



Inherent Bacterial Diversity and Enhanced Bioremediation of an Aged Crude Oil-contaminated Soil in Yorla, Ogoni Land Using Composted Plant Biomass

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Authors' contributions

This work was carried out in collaboration between all three authors. Author LS designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author GCO managed the analyses of the study and proof-read the manuscript. Author CJO wrote the second draft of the manuscript. All three authors read and approved the final version of manuscript.

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ABSTRACT

Aim: Inherent bacterial diversity and enhanced bioremediation of an aged crude oil-contaminated soil (ACOCS) in Yorla, Ogoni land were investigated using composted plant biomass of *Eichhornia crassipes* (EC), *Tithonia diversifolia* (TD) and *Cynodon dactylon* (CD) as biostimulants to enhance rate of crude oil biodegradation by autochthonous hydrocarbon utilizing microorganisms in the soil.
Study Design: An area of 50 m × 50 m was marked out in the ACOCS and EC, TD and CD (2,500 g each) were used to biostimulate 4,000g of ACOCS *in situ* in TPA (treatment plot A) through TPG

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(treatment plot G). TPA was un-amended while TPB, TPC, and TPD had EC, TD and CD added singly. TPE had EC and TD, TPF with EC and CD whereas; TPG had EC, TD, and CD combined.

Place and Duration of Study: The study was carried out in Yorla farm land in Khana L.G.A. in Rivers State. Age crude oil-contaminated soil was taken bi-weekly from each of the 7 treatment setup during the 70-day remediation study period that spanned 10 weeks (0, 14, 28, 42, 56 and 70).

Methodology: Soil samples were obtained using an auger and analyzed for their microbiological and physicochemical properties. Whole plant parts of EC, TD, and CD were collected and composted for 2 weeks before being used for biostimulation of resident crude oil-utilizing microbes.

Results: Results indicated reductions in total petroleum hydrocarbon (TPH) from 98,673 to 79,583 ppm (19% loss), 98,443 to 31,461 ppm (68% loss), 98,446 to 19,364 ppm (80% loss), 98,337 to 26,345 ppm (78% loss), 98,225 to 6,987 ppm (93% loss), 98,113 to 11,243 ppm (89% loss) and 93,867 to 1,002 ppm (99% loss) in TPA, TPB, TPC, TPD, TPE, TPF and TPG respectively. Gas chromatographic fingerprinting of ACOCS before treatment indicated the absence of *n*-alkanes within *n*-C₂ to *n*-C₈ region which is attributable to weathering processes. However, after treatment with the amendments, carbon lengths between *n*-C₉ to *n*-C₃₄ were significantly ($p > 0.05$) attenuated while the much heavier fractions (*n*-C₃₅ to *n*-C₄₅) showed a decreasing tendency for enhanced biodegradation thus, signifying their immobilization or possibility of being “lock-up” in particle pores.

Conclusion: Results suggest that composted *E. crassipes*, *T. diversifolia* and *C. dactylon* are potent biostimulants for enhanced degradation of residual hydrocarbons after aging of contaminated sites. These substrates could serve as potential candidates for rapid bioremediation of aged crude oil-contaminated soil hence, availing these long lost fields in for crop cultivation once again.

Keywords: Crude oil; bioremediation; biodegradation; potent biostimulants, crop cultivation.

1. INTRODUCTION

The over fifty years of crude oil exploration, production, and exploitation in Ogoni land have resulted in environmental and social-economic problems. The effects of crude oil pollution include threat to human health, toxic effect on biota, mobile and persistent in the ecosystem; pose fire or explosion hazards, financial and property value loss [1]. In order to ameliorate the negative impacts of crude oil pollution and guarantee sustainable living, enhanced remediation is needed.

To “remediate” means to solve a problem. Remediation has helped in restoring and rehabilitating the negative impact of crude oil-contamination on various environmental media [2]. Remediation has been defined as the management of environmental contaminants at a site so as to prevent, reduce or mitigate the damage of such contaminants to human health and/ or the natural environment [3].

However, remediation of crude oil-contaminated environment can lead to the quick recovery of the contaminated lands. Enhanced remediation involves a carefully controlled and monitored cleanup approach that achieves site-specific remedial objectives within a time frame that is

reasonable compared to that offered by other physical and chemical methods.

Currently, microbes are being used in solving the multitude of problems associated with crude oil-contaminated soils with the aid of their enzymes or metabolites via bioremediation [4,5,6]. In bioremediation, adapted microbes in the environment can rapidly reduce the impact magnitude of crude oil pollution on soil health by mineralizing the total petroleum hydrocarbon contaminants to CO₂, H₂O and biomass, making it less toxic and problematic to ecotype [7,8].

Invasive plants such as *Eichhornia crassipes*, *Tithonia diversifolia* and *Cynodon dactylon* (Plate 1 A, B, and C) are widespread in the Niger Delta, constituting a nuisance to ecosystems [8,9,10]. These plants can supply the limiting nutrients needed for microbial growth, raising their densities by orders of magnitude thereby, enhancing biodegradation [11,12]. In this study, inherent bacterial diversity and enhanced bioremediation of an aged crude oil-contaminated soil (ACOCS) in Yorla, Ogoni land was investigated using composted plant biomass of *E. crassipes* (EC), *T. diversifolia* (TD) and *C. dactylon* (CD) as biostimulants to enhance rate of crude oil biodegradation by resident microbes.



(A) Water hyacinth



(B) Mexican sunflower



(C) Bermuda grass

Plate 1. Pictures of (A) Water hyacinth (*E. crassipes*), (B) Mexican sunflower (*T. diversifolia*) and (C) Bermuda grass (*C. dactylon*)

2. MATERIALS AND METHODS

2.1 Study Area Description

The study was conducted in Yorla oilfield located in Kpean community (Fig. 1) in Khana Local Government Area of Rivers State. The oilfield was commissioned in 1973, and is located at latitude 4° 39' N; longitude 7° 26' E. The study site is a terrestrial environment with a patchy regenerating vegetation type dominated by

herbs, shrubs and scanty trees. Crude oil pollution is characteristic of the Yorla farm [13].

2.2 Sample Collection

Aged crude oil-contaminated soil (ACOCS) within 0-15 cm depth was collected from the different treatment plots (Plate 2) using manual soil auger. The soil was then transferred to the laboratory via polythene for microbiological, gas chromatographic and physicochemical analyses. Whole green plant samples of water hyacinth (*E. crassipes*), Mexican sunflower (*T. diversifolia*) and bermuda grass (*C. dactylon*) were collected, composted in container and allowed to decay for two weeks using the method of Röling et al. [14].

The composted plant biomass was then used to biostimulate the indigenous hydrocarbon degraders in the soil. The ACOCS were taken bi-weekly from the treatment plots to the laboratory for microbial and physicochemical analyses.

2.3 Description of Treatment Plots

An area of 50 m x 50 m was marked out on the study site. Seven treatment plots (TPA, TPB, TPC, TPD, TPE, TPF, and TPG) each containing 4000g of ACOCS (Table 1) were used for this experiment. TPA contained ACOCS only and without amendment. The TPA served as control and served as control to simulate natural attenuation processes.

Furthermore, TPB, TPC and TPD set-ups were stimulated singly with 2500 g of composted *E. crassipes* (Plot B), *T. diversifolia* (Plot C) and *C. dactylon* (Plot D) respectively. TPE, TPF and TPG were supplemented in combination with 2500 g of composted plant biomass as follows: Plot E: *E. crassipes* and *T. diversifolia*, Plot F: *E. crassipes* and *C. dactylon* and Plot G: *E. crassipes*, *T. diversifolia* and *C. dactylon* respectively (Table 1).

2.4 Determination of Macronutrients in Composted Green Plant Biomass

Each composted plant biomass was analyzed for nitrogen, phosphorus, potassium, calcium, and magnesium following the method of Olabode et al. [15]. Composted plant parts were analyzed and dried at 60°C to constant weight. Thereafter, samples were ground through a 0.2-mm mesh size [8,15].

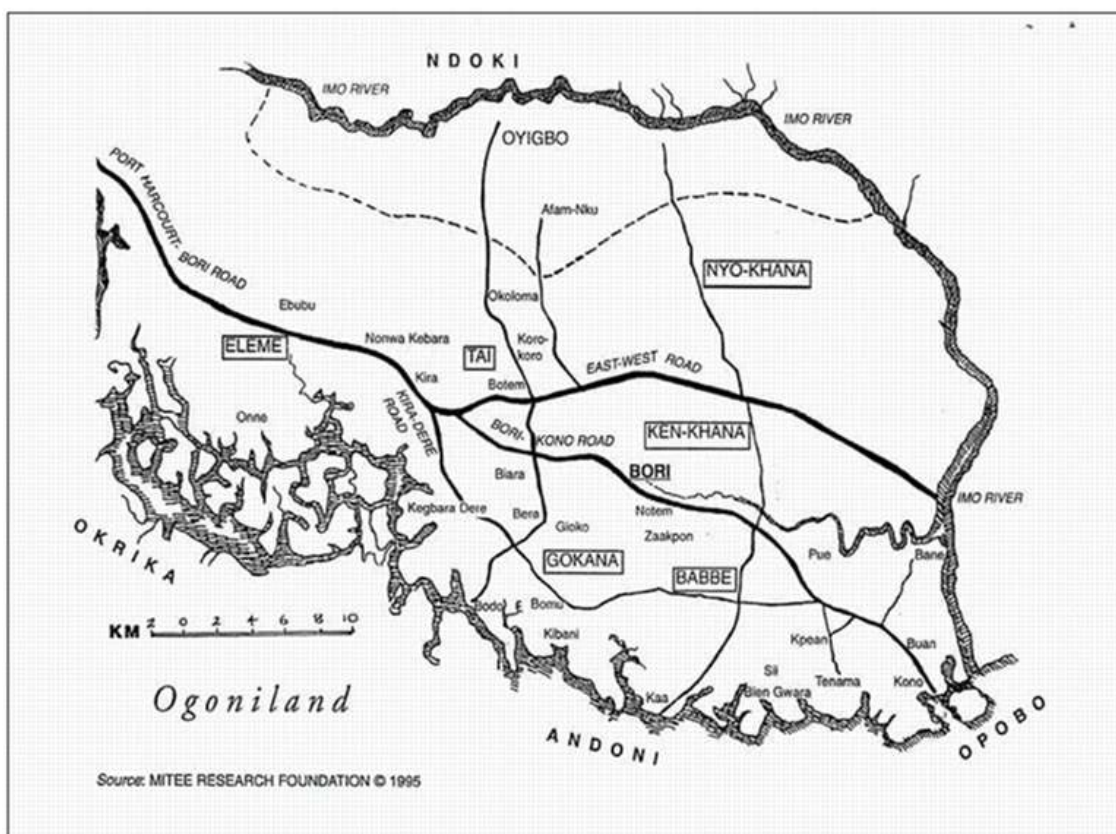


Fig. 1. Map of Ogoni showing study location in Kpean community in Khana local government Area of Rivers State

Table 1. Description of bio-treatment plots

Treatment	Treatment content
Plot A	ACOCS (4000g), control +Tilling
Plot B	ACOCS (4000g) + EC (2500g) +Tilling
Plot C	ACOCS (4000g) + TD (2500g)+Tilling
Plot D	ACOCS (4000g) + CD (2500g) +Tilling
Plot E	ACOCS (4000g) + EC (2500g) + TD (2500g) +Tilling
Plot F	ACOCS (4000g) + EC (2500g) + CD (2500g) + Tilling
Plot G	ACOCS (4000g) + EC (2500g) + TD (2500g) + CD (2500g) +Tilling

Key: g: gram. TPA-TPG: Treatment plots A, B, C, D, E, F and G, ACOCS: Aged crude oil contaminated soil, EC: *E. crasipes* (Water hyacinth), TD: *T. diversifolia* (Mexican sunflower), CD: *C. dactylon*(Bermuda grass)

The macronutrients in composted plants were determined by Kjeldahl digestion method in continuous flow auto-analyzer (ChemLab, UK). The concentration of orthophosphate, as soluble reactive phosphorus was measured by the malachite green–molybdate method (Olsen) while, nitrate was determined by direct ultra-violet spectrophotometry. The potassium content was analyzed by atomic absorption spectroscopy [15,16,17].

2.5 Soil Extraction and Gas Chromatographic Analysis

Each soil sample was extracted with a gas chromatograph (HP 5890, Hewlett Packard, Avondale, PA, USA) equipped with a flame ionization detector (FID). The residual total petroleum hydrocarbons (TPH) in the different treatment plots (5 g each) was extracted bi-weekly with 40 μ L of n-pentane (HPLC grade) by

sonicating the sample 5min at each extraction for 3 times. The pentane extract was centrifuged at 3000g for 5 min. The 3 organic phases were dried over Na₂SO₄, pooled together and adjusted to 150 mL after 32 µL of Cumene (Isopropyl benzene) was added as internal standard. Analysis was carried out using a Varian 1440 GC-FID (California, USA). A DB-1 column was used with the following dimensions: 30 m × 30.2 m; 0.25 µm film thickness; 0.32 i.d. Helium was the carrier gas at a flow rate of 1 mLmin⁻¹. Analyses were carried out in split injection mode using a split ratio 5: 1. The injection port was set at 250°C. The oven temperature was programmed from 40°C for 10 min, then 20°C min⁻¹ to 330°C, holding this temperature for 10min. The extractable TPH was quantified as described by Atlas and Cerniglia [16].

The biostimulation efficiency (B.E) of the various bio-treatment plots was calculated using the expression presented thus:

$$B.E = \frac{[\%TPH_c - \%TPH_t]}{\%TPH_c} \times \frac{[100]}{1}$$

Where,

%TPH_t = percentage degradation of residual crude oil (residual total petroleum hydrocarbon) in the biostimulated aged crude oil contaminated soil.

%TPH_c = percentage degradation of residual crude oil (residual total petroleum hydrocarbon) in the non-stimulated aged crude oil contaminated soil.



Plate 2. ACOCS in treatments undergoing bioremediation by biostimulation

2.6 Molecular Studies

The genomic DNA (gDNA) from natural soil corresponding to the seven treatment plots was extracted with the Wizard® gDNA purification kit (Promega, Madison, WI, USA) and Bio 101FP-120 FastPrep cell disruptor (Qbiogene, Inc. Canada), following the manufacturer's instructions. Polymerase chain reaction (PCR) was performed with the universal primer set 27F: GAGTTTGATCCTGGCTCAG and 1492R: GGTTACCTTGTTACGACT used for the 16S rRNA amplification. Partial nucleotide sequences of 16S rRNA gene corresponding to positions 37-1370 of *Escherichia coli* rRNA sequence were amplified by blasting against the GenBank database using the BLAST program.

Sequences with similarities > 97% were included in the phylogenetic analysis. Sequences used were deposited in the GenBank nucleotide sequence database under accession numbers ranging from KJ179809 to KJ179832. Denaturing gradient gel electrophoresis (DGGE) analysis was performed with 8% (w/v) polyacrylamide gels (ratio of acrylamide to bis-acrylamide 37.5 in 1 × TAE buffer (40 mM Tris-acetate, 1 mM Na-EDTA, pH 8.0) with a gradient ranging from 40 to 60% (where 100% denaturant was defined as 7 M urea and 40% formamide) at a constant voltage of 65 V and 60°C for 16 h (Bio-Rad Dcode System, USA).

2.7 Statistical Analysis of Data

Data obtained were subjected to statistical analysis to determine the significant difference among the data obtained using one-way analysis of variance (ANOVA). A value of $p > 0.05$ was considered significant.

3. RESULTS AND DISCUSSION

Results of the major macronutrients present in *E. crassipes* (EC), *T. diversifolia* (TD) and *C. dyctolon* (CD) are presented in Table 2. Total nitrogen (mg/kg) amounted to 11.76, 13.15 and 11.12 for EC, TD and CD respectively while organic matter (mg/kg) was highest in EC (68.12) followed by CD (54.64) and then TD (43.31). Total phosphorus (mg/kg) was higher in TD with a value of 4.26 followed by CD (3.62) and then EC (3.61). Furthermore, the concentration of total potassium in the plant-based organic compost showed values of 4.38 mg/kg, 3.16 mg/kg and 2.24 mg/kg for EC, CD and TD respectively.

The basic principle of enhanced remediation is to optimize environmental conditions so that microbial degradation of contaminants can take place rapidly and as completely as possible [18,16,17,19,20,21,22,23]. Data obtained indicated that the total petroleum hydrocarbon biodegradation (TPH) value of 98, 673 ppm in TPA on 0d was reduced slightly to 79, 583 ppm by 70d, representing 19% TPH loss.

This could be due to natural attenuation processes. Similar losses in TPH due to natural attenuation processes have been reported [24, 25, 26]. TPB-amended with water hyacinth had a residual TPH reduced from 98, 443 ppm on 0-day to 31461 ppm on 70-day, representing 68% loss. TPC had TPH reduced from 98, 446 to 19, 364 ppm, corresponding to 80% TPH loss.

TPD with TPH of 98, 337 ppm on 0-day (0d) had its TPH reduced by 78% to 26345 ppm after 70-day. TPE with TPH of 98, 225 ppm on 0d decreased by 93% to 6987 ppm after 70d. Also, TPF with TPH of 98, 113 ppm had its TPH

reduced by 89% to 11, 243 ppm. Lastly, TPG had TPH of 93, 867 ppm at the onset of the study on 0d and reduced to 1002 ppm after the 70d study. Again, this corresponds to 99% TPH reduction (Figs. 2 and 3).

The Department of Petroleum Resources (DPR) and Environmental Guidelines and Standards for the Petroleum Industry in Nigeria (EGASPIN) intervention and target values for TPH are 5000 ppm and 50 ppm respectively. The intervention value indicates the quality for which the functionality of soil for human, animal and plant life is threatened. Concentrations in excess of the intervention values correspond to serious contamination (DPR/EGASPIN, 2002). The target values indicate the soil quality required for sustainability or expressed in terms of remedial policy, the soil quality required for the full restoration of the soil's functionality for living organisms. The target values indicate the soil quality levels ultimately aimed for.

Table 2. Major elemental macronutrients in composted green plants biomass

Composted green plants biomass	Concentration (mg/kg)			
	Total nitrogen	Organic matter	Total phosphorus	Total potassium
EC (<i>Eichhorniacrassipes</i>)	11.76	68.12	3.61	4.38
TD (<i>Tithoniadiversifolia</i>)	13.15	43.31	4.26	2.24
CD (<i>Cynodondactylon</i>)	11.25	54.64	3.62	3.16

Key: EC: *Eichhornia crassipes*, TD: *Tithonia diversifolia*, CD: *Cynodon dactylon*

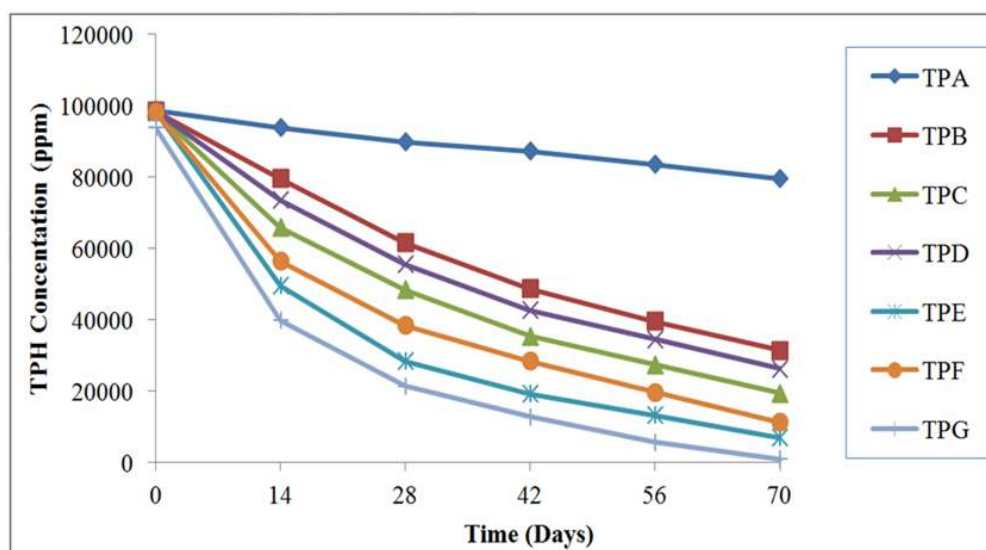


Fig. 2. Changes in total petroleum hydrocarbon (TPH) of aged crude oil-contaminated soil I various treatments during the study period

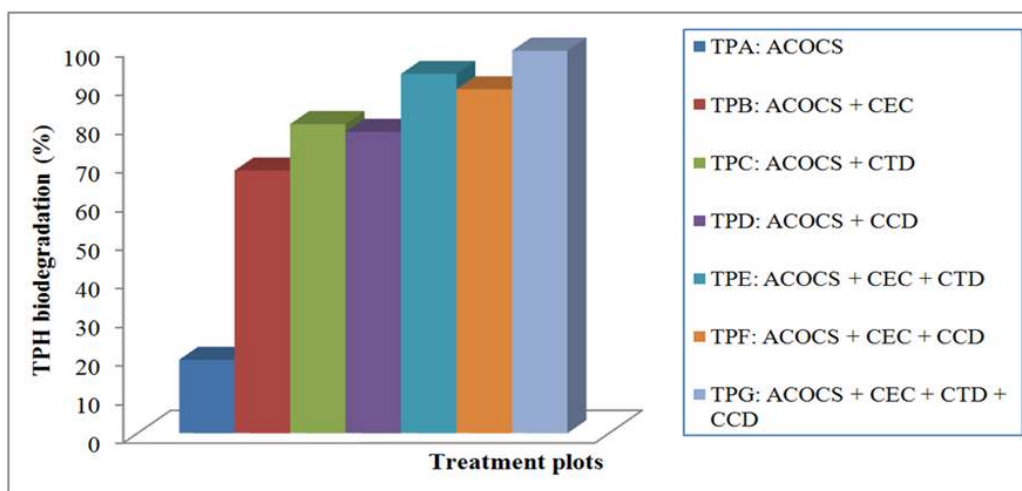


Fig. 3. Percentage biodegradation of total petroleum hydrocarbon (TPH) of ACOCS in the various treatments during the study period

TPH value of 6987 ppm (TPE) obtained after 70-day biostimulation is an indication that if the remediation days were increased, the DPR intervention limit could have been met while the TPH value of 1002 ppm recorded in treatment plot TPG that contained all three amendments met the DPR/EGASPIN recommended intervention limit.

Results obtained from the gas chromatographic fingerprinting (GCF) of the residual crude oil in the remediated soil environment indicated increased degradation of higher molecular weight *n*-alkanes within the *n*-C₂₈ to *n*-C₃₄ carbon length. Low molecular weight hydrocarbons from *n*-C₂ to *n*-C₈ were not detected, probably because carbon lengths <*n*-C₈ are volatile and may have escaped from the soil via weathering processes [27,28,29]. Results of GCF showed degradation of *n*-alkanes within the *n*-C₈–*n*-C₂₃ region. The approximate range of petroleum hydrocarbons in crude oil has been reported to range from *n*-C₅H₁₂ to *n*-C₁₈H₄₅ [30,31]. There was a significant ($p > 0.05$) attenuation of the heavy fractions of hydrocarbons between *n*-C₂₄ to *n*-C₃₄ chain lengths in the treated soil. After treatment with the amendments, carbon lengths between *n*-C₉ to *n*-C₃₄ were significantly ($p > 0.05$) attenuated while the much heavier fractions (*n*-C₃₅ to *n*-C₄₅) showed a decreasing tendency for enhanced biodegradation thus, signifying their possible immobilization and/or capability of being “lock-up” in particle pores [30,32,33].

The long-term aim of bioremediation study is to present a cost-effective design which reduce the

contaminant level to a level referred to “As Low As Reasonable and Practically Possible (ALARP)” that indicates that the contaminant level has been reduced to an acceptable level in the particular soil environment investigated. This study has demonstrated that composted green plant manures of *E. crassipes*, *T. diversifolia*, and *C. dactylon* can provide the limiting nutrients needed for enhanced biodegradation of petroleum hydrocarbons in aged crude oil contaminated soils.

3.1 Phylogenetic Relationships of 16S rRNA Sequences from the Plots

Sequencing of bacterial isolates showed that *Pseudomonas* was the dominant bacterial isolate in the treatment plots during the study period. The successfully sequenced *Pseudomonas* species had sequence similarity with isolates deposited in GenBank with percentage similarity of the bacterial isolates ranging from 91 to 100. Other microbes were identified as bacterial related to *Gordonia* sp. with 98% sequence similarity; *Brevundimonas naejangsanensis* with 99% sequence similarity and *Shewanella* sp. with 99% sequence similarity. More so, *Achromobacter* sp. had 96% sequence similarity, *Sphingobacterium* sp., 97% sequence similarity and *Bacillus* sp., 98% similarity. Furthermore, *Aquitalea magnusonii* had 96% sequence similarity, *Achromobacter* sp. had 100% sequence similarity and *Chromohalobacter salexigens* showed 94% sequence similarity.

The bacterium strain BH23 (KR261429) was not identified and could be a novel species resident in the environment. The unidentified bacterium strain BH23 and the uncommon isolates *Aquitalea magnusonii* and *Brevundimonas naejangsanensis* recovered would require further screening to ascertain their biodegradative capabilities. *Halomonas* sp. and *Marinobacter* sp. have been obtained from oil-contaminated soils [32,33,14,34,35,36,37,38].

Pseudomonas constituted the majority of all bacterial species isolated in the course of the experiment. This could be attributed to their effective competitive capabilities in a wide range of environments with ACOCS environment

inclusive [39,40,41,42,43,44,45,46,47,48,49,50]. *Pseudomonas*, *Acinetobacter*, and *Alcanivorax* genera have been isolated and identified from COCS [35,36,37] and their presence in this study corroborated their findings.

The National Center for Biotechnology Information (NCBI) accession numbers/percentage relatedness representing maximum identity score to GenBank closest relative are enclosed in parentheses (Fig.4). The numbers 1–33 represent 16S rRNA sequences of the dominant denaturing gradient gel electrophoresis (DGGE) bands.

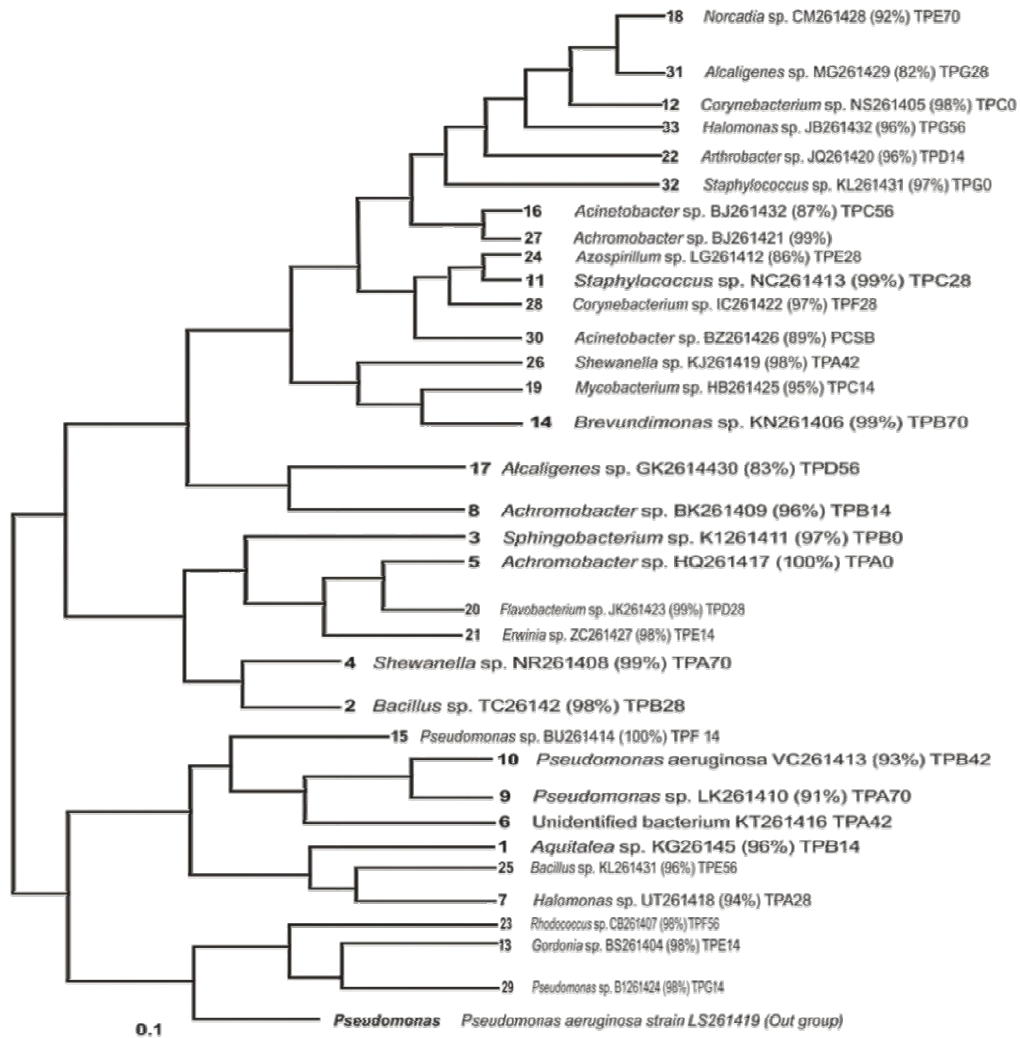


Fig. 4. Phylogenetic relationships of 16S rRNA sequences of bacterial species from the various treatment plots

Distance matrixes were calculated by Jukes and Cantor Model for single nucleotide substitution per sequence represented by a scale of 0.1 at the foot of the phylogenetic tree (Fig. 4). The tree is rooted with the 16S rRNA gene sequence of *Pseudomonas aeruginosa* strain LS261419 (out group).

4. SUMMARY AND CONCLUSION

The compostable green plant biomass acts as both nutrient supplement, bulking agents and bacterial biomass suppliers, supporting resident hydrocarbon utilizing microbes in mineralizing the residual crude oil in the soil. Results obtained from this study showed the order of biostimulation efficacy of the bio-treatments: TPG>TPE>TPF>TPC>TPD>TPB>TPA.

Data obtained from this study thus suggest that composted EC, TD and CD are potent biostimulants for enhanced degradation of residual heavy fractions of crude oil after aging of the contaminated soil environment. The results are maiden in demonstrating the biodegradation of petroleum hydrocarbons of aged crude oil-contaminated soil under natural environmental settings and may contribute substantially to sustainable agricultural outputs and activities.

These substrates could serve as potential candidates for rapid bioremediation of aged crude oil-contaminated soil *in situ* hence, availing these long lost fields in the Niger Delta for crop cultivation once again. These plants could also be used to make organic compost manures. More research attention is required facilitate the permanent remediation of aged crude oil-contaminated sites in Ogoni and the Niger Delta.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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