oxidase activity of sample. One gramme (1g) of the sample was transferred into a 50ml measuring flask and mixed with 10ml of 1% pyrogallol solution. The amended sample was incubated at 30^0C for 3 hours after which 0.5mol/l ether was added into the mixture in the flask and shaken. The gallic acid produced was extracted with ether and measured spectrophotometrically at 430nm.

Polycyclic Aromatic Hydrocarbon (PAH) in samples were assessed according to United States Environmental Protection Agency (US EPA) Method 8270 D for .Semi volatile Organic Compounds by Gas Chromatography / Mass Spectrometry [23].

2.5 Statistical Analysis

The results were subjected to analysis of variance (ANOVA) and Kruskal Wallis test on log-transformed data using Statistical Package for the Social Science (SPSS version 20.0, IBM Corp, USA). Results are presented as mean ± standard deviation with levels of significance maintained at 95% for each test.

3. RESULTS

3.1 Microbial Counts During Remediation of Oily Waste

The microbial counts of hydrocarbon utilizing bacteria, fungi and actinomycetes during the remediation of oily waste (Fig. 1) indicates higher counts with increase in remediation time. The results indicate that hydrocarbon utilizing bacterial count increased from 5.58 ± 0.05 to 5.96 ± 0.06 Log_{10}CFU g^{-1} in week one to five, with elevated value of 6.15 ± 0.03 to 6.79 ± 0.05 Log_{10}CFU g^{-1} (week 6 to 10) and a slight raise of 6.80 ± 0.01 to 6.81 ± 0.04 Log_{10}CFU g^{-1} in week 11 to 12. Similarly, hydrocarbon utilizing fungi increased from 3.51 ± 0.04 to 3.93 ± 0.01 Log_{10}CFU g^{-1} in week one to six, 4.11 ± 0.02 to 4.59 ± 0.07 Log_{10}CFU g^{-1} (week seven to ten) and slight increase of 4.64 ± 0.1 to 4.65 ± 0.03 Log_{10}CFU g^{-1} in week 11 and 12. Also, counts of

hydrocarbon utilizing actinomycetes increased from 2.45 ± 0.02 to 2.83 ± 0.2 Log_{10}CFU g^{-1}, 3.08 ± 0.05 to 3.99 ± 0.02 Log_{10}CFU g^{-1} and 3.72 ± 0.01 to 3.74 ± 0.1 Log_{10}CFU g^{-1} in week 1 to 6, 7 to 10, 11 and 12 respectively. There were 1.0 to 1.2 times higher microbial counts for the HUB, HUF and HUA in relation to time and these differences were significant at p = 0.05.However, there was minimal increase in microbial population in the control from week 1 to 7 (3.26 ± 0.2 to 3.74 ± 0.01 Log_{10}CFU g^{-1}) and a decrease in microbial load from weeks 8 to 12 (3.74 ± 0.2 to 3.54 ± 0.04 Log_{10}CFU g^{-1}) for hydrocarbon utilizing bacteria. Hydrocarbon utilizing fungi increased from week 1 to 7 (2.23 to 2.61 ± 0.2 Log_{10}CFU g^{-1}) and reduced from week 8 to 12 (2.53 to 2.34 ± 0.02 Log_{10}CFU g^{-1}). Also, there was a minimal increase from week 1 to 6 (1.79 ± 0.1 to 1.87 ± 0.05 Log_{10}CFU g^{-1}) and decrease from week 7 to 12 (1.72 ± 0.2 to 1.34 ± 0.03 Log_{10}CFU g^{-1}) was also observed in control treatment for hydrocarbon utilizing actinomycetes.

3.2 Microorganisms Associated with Remediated Waste

The microorganisms isolated from the remediated waste include species of the genera *Bacillus, Pseudomonas, Acinetobacter, Alcaligenes, Serratia, Penicillium, Aspergillus, Cladosporium, Rhodococcus, Nocardia* and *Streptomyces.*

3.3 Physicochemical Characteristics of Oily Waste

The oily waste was dark colored with clay texture. The chemical characteristics however indicate its pH as 5.5 ± 01, Total available Nitrogen of 0.02 ± 0.01%, Available Phosphorus of 0.16 ± 0.04 mg kg^{-1} and Total Petroleum Hydrocarbon of 89, 900 mg kg^{-1}. Among the heavy metals, Iron at 77.51 ± 0.06 mg kg^{-1} was higher than others and Cadmium with the least value of 0.7 ± 0.3 mg kg^{-1}. The level of some polycyclic aromatic hydrocarbon (PAH) associated with oily waste revealed Naphthalene with highest level of 62.16 ± 0.05 mg L^{-1} while Phenanthrene had least level of 0.86 ± 0.01 mg L^{-1}.

The physicochemical characteristics and PAH level of oily waste is as presented on Tables 1 and 2 respectively.
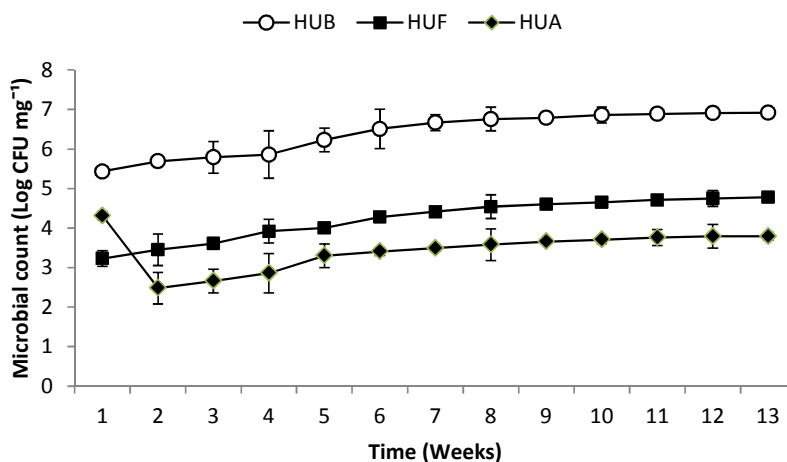
Fig. 1. Microbial counts during oily waste remediation
*(**HUB**-Hydrocarbon utilizing Bacteria, **HUF**- Hydrocarbon Utilizing Fungi, **HUA**-Hydrocarbon utilizing Actinomycetes)*

3.4 Chemical / Microbiological Characteristics of Goat Dung

The chemical characteristics of Goat dung used for the remediation studies is as presented on Table 3. The Goat dung revealed a pH of 7.3 ± 0.04, with considerable levels of Nitrogen and Available Phosphorus. The microbiological characteristics of the dung revealed some hydrocarbon degraders as constituent of the goat dung.

3.5 Changes in Total Petroleum Hydrocarbon (TPH) During Oily Waste Remediation

The percentage reduction in TPH during oily waste remediation (Fig. 2) shows 99.3% removal in the TPH content of the remediated waste. Initial Total petroleum hydrocarbon (ie TPH of unremediated oily waste value) was observed from week 1 to 2, rapid reduction of TPH from week 3 to 7 and slowed reduction from week 8 to 12 in the treatments .The control however, revealed very slow reduction from week 0 – 12.

3.6 Changes in pH of Oily Waste During Remediation

The changes in pH of oily waste during remediation indicates the pH ranged between 6.5 ± 0.02 and 7.1 ± 0.05 (Fig. 3).

The results revealed pH increase from week 0 – 4 and steady pH decrease from week 5 to 12 for the treatments while The control revealed steady initial pH from week 0 to 2 and a steady decrease from week 3 to 12.

3.7 Changes in Enzyme Activity during Oily Waste Remediation

The activities of Phenol Oxidase, Urease and Dehydrogenase during oily waste remediation are presented in Figs. 4, 5 and 6 respectively. The Urease and Dehydrogenase activities increased with increase in remediation time while Phenol Oxidase activity decreased with remediation time.

3.8 Changes in Polycyclic Aromatic Hydrocarbon (PAH) During Oily Waste Remediation

The changes in PAH of oily waste during remediation (Figs. 7–13) suggests there was remarkable decrease in PAH levels with increase in remediation time.

4. DISCUSSION

We used soil-goat dung stimulant to remediate oily waste contaminated soil for 12 weeks and the results indicated microorganisms associated with remediated oily waste were bacteria that belonged to the genera *Bacillus, Pseudomonas, Acinetobacter, Alcaligenes* and *Serratia.*

Table 1. Physicochemical characteristics of oily waste

Physical Appearance	pH	TN (%)	AV. P (mg kg^{-1})	TPH (mg kg^{-1})	Heavy metals (mg kg^{-1})							
					Fe	Ni	V	Mn	Zn	Cu	Co	Cd
Dark coloured with Clay texture	5.5 ± 0.1	0.02 ± 0.01	0.16 ± 0.04	89.9 x 10^3 (89,900 ± 0.01)	77.51 ± 0.06	31.02 ± 0.04	43.19 ± 0.3	15.12 ± 0.05	14.22 ± 0.2	18.05 ± 0.08	5.6 ± 0.5	0.7 ± 0.3

Key: TN – Total Nitrogen, Av.P – Available Phosphorus, TPH – Total Petroleum Hydrocarbon

Table 2. Levels of assessed Polycyclic Aromatic Hydrocarbon (PAH) associated with oily waste

Polycyclic Aromatic Hydrocarbon (PAH)	Level of PAH (mg L^{-1})
Naphthalene	62.16 ± 0.05
Fluorene	13.53 ± 0.4
Phenanthrene	0.86 ± 0.01
Anthracene	28.21 ± 0.5
Pyrene	4.81 ± 0.02
Chrysene	26.72 ± 0.3
Benzo(*b*) Fluoranthene	11.75 ± 0.3

Table 3. Chemical / Microbiological characteristics of Goat dung

Organic manure	Ph	Total Carbon (%)	Total Nitrogen (%)	Available Phosphorus (mg kg^{-1})
Goat dung	7.3 ± 0.04	19.8 ± 0.04	3.5 ± 0.3	14.6 ± 0.1
HUB (x10^{2}Cfu/g)	HUF (x10^{2}Cfu/g)	HUA (x10^{2}Cfu/g)	Hydrocarbonoclastic microbial genera associated with organic manure	
6.3±0.02	3.4±0.05	2.8±0.03	*Acinetobacter, Serratia Nocardia, Penicillium* Aspergillus	

Fig. 2. Percentage reduction in Total Petroleum Hydrocarbon of oily waste during remediation

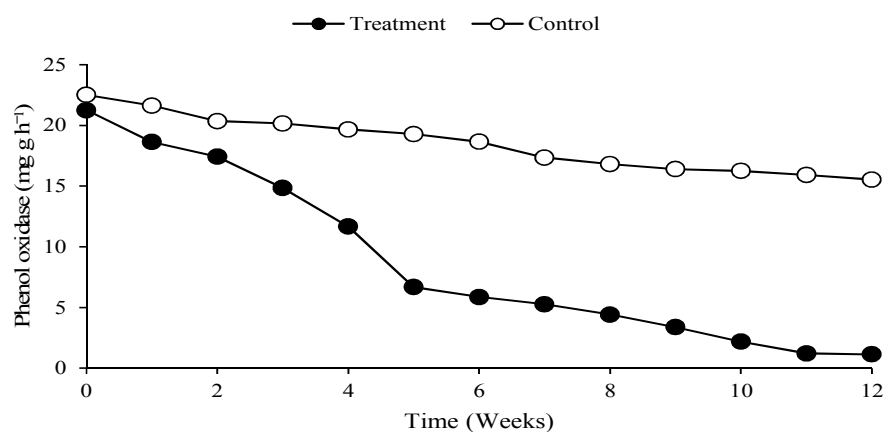**Fig. 3. Changes in pH of oily waste during oily waste remediation.**

Fig. 4. Phenol oxidase activity during oily waste remediation
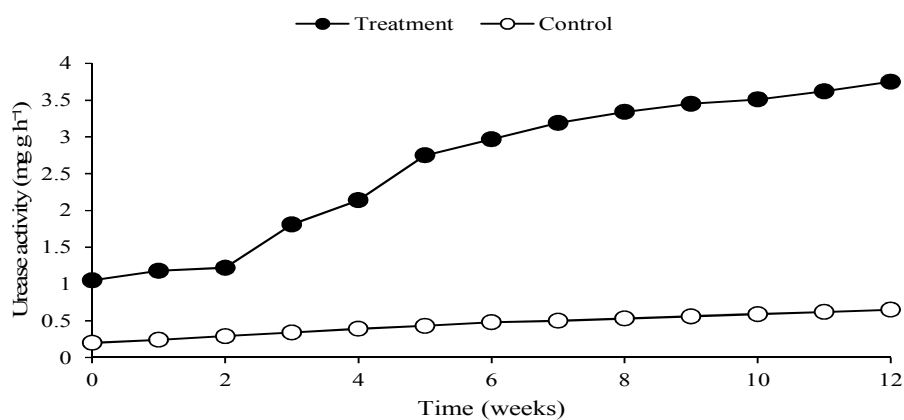

Fig. 5. Urease activity during oily waste remediation
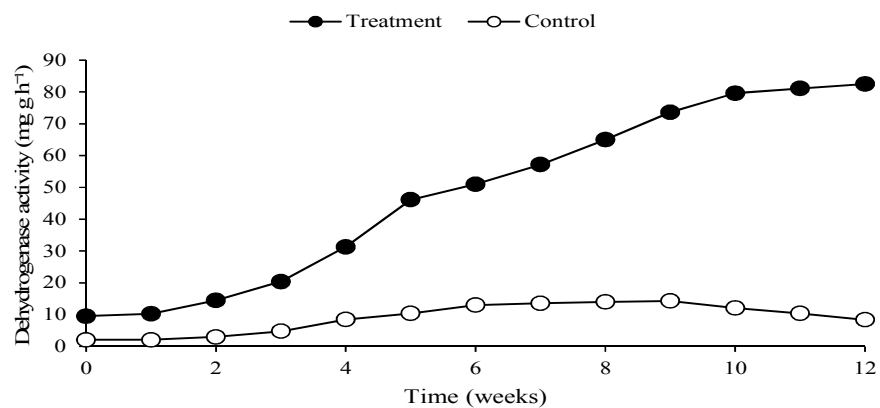
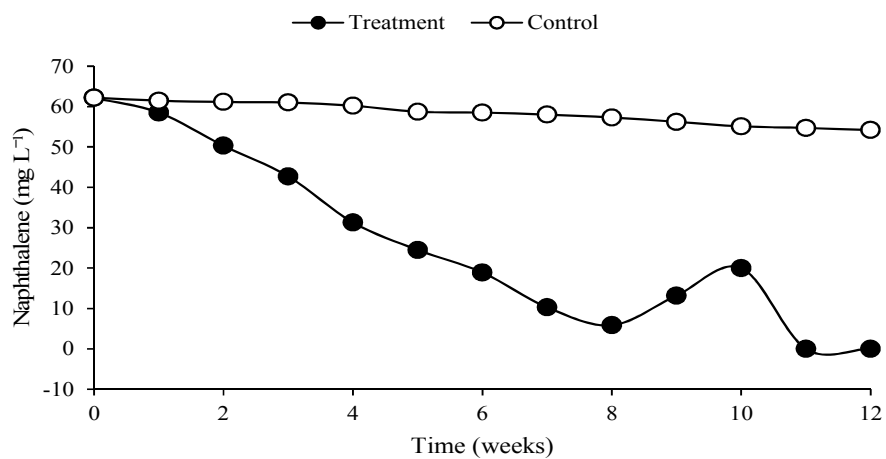**Fig. 6. Dehydrogenase activity during oily waste remediation**

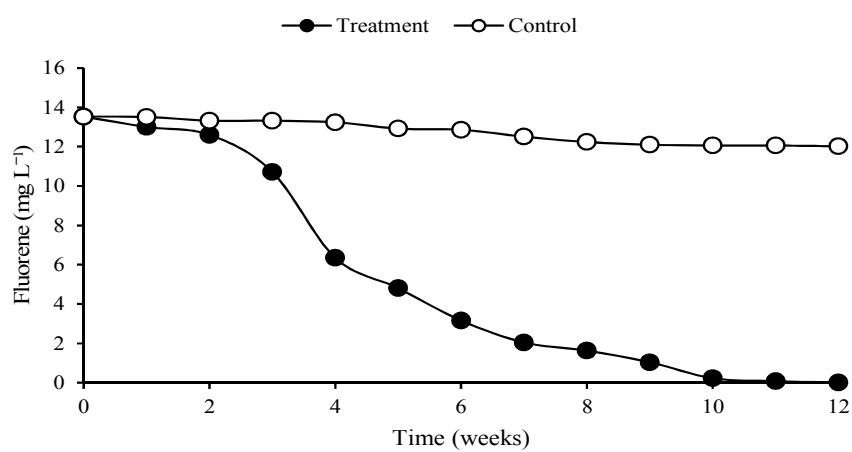**Fig. 7. Changes in Naphthalene level during oily waste remediation**

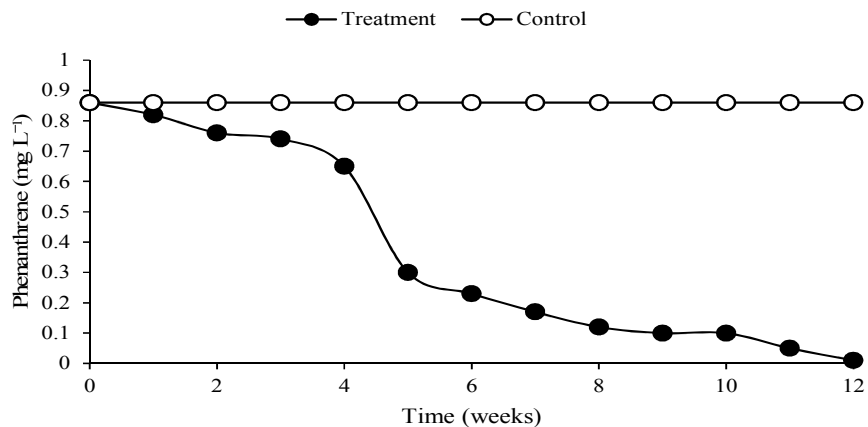**Fig. 8. Changes in Fluorene level during oily waste remediation**

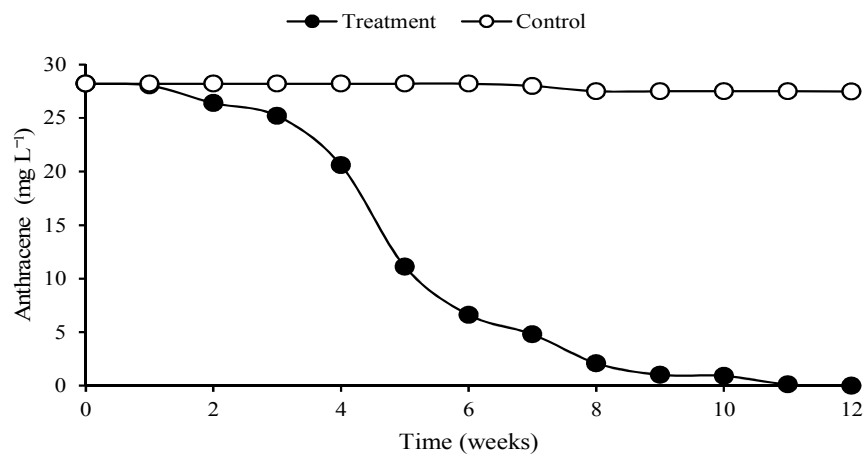**Fig. 9. Changes in Phenanthrene level during oily waste remediation**

Fig. 10. Changes in Anthracene level during oily waste remediation
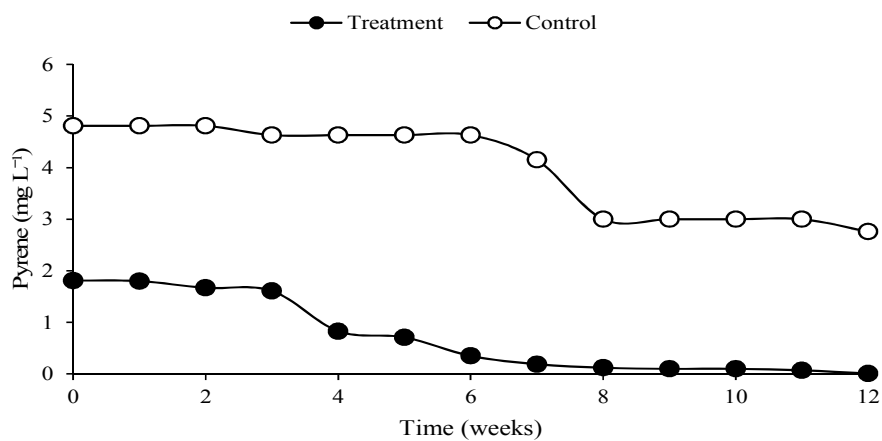

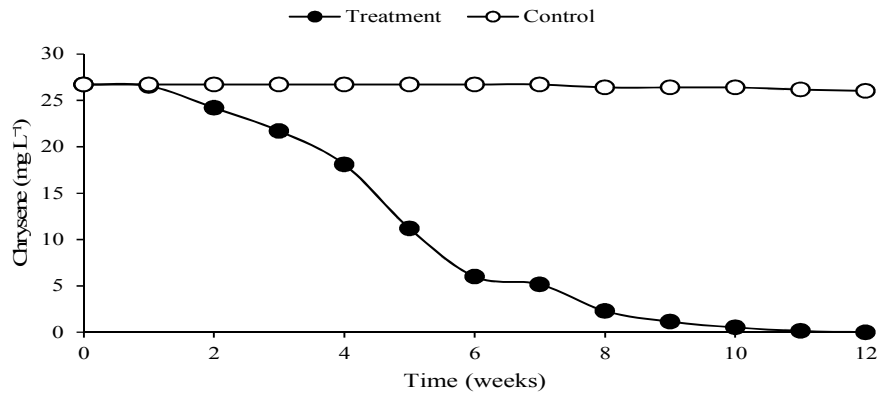**Fig. 11. Changes in Pyrene level during oily waste remediation**

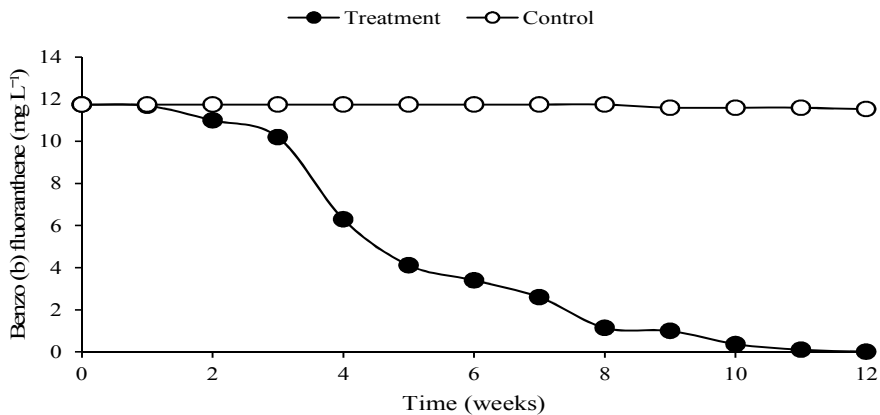**Fig. 12. Changes in Chrysene level during oily waste remediation**

Fig. 13. Changes in Benzo(*b*)fluoranthene level during oily waste remediation.

The actinomycetes were members of the genera *Rhodococcus, Nocardia* and *Streptomyces* and the fungi were *Penicillium, Aspergillus* and *Cladosprium.* These microbes have been implicated in hydrocarbon biodegradation by different reports [1,9,24,25]. The biodegradation of hydrocarbon is attributed to the presence of efficient hydrocarbon degradative enzyme systems and catabolic genes [26]. The most efficient degraders of hydrocarbon among the bacteria, actinomycete and fungi agree with other reports [1,9] for microbes involved in such activities. The coastal wetland soil which constitute a component of the soil-organic stimulant was the source of the hydrocarbon degraders involved in the remediation process because it is a complex ecosystem that harbors diverse microorganisms [27].

The availability of nutrient such as Nitrogen and Phosphorus play vital roles in the biodegradation of hydrocarbons in any environment [28,29]. The physicochemical characteristics of the oily waste in this study indicate low nitrogen and available phosphorus, as well as high Total petroleum hydrocarbon (Table 1). This imply high C:N and C:P ratio in the waste. Therefore, biodegradation of this oily waste if allowed to occur under natural condition wherever the waste is disposed will be very slow. Studies have shown the use of organic and inorganic nutrient amendment to enhance hydrocarbon biodegradation activities [1,9,30,31]. Though the use of organic manure amendment has been reported for remediation of contaminated soils, reports of their use in the remediation of oily waste is scarce. The use of goat dung for the remediation of oily waste was employed in this study.

The remediation of oily waste in this study for twelve weeks using non-impacted coastal wetland soil - organic (goat dung) nutrient

stimulant indicated changes in the microbial counts of the hydrocarbonoclastic organisms during the remediation process (Fig. 1).Generally, counts of hydrocarbonclastic bacteria during oily waste remediation increased with extended remediation time. The increase in microbial population is also attributed to the addition of the organic manure (goat dung) containing nutrients and even hydrocarbonoclastic microbes (Table 3) which stimulated and boost microbial (i.e. hydrocarbon degraders) population, growth and reproduction in the treatments. This result corroborates with other reports [1,9] on the use of organic amendments to enhance biodegradation activities. The initial phase depicts when the microorganisms adapted and elaborated the required enzymes to breakdown the complex mix of organic and oily waste. The phase of rapid growth, week 6 to 10 suggests production of surfactants and appropriate degradative enzymes which emulsified and transformed the oily waste into nutrients available for microbial growth and reproduction. However, the last phase of treatment in week 11 and 12 shows reduced microbial proliferation compared with the previous weeks. This suggests depletion of available nutrient and accumulation of waste metabolites [32,33] which altered the physicochemical composition of the growth substrate.

The total petroleum hydrocarbon reduction (Fig. 2) ranged between 4 and 99.3% with the pH that varied from 5.0 ± 0.02 and 7.3 ± 0.06 (Fig. 3) during oily waste remediation for the control and treatments respectively. The increase in pH at the initial remediation period of treatment which subsequently reduced with time is attributed to the addition of organic manure to the oily waste. The production of acidic intermediates during the biodegradation of hydrocarbons [7] contributed to the low pH in the treatments. The high removal rate of TPH in relation to remediation time suggests the breakdown of constituents of the complex hydrocarbons into simpler units by actively growing HUB, HUF and HUA in the medium. In addition, the changes in total petroleum hydrocarbon also occur in different phases. Low molecular hydrocarbons (e.g. naphthalene) are known to be degraded at the initial phase [26,32] followed by a period of maximum biodegradative activity in the different treatment cells. Thereafter, there was reduced biodegradation activities in the treatment cells and indicates that large molecular weight

hydrocarbons were degraded and often proceed at a slow rate [7,29,34].

There was high dehydrogenase and urease activity in relation to a low phenol oxidase activity with remediation time in the different treatment cells. The dehydrogenase activities in all the treatment cells correlated positively with the microbial counts. The phenol oxidase activity also showed positive correlations with the level of total petroleum hydrocarbon. The Urease activity however negatively correlated with total petroleum hydrocarbon levels in the treatment cells. The results are consistent with other studies [35,36] for activities of dehydrogenase, urease and phenol oxidase. However, the result differs with the findings in another study [36] which indicate reduced phenol oxidase activity and increased Total petroleum hydrocarbon concentration.

The levels of polycyclic aromatic hydrocarbons (PAHs) in the remediated oily waste was below the detectable limit (< 0.01) and indicates that the treatment enhanced degradation of PAHs. This result corroborates with other studies [25,30] on the efficiency of organic manure such as cow dung and poultry droppings in the remediation of contaminated environments. The detectable concentration of PAH in the control compared with the treatments at the end of twelve weeks remediation suggests that amendment with soil-organic nutrient stimulant enhanced biodegradation of hydrocarbons. Nutrient deficient medium is unable to support proliferation of microorganisms or stimulate microbial degradative potentials [9,30]. This inability translated into an inefficient degradation process and accumulation of PAH in the control in relation to the treatment.

5. CONCLUSION

The results of the remediation of oily waste using soil-goat dung stimulant indicate high efficacy to produce ecofriendly waste at a reduced cost compared to the expensive methods which involve the use of chemicals. Thus, soil-goat dung stimulant is a competitive option for efficient remediation of oily waste to minimize adverse effect on microorganisms and the environment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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