Original Research Article

Enhanced Phytoremediation Crude Oil Polluted Soil Using *Pseudomonas Fluorescens*.

ABSTRACT

Environmental pollution in the Niger Delta has been a huge concern. Bacteria have proven to be of great benefit in the degradation of petroleum hydrocarbons. This research is aimed at enhanced phytoremediation of crude oil polluted soil using *Pseudomonas fluorescens*. Soil was collected from oil spill polluted site at B-dere, Rivers State. Six (6) treatments consisting of contaminated soil (CS), uncontaminated soil (US), augmenting organisms *Pseudomonas fluorescens* (Pse), and phytoremediation grasses (Elbow buffalo grass (*Panicum subalbidum*) (PAN-G) and Sedge plant *Schoenoplectus senegalensis* (SCH-G)) were evaluated during a period of 28 days. Total Heterotrophic Bacteria (THB) and Hydrocarbon Utilizing Bacteria (HUB) were monitored. Physicochemical parameters monitored were pH, Temperature, Nitrogen, Phosphorus, Potassium and Total Hydrocarbon Content (THC). Baseline results of physicochemical parameters carried out in the uncontaminated soil were pH, temperature (^{o}C), Moisture content (%), Electrical conductivity (uS/cm), Total Organic Carbon (TOC), Soil Organic Matter, Total Hydrocarbon Content (THC), Nitrogen, Phosphorus and Potassium (mg/kg). The amount of hydrocarbon remediated and percentage bioremediation in the soil after 28 days were: CS+PSE+SCH (3454mg/kg; 85.28%) > CS + BAC + SCH-G (3264mg/kg; 80.59%) > CS +PSE + BAC + SCH-G (3210mg/kg; 79.26%) and CS + SCH-G had the lowest (434mg/kg: 62%). This research revealed that *Schoenoplectus senegalensis* (sedge plant) is a suitable plant species for phytoremediation of crude oil polluted soil than *Panicum subalbidum*. Moreso, *Schoenoplectus senegalensis* (sedge plant) has a higher phytoremediation potential when augmented with *Psuedomonas*. it is therefore recommended that *Schoenoplectus senegalensis* (sedge plant) in combination with augmenting organism (*Psuedomonas*) is best option for remediation of crude oil polluted.

Keyword: Phytoremediation, polluted soil, crude oil, Enhanced, *Pseudomonas fluorescens*

INTRODUCTION

Crude oil pollution is one of the major environmental problems effecting terrestrial and aquatic environments. Presently, approximately 80% of lands are affected by product of petroleum origin i.e., hydrocarbons and these products are used in oil and chemical industries as energy sources (Farraji *et al*., 2016). Crude oil makes a covering on the surface of soil and causes the retention of carbon dioxide produced by soil organisms. It also decreases the soil porosity by sticking the soil particles together. The amount of loss depends on the amount and grade of oil spilled (Garcia *et al.*, 2017).

The introduction of crude oil into the environment can partially or completely destroy its aesthetic value, which is referred to as oil pollution. The need for crude oil as an energy source

and a key raw material for businesses has increased, which has led to an increase in its production, transportation, and refining, which has led to severe environmental pollution (Anikwe *et al.,* 2017). Both aquatic and terrestrial have faced serious threats from environmental contamination. Crude oil is one of the contaminants that enter the environment through the activities of man during oil exploration and oil spill during transportation process (Borah *et al.,* 2016). Nigeria is one of the major oil producers in Africa. When crude oil is released into the environment, the components are deposited in the soil and surrounding water bodies, thereby, altering the normal composition of both biotic and abiotic components of the affected ecosystem (De Boer *et al.,* 2016).

Reduced agricultural land due to soil and water contamination affects crop productivity as well as aquatic life in the water bodies. When agricultural practices are carried out on polluted soil, it's possible for the plants to become toxic and the animals' health to be at risk (Diphare *et al.,* 2014). Microbes in these ecosystems react to crude oil contamination when it occurs. Crude oil contamination drastically enhances heavy metal concentration in soil and water bodies. Heavy metals such as zinc, chromium, nickel, mercury, iron and copper are components of crude oil, though in low concentrations (Escobar *et al.,* 2018). Although physical and chemical methods are occasionally used to remove oil spills, they are typically neither economical nor environmentally benign. For instance, burning waste causes air pollution, and burying it causes ground water poisoning. The spilt oil is simply burned in an incineration process, which has the negative effect of increasing the air concentrations of carbon dioxide, nitrogen oxides, and sulfur oxides (Errington *et al.,* 2018). The current problem of global warming is known to be due to the accumulation of CO_2 in the atmosphere.

MATERIALS AND METHODS

Study Area

The study was carried out in Rivers State university school farm in Port Harcourt Local Government ($4^o48'3.59496"$ N$6^o58'46.09848"$E) and B-Dere in Gokhana Local Government of Rivers state ($4^052''38''$N, $5^018'35.29''$E) all in Rivers state, Nigeria. These two sites are located in the South-South geopolitical zone of Nigeria where crude oil exploration take place. The B-Dere location have been implicated for crude oil spills as a result of the activities of antisanal refineries. The locations were selected due to the fact that they are sites known for various activities including bunkering/Local refining of crude oil.

Sampling Technique

Simple random sampling technique was used to collect the soil samples. From each soil samples, one bag was contaminated soil and the other was uncontaminated and they were collected by simple random sampling technique.

Sample Collection, and Processing

The collection of samples was done aseptically using auger apparatus. Soil samples were collected by adopting the Food and Agriculture Organization (FAO), (2002) guideline using a sterile auger. The soil samples for analysis were collected using sterile black polythene bags and it was transported to the laboratory of Microbiology Department, Rivers state university Port Harcourt, Nigeria. Processing of the soil samples began immediately upon it arrival at the Microbiology laboratory

Bacteriological Analysis of Samples

Serial tenfold dilution was done on the weighed soil sample with dilution factor from10^{-1} to 10^{-6}. Aliquot (0.1ml) of appropriate dilutions were spread plated in duplicates onto Nutrient and Mineral salt agar. The plates was incubated at 37^oC for 24 hours. The colonies formed on the plates were counted and described morphologically. Colonies formed on Nutrient Agar was used to estimate the total heterotrophic bacterial counts (THBC). Representative distinct colonies was

purified by sub-culturing on freshly prepared sterile nutrient agar plates and incubated at 37^{o}C for 24hours to obtain pure cultures.

Isolation and Enumeration of Crude oil Utilizing Bacteria

For the isolation of hydrocarbon utilizing bacteria, Mineral salt agar medium was used. The composition (g/L) of the mineral salt media are 0.2 MgSO4, 0.02 $CaCl_2$, 1.0 KH_2PO_4, 1.0 NH_4NO_3, 0.05 $FeCl_3$ and pH adjusted to 7-7.2. The Mineral salt agar (MSA) plates were inoculated in duplicate with 0.1ml aliquots of 10^{-6} dilution of each soil samples and incubated at 35 ^{o}C for 7 days. Colonies that appeared on the agar plates was counted after a week and resulted as the count of total hydrocarbon degrading bacteria for the four soil samples. The colonies counted were expressed as the colony forming unit (CFU) per gram soil.

Preparation Of Bacterial Suspension For Bioremediation Setup

Suspension of *Pseudomonas fluorescens* was prepared from 24hrs sub-cultured Petri plate. Two hundred milliliter (200ml) of nutrient media broth was transfer into Two hundred and fifty milliliter (250ml) conical flask and sterilized using an autoclave at 121^{o}c for 15minutes at 15psi, and allowed to cool at room temperature. Cicatrin (0.8g) was added to the broth. Pure cultures of the organism (*Pseudomonas fluorescens*) were picked from the culture plate and then incubated to the 250ml nutrient broth in conical flask until a turbid was form. The flask was cap with cotton wool. It was incubated at room temperature 28^{o}C for 48hrs.

Treatment of the Soil for Bioremediation.

The soil sample was treated for bioremediation as described by Nrior, (2014). In this method, 6 setups were made. Each basin contained;

1. 2500g of uncontaminated soil + *Panicum subalbidum* which served as control
2. 2500g of uncontaminated soil + *Schoenoplectus senegalensis*.
3. 2500g of contaminated soil + *Panicum subalbidum* + 250ml of bonny light crude oil
4. 2500g of contaminated soil + *Schoenoplectus senegalensis* + 250ml of bonny light crude oil
5. 2500g of contaminated soil + *Panicum subalbidum* + 250ml of bonny light crude oil + 50ml of *Pseudomonas fluorescens* broth.
6. 2500g of contaminated soil + *Schoenoplectus senegalensis* + 250ml of bonny light crude oil + 50ml of *Pseudomonas spp* broth.

This bioremediation set up was monitored for selected microbiological and physicochemical parameters from day 1 to 28 days, such as Hydrocarbon Utilizing Bacteria (HUB), Total Heterotrophic Bacteria (THB), Total Hydrocarbon Content (THC), Nitrogen, Potassium, Phosphorus, Soil Organic Matter, Moisture Content, Temperature and pH, respectively at 14 days' interval. One Hundred milliliter (100ml) of sterilized water was added to the set up two times weekly and agitated for proper aeration and adequate distribution of microorganisms.

Phytoremediation

Five pots of soil contaminated with 5% crude oil were prepared by mixing the soil uniformly with crude oil. Each pot contained 2500g of soil mixed with 250ml of crude oil. The soil was commercially available compost soil from Rivers state university school farm while the crude oil was Bonny light crude (API = 32.3o; sulfur content: 0.08%).

Soil Preparation And Application Of Crude Oil And Nutrients

Soils were collected in two places, one batch is a hydrocarbon-contaminated soil while the other batch is uncontaminated soil. Two thousand five hundred grams (2500g) of the contaminated soil were weighed into 8 batches while 2500g of the uncontaminated soil were weighed into 2 batches. However, different treatments were considered for each soil batch. In order to ensure

easy interpretation of these results, according to different bioremediation strategies, the uncontaminated soil was named as control

Soil Analysis

Soil sampling was conducted on the first week after planting and weekly subsequently for another 5 weeks during which soil samples were collected from each pot at a fixed radius from the plant. The samples collected were sieved with 2mm mesh to separate organic materials and particulate maters. Sixteen (16 g) of soil was collected after sieving for subsequent analysis. The soil was tested for the moisture content, pH and the crude oil concentration. Soil moisture content was tested to maintain a sufficient level of soil moisture for phytoremediation. pH was tested as plants were known to alter the pH of surrounding soil as phytoremediation occurred.

Data Processing and Analysis

Statistical Package for Social Sciences (SPSS) version 25 was used to statistically analyse the data obtained from counts and the measurement of the zones of inhibition. Descriptive statistics was used to summarize all data obtained. Analysis of variance (ANOVA) was carried out to test for significant difference ($p \leq 0.05$) in the bacterial counts from the various locations. Duncan multiple range test was used to separate the means where difference existed (Bewick *et al*., 2004).

RESULTS

Results of the bacterial population of soil samples are presented in Table 1. The result of analysis showed that the mean total heterotrophic bacterial count ranged from 2.35_to 4.15_cfu/g. The statistical analysis revealed that there was no significant difference ($p>0.05$) in the total heterotrophic count between the samples.

Results of the hydrocarbon utilizing bacterial ranged from 0.7_to 1.45_cfu/g. There was no significant difference ($p>0.5$) in the total hydrocarbon degrading microorganism count.

Table 1. Bacterial Population of soil samples.

Location	THB $X10^6$cfu/g	HUB $X10^5$cfu/g
B-Dere 1	3.65	1.15
B-Dere 2	2.35	1.45
B-Dere 3	3.7	0.7
B-Dere 4	4.15	1.15

Key: THB (Total Heterotrophic Bacterial), HUB (Hydrocarbon utilizing Bacterial)

Table 2. Bacterial Population Count Obtained From Soil Sample During Bioremediation Monitoring.

SET UP CODE	DAY 1 $X10^6$ CFU/g	DAY 14 $X10^6$ CFU/g	DAY 28 $X10^6$ CFU/g
US+ PAN-G	2.95	2.15	1.5
U S+ SCH-G	2.65	1.9	1.4
CS + PAN-G	1.7	1.3	8.5
CS + SCH-G	3.2	2.35	2.1
CS + PSE+ PAN-G	3.05	2.75	1.9
CS + PSE+ SCH-G	2.5	1.7	1.15

KEY: US (uncontaminated soil), CS (contaminated soil), Pse (*Pseudomonas spp*), PAN-G (*Panicum subalbidum*), SCH-G (*Schoenoplectus senegalensis*).

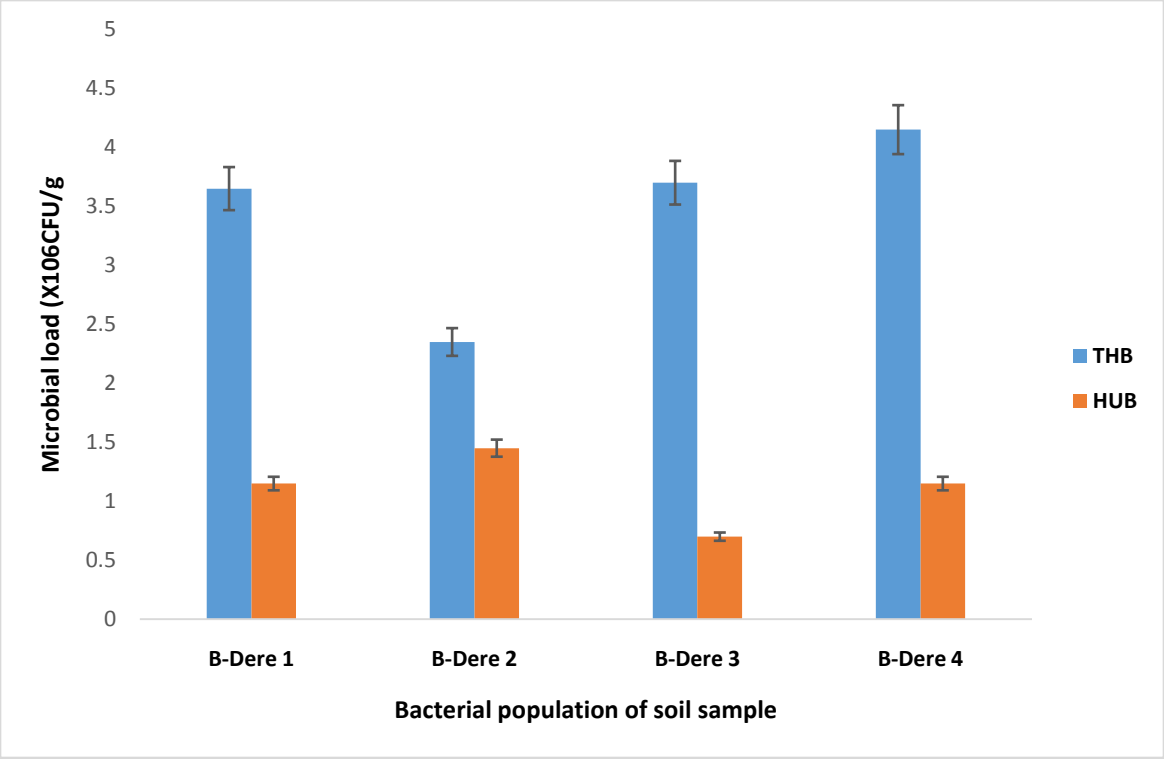

Fig. 1 Mean Bacterial Population of Soil Sample

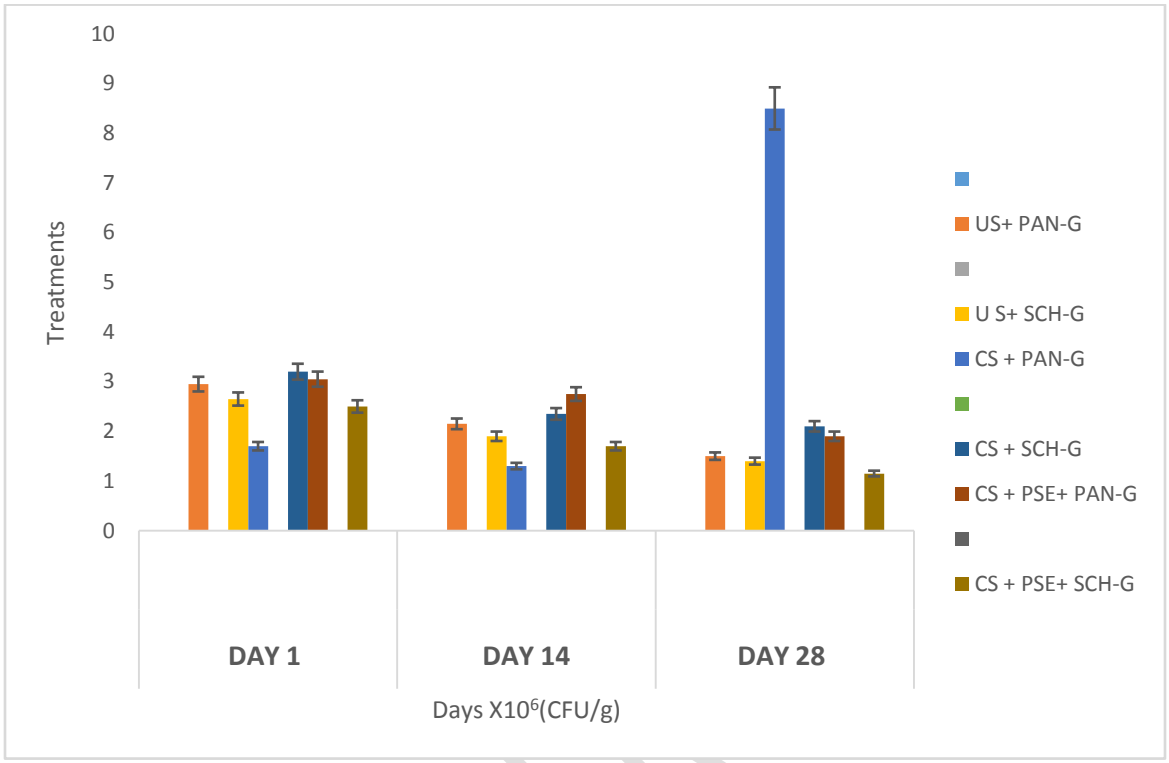

Fig. 2 Bacterial Population Count obtained from soil sample during Bioremediation monitoring.

Table 3. Mean Physicochemical Parameter of the Soil Sample During Bioremediation Monitoring

Treatment code	pH	Temperature	Nitrogen	Phosphorus	Potassium	Soil THC	Root THC
US+ PAN	6.48	27.83	66.18	0.48	5.38	442	9.87
U S+ SCH	6.69	27.47	65.51	0.47	4.13	490.67	6.07
CS + PAN-G	6.07	27.43	35.27	0.45	3.38	2429.33	118
CS + SCH-G	6.01	27.3	34.15	0.49	2.36	2346	290.67
CS + PSE+ PAN-G	6.07	27.3	32.36	0.46	2.54	2206	122.67
CS + PSE+ SCH-G	6.11	27.37	40.40	0.45	5.54	1942.67	243.33

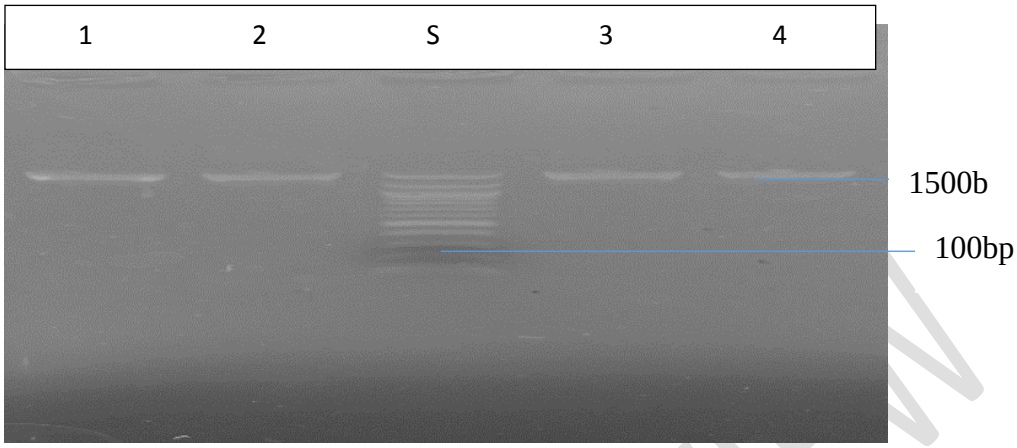

PLATE 1: Agarose gel electrophoresis of bacterial isolates. Lanes 1 – 4 represent 16SrRNA gene bands (1500bp). Lane S represents the 100bp Molecular ladder indicated at 100bp.

DISCUSSION

Crude oil pollution leads to variation in the composition of resident microorganisms in an ecosystem. Microbes make the major contribution to mineralization of crude oil pollutants. When crude oil contamination occurs, microbes in such habitat respond to the stimulus. One of the main environmental issues affecting both aquatic and terrestrial areas is crude oil contamination. The native microorganism population of an ecosystem can change as a result of crude oil contamination. Due to their capacity to tolerate the new stress, microorganisms, especially bacteria, respond favorably and continue to occupy their ecological niche. This adaptive measure enables the organism to source their nutrients from the composition of the crude oil. When the response is negative, the bacterial species are sensitive to the component of the crude oil, so they cannot withstands the stress, which may result in their complete elimination from the habitat (Anwar *et al.*, 2017).

The study revealed that total heterotrophic bacteria had the highest occurrence in the sample 4 (Rivers state university school farm) at both depths (0-15cm and 15-30cm) and it concurs with works done by Williams and Hakam (2016). Total Heterotrophic bacteria occurring as the highest organisms at both depth of the soil could be attributed to the tolerance of bacteria to wide variations of the soil properties such as nutrient content, moisture content, oxygen concentration and many other parameters of importance in this study. The results from this study followed the same trend for soil bacterial populations reported by Nrior and Ogbonna (2019). The isolates from soil samples have significant ability to utilize crude oil as a sole source of carbon and energy and the occurrence of these organism have been reported by Williams and Hakam (2016) and different researchers. When crude oil or other petroleum products leak into the environment, a chain of events occurs that causes the environment to deteriorate. Between 20 and 40% of the oil mass transforms into gases after the first few days; the volatile gases evaporate, leaving the heavier components (Williams *et al.*, 2018). Natural attenuation, which is a variety of physical, chemical, or biological processes that, under favorable conditions, act without human intervention to reduce the mass, toxicity, mobility, volume, or concentration of contaminants in soil also set in (Williams *et al*., 2018). After a few months, microorganism which are not able to utilize the hydrocarbons present in the soil either mutate or completely die off due to environmental stress. After a few years, the organisms in the environment fully adapt and reproduce, most of the crude oil components are also completely broken down. Due to these

factors, sites with older spills have lesser total petroleum hydrocarbon (TPH) content, lesser TPH contents implies higher chances for the proliferation of microorganisms (Nrior *et al*., 2019). The results from this study also showed a decrease in the heterotrophic bacterial counts with an increased in soil depth. This could be due to the higher availability of favorable growth factors such as utilizable organic matter and oxygen at the surface soil (0-15cm) than at the subsurface soil levels (15-30cm).

This study revealed as expected that the total heterotrophic bacterial had the highest occurrence while hydrocarbon utilizing bacterial were the least occurring in the soil sample (Table 1). Hydrocarbon degrading microorganism are ubiquitously distributed in crude oil contaminated soil environment. According to Williams and Barisi, (2018), population of hydrocarbon degraders normally constitute less than 1% of the total microbial communities, but when oil pollutants are present in an environment, the hydrocarbon-degrading populations increase, typically to 10% of the community. This study revealed that not all the members of the heterotrophic population could utilize the crude oil and petroleum products spilled in the soil environment, hence a decrease in the counts of hydrocarbon utilizing organisms compared to the heterotrophic microbial count (Anwar *et al.*, 2017). The high hydrocarbon utilizing bacterial counts could be attributed to the utilizable organic matter present in crude oil.

About 3 genera of bacteria were genetically identified from crude oil contaminated soil sample in this study across the location which *Pseudomonas* was the most occurring. The organisms isolated and identified in this study include *Peudomonas, Priestia megaterium* and *Bacillus spp*. These organisms have the ability to utilize crude oil as their sole source of carbon and energy. And the dominance of these organisms have been reported by different researchers as crude oil degraders (Nrior and Ogbonna, 2019; Williams and Barisi, 2018).

The main factor in the mineralization of crude oil contaminants is microbes. The ecological recovery of petroleum waste-contaminated places is accomplished through bioremediation, which employs the metabolic adaptability of microorganisms to breakdown harmful pollutants. Bacteria are typically chosen among microorganisms due to their quick metabolic rates, close proximity to multiple degradation pathways, and ability to undergo genetic manipulation to enhance bioremediation (Anwar et al., 2017). It further revealed that a combination of phytoremediation and Bioaugmentating agents creates more favorable conditions for biological activity to thrive and has shown to be effective, economical, eco-friendly and sustainable in remediating organic contaminants from contaminated soil.

The bacteria from the experimental soil used in this study belong to the genera; *Pseudomonas* spp. This is in line with the observations of various researchers who reported similar bacterial from crude oil contaminated soil. The results of the microbial evaluation of the study are shown in Table 2. Significant microbial counts for total heterotrophic bacteria counts were recorded on day 14 and day 28 of the bioremediation monitoring. The highest counts for each day were contaminated soil + *Pseudomonas* + *Schoenoplectus senegalensis*; contaminated soil + *Pseudomonas* + *Schoenoplectus senegalensis* and contaminated soil + *Panicum subalbidum* for Days 1, 14 and 28, respectively of the Bioremediation set up. The results of bacterial population count of the set up obtained revealed that the total heterotrophic bacterial generally increased during the study as the treatment progressed resulting in corresponding bioremediation with time (Day). The result is consistent with the reports of Nrior and Ogbonna (2019) who observed that Total Heterotrophic Bacteria and Hydrocarbon Utilizing Bacteria increased over time in a nutrient amended crude oil contaminated soil undergoing bioremediation with time (Day). This may also be as a result of increase in microbial activities in soil because of added nutrient. The

rates of chemical reactions and microbiological activities as well as biodegradation rates generally increased with temperature. Biodegradation mediated by indigenous microbial communities is a key process by which petroleum hydrocarbons are mineralized and removed from contaminated environments. Thus, microbial oil biodegradation is recognized as one of the most important methods for petroleum hydrocarbon remediation.

Phytoremediation using grass plant *Panicum subalbidum* (Elbow buffalo grass), Sedge plant (*Scoenoplectus senegalensis*) were carried out on Crude Oil polluted soil. Isolated microorganisms *Pseudomonas fluorescens* were used to augment the indigenous microbial population present in a crude oil contaminated soil to enhance microbial remediation in pari per sue with phytoremediation (uptake of Crude oil by test plants) over a period of 28 days. The Sedge plant (*Scoenoplectus senegalensis*) survive the first screening stage with crude oil contamination but died during the monitoring period of 28days because of the uptake of crude oil by the roots. The Elbow buffalo grass (*Panicum subalbidum*) survive after monitoring of 28 days with crude oil contamination.

Experimental transplants had an initial height of 16.7cm on the first 7 days of growth, Plant showed reduced growth whereas; plant in uncontaminated soil were in good condition. *Panicum subalbidum* (Elbow buffalo grass) indicated a high potential of adaptation in the contaminated soil as shown by the growth during 14 to 28 days regardless of the bio-organic in the contaminated soil compensating for the higher C/N ratio. The plant height increased significantly with time ($p=0.05$). The average plant height of *Panicum subalbidum* (Elbow buffalo grass) were 52.46 and 55.82cm respectively in pot 4 and pot 6 in comparison to 36.88cm in (uncontaminated plots) during the 28 days; while the *Schoenoplectus senegalensis* did not survive the crude oil contamination after 28days of monitoring. There was no significant difference of plant height between the contaminated and uncontaminated.

Root structure is considered just as important as root biomass concerning degradation process. Generally, the roots growing in uncontaminated soil were longer, and covered more surface area than those growing in contaminated soil. The result from this study indicates that under normal pH, oxygen and sufficient nutrients, phytoremediation of crude oil contaminated soil increased in each pot compared to the controls. Statistically there was no significant difference ($p<0.05$) in hydrogen ion concentration (pH) in various treatment pots.

CONCLUSION AND RECOMMENDATIONS

Petroleum hydrocarbon pollution is a worldwide threat to the environment and the remediation of oil contaminated soils is a major challenge for environmental research. Bioremediation is the biological treatment system to destroy or reduce the concentration of hazardous waste from contaminated sites. Crude oil contamination drastically enhances heavy metal concentration in soil and water bodies. It has been revealed that heavy metals accumulate in the soil, especially when there is an oil spillage. The absorption of these heavy metals is facilitated by low soil pH, which can be accelerated by bacteria products of metabolism and organic matter. Results revealed amount of hydrocarbon remediated and percentage (%) Bioremediation in the soil after 28 days of monitoring is higher in set up CS+PSE+SCH (3454mg/kg; 85.28%) and lowest in set up with US+SCH (434mg/kg: 62%) and the amount and percentage (%) phytoremediation uptake by the roots after 28 days of monitoring is higher in set up CS+BAC+SCH (632Mg/kg; 15.6%) and lowest in set up with US+SCH (12.2mg/kg; 1.74%).

This research revealed that is a suitable *Schoenoplectus senegalensis* plant specie for phytoremediation of crude oil contaminated soil. It can also be concluded from this study that the organism and test plant used in this study are readily available, natural, cost effective, eco-

friendly and effective. The use of *Schoenoplectus senegalensi* as efficient phytoremediation grass should be encouraged. Moreso, *Schoenoplectus senegalensis* (sedge plant) has a higher phytoremediation potential when augmented with *Psuedomonas.*

The use of *Panicum subalbidum* and *Schoenoplectus senegalensis* as efficient phytoremediation agents should be encouraged. Based on the findings from this research, the use of ecofriendly bioorganic (Biostimulants) and augmenting microbes as amendment options with phytoremediation plants should be encouraged to facilitate pollutant removal/ clean up. This study showed that *Schoenoplectus senegalensis* should be used as a suitable plant species for phytoremediation of crude oil contaminated soil.

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