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Biotreatability of Crude Oil Polluted Aquatic Environment Using Indigenous Hydrocarbon Utilizing Bacteria

Tudararo-Aherobo Laurelta[1*], Okotie Sylvester[2], Ataikiru Tega[1] and Stephen Avwerosuoghene[2]

[1]Department of Environmental Management and Toxicology, Federal University of Petroleum Resources, Effurun, Delta State, Nigeria.
[2]Department of Petroleum Engineering, Federal University of Petroleum Resources, Effurun, Delta State, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Author TL wrote the first draft of the manuscript and performed the graphical presentations. Authors OS and SA wrote the protocol and literature review, while Authors TL and SA managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The research aims to assess the biodegradability of crude oil polluted aquatic environment using indigenous hydrocarbon degrading bacteria.
Place and Duration of Study: The research was conducted in the Environmental Management and Toxicology Laboratory, Federal University of Petroleum Resources, Effurun, Delta State.
Methodology: Hydrocarbon degrading bacteria species were isolated from hydrocarbon contaminated soils, screened and used for the degradation of crude oil. 5% and 10% crude oil were used to spike the test microcosm. Physicochemical parameters such as, pH, turbidity, total petroleum hydrocarbon (TPH) and bacterial counts of the bioremediated crude oil contaminated water were monitored on Day 0, 7 and 14. The biodegradation of the crude oil was done with the various bacteria isolates singly and as a consortium. Standard methods of American Public Health Association (APHA) and American Society for Testing and Materials (ASTM) were used for the analysis.

**Corresponding author: E-mail: tudararo.aherobo@fupre.edu.ng;*

Results: The isolates identified and used for the biodegradation process were, *Azomonas sp., Enterococcus sp., Klebsiella sp.* and *Rhizobactersp*. On day 14, in the microcosms with 5% crude oil contamination, *Azomonas sp*. recorded the highest turbidity reading of 328 ± 2.0 NTU, while *Rhizobacter sp*. recorded the least with 57.67 ± 0.58 NTU. The bacterial countswere between 7.68 ± 0.002 CFU/ml and 8.05 ± 0.10x 10^7 CFU/ml for *Rhizobacter sp.* and *Azomonas sp*. respectively.The crude oil was also degraded most in the microcosm treated with *Azomonas sp*. with a residual TPH concentration of 0.0013± 0.005 mg/l.For the 10% crude oil contaminated microcosms, TPH was also biodegraded most by *Azomonas sp.* with a value of 0.0026 ± 0.002mg/l. Turbidity readings were between 82 ± 1.0 NTU and 375.33 ± 0.57 NTU for *Rhizobacter sp.* and *Azomonas sp.* respectively. Bacterial counts were between (7.71± 0.012)x 10^7CFU/ml – (8.13± 0.001) x 10^7CFU/ml for *Rhizobacter sp.* and *Azomonassp.* respectively.
Conclusion: There wasincreased microbial countsand decrease of residual crude oil concentration, indicating degradation of the crude oil by all the isolates.However, *Azomonas sp.* recorded the highest TPH degradation for both the 5% and 10% crude oil contaminated microcosms.Thus, findings from the research indicate that hydrocarbon degrading bacteria exist in our environment and can be used in the remediation of aquatic polluted environment.

Keywords: Crude oil; aquatic pollution; biotreatability; indigenous bacteria species.

1. INTRODUCTION

Hydrocarbons are naturally occurring, flammable organic compounds in crude oil found in geologic formations beneath the earth's surface. Crude oil refers to hydrocarbon mixtures that are liquid at normal atmospheric pressure and temperature. Petroleum hydrocarbons are widespread industrial pollutants that are released into the environment through crude oil transporting, storing, accidental leaking, petroleum refining, and wastewater irrigation. These pollutants alert diversity of microbial community and soil enzymes [1,2].

The behavior of pollutants in the environment is influenced primarily by the nature and amount of the contaminants present and the interaction between chemical, geochemical, and biological factors [3]. Among biological factors, the diversity of microbial species and their metabolic capabilities constitute an important source of biocatalysis [4]. The structure and dynamics of the indigenous microbial communities are major characteristics influencing biodegradation [3]. The degradation of complex pollutant mixtures such as petroleum requires a combination of different bacterial taxa that can degrade a broader spectrum of hydrocarbons than any single bacterial species alone [5]. Several studies of contaminated sites have shown that the impact of contamination on bacterial communities is dependent on the previous pollution history [3,5,6].

Bioremediation can be defined as a biotechnological technique using microorganisms to breakdown or neutralize a contaminant from a polluted area. It is based on the ability of certain microorganisms to convert, modify and utilize toxic pollutants in order to obtaining energy and biomass production in the process [7]. Bacteria, archaea and fungi are typical prime bioremediators [8].

Many strategies of bioremediation can be applied in terms of soil/water waste treatment [9]. These include, Bioattenuation [10], biostimulation [11,12] and bioaugmentation [13]. Bioremediation technology utilizes microorganisms to degrade toxic pollutants to harmless products such as CO_2, H_2O, and other inorganic compounds and these processes are environmentally safe and cost efficient [14]. It has been reported that roughly 25% of all petroleum-contaminated land is being bioremediated using natural attenuation (do nothing) thus understanding the importance of microorganisms in remediation strategies [15]. Bioremediation is simple, less labor intensive, eco-friendly and sustainable [16], nonintrusive, contaminants are destroyed, not simply transferred to different environmental media and potentially allowing for continued site use [17,18].

Giving the effectiveness of indigenous microorganisms in remediating hydrocarbon pollutes sites and the sustainable and cost effective advantages of this strategy over chemical or physical strategies, this research aimed at isolating and identifying the biodegradative potentials of indigenous hydrocarbon utilizing bacteria in laboratory microcosms, with the view of creating a microbial bank for prompt application to contaminated

sites for effective and timely clean-up of polluted site.

2. MATERIALS AND METHODS

2.1 Collection of Hydrocarbon Contaminated Soils

Hydrocarbon contaminated soil samples were collected from automobile mechanic workshops and diesel generator sites. Composite samples were collected from FUPRE diesel generator site at Ugbomro (latitude 5°56'92', longitude 5°83'49'") in Uvwie LGA and automobile mechanic workshop at Okwagbe town (latitude 6°23'58'', longitude 5°55'11'') in Ughelli South LGA, Delta state. The soil samples were kept in clean polythene bags and kept at 4^{O}c until required for use.

2.2 Collection of Crude Oil

Two (2) litres of crude oil was collected from Oredo flow station located at Ologbo in Oredo LGA, Edo state (latitude 5°34'12'', longitude 5°50'26) in 1L glass bottle.

2.3 Isolation and Selection of Crude Oil Degrading Bacteria

The procedure of Bhattacharya *et al*. [19] was adopted for this study. Bushnell-Haas (BH) media with the following composition (g/L): K_2HPO_4 (1.0 g), KH_2PO_4 (1.0 g), NH_4NO_3 (1.0 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $FeCl_3 \cdot 6H_2O$ (0.05 g), $CaCl_2 \cdot 2H_2O$ (0.02 g), was used as an enrichment medium with 1% crude oil (v/v) as the sole carbon source to isolate the crude oil degrading bacteria from the hydrocarbon contaminated soils. Soil samples (10 g) were added to 50 mL BH media in 250 mL Erlenmeyer culture flasks. It was then incubated at 28 ± 2°C for 7 days. After 7 days incubation, the bacteria cultures were isolated as single colonies into petri dishes containing nutrient agar (NA) media by streak-plate method. The pure bacteria isolates were maintained in slant cultures by preserving at 4°C and sub cultured at 2 weeks interval to maintain its viability.

2.4 Screening Test for Biodegradability Potential of Hydrocarbon Degrading Bacteria

The isolated bacteria cultures were screened for effective crude oil degradation. Fresh overnight cultures suspended in BH medium were used as inoculum. The isolates were aseptically inoculated into the BH medium in culture flasks with 2% (v/v) crude oil as carbon source. The flasks were then incubated at 30°C for 7 days. After completion of the incubation period, the growth of the bacteria isolates were assessed by turbidity readings [20], while the residual oil was measured to evaluate the degradation efficiency of the isolated microorganism. All the experiments were performed in triplicates, and a control devoid of the bacterial isolates was prepared along with the test experiment. Isolates with high turbidity (high growth) and crude oil degradability were used for the crude oil bioremediation study.

2.5 Identification of Selected Bacteria Isolates

The screened and selected crude oil degrading bacteria were identified by cultural, morphological and biochemical characteristics, following the method described by Holt *et al.* [21].

2.6 Crude Oil Biodegradability Studies

The crude oil contaminated microcosms were treated with variable culture conditions which includes incubation period (0, 7 & 14 days) and oil concentration (5 & 10 % v/v). This was done to study the crude oil degradation ability of the selected and screened culture, according to the method of Bhattacharya *et al*. [19]. Five (5% v/v, 50000 mg/L) and 10% v/v (100000 mg/L) of crude oil in 500 mL of BH medium were used for the biodegradation study. In addition, a control without bacterial isolates were prepared along with the test treatments. The set up was incubated for a period of 14 days, at an incubation temperature of 28 ± 2°C. Biodegradation of the crude oil at the two test concentrations were assessed and monitored for on days 0, 7 and 14, by sampling 100 mL of the culture media. The physicochemical parameters (pH and turbidity), residual TPH and bacterial countswere monitored weekly according to APHA method [22].

2.7 Biodegradation Monitoring Analysis

2.7.1 Residual total petroleum hydrocarbon

DichloroMethane (DCM) was used as the solvent for the extraction process. A 420nm Bio-board Smart Spec 3000 was used for the analysis. The sample to be analyzed was mixed in the original sample bottle by shaking. 50ml of sample was

measured and poured into the separating funnel after which 10 ml of dichloromethane was then added and corked. The mixture was mixed by shaking vigorously for 2 minutes while releasing pressure. The sample was then allowed to stand and settle till the bubbles disappeared. Sodium sulphate was used to remove water from the organic extract. The extracts were collected in 10ml vials and analyzed for TPH using a 420nm BIO-RAD smart spec 3000.

2.8 Determination of Bacteria Growth

This was determined by turbidity measurements of the inoculated and un-inoculated cultures on day 0, 7 and 14 using a Hach Ratio Turbimeter [22]. Bacterial growth was also determined as total heterotrophicbacterial count (THBC), according to APHA method [22]. 0.1 mL of fungizone was added to the nutrient agar to inhibit fungal growth. The plates were then incubated at 28± 2°C for 24 h. Results were expressed as CFU/ml.

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of Crude Oil Degrading Bacteria from Contaminated Soil

Four bacteria isolates with crude oil biodegradative ability were isolated during this study. The bacteria identified were species *Enterococcus sp, klebsiella sp, Rhizobacter sp and Azomonas sp* as shown in Table 1. Several researchers have reported these isolates aspotential hydrocarbon degraders from different environments [23,24,25].

3.2 Mean Changes in pH during Bioremediation of Crude Oil Contaminated Water

The mean pH values for the treatments with single isolates and the consortium (a combination of the four isolates)with 5% crude oil contamination are shown in Fig. 1. Themean pH values of the treatments on day 0 ranged from 6.36 ± 0.01(*Rhizobacter sp.*) - 6.62 ± 0.002(Consortium). On day 7, *Klebsiella sp.* recorded the lowest pH value, (5.85 ± 0.005) while *Azomonas sp.* recorded the highest pH value (6.37 ± 0.031). On day 14, the pH of the treatments dropped, with *Azomonas sp.* recording the lowest value of 5.57 ± 0.012 and *Enterococcus sp.* the highest pH value of 6.14 ± 0.006 (Fig. 1).

The pH values in the different treatments with 10% crude oil contamination are as shown in Fig. 2. On day 7, the control had the highest pH (6.38), followed by *Enterococcus sp.*(6.26) and *Azomonassp*. had the least pH (5.80). Again, the control setup had the highest pH (6.24), followed by *Rhizobacter sp*(6.10) and *Klebsiella sp* had the least pH value(5.48) at day 14.*Obahiagbon et al.,* [26] in their study reported that high acidic and alkaline pH values have negative effects on bioremediation, thus the pH values recorded for the different microcosms were within the ideal pH range supporting microbial hydrocarbon degradation.

Table 1. Biochemical identification of bacterial isolates

Parameters	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Gram reaction	+	-	-	-
Shape	Rod	Rod	Rod	Cocci
Lactose	+	+	+	+
Glucose	+	+	+	+
Oxidase	-	+	-	+
Methyl red	-	-	+	+
Vogues Proskaeur	+	-	+	+
Urease	+	+	+	+
Citrate	+	-	+	-
Indole	-	-	-	-
Hydrogen sulphide	-	+	-	-
Yellow pigment	+	+	+	*Brown-black /Blue-white fluorescence*
Catalase	-	+	+	+
Identified isolate	*Enterococcus sp.*	*Rhizobacter sp.*	*Klebsiella sp.*	*Azomonas sp.*

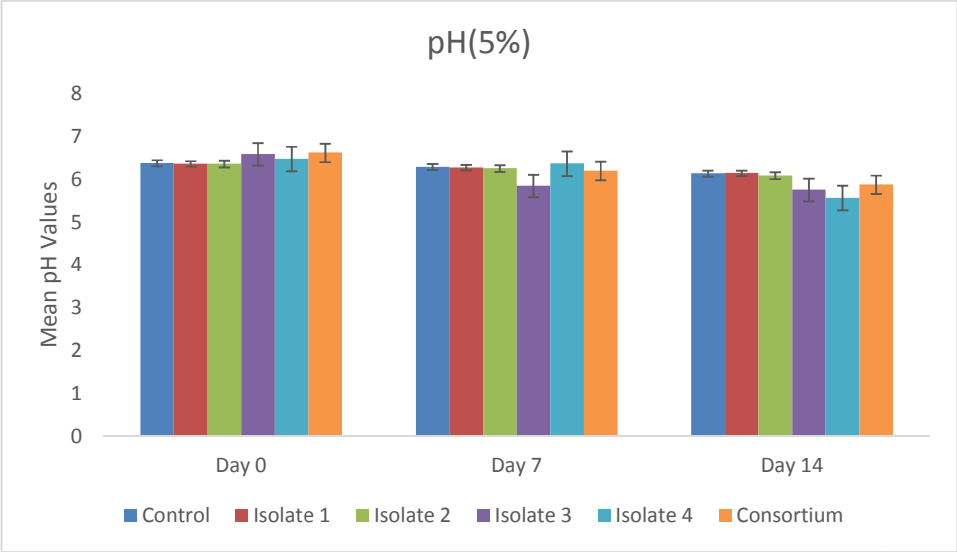

Fig. 1. Mean pH values of microcosmswith 5% crude oil contamination
Key: Isolate 1: Enterococcussp.; Isolate 2: Rhizobactersp.; Isolate 3: Klebsiellasp.; Isolate 4: Azomonassp.; Consortium: a combination of four isolates

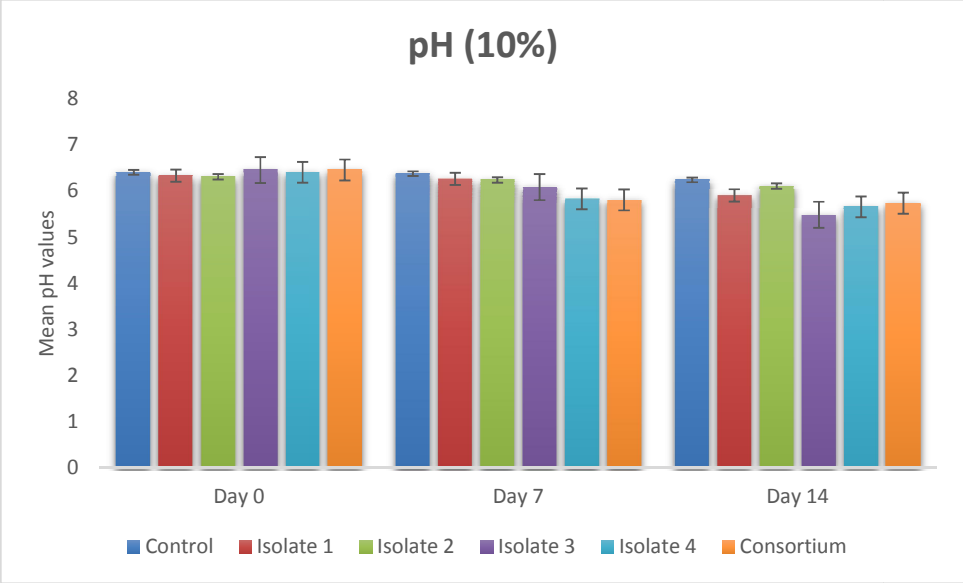

Fig. 2. Mean pH values in the experimental setup with 10% crude oil contamination
Key: Isolate 1: Enterococcussp.; Isolate 2: Rhizobactersp.; Isolate 3: Klebsiellasp.; Isolate 4: Azomonassp.; Consortium: a combination of four isolates

3.3 Changes in Turbidity and Bacteria Biomass

Turbidity values for the5% crude oil contaminated microcosmsare shown in Fig. 3. On day 7, *Azomonas sp.* and consortium had the highest value (119.33 ± 1.15 NTU) and least value(19.67 ± 0.58NTU), respectively. At the end of the study (day 14), *Azomonas sp.* and *Rhizobacter sp*. had the highest (328 ± 2.0 NTU) and lowest (57.67 ± 0.58 NTU) values respectively (Fig. 3).

In the 10% crude oil contaminated microcosms, (Fig. 4), on day 14, turbidity valuesobtained were in the following decreasing sequence, *Azomonas*

sp (375.33 ± 0.58 NTU)>*klebsiella sp.* (335 ± 0.58 NTU)>*Enterococcus sp.* (186 NTU)>Consortium (158 NTU) >*Rhizobacter* sp (62 NTU).

Bacteria growth can also be determined or measured by turbidity since absorbance (amount of light absorbed by the bacteria cells) is directly proportional to cell concentration, thus the higher the number of cells, the higher the turbidity [27]. The high crude oil biodegradation recorded for the selected bacteria isolates is attributed to the biodegradative activities of the bacteria isolate inoculated into the test culture medium in relation to the control. This is similar with observation in studies by Diaz-Ramirez et al., [28].

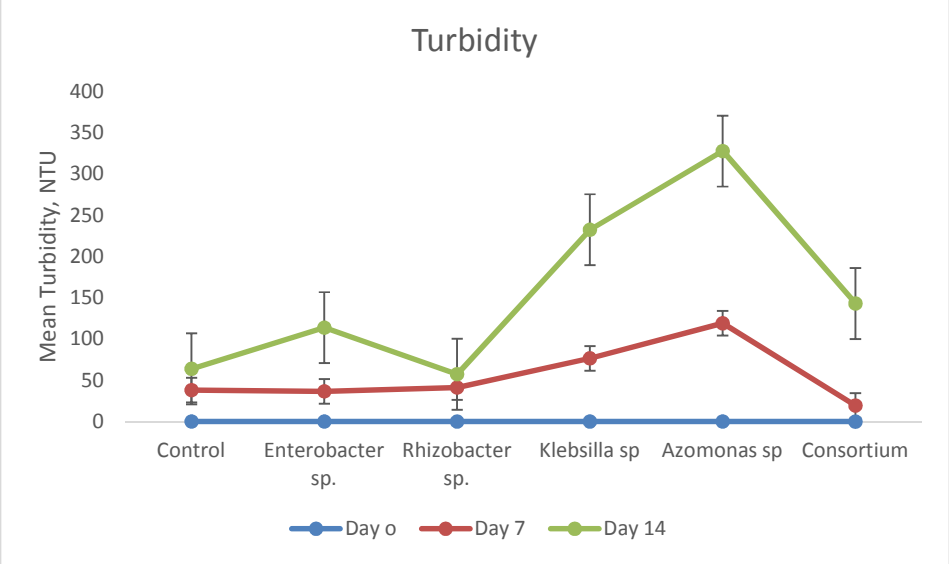

Fig. 3. Mean turbidy values of microcosms with 5% Crude oil contamination
Key: Isolate 1: Enterococcussp.; Isolate 2: Rhizobactersp.; Isolate 3: Klebsiellasp.; Isolate 4: Azomonassp.; Consortium: a combination of four isolates

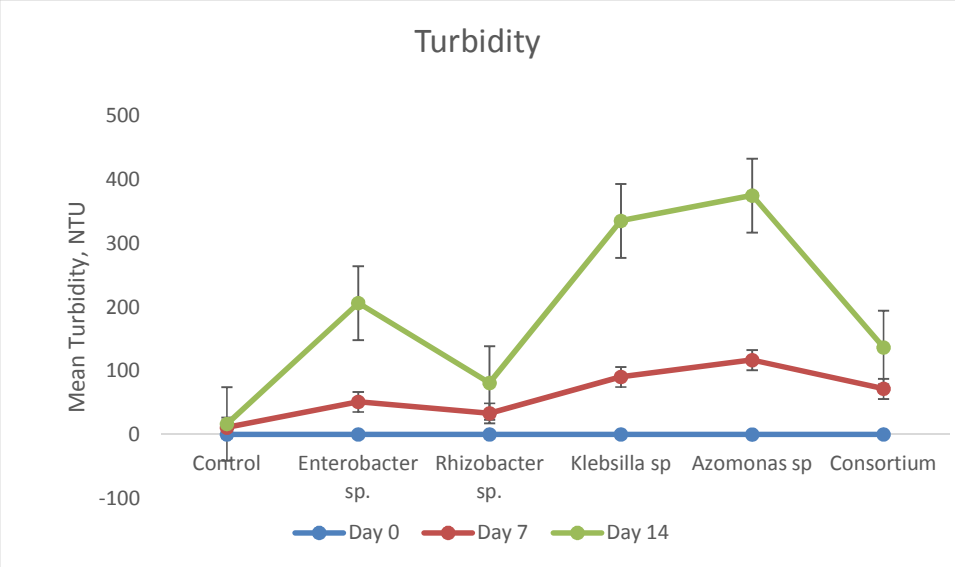

Fig. 4. Mean turbidity values of microcosms with 10% Crude oil contamination
Key: Isolate 1: Enterococcussp.; Isolate 2: Rhizobactersp.; Isolate 3: Klebsiella sp.; Isolate 4: Azomonassp.; Consortium: a combination of four isolates

3.4 Mean Residual Total Petroleum Hydrocarbon Concentration in Bioremediated Crude Oil Contaminated Water

On day 14, in the microcosm with isolates, residual TPH concentration was between 0.0087± 0.001 mg/l and 0.0013 ± 0.001 mg/l for K*lebsiella* sp. and *Azomonas sp.* respectively. The lowest TPH degradation was however recorded inthe controlmicrocosm with the highest residual TPH concentration of 0.0261±0.026mg/l (Fig. 5). The residual TPH at the end of the test period (Day 14) were in this increasing sequence; *Azomonas sp*. (0.0013 ± 0.001mg/l) >*Enterococcus s*p. (0.0034 ± 0.001mg/l) >*Rhizobacter sp*, (0.0044 ± 0.001 mg/l) >Consortium (0.0071 ± 0.001 mg/l) > K*lebsiella* sp. (0.0087± 0.001 mg/l) with the control showing the least degradation (0.0261 ±0.026 mg/l).

In the microcosms with 10% crude contamination*, Azomonas sp*. recorded the least residual total hydrocarbon petroleum on day 7 (0.020 ±0.002 mg/l) and day 14. A reverse trend was observed for the control (Fig. 6). There was a progression in the decrease of total petroleum hydrocarbon (TPH) for the 10% crude oil test setup from Day 0 to 14 but at a much slower rate than the 5% test set up. The degradation sequence for the 10% crude oil contaminatedmicrocosms on Day 14 were in following decreasing sequence; *Rhizobacter sp* (0.0178 ±0.002 mg/l) >Consortium (0.0081 ± 0.001mg/l), *Enterococcussp*. (0.0060 ± 0.002 mg/l) >*Klebsiella sp*. (0.0040 ± 0.003 mg/l) >*Azomonas sp* (0.0010 ± 0.001mg/l) with the control showing the least degradation (0.2870 ± 0.003mg/l).

Azomonassp. degraded the crude oil most for the 5% and 10% crude oil contaminated microcosms. This corroborates with the high turbidity values recorded by *Azomonas sp.* at both concentrations. In comparing the amount of crude oil degraded by the bacterial isolates in both concentrations of crude oil tested, it was observed that the percentage degradation of crude oil decreased with increasing oil concentration possibly due to the presence of highly persistent aromatic alkanes as reported by Jain et al., [29]. According to Okerentugba et al., [30], microbial communities exposed to hydrocarbons adapt to this exposure through selective enrichment and genetic changes resulting in an increase in hydrocarbon-degradation. This pre-exposure of microbes make them better suited to degrade the pollutant through higher growth, reproduction and more efficient metabolism thus maximizing the rate of hydrocarbon removal from such environments [30].

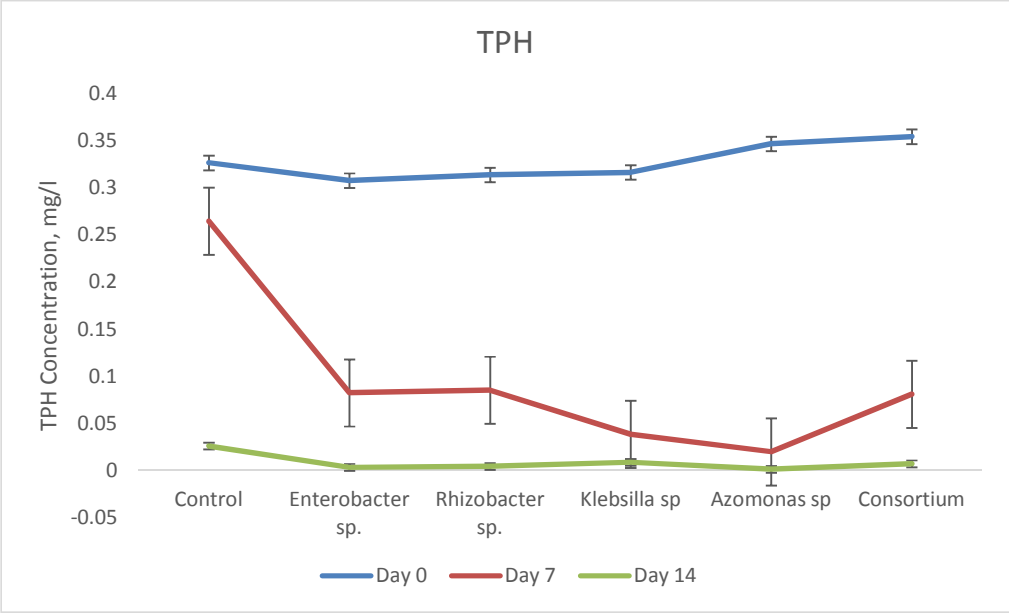

Fig. 5. Mean concentration of residual total petroleum hydrocarbon (TPH) with 5% crude oil contamination

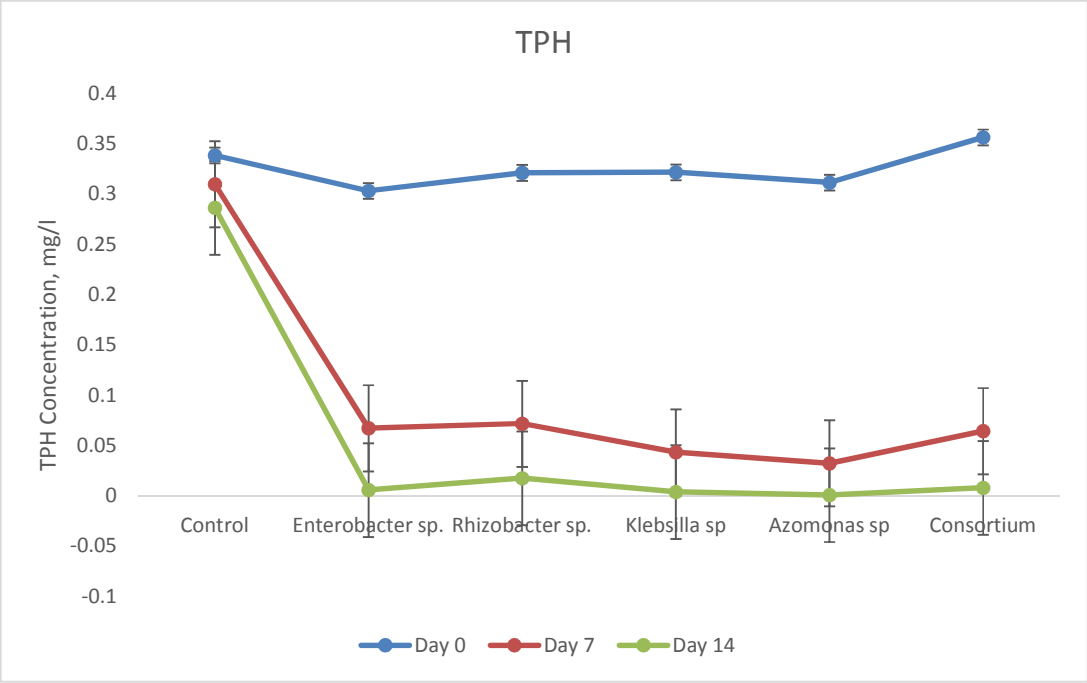

Fig. 6. Mean concentration of residual total petroleum hydrocarbon (TPH) with 10% crude oil contamination

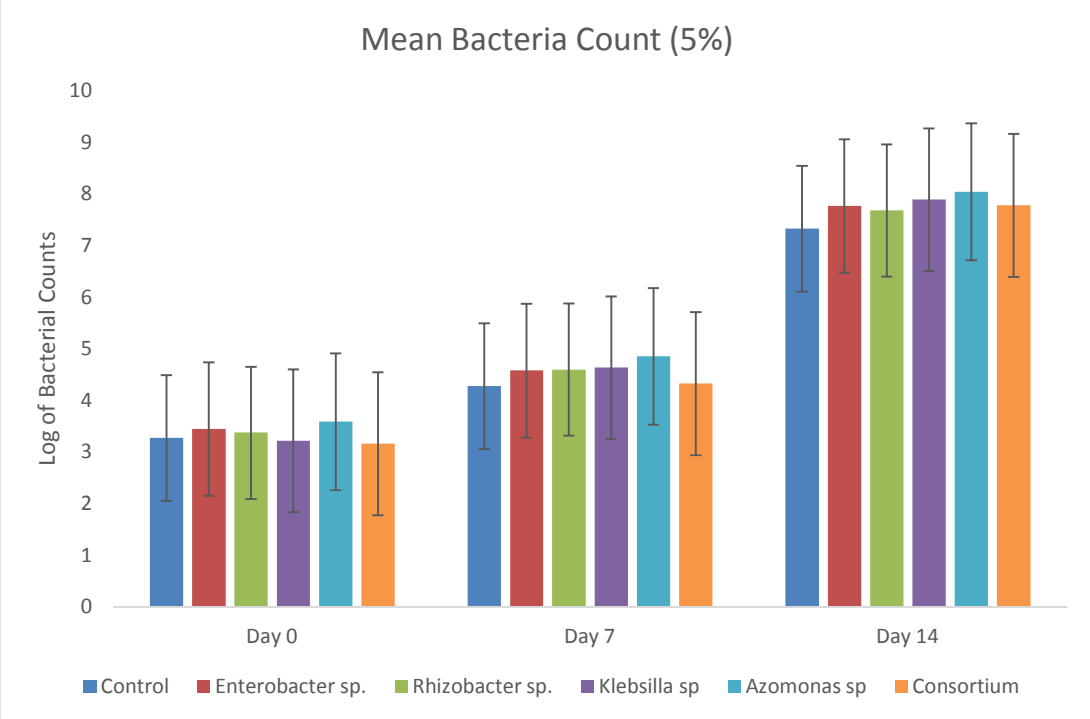

Fig. 7. Mean Hydrocarbon degrading Bacterial counts with 5% crude oil contamination

Key: Isolate 1: Enterococcus sp.; Isolate 2: Rhizobacter sp.; Isolate 3: Klebsiella sp.; Isolate 4: Azomonas sp.; Consortium: a combination of four isolates

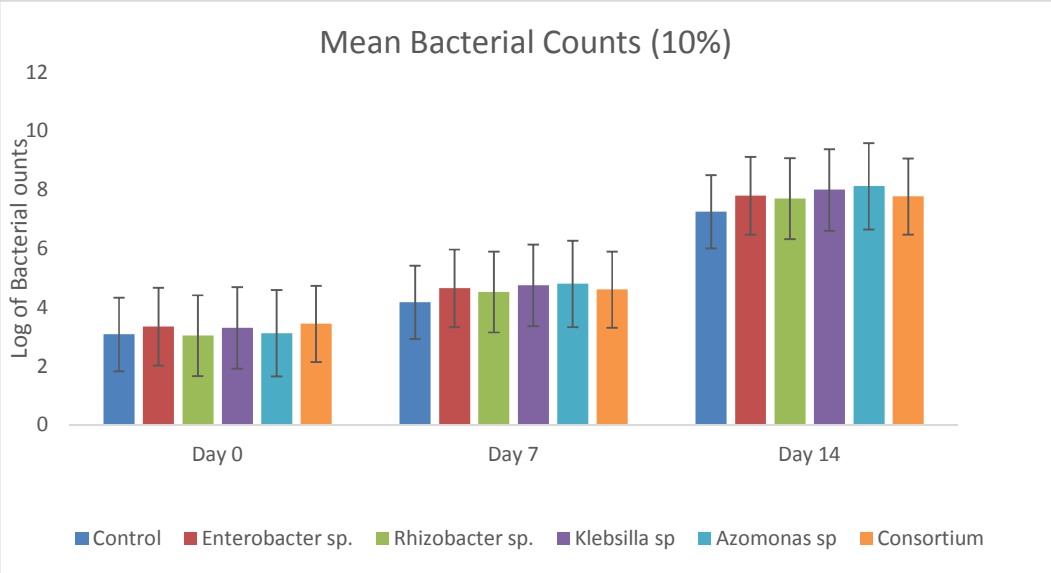

Fig. 8. Mean hydrocarbon degrading bacterial counts with 10% crude oil contamination
Key: Isolate 1: Enterococcussp.; Isolate 2: Rhizobactersp.; Isolate 3: Klebsiellasp.; Isolate 4: Azomonassp.; Consortium: a combination of four isolates

3.5 Mean Changes in Microbial Count during Bioremediation of Crude Oil Contaminated Water

In the 5% crude oil contaminated microcosms (Fig. 7), *Azomonas* recorded the highest count of (8.05 ± 0.103) x 10^7CFU/ml, followed by *Klebsiella sp*. (7.89 ± 0.002) x 10^7CFU/ml), while the control had the least (7.73± 0.002 x 10^7CFU/ml) at the end of the incubation period (day 14). The high count of *Azomonas sp*. corroborates with the high turbidity reading it recorded.

For the microcosms contaminated with 10% crude oil, *Azomonas sp.*recorded the highest count of (8.12 ± 0.001) x 10^7CFU/ml while the control was the least at the end of the incubation period (day 14) (Fig. 8).This response by *Azomonas sp*. was similar to studies by Diaz-Ramirez *et a*l., [28].

The numerous reports and cases of oil spill though devastating can be resolved with proper manipulation of environmental conditions, crude oil concentrations, time, temperature and the appropriate microorganisms in density and diversity [24]. Indigenous hydrocarbon utilizing microorganisms are readily available in hydrocarbon contaminated soils and water in the Niger Delta and could be cultivated in a large scale to be promptly used to clean up crude oil contaminated aquatic and terrestrial environments [31]. Bioaugmentation requires low cost, small amounts of added biomass, and do not require extreme manipulation of environmental conditions to allow introduced microorganisms to be effective [32]. An immediate increase in the population density of the microorganisms could ensure the rapid degradation of the pollutants. Diaz-Ramirez *et a*l., [28] in their study used native and exogenous microbial inoculum from a Mexican soil during a laboratory study and reported that the number of viable counts increased with bioaugmentation. This could be attributed to the production of intracellular and extracellular organic compounds (enzymes and biosurfactants) which improved the hydrocarbon degradation rate by increasing uptake of hydrocarbons and in their metabolism to yield biomass, energy, water and carbon dioxide [32].

4. CONCLUSION

From the findings of this research,Bacteria species having the potential to degrade crude oil exist in our environment. Soil samples from hydrocarbon contaminated sites could be used for the isolation of the Bacteria with hydrocarbon degradation ability as obtained in this research.

The isolates degraded the crude oil to various degrees. The degradation sequence for the 5%

test microcosms was as follows: *Azomonas sp*. >*Enterococcus sp*. >*Rhizobacter sp*.> Consortium >*Klebsiella sp.*>control, while for the 10% test microcosms, the sequence was as follows: *Azomonas sp.*>*Klebsiella sp.*>*Enterococcussp*.>Consortium>*Rhizobacter sp.*>control. The Bioaugmented microcosms enhanced the biodegradation of crude oil more than natural attenuation (control) in all the test microcosms.These isolates could be stored in a genome bank,from where they can be activated and deployed to promptly remediate a crude oil impacted site.The findings from this research could also aid the regulators of the oil and gas industry in Nigeria, to review their remediation policy to include bioaugmentation using indigenous bacteria species as a remediation strategy, to promptly treat a crude oil impacted site and protect the fragile biodiversity of the Niger Delta.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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