Remediation of Hydrocarbon Polluted Soil Offsets Methanogenic Microbial Communities and Improves Soil Recovery of Crude Oil-Polluted Site

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Abstract

Hydrocarbon pollution amplified by artisanal refining has led to the devastating destruction of farmlands and fishing settlements of subsistence farmers in the Niger Delta. The relatively slow natural attenuation of these polluted sites is the motivation for this study. The natural response and the influence of intermittent tillage and nutrient addition on bacterial community ecological functions were investigated using 16S rRNA metabarcoding. Representative soil samples were drawn from the surface (0 – 15cm) and subsurface (1m and 1.5m depths) of the polluted site pre-remediation and during remediation on Days 0, 14, 49, 70 and 91. Nutrient in the form of poultry droppings was added to the polluted soil while aeration was improved by tillage at 3-weeks interval throughout the duration of remediation. Total petroleum hydrocarbon was reduced from **93,720** mg kg⁻¹ on timepoint zero to 9,029.76 mg kg⁻¹ on day 91. Alpha diversity analysis revealed that the proportionality (evenness) of bacterial species significantly reduced during remediation. The bacterial community structure during remediation was significantly different compared to the structure pre-remediation. Spearman's rank correlation revealed that soil pH, total phosphorus and total potassium were the chemical factors that influenced diversity. During remediation, the most responsive bacterial phyla were Proteobacteria and Cyanobacteria while Hydrogenedentes and Spirochaetes were among biomarkers pre-remediation. PICRUST2-based functional prediction revealed that pre-remediation, pathways for methanogenesis and terpenoids biosynthesis were differentially abundant while high energy-yielding TCA cycle and pathways for both fatty acid biosynthesis and the degradation of aromatic hydrocarbons were differentially abundant during remediation. Overall, the addition of poultry droppings and intermittent tillage improved nutrient availability and this subsequently led to a significant change in the bacterial community structure and the rate of hydrocarbon sequestration. The authors concluded that incorporation of poultry droppings by tillage may serve as a suitable remediation method to reduce hydrocarbon in oil-polluted Niger Delta soils.

1. Introduction

Fossil fuel plays an important role and provides for the majority of the world's energy need. Consequently, this has led to increased exploration for crude oil in both onshore and offshore environments [1]. However, the activities of the oil and gas industry can often lead to severe environmental consequences through the unexpected release of crude oil into the environment [2, 3]. The reasons for the spills are enormous, but the major causes include equipment failure, human error, accident and sabotage [4]. The high incidence and environmental impact of crude oil pollution are of major concern. For instance, the release of approximately 4.9 million barrels of crude oil into the environment from the Gulf of Mexico's Deep-Water Horizon oil spill incident, led to the loss of human lives and also had severe environmental and economic consequences [5]. Similarly, the increasing activities of artisanal crude oil refiners in the Niger Delta has led to loss of lives, destruction of farmlands, fishing settlements, recreational beaches, the marine environment and the biodiversity thereof [6, 7].

Currently, there are several chemical and physical methods for the recovery of hydrocarbon polluted sites that are generally expensive and are not able to remove trace quantities of pollutant. However, microbial-mediated remediation (bioremediation) is considered the most appropriate method for the clean-up of crude oil-polluted soils [8]. Unlike chemical and physical methods that can lead to a significant reduction of soil quality and has the potential to cause secondary environmental contamination; bioremediation is considered to be cost-effective, non-toxic, efficient in reducing the recovery half-life and can lead to complete mineralization of the pollutant, thereby improving the soil overall quality [9]. The process of bioremediation depends on the natural ability of microorganisms to utilize hydrocarbons as sources of carbon and energy [10]. Several bacterial and fungal genera are established degraders of various fractions of hydrocarbons [11]. For example, bacterial members including *Alcanivorax*, *Pseudomonas*, *Bacillus*, *Sphingomonas* and *Mycobacterium* are some of the well-studied and frequently recovered degraders of saturates and aromatic hydrocarbons [11–13]. Notably, *Alcanivorax* is a known degrader of both short and long-chain alkanes [14] while *Pseudomonas* has been demonstrated to degrade a wide range of saturates and aromatic hydrocarbons [12].

Hydrocarbon degradation by microorganisms can occur either under oxic or anoxic conditions, and this largely determines the microbial community composition, response rate and overall functional potential [15–17]. Anaerobic hydrocarbon degradation can occur under methanogenic, nitrate-reducing, sulphate-reducing and iron-reducing conditions [18]. Methanogenic hydrocarbon degradation has been demonstrated in both field and laboratory studies [15, 17, 19, 20] and has been reported to occur in diverse environments including under microaerobic conditions like water droplets obtained from a water-in-oil emulsion [21] and in crude oil-polluted sites where hydrocarbon represents a significant portion of the organic matter [22]. Methanogenesis occurs mainly

through carbon dioxide reduction by hydrogenotrophic methanogens (utilize hydrogen as electron donor while reducing carbon dioxide to methane) or through acetoclastic methanogenesis (utilize acetate as a terminal electron acceptor or convert acetate to methane and bicarbonate). Further, some other archaeal genera like *Methanolobus*, *Methanosphaera* and *Methanosalsum* (methylotrophic methanogens) rather utilize methylated compounds like methylamines or methanol for methanogenesis [15, 23]. Methanogenic hydrocarbon degradation is an important process for carbon fixation. However, anaerobic hydrocarbon degradation is known to be relatively slow due to low energy yield and a correspondingly slow rate of cell multiplication [18].

The relatively slow rate of bioattenuation and the paucity of information regarding the natural recovery process of most crude oil-polluted artisanal refining sites make this study important and timely. Also, considering the difficulty in assessing most artisanal refining sites and the unavailability of funds for clean-up processes; since the spill is not coming from the major oil and gas companies, microbe-dependent degradation seems the only feasible approach for recovery. Consequently, this study investigated microbial response to the long-term presence of hydrocarbons in an artisanal refining site and also investigated the response of the microbial communities to nutrient amendment and intermittent tillage. We hypothesized that: (1) the slow natural recovery rate of artisanal refining sites is largely due to lack of nutrients and oxygen for aerobic hydrocarbon activation and (2) that the addition of nutrients and tillage of the crude oil polluted soil will increase aeration and change the compositional and functional diversity of the indigenous microbial communities involved in hydrocarbon degradation and energy metabolism. The overall aim is to provide information for future intervention and recovery of several artisanal refining sites within the Niger Delta, using a cost-effective approach.

2. Material And Methods

2.1 Site description

An oil-polluted soil with a history of decades of artisanal refining activities was selected for this study (Fig. 1A). The site is situated in Tombia, Degema Local Government Area of Rivers State, Nigeria with GPS coordinates 4°47'41.1"N 6°51'47.2"E – 4°47'42.4"N 6°51'46.4"E (Fig. 1D – 1E). The refining site is within the tropical rainforest and temperature ranges from 25°C – 35°C. Surrounded by the heavily oil-polluted site are mangroves and interconnected creeks with a daily tidal flow from brackish water. Wastes from the refining activities are directly channelled into the surrounding water bodies and besides, during the dry-wet cycles of the wetland, elevated water levels can reach most of the open-underground oil storage pits within the refining site. These features make most artisanal refining sites the current leading source of petroleum hydrocarbon pollution in the Niger Delta.

2.2 Sample collection and site remediation

To determine the physical and microbiological characteristics of the site, composite samples were obtained from both the surface (0–15 cm) and subsurface (30 cm – 1 m and 1 m – 1.5 m) of the oil-polluted soil using a soil auger. Fifteen surface samples were first collected from different points of the polluted site while 3 samples each were collected from different points of the subsurface (30 cm – 1 m and 1 m – 1.5 m) of the soil. The samples were subsequently homogenized according to the depth of sampling prior to chemical and microbiological analysis. Samples were further collected from an unpolluted vegetation rich site 1000 meters away from the polluted site. The unpolluted soil sample served as a control in order to investigate the effect of hydrocarbons on soil chemical properties.

Remediation of the site commenced with site mapping (1,350 m²), clearing, excavation of the most polluted points and ridge making (Fig. 1B). Nutrient addition to the crude oil polluted soil involved the application of 1.00 kg m⁻² of poultry droppings to the site. Remediation was monitored for 91 days with intermittent tillage (3-weeks interval) of the soil to increase aeration. Composite samples were drawn on day 0, day 14, day 49, day 70 and day 91. All the samples obtained both pre-remediation and during remediation were safely transferred to the laboratory in ice-packs for both physicochemical and microbiological analyses.

2.2 Determination of soil physical and chemical properties

The soil physical properties investigated included the soil-type, soil colour, and percentage gravel, sand and fines. Among the soil chemical parameters investigated were pH (determined using Metrohn automated probe analyzer), moisture content (determined by the drying method), total nitrogen (TN), total phosphorus (TP), total potassium (TK) content, Pb, Ni, Cd and Cu concentrations

(ASTM D 1691, 3559 and 1886), electrical conductivity (conductivity meter) and the soil organic matter content. The petroleum hydrocarbon component in the oil-polluted soil was determined using targeted GC-FID (gas chromatography-flame ionization detector) analysis.

2.3 Determination of petroleum hydrocarbon components

Total petroleum hydrocarbons (TPH) were extracted from the soil samples using the EPA 418.1 method. For TPH analysis, 10 millilitres of the organic extract were filtered and transferred into GC vials and analysed by gas chromatography-flame ionization detector (GC-FID) equipped with an HP 7673 FID detector, autosampler and a fused silica capillary column. The detector and injector temperatures were set at 320°C and 250°C respectively. The oven temperature was set to run from 40°C for 3mins to 300°C. Helium served as the carrier gas at a velocity of 38 cm sec⁻¹. Agilent Chemstation chromatography (Version 10) was used for data handling and analysis. To quantify TPH, the peak area was determined using forced line integration using the Agilent Chemstation between n-C6 (n-hexane) through n-C40 (tetracontane).

2.4 Genomic DNA extraction from soil, amplification and sequencing

The total genomic DNA of the soil samples were extracted using Power Soil™ DNA extraction kit following the manufacturer's instructions. Quantification of the extracted DNA was done using a Qubit fluorometer (Invitrogen, Carlsbad, CA, United States). PCR primer pairs 341F (CCTACGGGNGGCWGCAG) and 805R (GGACTACHVGGGTWTCTAAT) [24] targeting the V3 – V4 hypervariable region of the 16S rRNA gene was used for DNA amplification. Illumina-MiSeq sequencing of the PCR amplicons was done at the Agricultural Research Council (ARC), Pretoria, South Africa. DNA amplicons from PCR were first checked in 1% agarose gel. Initial purification of the amplicons was done using AMPure XP beads (Beckman Coulter, Brea, CA, United States) according to the manufacturer's instruction. After unique indexing of the amplicons and the addition of the Illumina sequencing adapters, added purification was done with the AMPure XP beads. The purified product was then normalized to equal concentration, denatured and loaded onto a MiSeq V3 cartridge for a paired-end sequencing run on the Illumina MiSeq sequencer (Illumina Inc, San Diego, CA, United States).

2.5 Bioinformatics and diversity analysis

Raw Illumina MiSeq sequences for all the samples were processed using QIIME v2019.4 [25]. DADA2 [26] was used for sequence denoising, removal of low-quality reads, marginal sequences and clustering of sequences into amplicon sequence variants (ASVs). Further clustering of the sequences into Operational Taxonomic Units (OTUs) at 97% relatedness was done using VSEARCH [27] open-reference OTU picking strategy and the SILVA v132 [28] served as the reference database. Taxonomy was assigned to the representative sequences using VSEARCH consensus taxonomy classifier while applying the default parameters. Before the analysis of diversity, the OTU count table was first filtered of singletons and normalized to the minimum library size. Diversity analysis was computed in R using *microbiome* [29], *ampvis2* [30], *ape* [31] and *vegan* [32] R packages.

The microbial community functional attributes pre-remediation and during remediation was determined using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) [33]. PICRUSt2 enables the prediction of functional profiles using community-based 16S rRNA data. The outputs of the prediction are the enzymatic (gene families) and MetaCyc pathway profile of the 16S rRNA representative sequences. The predicted pathways were subsequently used to infer differences in microbial community function pre-remediation and during remediation.

2.6 Statistical analysis

Unless otherwise stated, all statistical analysis was performed between sample groups obtained pre-remediation and those obtained during remediation using R v3.6.1 [34]. The soil physicochemical parameters and diversity metrics were compared using the Kruskal-Wallis rank-sum test. Spearman's rank correlation was also performed to determine the relationship between soil chemical properties and alpha diversity using the *agricolae* [35] R package. Differential abundance testing of taxonomic composition pre-remediation and during remediation was done using *Deseq2* [36] and *lefse* [37].

Determination of differences in microbial community structure was based on Bray-Curtis dissimilarities while visualization was done using Principal Coordinates Analysis (PCoA). Significance testing of differences in multivariate space was based on permutational multivariate analysis of variance (PERMANOVA) using the *vegan* package. To test the significance of within-group differences, a further permutational test for homogeneity of multivariate dispersion (PERMDISP) was done. Constrained

redundancy analysis (RDA) was performed on Hellinger transformed soil chemical and microbiological parameters using *ampvis2* R package. The significance of RDA was tested using the *vegan* function `anova.cca`.

Differentially abundant pathways pre-remediation and during remediation was determined using *STAMP* [38]. Pathways with Benjamini-Hochberg FDR-adjusted Welch's t-test ($p \leq 0.05$) and effect size (> 2) were considered as biomarkers.

3. Results

3.1. Physical and chemical signatures of the oil-polluted site

The results of the physicochemical parameters of the artisanal refining site and the unpolluted soil are presented in Table 1. All analysed baseline samples had extractable total petroleum hydrocarbon (TPH) concentration above the minimum intervention limit ($5,000 \text{ mg kg}^{-1}$) as stipulated in EGASPIN (Environmental Guidelines and Standards for the Petroleum Industry in Nigeria) by Nigeria's Department of Petroleum Resources [39]. The soil was polluted with varying fractions of hydrocarbons ranging from $n\text{-C}_8$ to $n\text{-C}_{40}$. Further, n -alkanes with a carbon chain length between $n\text{-C}_{16}$ to $n\text{-C}_{33}$ formed the bulk of hydrocarbons across the soil gradients investigated (Fig. S1). TPH concentration was $490,631.30 \text{ mg kg}^{-1}$ in the surface (0–15 cm), $320,971.60 \text{ mg kg}^{-1}$ at 1m depth and $81,434.86 \text{ mg kg}^{-1}$ at 1.5m depth. Analysis of TPH revealed that there was a significant reduction in hydrocarbon concentration after 91 days of remediation. Following the addition of poultry droppings to the polluted soil and subsequent ridge making, TPH concentration was $93,720 \text{ mg kg}^{-1}$ on day zero and reduced to $9,029.76 \text{ mg kg}^{-1}$ on day 91.

Table 1
Physicochemical and sequence properties of the crude oil polluted soil pre-remediation and during remediation

Parameter	Control	Pre-remediation			During remediation				
	0–30 cm Unpolluted soil	0–15 cm depth	1 m depth	1.5 m depth	Day 0	Day 14	Day 49	Day 70	Day 91
TPH (mg kg ⁻¹)	764.94	490, 631.30	320, 971.60	81, 434.86	81,000.43	49,000.53	19,000.51	11,000.33	9,029.76
Total nitrogen (%)	ND	0.0022	0.00082	0.00068	0.0179	0.0582	0.0631	0.0593	0.0458
EC (μS cm ⁻¹)	60	120	130	100	0.10	0.01	0.03	20.00	0.00
Salinity (ppm)	19.2	27.8	83.2	64.0	64.0	6.40	19.20	ND	ND
pH	6.9	6.1	6.2	6.5	6.4	6.5	6.9	6.6	6.9
Organic matter content (%)	2.1675	6.1286	2.7972	2.9952	10.345	25	8.828	7.363	18.302
Total phosphorus (%)	ND	0.00302	0.00158	0.00104	0.0118	0.0179	0.0199	0.0227	218
Total potassium (%)	ND	0.00082	0.00031	0.000221	0.00159	0.00182	0.00135	0.00148	9.7
Moisture content (%)	4.01	19.61	16.07	8.03	5	35.48	3.087	4.214	1.86
Soil-type	Gravelly sand	Gravelly sand	Gravelly sand	Gravelly sand	Gravelly sand	Gravelly sand	Gravelly sand	Gravelly sand	Gravelly sand
Soil colour	Light brown	Light brown	Light brown	Light brown	Light brown	Light brown	Light brown	Light brown	Light brown
Gravel (%)	5.40	5.20	4.90	4.90	5.20	5.20	5.20	5.20	5.20
Sand (%)	95.60	94.70	93.22	93.61	94.70	94.70	94.70	94.70	94.70
Fines (Silt + clay) (%)	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Quality sequences	NA	11,992	10,287	11,727	3977	2,793	1,489	2,536	621
*ND = Not determined; NA = Not applicable									

Furthermore, the results of nutrient analysis pre-remediation and during remediation is presented in Table 2. During remediation, nutrient analysis revealed that the addition of poultry droppings significantly (Fisher's LSD $p < 0.05$) affected the soil chemical parameters. Total nitrogen, total phosphorus, total potassium and the soil pH significantly ($p < 0.05$) increased during the period of remediation than pre-remediation (Table 2), while moisture content and organic matter content were not significantly affected by the remediation approach. Correlation analysis revealed that pH correlated with nitrogen ($r = 0.90$, $p = 0.002$) and phosphorus ($r = 0.88$, $p = 0.004$), nitrogen correlated with phosphorus ($r = 0.95$, $p = 0.002$) and potassium ($r = 0.75$, $p = 0.030$) while phosphorus correlated with potassium ($r = 0.76$, $p = 0.029$).

Table 2
Changes in soil chemical parameters after 91 days of site remediation

Parameter	Mean (Pre-remediation)	Mean (During-remediation)	p-value (FDR-adjusted)
Moisture (%)	14.57 ± 5.93	9.93 ± 14.33	0.62
Organic matter content (%)	3.97 ± 1.87	13.97 ± 7.47	0.07
Total nitrogen (%)	0.0012 ± 0.00	0.0489 ± 0.01	0.005**
Total phosphorus (%)	0.0019 ± 0.00	0.0188 ± 0.00	0.000***
Total potassium (%)	0.0005 ± 0.00	0.0014 ± 0.00	0.005**
pH	5.97 ± 0.15	6.58 ± 0.19	0.003**

3.2. Bacterial community diversity

A total of 75,137 high-quality sequences were denoised and clustered into 5,029 OTUs based on 97% sequence similarity. After taxonomic classification of OTUs and elimination of non-bacterial phylotypes, rarefaction of sequence reads to the minimum library size was sufficient to determine the bacterial diversity of all samples and sample groups (Fig S2). The number of unique OTUs was higher at the soil surface (0–15 cm) than the subsurface (1 M and 1.5 M depths) soil samples (Fig S3A). Also, a higher proportion of the OTUs was shared across vertical samples than the percentage unique to each sampling depth. Further comparison of the soil samples obtained pre-remediation to those obtained during remediation revealed that unique OTUs were higher pre-remediation than during remediation (Fig S3B).

Observed OTUs and Chao1 diversity indices revealed higher species richness pre-remediation compared to during-remediation, though the observed changes in bacterial richness were not significant (Wilcoxon $p > 0.05$) (Fig. 2A and 2B). Similarly, the species diversity (Shannon-Wiener) was lower during remediation (Fig. 2C) while the proportionality of bacterial species (evenness) pre-remediation was significantly ($p < 0.05$) higher than during the period of remediation (Fig. 2D). This finding suggests that the addition of nutrients and intermittent tillage exerts selective pressure on bacterial species. Spearman's rank correlation to gain insight into factors that influenced alpha diversity pre-remediation and during remediation revealed that species diversity (Shannon) significantly correlated with moisture content ($r = 0.78$, $p = 0.046$) and inversely with total phosphorus ($r = -0.80$, $p = 0.018$). Species proportionality (Evenness) significantly correlated inversely with total nitrogen ($r = -0.74$, $p = 0.037$), total phosphorus ($r = -0.82$, $p = 0.013$) and pH ($r = -0.74$, $p = 0.036$) while species richness (Observed and Chao1) significantly correlated positively with moisture content ($r = 0.82$, $p = 0.012$) and inversely with total phosphorus ($r = -0.69$, $p = 0.056$).

Bray-Curtis dissimilarity between soil bacterial communities revealed differentiation in the community structure pre-remediation and during remediation (Fig. 2E). Further, it was observed that samples obtained during the early stages of remediation (Day 0 – Day 49) clustered together within the ordination space while the samples collected between day 70 and 91 clustered separately. The observed bacterial community differences in multivariate space pre-remediation and during remediation was significant (PERMANOVA $R^2 = 28.79\%$, $p = 0.04$; PERMDISP $p = 0.24$).

3.3. Taxonomic profile and biomarkers

The taxonomic analysis revealed that Proteobacteria was the most abundant phylum pre-remediation however, during remediation, the relative abundance increased significantly (+ 38%). Other relatively abundant phyla included Chloroflexi, Actinobacteria, Firmicutes, Acidobacteria, Bacteroidetes and Euryarchaeota (Fig. 3A). Additionally, Chloroflexi and Acidobacteria were relatively more abundant in the crude oil-polluted soil pre-remediation while the relative abundance of Cyanobacteria, Actinobacteria and Proteobacteria were higher during remediation. Differential abundance analysis revealed that Cyanobacteria and Proteobacteria were differentially (FDR-adjusted $p < 0.05$) abundant during remediation than pre-remediation while Hydrogenedentes, Spirochaetes, Armatimonadetes, Caldiserica, Cloacimonetes and Defferibacteres were differentially abundant pre-remediation (Fig. 3B).

At the genus taxonomic rank, 17 phylotypes had at least 1% relative abundance either during remediation or pre-remediation (Fig. 4A). *Sphingopyxis*, *Acidocella*, *Corynebacterium*, *Candidatus Solibacter*, *Smithella*, *Anaerolinea*, *Sulfuritalea*, *Mycobacterium*

and *Methanosaeta* were among the most abundant genera. The genus *Acidocella* was dominant in the soil both in pre-remediation and during remediation stages. *Sphingopyxis*, *Sphingomonas*, *Sulfuritalea*, *Corynebacterium*, *Mycobacterium*, *Pseudolabrys*, *Extensimonas*, *Bradyrhizobium*, *Azospirillum* and *Mesorhizobium* were all more relatively abundant during remediation while *Candidatus Solibacter*, *Smithella*, *Anaerolinea* and *Methanosaeta* were relatively more abundant in the soil pre-remediation. With differential abundance testing, a total of 10 phylotypes were found to be differentially (FDR-adjusted $p < 0.05$) (Fig. 4B). The phylotypes were all more abundant pre-remediation and included *Syntrophus*, *Syntrophobacter*, *Smithella*, SCADC1 – 2–3 (Peptococcaceae), *Pelolinea*, *Methanosaeta*, *Leptolinea*, *Desulfobacca*, *Caldisericum* and *Bryobacter*.

3.4 Microbial species-environment interaction

The relationship between microbial species and environmental variables was significant ($p = 0.031$) as determined using constrained redundancy analysis (RDA) (Fig. 5). The constrained variables explained 26.30% of the bacterial community variation. The environmental variables that significantly ($p < 0.05$) fitted into the RDA model included phosphorus ($r^2 = 0.98$, FDR-adjusted $p = 0.002$), pH ($r^2 = 0.83$, $p = 0.009$) and potassium ($r^2 = 0.87$, $p = 0.008$). Notably, these soil chemical parameters all strongly correlated with the centroid of the samples obtained during remediation and thus indicates that they responded the most to the remediation approach. For the bacterial phylotypes, it was observed that *Ferrovibrio* correlated significantly with potassium while pH and phosphorus correlated significantly with *Sphingopyxis*.

3.5 Bacterial community function pre-remediation and during remediation

A total of 6830 predicted KEGG orthologs were collapsed into 412 MetaCyc metabolic pathways. Investigation of differentially abundant pathways (FDR-adjusted $p \leq 0.05$ and effect size > 2) between the samples obtained pre-remediation and during remediation revealed that there were 19 differentially abundant pathways (Fig. 6). Based on effect sizes, the topmost differentially abundant pathways pre-remediation were incomplete reductive TCA cycle, adenine and adenosine salvage III pathway, purine nucleobases degradation I (anaerobic), reductive acetyl coenzyme A pathway and methanogenesis from acetate pathway. During remediation, mycolate biosynthesis pathway, oleate biosynthesis IV (anaerobic), heme biosynthesis I (aerobic), TCA cycle VII (acetate-producers) and 4-methylcatechol degradation (ortho cleavage) were among the biomarker pathways. Most notably, the functional inference indicated that pathways associated with methanogenic processes (coenzyme M biosynthesis I, methanogenesis from acetate, reductive acetyl coenzyme A pathway II and incomplete reductive TCA cycle) and terpenoids biosynthesis (mevalonate pathway I, mevalonate pathway II and superpathway of geranylgeranyldiphosphate biosynthesis I) were biomarkers pre-remediation while pathways for the biosynthesis of fatty acids and aromatic compound degradation (4-methylcatechol degradation (ortho cleavage), GDP-D-glycero- α -D-manno-heptose biosynthesis, superpathway of fatty acid biosynthesis initiation, mycolate biosynthesis, stearate biosynthesis II, (5Z)-dodecenoate biosynthesis, oleate biosynthesis IV (anaerobic) and palmitoleate biosynthesis I) were most important in differentiating the microbial community function on commencement of remediation.

4. Discussion

Artisanal refining is the leading source of hydrocarbon pollution in the Niger Delta with severe consequences on farmlands, fishing settlements, marine biodiversity and key biogeochemical processes. Due to slow natural recovery processes (possibly the result of unfavourable environmental conditions), most artisanal refining sites remain highly polluted even after several years of abandonment. This study, therefore, investigated the natural response of microbial communities to the presence of hydrocarbons in an artisanal refining site and how nutrient addition and intermittent tillage affects microbial alpha diversity, community structure, potential function and the overall rate of hydrocarbon sequestration.

The availability of nutrients has been demonstrated to influence the rate of hydrocarbon biodegradation [9, 40]. In this study, nutrient and oxygen availability appeared to be one of the rates limiting factors that affect biodegradation and thus likely explains the slow rate of bioattenuation in the oil-polluted site. The application of poultry droppings resulted in a significant increase in soil pH, total nitrogen, total phosphorus and total potassium. This finding implies that these soil chemical parameters were the most responsive to the remedial approach. Soil pH, nitrogen, phosphorus and potassium are important chemical factors that exert a strong influence on both the relative abundance and structure of bacterial communities [41]. In hydrocarbon impacted soils, slightly

acidic to alkaline pH (6.5–8.0) is considered optimal for biodegradation [42], therefore, the improved rate of hydrocarbon degradation corresponds to the mean increase in the soil pH during remediation (Table 2). Furthermore, there is evidence that the influence of pH on bacterial community structure and abundance has a corresponding effect on the availability of soil nutrients including nitrogen, phosphorus and potassium [41]. Notably, pH significantly correlated positively with nitrogen and phosphorus, suggesting that these soil chemical features were directly impacted by a significant change in the soil pH during remediation.

In this study, 16S rRNA metabarcoding was used to investigate the diversity of microbial communities across vertical samples of the artisanal refining site and during remediation of the crude oil impacted site. A higher number of unique OTUs were present in the site pre-mediation than during remediation. Similarly, the number of unique OTUs were higher at the surface soil of the site compared to the subsurface, though the high number of shared OTUs across vertical samples suggest that conditions were not largely different across depths. These observations indicate that the intermittent tillage and addition of nutrients to the polluted site significantly impacted bacterial species diversity (diversity decreased) while the high concentration of hydrocarbons across vertical samples created a seemingly common condition irrespective of soil depth. Furthermore, the observed significant decrease in the proportionality of bacterial species during remediation suggest that improved aeration through tillage and the supply of nutrients may have reduced the dominance of some category of bacterial species as diversity indicators inversely correlated with nitrogen, phosphorus and potassium. Similar to our findings, Bonaglia et al. [43] reported that bacterial alpha diversity of a PAH contaminated sediment was higher at timepoint zero than during remediation irrespective of treatment-type.

Comparison of the bacterial community structure pre-remediation and during remediation revealed significant ($p < 0.05$) differences. Significant changes in the assembly of bacterial communities is an indication of a potential functional change driven by environmental and soil chemical dynamics. For this study, RDA analysis revealed that phosphorus, potassium and pH were the soil chemical parameters that influenced the assembly of bacterial communities. Koshlaf et al. [44] reported that the addition of nutrients to an oil-polluted soil led to a significant change in the bacterial community structure after 2–4 months of remediation while Obieze et al. [9] demonstrated that potassium, pH and phosphorus were among important chemical factors that influence the assemblages of bacterial communities during hydrocarbon remediation. The significant correlation of these soil chemical parameters with the centroid of the samples drawn during remediation implies that their availability can significantly affect the recovery rate of artisanal refining sites.

Cyanobacteria and Proteobacteria were differentially (FDR-adjusted $p < 0.05$) abundant during remediation while Hydrogenedentes, Spirochaetes, Armatimonadetes, Caldiserica, Cloacimonetes and Deferribacteres were differentially abundant pre-remediation. Proteobacteria are copiotrophs thus their significant increase during remediation is in response to the addition of nutrients to the oil-polluted soil. Also, several species of this phylum are established hydrocarbon degraders [7, 45] and may have increased in relative abundance due to more favourable environmental conditions during remediation. Meanwhile, Cyanobacteria are photosynthetic microorganisms that exist in diverse environments and are known to quickly adapt to fluctuating environmental nutrient conditions [46, 47]. They also promote soil stability and some species are capable of carbon and nitrogen fixation (Diazotrophic species) [46]. For this study, their increase in relative abundance may have contributed to the increased availability of nitrogen for the multiplication of hydrocarbon-degrading microorganisms. At the genus taxonomic rank *Syntrophus*, *Syntrophobacter*, *Smithella*, *Pelolinea*, *Methanosaeta*, *Leptolinea*, *Bryobacter*, *Desulfobacca* and *Caldisericus* were differentially abundant pre-remediation. The detection of established syntrophic, methanogenic, sulfate-reducing and nitrogen metabolizing bacterial and archaeal species as biomarkers suggest that prior to remediation, methanogenic hydrocarbon degradation was one of the main routes for carbon sequestration. Methanogenic hydrocarbon degradation is an important process in the biogeochemical carbon cycle [48]. Several reports have demonstrated that the mineralization of hydrocarbons through methanogenesis involves a syntrophic relationship beginning with the initial activation of hydrocarbon substrates and subsequent formation of intermediates for methane production [49–53]. The community is said to either comprise hydrogenotrophic methanogens (*Candidatus* Methanoregula, *Methanolinea*, *Methanospirillum*), methylotrophic methanogens (*Methanlobus*) and/or acetotrophic methanogens (*Methanosaeta*) while the hydrocarbon activators usually include *Deltaproteobacteria* (*Syntrophus*, *Smithella*, *Desulfovibrio*, *Geobacter*) or other *Proteobacteria*, *Verrucomicrobia* or *Firmicutes* bacterial groups [54, 55]. Similar to this study, Liang et al. [56] detected *Methanosaeta* among the dominant archaea in an alkane-dependent methanogenic culture while *Smithella* was recently reported to be responsible for the oxidation of long-chain alkanes (C_{16} – C_{20}) through fumarate addition during methanogenic hydrocarbon degradation [19].

PICRUSt2 predicted pathways revealed that there were significant differences in the microbial community function pre-remediation compared to the samples obtained during remediation. The differences in function corroborate the differences in the core microbiome pre-remediation and during remediation. Among pathways differentially abundant pre-remediation were those associated with methanogenesis and the biosynthesis of terpenoids. The detection of pathways for the biosynthesis of terpenoids indicates that methanogenic archaeal species were major contributors to the microbial community function as terpenoids are major components of methanogenic bacterial cells membrane [57, 58]. Meanwhile, the detection of several pathways associated with methanogenesis pre-remediation suggests that decades of artisanal refining in the Niger Delta can transform the site into a reservoir for methanogenic hydrocarbon degradation. The dominating presence of acetoclastic methanogens (*Methanosaeta*) and syntrophic hydrocarbon activating bacterial species (*Smithella*, *Synthrophus*) pre-remediation further establishes this fact. Roy et al. [22] had earlier suggested that under anoxic conditions, the activation of hydrocarbons and subsequent production of intermediates such as acetate, formate, hydrogen and methanol can trigger methanogenic hydrocarbon degradation. Furthermore, in sites where hydrocarbons make up a greater proportion of the organic matter, methanogenic hydrocarbon degradation may become the main carbon sequestration route [22]. Keeping this in mind together with our findings, we conclude that the characteristic low energy yield associated with hydrocarbon degradation under anoxic conditions is one of the reasons for the prolonged natural recovery of crude oil polluted artisanal refining sites in the Niger Delta.

5. Conclusion

This study revealed that alpha diversity and the assembly of bacterial communities before remediation and during remediation is influenced by environmental conditions and soil chemical parameters. Soil pH, total nitrogen, total phosphorus and total potassium were the most responsive chemical parameters. Differentially abundant bacterial genera pre-remediation comprised established syntrophic, methanogenic, sulphate and nitrate-reducing bacterial species that suggest methanogenic hydrocarbon degradation was one of the main routes for hydrocarbon sequestration. This was further confirmed by the differential abundance of pathways associated with methanogenesis pre-remediation. Overall, this study demonstrates that nutrient addition and intermittent tillage is required to trigger a microbial community change that supports higher energy yield and a corresponding higher rate of hydrocarbon degradation in artisanal refining sites.

Declarations

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Competing interests

Authors declare that there are no conflicts of interest.

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Not applicable

Availability of data and materials

The sequence reads from this study has been deposited in the sequence read archive (SRA) under the BioProject ID PRJNA726818

Authors' contributions

CCO, CBC, RA and OA conceived and designed the research. CCO, KN and RS conducted the experiments. RA, KN and RS provided new reagents. CCO analysed the data. CCO wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Crude oil-polluted artisanal refining site pre-remediation (A) and during remediation (B)

Figure 2

Index of alpha diversity (Observed OTUs, Chao1, Shannon-Wiener and Evenness-pielou) pre-remediation and during remediation (A – D) and Principal coordinate analysis (PCoA) plot based on Bray-Curtis dissimilarities (E)

Figure 3

Average relative abundance of phylotypes at the phylum taxonomic rank (A) and biomarker phyla pre-remediation and during remediation (B)

Figure 4

Average relative abundance of bacterial genera (A) and biomarkers at the genus taxonomic rank pre-remediation and during remediation (B)

Figure 5

Constrained redundancy analysis (RDA) explaining bacterial species – environment relationship pre-remediation and during remediation

Figure 6

Differentially enriched MetaCyc pathways based on PICRUST2 bacterial community functional prediction

Supplementary Files

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