Inherent Bacterial Diversity and Enhanced Bioremediation of an Aged Crude Oil-
Contaminated Soil in Yorla, Ogoni land using Composted Plant Biomass

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 an enhanced rate of crude oil biodegradation by resident microbes.

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 through TPG. 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 contained EC, TD, and CD in combination.

Place and Duration of Study: The study was carried out in Yorla farmland in Ogoni land. The ACOCS were taken bi-weekly from each of the 7 treatment plots setup (TPA through TPG) during the 70-day study period, spanning 10 weeks (weeks 0, 14, 28, 42, 56 and 70).

Methodology: Soil sample was obtained using an auger and analyzed for its microbiological and physicochemical properties. Bacterial diversity in soil was captured using the molecular tool. Whole plants of EC, TD, and TD were collected and composted in a contained for 2 weeks.

Results: Results indicated reductions in total petroleum hydrocarbon (TPH) from 98,673 to 79,583ppm (19% loss), 98,443 to 31,461ppm (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*-C$_2$ to *n*-C$_8$ region which is attributable to weathering processes during aging. However, after treatment with the aforementioned amendments, carbon lengths between *n*-C$_9$ to *n*-C$_{34}$ were significantly ($p > 0.05$) attenuated while the much heavier fractions (*n*-C$_{35}$ to *n*-C$_{45}$) showed a decreasing tendency for enhanced attenuation thus, signifying their non-bio-availability and possible immobilization in particle pores.

Conclusion: Results suggest that composted EC, TD and CD are potent biostimulants for enhanced degradation of residual heavy fractions of crude oil after aging of contaminated sites. These substrates could serve as potential candidates for rapid bioremediation of ACOCS *in situ* hence, availing these long lost fields in Ogoni for crop cultivation once again.

Keywords: Crude oil, bioremediation, Ogoni, bacterial diversity, Potent biostimulants.

1. INTRODUCTION

The over fifty years of crude oil exploration, production, and exploitation in Ogoni land have resulted in environmental and social-economic problems. Crude oil contaminants constitute threat to human health, safety, and environment,

Other negative impacts of crude oil pollution include: toxic to biota, mobile and persistent in the ecosystem, pose fire or explosion hazards, financial and property value loss [1].

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 is 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
Remediation can also 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 [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 CO$_2$, H$_2$O 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 ecosystem [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 enhanced rate of crude oil biodegradation by resident microbes.

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

Study Area Description

The study was conducted in Yorla oilfield located around Kpean community (Fig. 1) in Khana Local Government Area of Rivers State. The oilfield was commissioned in 1973, 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 area [13].

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 (\textit{E. crassipes}), Mexican sunflower (\textit{T. diversifolia}) and bermuda grass (\textit{C. dactylon}) were collected, composted and allowed to decay for 2 weeks before being used to biostimulate the indigenous hydrocarbon degraders in the soil. The ACOCS were taken bi-weekly from each of the treatment plots during the 70-day study period.

Description of Treatment Plots

An area of 50 m × 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 represented natural attenuation. Furthermore, TPB, TPC and TPD set-ups were stimulated singly with 2500g of composted \textit{E. crassipes} (Plot B), \textit{T. diversifolia} (Plot C) and \textit{C. dactylon} (Plot D) respectively. TPE, TPF and TPG were supplemented in combination with 2500g of composted plant biomass which was allowed to decay in a clean container for 2 weeks as follows: Plot E: \textit{E. crassipes} and \textit{T. diversifolia}, Plot F: \textit{E. crassipes} and \textit{C. dactylon} and Plot F: \textit{E. crassipes}, \textit{T. diversifolia} and \textit{C. dactylon} (Table 1).
Plate 2: ACOCS in treatments undergoing bioremediation by biostimulation

Table 1: Content of Bio-treatment Plots

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plot A</td>
<td>ACOCS (4000g), control +Tilling</td>
</tr>
<tr>
<td>Plot B</td>
<td>ACOCS (4000g) + CEC (2500g) +Tilling</td>
</tr>
<tr>
<td>Plot C</td>
<td>ACOCS (4000g) + CTD (2500g) +Tilling</td>
</tr>
<tr>
<td>Plot D</td>
<td>ACOCS (4000g) + CCD (2500g) +Tilling</td>
</tr>
<tr>
<td>Plot E</td>
<td>ACOCS (4000g) + CEC (2500g) + CTD (2500g) +Tilling</td>
</tr>
<tr>
<td>Plot F</td>
<td>ACOCS (4000g) + CEC (2500g) + CCD (2500g) +Tilling</td>
</tr>
<tr>
<td>Plot G</td>
<td>ACOCS (4000g) + CEC (2500g) + CTD (2500g) + CCD (2500g) +Tilling</td>
</tr>
</tbody>
</table>


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. [14]. 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, 14].

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 [14, 16, 17].

Soil Extraction and Gas Chromatographic Analysis

Each soil sample for gas chromatographic analysis was extracted with methylene chloride and an aliquot of the extract injected into a gas chromatograph (HP 5890, Hewlett Packard, Avondale, PA, USA) equipped with a flame ionization detector (FID). The extractable TPH was quantified using the method of Atlas and Cerniglia (1995). Biostimulation efficiency (B.E) was calculated using the expression below:
Where,

\[ \%TPH_t = \frac{\text{percentage degradation of residual crude oil (residual total petroleum hydrocarbon) in the biostimulated aged crude oil contaminated soil.}}{\%TPH_c} \times \left[ \frac{100}{1} \right] \]

\[ \%TPH_c = \text{percentage degradation of residual crude oil (residual total petroleum hydrocarbon) in the non-stimulated aged crude oil contaminated soil.} \]

**Molecular Studies**

Total genomic DNA (gDNA) from natural soil corresponding to the seven treatment plots was extracted with the Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA) and Bio 1011FP-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 37.5:1) 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).

**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 \( \rho >0.05 \) was considered significant.

**3. RESULTS AND DISCUSSIONS**

Results of the major elemental macronutrients present in *E. crassipes* (EC), *T. diversifolia* (TD) and *C. dyctolon* (CD) are presented in Table 2. TD compostable green plant manure had essential nutrients (in mg/kg) values of 4.06 (organic carbon), 8.96 (total nitrogen), 0.45 (C/N ration), 68.12 (organic matter), 5.12 (total phosphorus) and 4.38 (total potassium) respectively.

EC had (in mg/kg) organic carbon (2.06), total nitrogen (4.16); C/N ration (0.49), organic matter (43.31), total phosphorus (2.45) and total potassium (2.24) respectively. CD had major limiting nutrients (in mg/kg) including organic carbon (3.11), total nitrogen (5.26), C/N ration (0.59), organic matter (54.64), total phosphorus (3.84) and total potassium (3.16) respectively in its composite samples.
Table 2: Major Elemental Macronutrients in Composted Green Plants Biomass

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Organic Carbon (mg/kg)</th>
<th>Total Nitrogen (mg/kg)</th>
<th>C/N Ratio</th>
<th>Organic matter (mg/kg)</th>
<th>Total Phosphorus (mg/kg)</th>
<th>Total Potassium (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC</td>
<td>2.06</td>
<td>4.16</td>
<td>0.49</td>
<td>43.31</td>
<td>2.45</td>
<td>2.24</td>
</tr>
<tr>
<td>TD</td>
<td>4.01</td>
<td>8.96</td>
<td>0.45</td>
<td>68.12</td>
<td>5.12</td>
<td>4.38</td>
</tr>
<tr>
<td>CD</td>
<td>3.11</td>
<td>5.26</td>
<td>0.59</td>
<td>54.64</td>
<td>3.84</td>
<td>3.16</td>
</tr>
</tbody>
</table>

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

The basic principle of enhanced remediation is superficial to optimize environmental conditions so that microbial degradation of contaminants can take place rapidly and as completely as possible [15, 16, 17, 18, 19, 20, 21, 22]. 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 [23, 24, 25]. TPB-amended with water hyacinth had a residual TPH value loss of 98, 443 ppm on 0d and 31461 ppm after 70d, representing 68% removal. TPC had TPH reduced from 98, 446 to 19, 364 ppm, corresponding to 80% total petroleum hydrocarbon loss.

TPD with TPH of 98, 337 ppm on 0-day (0d) 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 was degraded 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 values indicate the quality for which the functionality of soil for human, animal and plant life are, or threatened with being seriously impaired. 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.

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 \( n-C_{28} \) to \( n-C_{34} \) carbon length. Low molecular weight hydrocarbons from \( n-C_2 \) to \( n-C_4 \) were not detected, probably because carbon lengths \( \leq n-C_8 \) are volatile and may have escaped from the soil via weathering processes [26, 27, 28]. Results of GCF showed degradation of \( n \)-alkanes within the \( n-C_8 \)–\( n-C_{23} \) region. The approximate range of petroleum hydrocarbons in crude oil has been reported to range from \( n-C_5H_{12} \) to \( n-C_{18}H_{45} \) [29, 30]. There was a significant \(( \rho > 0.05 \) attenuation of the heavy fractions of hydrocarbons between \( n-C_{24} \) to \( n-C_{34} \) chain lengths in the treated soil. There was increasing degradation of the fractions \( n-C_{35} \) to \( n-C_{45} \) of petroleum hydrocarbon and the remaining fractions signify their possibility of being ‘locked up’ in particle pores [29, 31, 31, 32].

The long-term aim of bioremediation study is to present a cost-effective designs 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.
Phylogenetic Relationships of 16S rRNA Sequences from the Plots

Sequencing showed that *Pseudomonas* was dominant bacteria isolates in the treatment plots during the study period that were successfully sequenced and had similarity with isolates deposited in GenBank which ranged from 91% to 100%.

The isolates were identified as microbes related to *Gordonia* sp. with 98% sequence similarity; *Brevundimonas naejangsanensis* with 99% sequence similarity and *Shewanella* sp. with 99% sequence similarity. Others are *Achromobacter* sp., 96% sequence similarity, *Sphingobacterium* sp., 97% sequence similarity and *Bacillus* sp., 98% similarity. Furthermore, *Aquitalea magnusonii* had 96% sequence similarity, *Achromobacter* sp., 100% similarity and *Chromohalobacter salexigens*, 94% 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 got from oil-contaminated soils [31, 32, 33, 34, 35, 36, 37, 38].

*Pseudomonas* constituted the majority of all species isolated in the course of the experiment. This could be attributed to their effective competitive capabilities in a wide range of environments with COCS environment inclusive. *Pseudomonas, Acinetobacter, and Alcanivorax genus* 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. The numbers 1–15 represent 16S rRNA sequences of the dominant denaturing gradient gel electrophoresis (DGGE) bands. 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).
Fig. 4: Phylogenetic relationships of 16S rRNA sequences from the various treatments.

The tree is rooted with the 16S rRNA gene sequence of Pseudomonas aeruginosa strain LS261419. The use of denaturing gradient gel electrophoresis (DGGE) technique with conventional culture-based methods during biostimulation showed alteration in the dominant bacterial community, confirming the choice of E. crassipes, T. diversifolia and C. dactylon as a nitrogen source.
4. SUMMARY AND CONCLUSION

The compostable green plant biomass acts as both 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 various 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 pioneering in observing biodegradation of petroleum hydrocarbons of aged crude oil-contaminated soil under natural environmental settings and may contribute substantially to sustainable agricultural outputs and activities that will support a healthy citizenry in crude oil impacted areas.

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 or be spread in farms to improve soil fertility and sustainable agriculture. More research attention is required to permanently remediate aged crude oil-contaminated sites in Ogoniland.

Competing interests

Authors have declared that no competing interests exist.

REFERENCES


