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ARTICLE

Field-scale biostimulation shifts microbial community composition and improves soil pollution recovery at an artisanal crude oil refining site

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ABSTRACT

Hydrocarbon pollution has led to the destruction of farmlands and fishing settlements in the Niger Delta. In this study, DNA metabarcoding was used to investigate bacterial response during the remediation of an artisanal refining site. Total petroleum hydrocarbons reduced from 93,720 mg kg^{-1} on timepoint zero to 9,029.76 mg kg^{-1} on day 91. Bacterial species distribution significantly reduced during remediation. The most responsive phyla to the remediation approach were *Proteobacteria* and *Cyanobacteria*. Pathways for hydrocarbon degradation were differentially abundant during remediation. Overall, nutrient addition and intermittent tillage improved bacterial energy yield and the degradation of hydrocarbons.

KEYWORDS

Biostimulation; bioremediation; oil-pollution; biodegradation; methanogenesis; biodiversity

Introduction

Fossil fuel provides for most of the world's energy need. This has led to increased exploration for crude oil in both onshore and offshore environments [1]. The activities of the oil and gas industry can often lead to severe environmental consequences through the release of crude oil into the environment [2]. The major causes of oil spills include equipment failure, human error, accident and sabotage [3]. The consequences can be enormous. For instance, the release of approximately 4.9 million barrels of crude oil from the Gulf of Mexico's Deep-Water Horizon oil spill incident led to the loss of human lives and severe environmental and economic damage [4]. Similarly, the increasing activities of artisanal crude oil refiners in the Niger Delta have led to loss of lives, destruction of farmlands, fishing settlements, recreational beaches, the marine environment and biodiversity [5].

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Currently, there are several expensive chemical and physical methods for the recovery of hydrocarbon polluted sites. They cannot remove trace quantities of pollutant. Microbial-mediated remediation, which optimises environmental parameters and adds nutrients (bioremediation) is considered the most appropriate method for the clean-up of crude oil-polluted soils [6]. Unlike chemical and physical methods that can significantly reduce soil quality and can cause secondary environmental contamination, bioremediation is cost-effective, non-toxic, efficient in reducing the recovery half-life and can lead to mineralisation of the pollutant, thereby improving the soil quality [7]. The process of bioremediation depends on the natural ability of microorganisms to use hydrocarbons as sources of carbon and energy [8]. Several bacterial and fungal genera are established degraders of various fractions of hydrocarbons [9]. Notably, *Alcarnivorax* is a known degrader of both short and long-chain alkanes [10] and *Pseudomonas* has been demonstrated to degrade a wide range of saturated and aromatic hydrocarbons [11].

The degradation of hydrocarbons by microorganisms can occur under aerobic, microaerobic or anaerobic conditions. And these conditions largely influence the microbial community composition, including their response rate and overall community functions. Anaerobic hydrocarbon degradation can occur under methanogenic, nitrate-reducing, sulphate-reducing and iron-reducing conditions [12]. The occurrence of anaerobic methanogenic hydrocarbon degradation has been demonstrated in both laboratory and field studies [13,14]. There are also reports that methanogenic hydrocarbon degradation occurs under microaerobic conditions, such as water droplets [15], and in crude oil-polluted sites, where hydrocarbon represents a significant portion of the organic matter [16]. Methanogenesis occurs mainly through (i) carbon dioxide reduction, as observed in hydrogenotrophic methanogens (using hydrogen as electron donor while reducing carbon dioxide to methane); (ii) through acetate reduction, as in acetoclastic methanogens (using acetate as a terminal electron acceptor or converting acetate to methane and bicarbonate); and (iii) through the use of methylated compounds like methanol, as observed in methylotrophic methanogens such as *Methanolobus, Methanosphaera* and *Methanosalsum* [17,18]. Although methanogenic hydrocarbon degradation is an important process for carbon fixation, anaerobic hydrocarbon degradation is relatively slow as a result of low energy yield and a correspondingly slow rate of cell multiplication [12].

The relatively slow rate of natural degradation of hydrocarbons (bioattenuation) and the paucity of information about the natural recovery process of most crude oil-polluted artisanal refining sites make this study timely. It is difficult to assess most artisanal refining sites and there are no funds available for clean-up. Microbe-dependent degradation seems the only practical option. 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 hypothesised that the slow natural recovery rate of artisanal refining sites is largely caused by lack of nutrients and oxygen for aerobic hydrocarbon degradation. The specific objectives were to (1) determine the bacterial diversity and compositional structure of the artisanal refining site at different depths and (2) to determine the effect of nutrient addition on bacterial diversity and community function during remediation. 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.

Materials and methods

Site description

An oil-polluted soil with a history of decades of artisanal refining activities was selected for this study (Figure 1(a)). 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. 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.

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 sampler. Fifteen surface samples were first collected from different points of the polluted site and 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

Figure 1. Crude oil-polluted artisanal refining site pre-remediation (a) and during remediation (b).

were subsequently homogenised according to the depth of sampling prior to chemical and microbiological analysis. Samples were further collected from an unpolluted vegetation rich site 1000 metres 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 m^2), clearing, excavation of the most polluted points and ridge making (Figure 1(b)). Nutrient was added to the crude oil polluted soil by the application of 1.00 kg m^{-2} of poultry droppings to the site. Remediation was monitored for 91 days, with intermittent tillage at 3-weeks interval and mobilising the soil from the surface to 100 cm depth in order to increase aeration. Soil composite samples were collected on day 0, day 14, day 49, day 70 and day 91. All the samples, collected both on pre-remediation and during remediation, were transferred to the laboratory in ice-packs for both physicochemical and microbiological analyses.

Determination of soil physical and chemical properties

The soil physical properties investigated included the soil-type, soil colour, and percentage gravel, sand and fines (slit and clay). Among the soil chemical parameters investigated were pH (determined using Metrohn automated probe analyser), moisture content (determined by the drying method), total nitrogen, electrical conductivity (conductivity metre) and the soil organic matter content. The petroleum hydrocarbon components in the oil-polluted soil were determined using targeted GC-FID analysis.

Determination of petroleum hydrocarbon components

The total petroleum hydrocarbons (TPH) were extracted from 10 g of the soil samples using Dichloromethane as extraction solvent. TPH determination from the soil extract was based on USEPA 8015D method [19]. Briefly, 10 ml of the organic extract were filtered and transferred into gas chromatography vials and analysed by gas chromatography-flame ionisation detector (GC-FID; Agilent 7890A, Agilent Technologies) equipped with an HP 7673 FID detector, autosampler and a fused silica capillary column. Calibration was done using Hydrocarbon Window Defining Standard (AccuStandard®), for the detection of n-C8 (n-Octane) through n-C40 (n-Tetracontane) hydrocarbons. 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 3 mins 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.

Genomic DNA extraction from soil, amplification and sequencing

The total genomic DNA of the soil samples were extracted using MoBio PowerSoil™ 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 341 F (CCTACGGGNGGCWGCAG) and 805 R (GGACTACHVGGGTWTCTAAT) targeting the V3 – V4 hypervariable region of the 16S rRNA gene were used for DNA amplification. Illumina-MiSeq sequencing of the PCR amplicons was conducted at the Agricultural Research Council –

Biotechnology Platform, Onderstepoort, Tshwane, 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 normalised 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).

Bioinformatics and diversity analyses

Raw Illumina MiSeq sequence reads for all the samples were processed using QIIME v2019.4 [20]. DADA2 [21] was used for sequence denoising, removal of low-quality reads, marginal sequences and clustering of sequences into amplicon sequence variants. Further clustering of the sequences into Operational Taxonomic Units (OTU) at 97% relatedness was done using VSEARCH open-reference OTU picking strategy and the SILVA v132 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, OTUs that appeared only once were filtered off the count table and the data were subsequently normalised to the minimum library size.

The microbial community functional attributes pre-remediation and during remediation were determined using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) [22]. 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.

Statistical analyses

Unless otherwise stated, all statistical analyses were performed between sample groups obtained pre-remediation and those obtained during remediation using R v3.6.1 [23]. The soil physicochemical parameters and diversity matrices 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* R package. Differential abundance testing of taxonomic composition pre-remediation and during remediation was done using *Deseq2* and *lefse*.

Determination of differences in microbial community structure was based on Bray-Curtis dissimilarities. Visualisation 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 were determined using *STAMP* [24]. Pathways with Benjamini-Hochberg FDR-adjusted Welch's t-test ($p \leq 0.05$) and effect size (>2) were considered as biomarkers.

Results

Physical and chemical characteristics of the oil-polluted site

Table 1 shows the results of the physicochemical parameters of the artisanal refining site and the unpolluted soil. All analysed baseline samples had extractable TPH concentration above the minimum intervention limit (5,000 mg kg^{-1}) as stipulated in the Environmental Guidelines and Standards for the Petroleum Industry in Nigeria [25]. The soil was polluted with varying fractions of hydrocarbons ranging from n-C_8 to n-C_{40}. Further, n-alkanes with a carbon chain length between n-C_{16} to n-C_{33} formed the bulk of hydrocarbons across the soil gradients investigated (Figure 2). TPH concentration was 490,631 mg kg^{-1} in the surface (0 – 15 cm), 320,971.60 mg kg^{-1} at 1 m depth and 81, 434.86 mg kg^{-1} at 1.5 m 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 mg kg^{-1} on day zero and reduced to 9,029.76 mg kg^{-1} on day 91.

Table 2 shows the results of nutrient analysis pre-remediation and during remediation. 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 and the soil pH significantly ($p < 0.05$) increased during the period of remediation above levels prior to remediation (Table 2). The moisture content and organic matter content were not significantly affected by the remediation approach. Spearman's rank correlation analysis revealed that pH correlated with nitrogen ($r = 0.90$, $p = 0.002$).

Bacterial community diversity

A total of 75,137 high-quality sequences were denoised and clustered into 5,029 OTUs based on 97% sequence similarity. 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 (Figure 3(a)). The OTUs shared between the surface and sub-surface soils decreased with increasing depth. Overall, 88 OTUs were shared across vertical samples, and 72 ASVs were shared between the samples obtained pre-remediation and those obtained during remediation (Figure 3(b)).

Observed OTUs and Chao1 diversity indices revealed higher species richness pre-remediation than during remediation; but the observed changes in bacterial richness were not significant (Wilcoxon $p > 0.05$) (Figure 4(a,Figure 4b)). Similarly, species diversity (Shannon-Wiener index) was lower during remediation (Figure 4(c)). Species distribution (Pielou's evenness) pre-remediation was significantly

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
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

*ND = Not determined

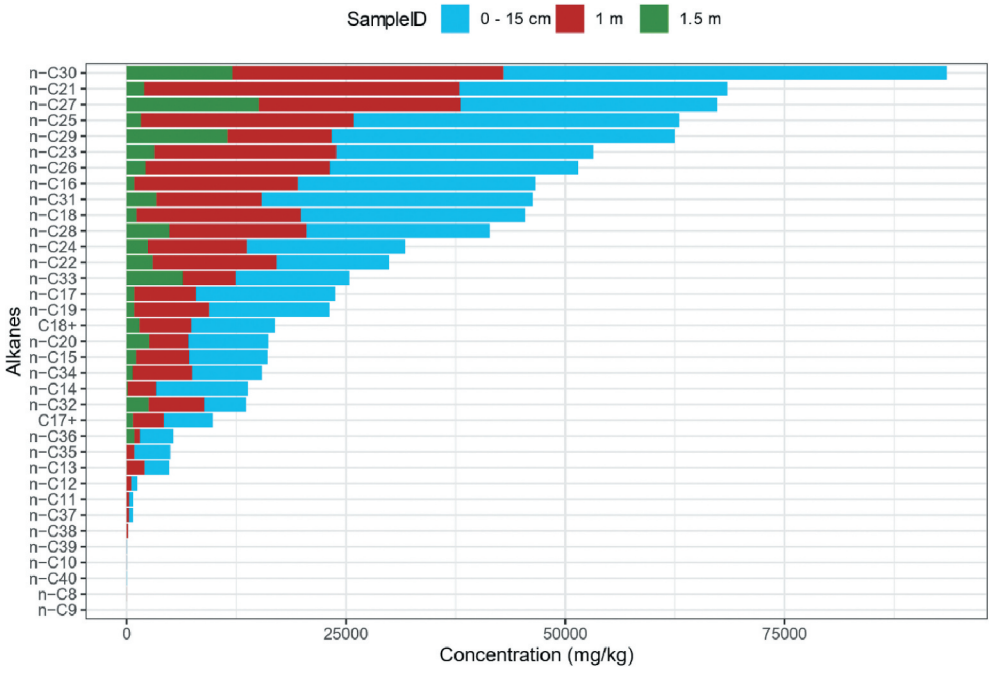

Figure 2. Depth distribution and concentration of n-alkanes detected in the artisanal crude-oil refining site 0–15 cm = 0–15 cm depth; 1 m = 1 m depth; 1.5 m = 1.5 m depth.

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

Parameter	Mean and standard deviation (Pre-remediation)	Mean and standard deviation (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**
pH	5.97 ± 0.15	6.58 ± 0.19	0.003**

($p < 0.05$) higher than during the period of remediation (Figure 4(d)). To determine the influence of soil chemical parameters on bacterial alpha diversity, we performed Spearman's rank correlation analysis and found that Shannon-Wiener index significantly correlated with moisture content ($r = 0.78$, $p = 0.046$). Pielou's evenness significantly correlated inversely with total nitrogen ($r = -0.74$, $p = 0.037$) and pH ($r = -0.74$, $p = 0.036$). Richness (Observed OTUs and Chao1) significantly correlated positively with moisture content ($r = 0.82$, $p = 0.012$).

Bray-Curtis dissimilarity between soil bacterial communities revealed differentiation in the community structure pre-remediation and during remediation (Figure 4(e)). Further, it was observed that samples obtained during the early stages of remediation (Day 0 – Day 49) clustered together within the ordination space. The samples collected between day 70 and 91 clustered separately. The observed bacterial community differences in multivariate space pre-remediation and during remediation were significant (PERMANOVA $R^2 = 28.79\%$, $p = 0.04$; PERMDISP $p = 0.24$).

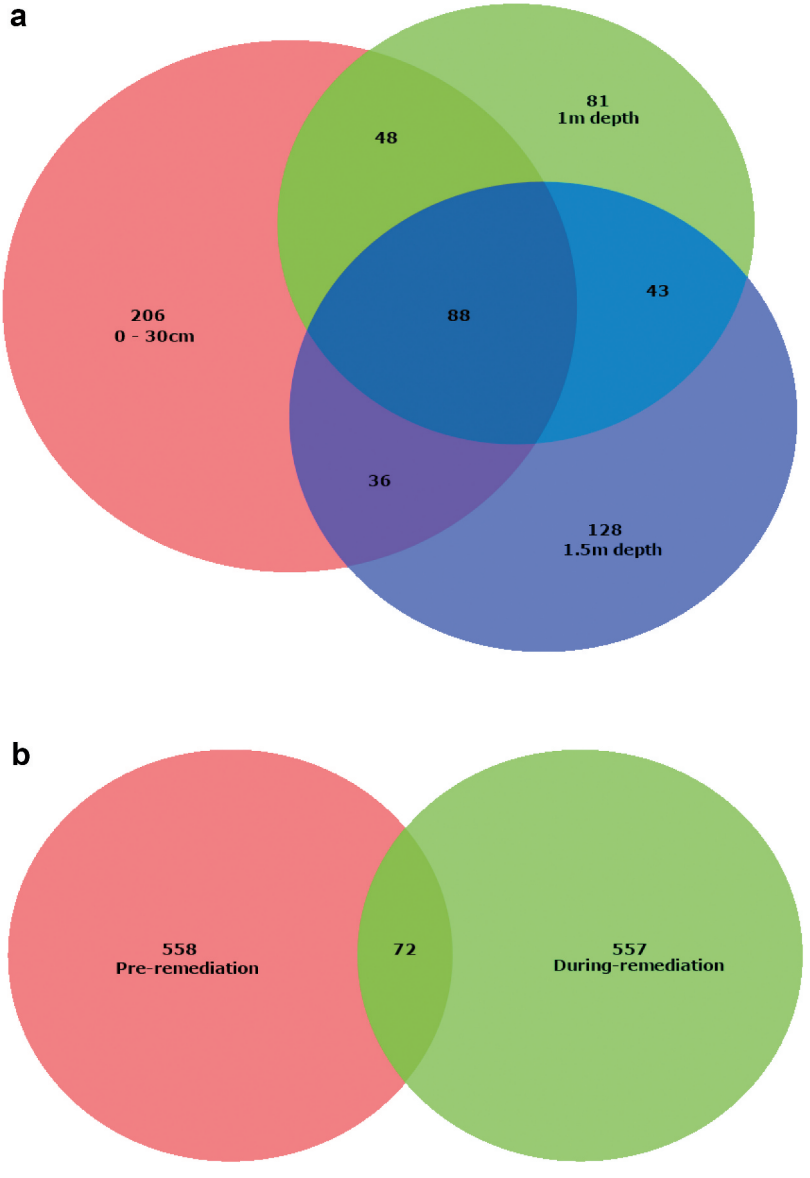

Figure 3. Shared and unique OTUs across vertical samples (a) and between OTUs pre-remediation and during remediation (b). Venn diagram was computed using the DeepVenn online tool (http://www.deepvenn.com/).

Taxonomic profile and biomarkers

The taxonomic analysis revealed that the most abundant phylum pre-remediation and during remediation was *Proteobacteria*. The relative abundance increased significantly (+38%) following nutrient addition. Other relatively abundant phyla included *Chloroflexi, Actinobacteria, Firmicutes, Acidobacteria, Bacteroidetes* and *Euryarchaeota* (Figure 5(a)). Furthermore, *Chloroflexi* and *Acidobacteria* were relatively more abundant in the crude oil-polluted site pre-remediation. *Cyanobacteria, Actinobacteria* and

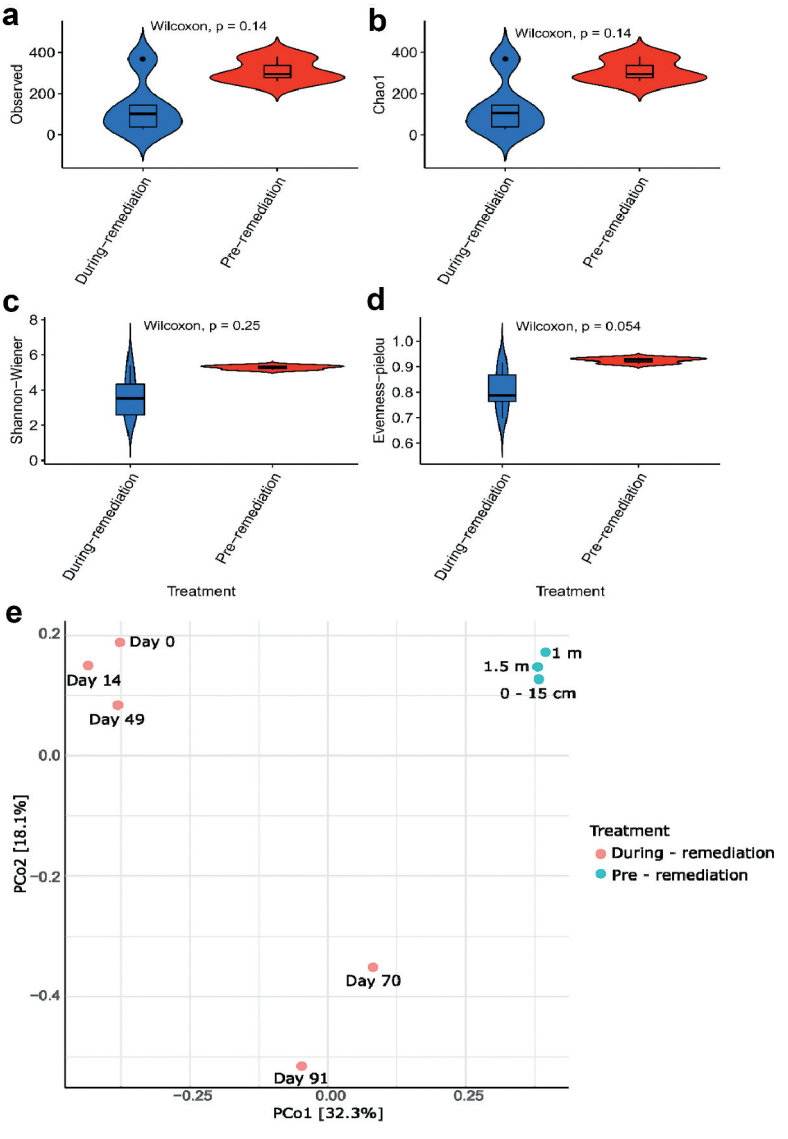

Figure 4. Index of alpha diversity (Observed OTUs, Chao1, Shannon-Wiener and Evenness-pielou) pre-remediation and during remediation (a – d) and PCoA plot based on Bray-Curtis dissimilarities (e).

Proteobacteria showed higher relative abundance during remediation. Differential abundance analysis revealed that *Cyanobacteria* and *Proteobacteria* were differentially (FDR-adjusted $p < 0.05$) abundant during remediation, not pre-remediation, whereas *Hydrogenedentes, Spirochaetes, Armatimonadetes, Caldiserica, Cloacimonetes* and *Deferribacteres* were differentially abundant pre-remediation (Figure 5(b)).

At the genus taxonomic rank, 17 phylotypes had at least 1% relative abundance either during remediation or pre-remediation (Figure 6(a)). *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 pre-remediation and during remediation.

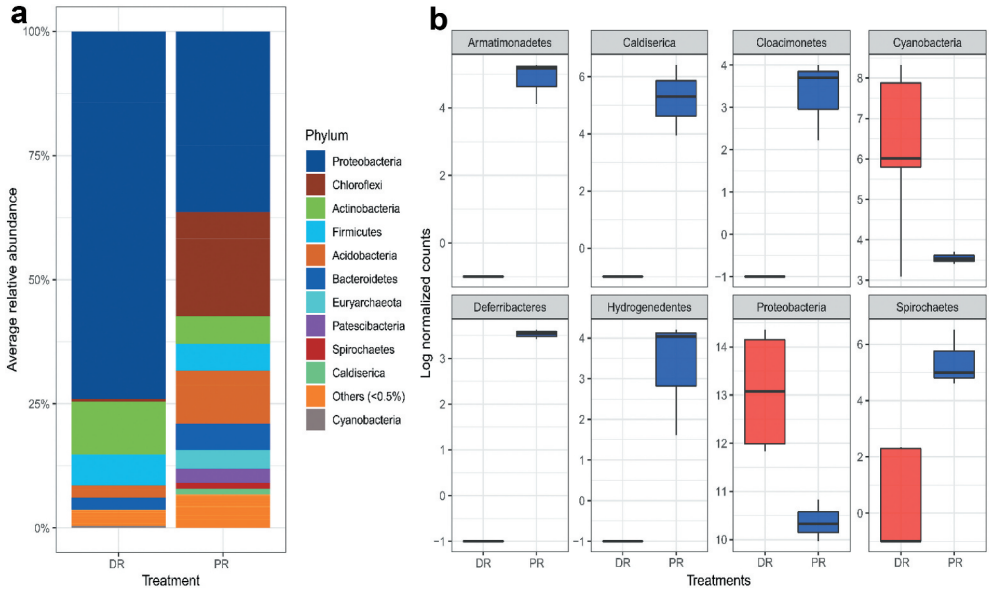

Figure 5. Average relative abundance of phylotypes at the phylum taxonomic rank (a) and biomarker phyla pre-remediation and during remediation (b).

Sphingopyxis, Sphingomonas, Sulfuritalea, Corynebacterium, Mycobacterium, Pseudolabrys, Extensimonas, Bradyrhizobium, Azospirillum and *Mesorhizobium* were relatively more abundant during remediation. *Candidatus Solibacter, Smithella, Anaerolinea* and *Methanosaeta* were relatively more abundant in the site pre-remediation. Following differential abundance testing, a total of 10 phylotypes were found to be differentially (FDR-adjusted $p < 0.05$) abundant pre-remediation (Figure 6(b)). They include *Syntrophus, Syntrophobacter, Smithella*, SCADC1 − 2 − 3 (*Peptococcaceae), Pelolinea, Methanosaeta, Leptolinea, Desulfobacca, Caldisericum* and *Bryobacter*.

Microbial species-environment interaction

The relationship between microbial species and environmental variables was significant ($p = 0.031$) as determined using RDA (Figure 7). The constrained variables explained 26% of the bacterial community variation. The environmental variables that significantly ($p < 0.05$) fitted into the RDA model included pH ($r^2 = 0.84$, $p = 0.007$) and nitrogen ($r^2 = 0.88$, $p = 0.006$). Notably, these soil chemical parameters all correlated with the centroid of the samples obtained during remediation; thus indicating that they responded the most to the remediation approach. For the bacterial phylotypes, it was observed that *Ferrovibrio* and *Sphingopyxis* correlated with both pH and nitrogen.

Bacterial community functioning at 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-

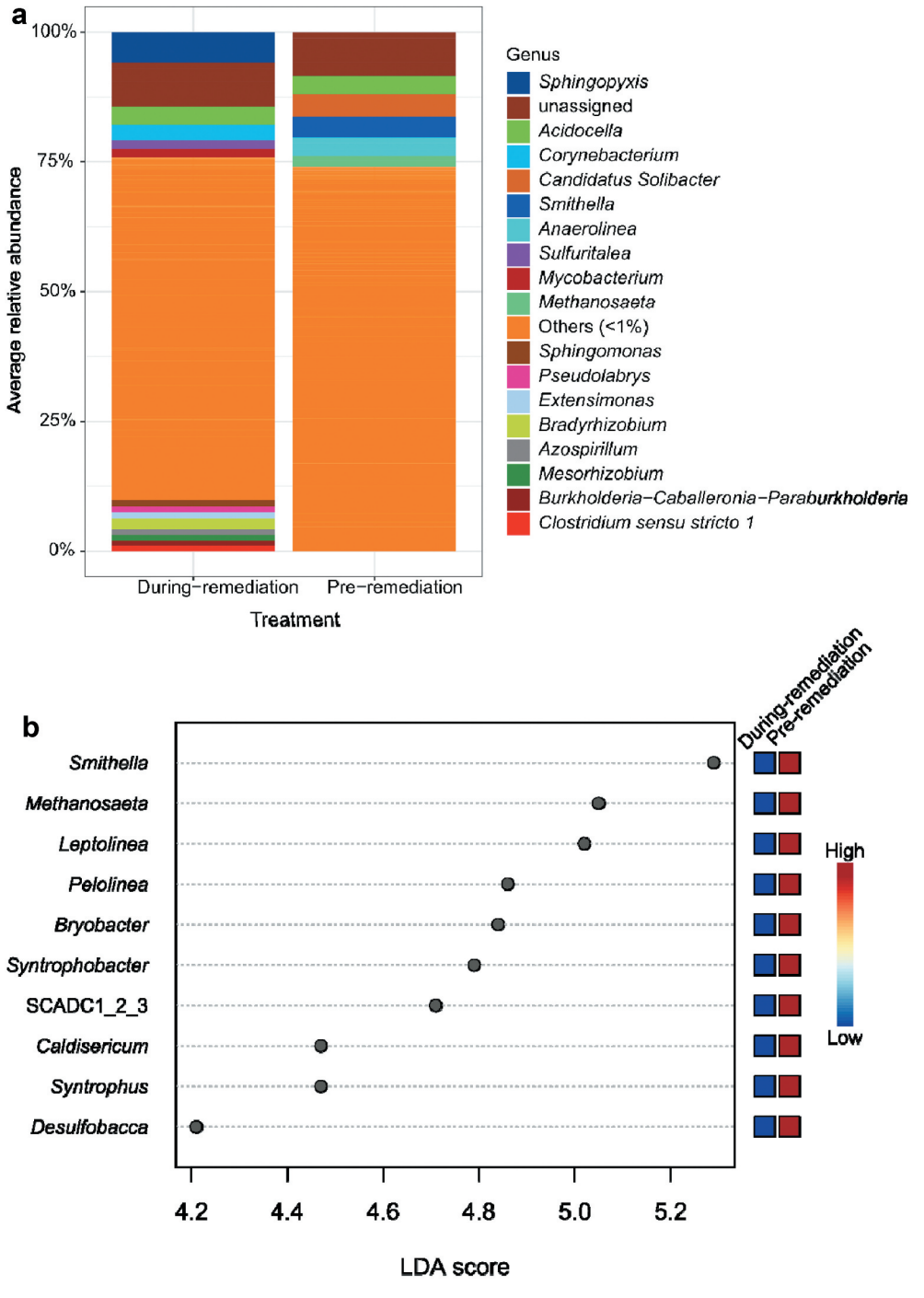

Figure 6. Average relative abundance of bacterial genera (a) and biomarkers at the genus taxonomic rank pre-remediation and during remediation (b).

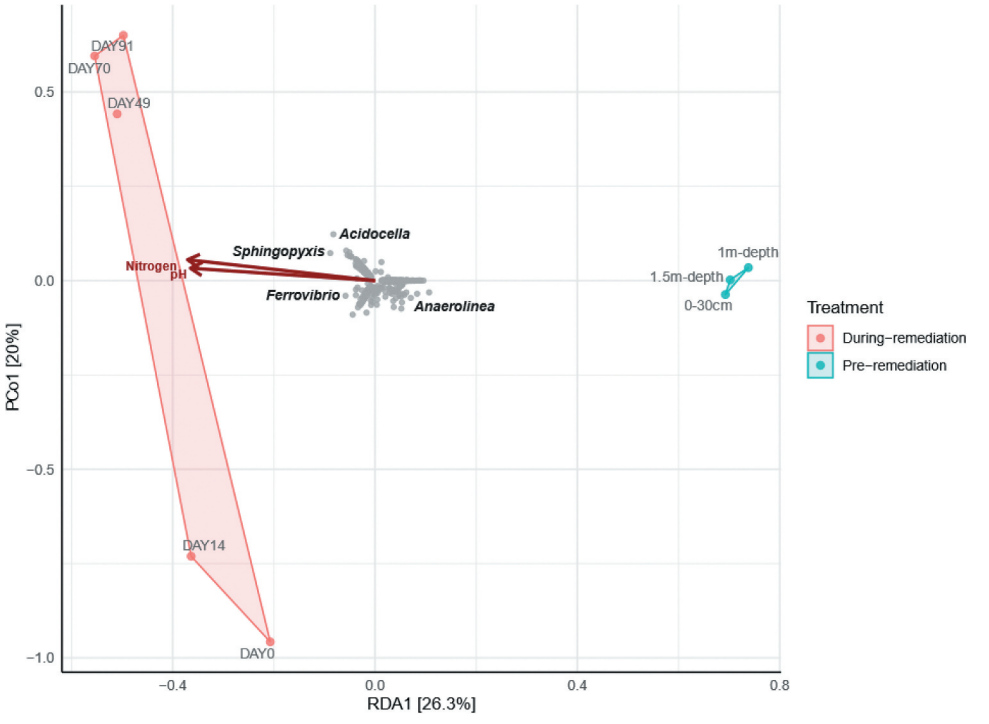

Figure 7. RDA plot explaining bacterial species – environment relationship pre-remediation and during remediation.

remediation and during remediation revealed that there were 19 differentially abundant pathways (Figure 8). 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), haem 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. 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.

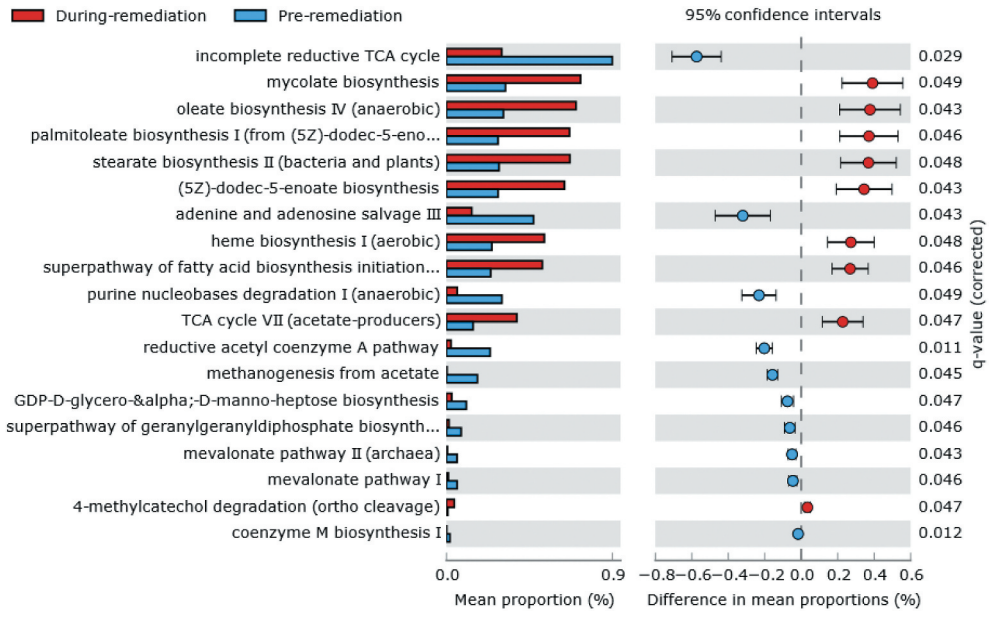

Figure 8. Differentially enriched MetaCyc pathways based on PICRUSt2 bacterial community functional prediction.

Discussion

Artisanal refining is the leading source of hydrocarbon pollution in the Niger Delta with severe effects on farmlands, fishing settlements, marine biodiversity and key biogeochemical processes. Because natural recovery is slow (possibly as a result of unfavourable environmental conditions for microbial community development), 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 degradation.

The availability of nutrients has been demonstrated to influence the rate of hydrocarbon biodegradation [7]. In this study, nutrient and oxygen availability appeared to be factors limiting the degradation rate. This probably explains the slow rate of bioattenuation in the oil-polluted site. The application of poultry droppings resulted in a significant increase in soil pH and total nitrogen available and prompted the microbial community changes. This effect shows that these soil chemical parameters were the most responsive to the remedial approach. Soil pH and nitrogen are important chemical factors that exert a strong influence on both the relative abundance and structure of bacterial communities [26]. In hydrocarbon impacted soils, slightly acidic to alkaline pH (6.5–8.0) is considered optimal for biodegradation [27]. Therefore, the improved rate of hydrocarbon degradation corresponds to the 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 [26]. Notably, pH significantly correlated positively with nitrogen, suggesting that these soil chemical factors were influenced by the 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. The number of unique OTUs was higher at the surface soil of the site than the subsurface, but the high number of shared OTUs across vertical samples suggests that conditions were not greatly different across depths. These observations indicate that depth influenced diversity. The high concentration of hydrocarbons across vertical samples created conditions for the proliferation of some OTUs, irrespective of soil depth. Furthermore, the observed significant decrease in bacterial species distribution during remediation suggests that improved aeration, through tillage and the supply of nutrients may have reduced the dominance of some categories of bacteria, as diversity indicators inversely correlated with nitrogen. Similar to our findings, Bonaglia et al. [28] reported that bacterial alpha diversity of a PAH contaminated sediment was higher at timepoint zero than during remediation, regardless of treatment-type. For this study, the decrease in diversity is attributable to the likely effect of aeration on both indigenous methanogens and other anaerobic phylotypes, which were possibly introduced as part of the gut microbiota from the poultry droppings.

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 functional change driven by environmental and soil chemical dynamics. For this study, RDA analysis revealed that nitrogen and pH were the soil chemical parameters that influenced the assembly of bacterial communities. Koshlaf et al. [29] 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. Obieze et al. [7] 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. *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. Several species of this phylum are established hydrocarbon degraders [30] 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 adapt quickly to fluctuating environmental nutrient conditions [31]. They also promote soil stability and some species are capable of carbon and nitrogen fixation (diazotrophic species) [32]. At the genus taxonomic rank *Syntrophus, Syntrophobacter, Smithella, Pelolinea, Methanosaeta, Leptolinea, Bryobacter, Desulfobacca* and *Caldisericus* were differentially abundant pre-remediation. The detection of these established syntrophic, methanogenic, sulphate-reducing and nitrogen metabolising bacterial and archaeal species as biomarkers suggests 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. Several reports have demonstrated that the mineralisation of hydrocarbons through methanogenesis includes a syntrophic relationship beginning with the initial activation of hydrocarbon substrates and subsequent formation of intermediates for methane production [33,34]. The community is said to comprise hydrogenotrophic methanogens (*Methanoregula, Methanolinea, Methanospirillum*), methylotrophic methanogens (*Methanolobus*) and/or acetoclastic methanogens (*Methanosaeta*). The hydrocarbon activators usually include *Deltaproteobacteria* (*Syntrophus, Smithella, Desulfovibrio, Geobacter*) or other *Proteobacteria, Verrucomicrobia* or *Firmicutes* bacterial groups [35,36]. Similar to this study, Liang et al. [37] detected *Methanosaeta* among the dominant archaea in an alkane-dependent methanogenic culture, and *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 [38]. Although the 16S rRNA primer used for this study is more specific for bacteria than archaea, our results provide a useful survey of the microbial community composition of long-term hydrocarbon impacted sites in the Niger Delta.

PICRUSt2-based 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 [39,40]. Furthermore, 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 supports this observation. Roy et al. [16] 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 route for carbon release [16]. Meanwhile, a high abundance of pathways associated with methanogenesis was not observed in a previous study of a conventional crude oil spill sites in the Niger Delta [41]. 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.

Conclusion

This study revealed that soil bacterial diversity and community composition depended upon soil chemical parameters, and nutrient addition and intermittent tillage reduced microbial diversity. Pre-remediation, diverse and abundant bacterial genera comprised

established syntrophic, methanogenic, sulphate and nitrate-reducing bacterial species that suggest methanogenic hydrocarbon degradation as one of the main routes for hydrocarbon degradation at that time. This was further confirmed by the differential abundance of pathways associated with methanogenesis at pre-remediation. Overall, this study demonstrated that nutrient addition and intermittent tillage was required to trigger a microbial community change able to support higher energy yield and a corresponding higher rate of hydrocarbon degradation. This method is useful for the remediation of contaminated soils in artisanal refining sites. Although there are several important findings from this study, the unavailability of a proper control (a corresponding oil-polluted site without nutrient addition or tillage) limits some of our observations, and should be considered in future investigations.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The sequence reads from this study have been deposited in the sequence read archive under the BioProject ID PRJNA726818

References

[1] Nikolova, C. and Gutierrez, T., 2020, Use of microorganisms in the recovery of oil from recalcitrant oil reservoirs: Current state of knowledge, technological advances and future perspectives. *Frontiers in Microbiology* **10**, 2996. doi: 10.3389/fmicb.2019.02996

[2] Han, Y., Nambi, I.M., and Prabhakar, C.T., 2018, Environmental impacts of the Chennai oil spill accident – A case study. *Science of the Total Environment* **626**, 795–806. doi: 10.1016/j.scitotenv.2018.01.128

[3] Obieze, C.C., Chikere, C.B., Adeleke, R., and Akaranta, O., 2019, Formulation and evaluation of slow-release fertilizer from agricultural and industrial wastes for remediation of crude oil-polluted soils. Presented at the 2019 Society for Petroleum Engineers - SPE Nigeria Annual International Conference and Exhibition, Lagos, 5 – 7 August.

[4] Atlas, R.M. and Hazen, T.C., 2011, Oil biodegradation and bioremediation: A tale of the two worst spills in U.S. history. *Environmental Science and Technology* **45**(16), 6709–6715. doi: 10.1021/es2013227

[5] Bebeteidoh, O.L., Kometa, S., Pazouki, K., and Norman, R., 2020, Sustained impact of the activities of local crude oil refiners on their host communities in Nigeria. *Heliyon* **6**(6), e04000. doi: 10.1016/j.heliyon.2020.e04000

[6] Ortega, M.F., Guerrero, D.E., García-Martínez, M.J., Bolonio, D., Llamas, J.F., Canoira, L., et al., 2018, Optimization of Landfarming Amendments Based on Soil Texture and Crude Oil Concentration. *Water, Air and Soil Pollution* **229**(7), 229–234. doi: 10.1007/s11270-018-3891-1

[7] Obieze, C.C., Chikere, C.B., Selvarajan, R., Adeleke, R., Ntushelo, K., and Akaranta, O., 2020, Functional attributes and response of bacterial communities to nature-based fertilization during hydrocarbon remediation. *International Biodeterioration and Biodegradation* **154**, 105084. doi: 10.1016/j.ibiod.2020.105084

[8] Weiland-bräuer, N., Fischer, M.A., Schramm, K., and Schmitz, R.A., 2017, Polychlorinated Biphenyl (PCB) - Degrading Potential of Microbes Present in a Cryoconite of Jamtalferner Glacier. *Frontiers in Microbiology* **8**, 1105. doi: 10.3389/fmicb.2017.01105

[9] Chikere, C.B., Tekere, M., and Adeleke, R., 2019, Enhanced microbial hydrocarbon biodegradation as stimulated during field-scale landfarming of crude oil-impacted soil. *Sustainable Chemistry and Pharmacy* **14**, 100177. doi: 10.1016/j.scp.2019.100177

[10] Marietou, A., Chastain, R., Beulig, F., Scoma, A., Hazen, T.C., and Bartlett, D.H., 2018, Corrigendum: The effect of hydrostatic pressure on enrichments of hydrocarbon degrading microbes from the Gulf Of Mexico following the Deepwater Horizon oil spill. *Frontiers in Microbiology* **9**, 1050. doi: 10.3389/fmicb.2018.01050

[11] Varjani, S.J. and Upasani, V.N., 2016, Biodegradation of petroleum hydrocarbons by oleophilic strain of Pseudomonas aeruginosa NCIM 5514. *Bioresource Technology* **222**, 195–201. doi: 10.1016/j.biortech.2016.10.006

[12] Dhar, K., Subashchandrabose, S.R., Venkateswarlu, K., Krishnan, K., and Megharaj, M., 2020, Anaerobic microbial degradation of polycyclic aromatic hydrocarbons: A comprehensive review. *Reviews of Environmental Contamination and Toxicology* **251,** 25–108 doi: 10.1007/398_2019_29.

[13] Laso-Pérez, R., Hahn, C., Van Vliet, D.M., Tegetmeyer, H.E., Schubotz, F., Smit, N.T., et al., 2019, Anaerobic degradation of non-methane alkanes by "Candidatus Methanoliparia" in hydrocarbon seeps of the Gulf Of Mexico. *MBio* **10**, 1–20. doi:10.1128/mBio.01814-19

[14] Ye, Q., Liang, C., Chen, X., Fang, T., Wang, Y., and Wang, H., 2019, Molecular characterization of methanogenic microbial communities for degrading various types of polycyclic aromatic hydrocarbon. *Journal of Environmental Sciences* **86**, 97–106. doi: 10.1016/j.jes.2019.04.027

[15] Meckenstock, R.U., Von Netzer, F., Stumpp, C., Lueders, T., Himmelberg, A.M., Hertkorn, N., et al., 2014, Water droplets in oil are microhabitats for microbial life. *Science* **345**(6197), 673–676. doi: 10.1126/science.1252215

[16] Roy, A., Sar, P., Sarkar, J., Dutta, A., Sarkar, P., Gupta, A., et al., 2018, Petroleum hydrocarbon rich oil refinery sludge of North-East India harbours anaerobic, fermentative, sulfate-reducing, syntrophic and methanogenic microbial populations. *BMC Microbiology* **18**(1), 1–22. doi: 10.1186/s12866-018-1275-8

[17] Blake, L.I., Tveit, A., Øvreås, L., and Head, I.M., 2015, Response of methanogens in Arctic sediments to temperature and methanogenic substrate availability. *PLoS One* **10**(6), e0129733. doi: 10.1371/journal.pone.0129733

[18] Jiménez, N., Richnow, H.H., Vogt, C., Treude, T., and Krüger, M., 2016, Methanogenic hydrocarbon degradation: Evidence from field and laboratory studies. *Journal of Molecular Microbiology and Biotechnology* **26**(1–3), 227–242. doi: 10.1159/000441679

[19] US EPA, 2003, Method 8015D (SW-846): Nonhalogenated Organics Using GC/FID Revision 4. Available online at (accessed January 26, 2020): https://www.epa.gov/sites/default/files/2015-12/documents/8015d_r4.pdf

[20] Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., et al., 2019, Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology* **37**(8), 852–857. doi: 10.1038/s41587-019-0209-9

[21] Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., and Holmes, S.P., 2016, DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods* **13**(7), 581–583. doi: 10.1038/nmeth.3869

[22] Douglas, G.M., Maffei, V.J., Zaneveld, J.R., Yurgel, S.N., Brown, J.R., Taylor, C.M., et al., 2020, PICRUSt2 for prediction of metagenome functions. *Nature Biotechnology* **38**(6), 685–688. doi: 10.1038/s41587-020-0548-6

[23] Team, R.C., 2013, R: A language and environment for statistical computing.

[24] Parks, D.H., Tyson, G.W., Hugenholtz, P., and Beiko, R.G., 2014, STAMP: Statistical analysis of taxonomic and functional profiles. *Bioinformatics* **30**(21), 3123–3124. doi: 10.1093/bioinformatics/btu494

[25] Department of Petroleum Resources (DPR), 2002, Environmental Guidelines and Standards for the Petroleum Industry in Nigeria (EGASPIN). Available online at (accessed January 26, 2020): https://www.ngfcp.dpr.gov.ng/media/1066/dprs-egaspin-2002-revised-edition.pdf

[26] Neina, D., 2019, The role of soil pH in plant nutrition and soil remediation. *Applied and Environmental Soil Science* **2019**, 1–9. doi: 10.1155/2019/5794869

[27] Vidali, M., 2001, Bioremediation. An overview. *Pure and Applied Chemistry* **73**(7), 1163–1172. doi: 10.1351/pac200173071163

[28] Bonaglia, S., Broman, E., Brindefalk, B., Hedlund, E., Hjorth, T., Rolff, C., et al., 2020, Activated carbon stimulates microbial diversity and PAH biodegradation under anaerobic conditions in oil-polluted sediments. *Chemosphere* **248**, 126023. doi:10.1016/j.chemosphere.2020.126023

[29] Koshlaf, E., Shahsavari, E., Haleyur, N., Osborn, A.M., and Ball, A.S., 2020, Impact of necrophytoremediation on petroleum hydrocarbon degradation, ecotoxicity and soil bacterial community composition in diesel-contaminated soil. *Environmental Science and Pollution Research* **27**(25), 31171–31183. doi: 10.1007/s11356-020-09339-2

[30] Meeboon, N., Leewis, M.C., Kaewsuwan, S., Maneerat, S., and Leigh, M.B., 2017, Changes in bacterial diversity associated with bioremediation of used lubricating oil in tropical soils. *Archives of Microbiology* **199**(6), 839–851. doi: 10.1007/s00203-017-1356-3

[31] Kumar, C., Chatterjee, A., Wenjing, W., Yadav, D., and Singh, P.K., 2020, Cyanobacteria: Potential and role for environmental remediation. In P. Singh, A. Kumar and A. Borthakur (Eds) *Abatement of Environmental Pollutants: Trends and Strategies* (Amsterdam: Elsevier) pp. 193–202.

[32] Singh, J.S., Kumar, A., and Singh, M., 2019, Cyanobacteria: A sustainable and commercial bio-resource in production of bio-fertilizer and bio-fuel from waste waters. *Environmental and Sustainability Indicators* **3**, 100008. doi: 10.1016/j.indic.2019.100008

[33] Fowler, S.J., Dong, X., Sensen, C.W., Suflita, J.M., and Gieg, L.M., 2012, Methanogenic toluene metabolism: Community structure and intermediates. *Environmental Microbiology* **14**(3), 754–764. doi: 10.1111/j.1462-2920.2011.02631.x

[34] An, D., Brown, D., Chatterjee, I., Dong, X., Ramos-Padron, E., Wilson, S., et al., 2013, Microbial community and potential functional gene diversity involved in anaerobic hydrocarbon degradation and methanogenesis in an oil sands tailings pond. *Genome* **56**(10), 612–618. doi: 10.1139/gen-2013-0083

[35] Strąpoć, D., Mastalerz, M., Dawson, K., Macalady, J., Callaghan, A.V., Wawrik, B., et al., 2011, Biogeochemistry of microbial coal-bed methane. *Annual Review of Earth and Planetary Sciences* **39**(1), 617–656. doi: 10.1146/annurev-earth-040610-133343

[36] Kleinsteuber, S., Schleinitz, K.M., and Vogt, C., 2012, Key players and team play: Anaerobic microbial communities in hydrocarbon-contaminated aquifers. *Applied Microbiology and Biotechnology* **94**(4), 851–873. doi: 10.1007/s00253-012-4025-0

[37] Liang, B., Wang, L.Y., Mbadinga, S.M., Liu, J.F., Yang, S.Z., Gu, J.D., et al., 2015, Anaerolineaceae and Methanosaeta turned to be the dominant microorganisms in alkanes-dependent methanogenic culture after long-term of incubation. *AMB Express* **5**(1), 1–13. doi: 10.1186/s13568-015-0117-4

[38] Ji, J.H., Liu, Y.F., Zhou, L., Mbadinga, S.M., Pan, P., Chen, J., et al., 2019, Methanogenic degradation of long n-alkanes requires fumarate-dependent activation. *Applied and Environmental Microbiology* **85**, e00985–19. doi:10.1128/AEM.00985-19

[39] D'Haeze, W., 2002, A hyperthermophilic methanogen sequenced. *Genome Biology* **3**, 1–4.

[40] Ourisson, G. and Nakatani, Y., 1994, The terpenoid theory of the origin of cellular life: The evolution of terpenoids to cholesterol. *Chemistry and Biology* **1**(1), 11–23. doi: 10.1016/1074-5521(94)90036-1

[41] Chikere, C.B., Tekere, M., and Adeleke, R., 2020, Microbial communities in field-scale oil-polluted soil remediation using 16S rRNA amplicon sequencing. *International Journal of Environmental Studies* **78**(3), 410–426. doi: 10.1080/00207233.2020.1817276