

Metagenomics insight into microbial dynamics in field-scale remediation of crude oil-polluted soil

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Abstract

Background: The frequency of crude oil pollution has been on the increase following increased exploration, exploitation and production of energy from fossil fuel. Bioremediation has been shown to be eco-friendly and cost-effective method of oil spill remediation. In the Niger Delta, Landfarming has been the most used technique. The aim of this research was to employ metagenomic techniques to understand microbial dynamics during field-scale remediation in the Niger Delta in order to improve and reduce the time of remediation.

Results: The surface (0.0 – 0.5m) sample had an extractable TPH value of 6231 mg/kg. The subsurface samples from 1m, 1.5m and 2.0m depths had extractable TPH concentration of 4836 mg/kg, 9112 mg/kg and 7273 mg/kg respectively. Proteobacteria dominated the soil microbial profile in all the samples studied as it made up at least 50% of each sample and mostly comprised of the class Alphaproteobacteria with variation only on day 18 and 36 which was mostly dominated by the class Gammaproteobacteria and Betaproteobacteria. Alpha diversity analysis revealed the presence of crude oil in the soil reduced microbial diversity. Principal coordinate analysis showed the microbial structure continually changed following changes in the chemical composition of the soil. *Mycobacterium*, *Burkholderia*, *Rhodoplanes*, *Methylobacterium* and *Bacillus* were the core OTUs detected during the period of remediation. Significant variation in pathway abundance particularly pathways for propanoate degradation, benzoate degradation, naphthalene degradation, fatty acid metabolism, polycyclic aromatic hydrocarbon degradation and degradation of xenobiotics were observed when the unpolluted soil was compared to the samples obtained during remediation.

Conclusions: The findings from this study will greatly advance an already preferred landfarming oil spill recovery technique in the Niger Delta.

Introduction

The anthropogenic release of pollutants particularly crude oil into the environment remains a major environmental challenge. The increasing demand for petroleum products worldwide has led to increased exploration and production with an attendant adverse effect on the environment. Crude oil is a complex mixture of hydrocarbons and other chemical compounds that are considered persistent and toxic to the environment [1]. The release of crude oil into the environment can be during exploration, production, transportation and processing into usable products [1]. Equipment failure, human error and sabotage are mostly responsible for crude oil spills. Some of the major spills include the Amoco Cadiz spillage [2], the Exxon Valdez Spillage [3] and more recently, the Deep water horizon oil spill [4], the Texas city “Y” spill [5] and the Chennai oil-spill in India [6]. In the Niger Delta region of Nigeria where oil exploration began in the 1950’s, the impact of oil activities on the environment has been a sad tale with clear evidence of loss of agricultural land, aquaculture and biodiversity [7, 8].

The natural ability of microorganisms to degrade hydrocarbons has been demonstrated to be cost effective and environmentally friendly. Approaches to bioremediation includes biostimulation and bioaugmentation. Biostimulation involves the optimization of environmental and physicochemical factors in such a way that it favours the proliferation of hydrocarbon degrading microorganisms and thus the removal rate of the oil contaminant [1]. On the other hand, bioaugmentation is the introduction of established hydrocarbon degrading microorganisms to the polluted environment with the aim of improving the rate of degradation [9]. One very viable technique that explores biostimulation is landfarming. A recent study investigated this approach using oil-polluted soil obtained from the Niger Delta and observed significant reduction in the hydrocarbon content after 110 days [10]. Landfarming involves the enhancement of several environmental and chemical parameters to optimise the activities of indigenous hydrocarbon degrading microorganisms. During landfarming, hydrocarbons are lost through biodegradation and volatilization and the process involves regular tilling, dilution of the hydrocarbon pollutant using less contaminated soil and the addition of fertilizers, enzymes and biosurfactants [1]. Manipulating parameters such as nutrients, pH, moisture and oxygen has been demonstrated to reduce the half-life of hydrocarbon removal from contaminated soils [11–13]. It has been severally applied both in the Niger Delta and other parts of the world [12, 14, 15]. In the Niger Delta, the use of landfarming is particularly advantageous because it requires less machinery, the technology can be easily transferred to the local indigenous people, the impact on the environment is less detrimental, it has been found to be very effective in the Niger Delta in reducing the deleterious effect of oil spills and lastly it creates employment opportunities for the locals in the Niger Delta [12].

The advent of high through-put sequencing platforms and major advances in bioinformatics has revolutionised microbial ecology and brought to the fore microbial responses to the presence of pollutants. Since landfarming is an established approach for hydrocarbon remediation in the Niger Delta, understanding the microbial response to landfarming is important in order to further improve the process. It is also important for predicting future microbial and chemical responses during landfarming of oil-polluted soils in the tropical rainforest zone [16–19]. This study is the first in-situ study in the Niger Delta detailing the response of bacterial communities prior to land farming, during-landfarming and post-landfarming of an oil-polluted soil using high-throughput bacterial sequencing techniques. The study also investigated the functional attributes of the bacterial community throughout the entire period of landfarming using a predictive approach. Understanding these events during in-situ field-scale landfarming is vital for predicting bacterial community responses to the presence of hydrocarbons in the Niger Delta and also in optimizing the process of landfarming as a green technology for the recovery other hydrocarbon impacted sites.

Results

Physicochemical analysis

The soil samples obtained for characterization of the spill site showed the concentration of TPH and PAHs at different depths of the soil analysed. The surface (0.0 – 0.5) sample had an extractable TPH

value of 6231 mg/kg. The subsurface samples from 1m, 1.5m and 2.0m depths had extractable TPH concentration of 4836 mg/kg, 9112 mg/kg and 7273 mg/kg respectively. Results of other physicochemical analysis including pH, arsenic, barium, cadmium, cobalt, copper, lead, mercury, nickel, total chromium and zinc are shown in Table 1.

The concentration of polycyclic aromatic hydrocarbons was low and was only detected in the surface sample and the 1m depth samples. The surface sample had a PAH concentration of 0.13 mg/kg while the subsurface sample had PAH concentration of 1.36 mg/kg. The pristine soil TPH was 479.7mg/kg while TPH for days 0, 09, 18, 36 and 56 was 8635.68 mg/kg, 6125.7 mg/kg, 4171.9 mg/kg, 2435.2 mg/kg and 677.2 mg/kg respectively.

Microbial dynamics and abundance during remediation

A total 1,211 bacterial species was detected for all the samples. The bacterial metagenome for all the samples was dominated by Proteobacteria for both oil polluted and unpolluted soils (Fig. 1), however the unpolluted soil harboured the least Proteobacteria abundance (49.7%). The abundance of Proteobacteria continuously increased from day 0 to day 36. Proteobacteria mostly of the class Alphaproteobacteria made up 49.7% of the originally polluted soil sample prior to remediation. On day 0 Proteobacteria alone made up 65% of the soil microbiome and increased to 67%, 69% and 74% on days 09, 18 and 36 respectively. While Alphaproteobacteria dominated on days 0 and 09, a rapid increase to the dominance of Gammaproteobacteria and Betaproteobacteria was observed on days 18 and 36. Alphaproteobacteria later dominated once more on day 56 as the oil concentration declined to below the intervention value (Fig 2.). As the dominance of Proteobacteria increased during the period of remediation, there was also a simultaneous decrease in the abundance of Actinobacteria from 36% prior to intervention by Landfarming to 3.4% on day 56. Apart from Proteobacteria and Actinobacteria, other bacterial phyla's detected includes Firmicutes, Acidobacteria, Bacteroidetes and Planctomycetes. The complete list of the detected bacterial phyla is shown in Fig. 2. The core microbiome detected during the period of remediation include *Mycobacterium*, *Burkholderia*, *Rhodoplanes*, *Methylobacterium* and *Bacillus*. A comparison of the crude oil polluted soils to the pristine soil showed 19 bacterial genera were differentially represented in the polluted soils and they include *Clostridium*, *Bacillus*, *Staphylococcus*, *Lactobacillus*, *Parachlamydia*, *Phenylobacterium*, *Edaphobacter*, *Rhodoplanes*, *Burkholderia*, *Jeotgalicoccus*, *Desulfosporosinus*, *Methylobacterium*, *Desulfotomaculum*, *Acidocella*, *Deinococcus*, *Mycobacterium*, *Propionibacterium*, *Ochrobactrum*, *Azospirillum*.

Diversity analysis following oil spill and during remediation

Analysis of the effect of the oil spill on microbial diversity revealed the presence of hydrocarbons reduced microbial diversity. Effect of hydrocarbons on bacterial diversity was inferred using Chao1 diversity index. The result of our analysis showed the polluted soil samples prior to any sort of intervention was the least diverse (Fig. 3). The hydrocarbon concentration in the soil significantly reduced bacterial diversity compared to the pristine soil which represents the soil true diversity prior to the oil spill. Following the commencement of intervention by Landfarming remediation, the diversity continued changing and

increasing as the concentration of hydrocarbons in the soil reduced. Principal coordinates analysis (PCoA) saw a close clustering of the polluted soil prior to remediation and a wider spread of the polluted soil samples on the PCoA plot (Fig. 4) during Landfarming which is indicative of a continuous changing dynamic of the soil microbiome as different fractions of the hydrocarbons present in the soil were degraded.

Predictive microbial community functional response during remediation

To determine the functional responses of the bacterial community to the environmental stress caused by the presence of the crude oil spill, the GREENGENES classified bacterial species were subjected to further analysis using PICRUST. 6,909 KEGG orthologs were detected for the entire samples. The mean abundance of pathways detected was 427,917.46. The oil polluted samples were all compared to the pristine soil for differentially represented KEGG pathways. Thirty five pathways were found to be differentially represented between the pristine soil the polluted soil prior to Landfarming. The pathways were mainly pathways for hydrocarbons degradation and they include naphthalene degradation, polycyclic aromatic hydrocarbon degradation, benzoate and aminobenzoate degradation pathways among others (Fig. 6a – 6c). As remediation commenced mostly the same predicted pathways remained significantly differentially represented from day zero to day 18 after which a decline in the degradative pathways was observed. On days 36 and 56 only pathway for transporters and pathways for phosphonate and phosphinate metabolism were differentially represented.

Discussion

This study provides an insight on microbial diversity and dynamics during field scale Landfarming of an oil polluted site in the Niger Delta of Nigeria. Landfarming has been the preferred and most used remediation technique for the recovery of oil polluted sites in the Niger Delta. The essence of this study was to provide details on the impact of the intermittent oil discharge in the environment on bacterial diversity and to determine their responses and how it could be exploited to enhanced recovery of the oil polluted sites. Chemical and microbiological properties of the polluted soil was monitored throughout the period of Landfarming to make useful conclusions that will improve the environmental recovery of crude oil polluted soils in the Niger Delta and beyond.

Soil chemical analysis for hydrocarbon concentration revealed the polluted soil required intervention as the obtained soil extractable TPH ranged from 4836 mg/kg to 9112 mg/kg which is above DPR's intervention limit for oil pollution in Nigeria. The soil extractable PAH was below the intervention limit as poly aromatic hydrocarbons was only detected in very minimal concentration and in just two of the oil polluted soil samples. The reason for the low PAH concentration is connected to the properties of the spilled oil as the oil was known to contain a very small fraction of aromatic hydrocarbons. The high TPH prompted the intervention by Landfarming and provided an opportunity to gain an in-situ insight into bacterial community structure, dynamics and response during oil spill in the Niger Delta using high through-put sequencing techniques. As remediation progressed, the soil extractable TPH continued to

reduce in response to the remedial action undertaken during a 56 day period. It was observed that the most decreases in TPH was achieved within the first 36 days of remediation which is typical of soil undergoing bioremediation. A similar observation has been made by several researchers [12, 20]. In this study approximately 72% of the total extractable TPH was removed within the first 36 days of remediation.

Diversity analysis of both the pristine and oil polluted soils showed the environmental stress posed by the release of crude oil into the environment during the spill greatly impacted bacterial diversity and richness. It has been severally reported that the presence of hydrocarbons in the environment reduces diversity and drives a selection of bacterial species with the capacity to degrade hydrocarbons [21–23]. In all the samples analysed prior to and during remediation, the unpolluted soil samples had the most diversity and richness of bacterial species. The diversity continued to change during remediation as different hydrocarbon fractions were removed. This was most represented in the PCoA plot where the samples obtained during the period of remediation spread across different points indicating a changing microbial structure following the depletion of various fractions of hydrocarbons and an overall reduction in crude oil concentration in the soil. [24] also reported the potential influence the degradation of different hydrocarbon fractions in crude oil polluted soils could have in determining the microbial community structure. [25] also studied the influence of reduction in different hydrocarbon fractions during bioremediation on bacterial community dynamics and diversity. Their finding revealed significant changes in microbial structure as remediation progressed.

In this study the phylum Proteobacteria dominated in all the soil samples. Soil samples are known to be mostly dominated by Proteobacteria [26–28] however in crude oil polluted soils they are known to be even more abundant [21, 29]. Alphaproteobacteria dominated the early stages of remediation, however as remediation progressed the abundance of gammaproteobacteria and betaproteobacteria also increased. This finding correspondce with the study of [29] which revealed the dominance of Alphaproteobacteria during the early stages of bioremediation. [25] in another study of microbial community dynamics during bioremediation revealed gammaproteobacteria became dominant after 15 weeks of bioremediation while there was a corresponding reduction in the abundance of alphaproteobacteria, betaproteobacteria and deltaproteobacteria after the 15 weeks of remediation. This phenomenon commonly refered to as “gamma-shift” has been frequently reported during bioremediation and in soils with changing hydrocarbon concentration [25, 30, 31]. A number of studies have also reported gammaproteobacteria to be actively involved in hydrocarbon degradation [25, 32, 33]. The loss of approximately 72% of the ETPH within the period of dominance of gammaproteobacteria is an indication of their role in the degradation of hydrocarbons. This was also confirmed from the results of the predictive functional profile during the period of remediation as the pathways for hydrocarbon degradation significantly increased between day 0 and 18.

Mycobacterium, Burkholderia, Rhodoplanes and Bacillus were the core microbiome detected during this study. All of these organisms have been severally reported to be involved in the degradation of various types of hydrocarbons [28, 34–37] including the degradation of alkanes and aromatic fractions of

hydrocarbons. Most of the organisms found to be differentially represented in the oil polluted soils have also been severally reported to be potential hydrocarbon degraders and thus their presence in the oil polluted soil could be linked to the role they played in hydrocarbon stressed environment.

Predictive functional profiling of the soil samples revealed several pathways involved in the degradation of various hydrocarbon fraction was significantly abundant in the oil polluted soil prior to remediation and during the early stages of remediation. Naphthalene degradation, polycyclic aromatic hydrocarbon degradation, benzoate and aminobenzoate degradation pathways were among the significantly differential pathways in the oil polluted soils. It was observed that the most relevant pathways for hydrocarbon degradation was particularly higher during the early stages of remediation (Day 0–18). This period of our remediation experimentation coincides with the period the most drastic hydrocarbon loss was recorded and it was also within the period there was a major change in bacterial structure from the dominance of Alphaproteobacteria to that of mostly Gammaproteobacteria. The predicted increase in specific pathways responsible for hydrocarbon degradation could be said to be responsible for the major hydrocarbon loss during the early stages of bioremediation. The reduction in abundance of these degradation pathways during the later stages of Landfarming and a subsequent resurgence of Alphaproteobacteria (day 36–56) is also a confirmation of the important role these pathways play during bioremediation. Several studies have employed the use of predictive methods to profile crude oil polluted soils and several other environments [38–41]. Using predictive functional profiling, Bao et al. [38] was able to reveal that majority of differentially detected functional genes responsible for both saturates and aromatic hydrocarbon degradation in their study were associated with oil polluted soils. Bell et al. [16] in another study observed a 4 to 4.5% increase in genes responsible for Xenobiotics Biodegradation and Metabolism in crude oil amended treatments during a remediation experimentation.

Conclusion

The success of bioremediation is dependent on soil microbiome features such as abundance, functional diversity and the soil microbial structure. Hence the understanding of microbial dynamics during remediation is very important for developing more advanced technique for accelerated biodegradation. This study provides an insight into microbial dynamics during field-scale landfarming of crude oil polluted soils. The findings made can be used to design accelerated engineered landfarming processes for the clean-up of oil spills in the Niger Delta and beyond.

Methodology

Sampling and site description

The spill site where this study was conducted is located in *Ibaa community* in Emohua Local Government Area in Rivers State, Nigeria (GPS coordinates E 6.804116; N 4.969581). The spill was first reported on the 8th of May, 2016 and it was as a result of an operational error on the Ibaa Manifold 8" Header at Ibaa

operated by Shell Nigeria LTD. An estimated 304 bbl of crude oil was released into the surrounding environment prompting remediation using the Landfarming technique.

Samples were drawn from 0.0 – 0.5cm, 1m, 1.5m and 2m depths for both metagenomic and physicochemical analysis following the spill. Samples were collected using soil auger and transferred in sterile containers to the laboratory for analysis. As remediation of the spill site commenced, samples were collected on days 0, 9, 18, 36 and 56 to monitor reduction in pollutant concentration and the role of the indigenous microbiome to the changing soil chemistry as well as the bacterial responses to the spill.

Physicochemical analysis

The residual TPH and PAHs were extracted from the soil samples and quantified using gas chromatograph - flame ionization detector (GC-FID) and Gas chromatograph – Mass spectrophotometer (GC-MS), (an Agilent 6890GC, Agilent technologies, Wilmington, USA GC equipped with 5975B MSD and MSD chemstation version D. 03.00), according to the methods of USEPA 8270 and USEPA 8015, respectively. The carrier gas was helium and the column with catalogue number HP-5(19091J-413). Physicochemical parameters such as pH, nitrate, moisture content, phosphate, potassium, electrical conductivity, total organic carbon, zinc, nickel and lead were determined according to methods of APHA 4500 and ASTM D1691, respectively.

Genomic DNA extraction and sequencing

Microbial composition and structure was determined by the extraction and analysis of total genomic DNA and sequencing of 16S rRNA genes (Bacteria and Archaea) from both the hydrocarbons polluted and unpolluted soils. The DNA was extracted from soil samples with a Zymo Research DNA extraction kit (ZymoResearch CA, USA). The primer pairs 27F (51AGAGTTTGATCMTGGCTCAG-31) and 1492R (51GGGTTACCTTGTTACGACTT31) were used for PCR based 16S rRNA gene amplification and sequencing on an Illumina MiSeq platform (Illumina Inc. CA, USA). Sequencing was conducted using v3 chemistry (paired-end 300 bp reads) according to standard protocols.

Sequence processing and analysis

Illumina MiSeq sequences were processed using QIIME v2018.6[42]. Demultiplexed fastq reads was imported into QIIME using the CASAVA 1.8 format for paired-end sequences. Chimeric sequences, marginal sequence errors and noisy sequences were filtered using DADA2 [43]. Dereplicated sequences were further clustered into operational taxonomic units using the GREENGENES database at 97% similarity while employing the VSEARCH open reference OTU picking technique [44]. The clustered sequences were assigned taxonomy using q2-feature-classifier plugin [45] and the Naïve Bayes classifier that was trained on the Greengenes (May 2013 release) 99% OTUs. Alpha and Beta diversity analysis was performed in R using the Phyloseq package [46]. Alpha diversity was calculated by Shannon's diversity index, observed OTUs, Chao1, and Simpson diversity index. Principal coordinate analyses (PCoA) was performed based on Bray-Curtis distance and determination of statistical significant difference in

diversity was performed using Permutational multivariate analysis of variance (PERMANOVA).. Exploration of the core bacterial taxa's in the oil polluted soils as remediation progressed, the determination of differentially abundant bacterial taxa's and the detection of the dynamics of the bacterial community structure was all achieved using the MicrobiomeAnalyst [47].

Functional profile determination

The OTUs classified using the GREENGENES reference database (May, 2013 release) were subjected to functional genes and pathways prediction using PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) [48]. The Kyoto Encyclopaedia of Genes and Genomes (KEGG) was used to obtain the relative abundance of gene families in both the unpolluted soil and the samples drawn throughout the entire period of landfarming remediation, based on a constructed phylogenetic workflow of 16S rRNA marker gene sequences. The classified OTUs were first normalised by copy number by dividing each OTU by the known 16S copy number abundance. The normalised OTUs were then used for metagenome predictions and subsequent collapse of the metagenomes into pathways. The Nearest Sequenced Taxon Index (NSTI) value, was used to validate the reliability of predicted metagenomes and functional pathways. The predicted metagenomes and functional pathways were then subjected to statistical analysis using STAMP to determine differences and similarities in genes and pathway abundance as remediation by landfarming progressed. The binomial test and odds ratio was calculated to determine differentially represented pathways in the unpolluted sample, the polluted samples and the samples obtained during remediation. Benjamini–Hochberg adjusted p-value [49] was calculated to control the false discovery rate (FDR) in multiple testing. Pathways with odds ratio ≥ 1 and FDR corrected p-value ≥ 0.05 were considered significantly enriched, while the significantly over-represented pathways satisfied an odds ratio of ≥ 2 and FDR corrected p-value ≥ 0.05 .

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Datasets generated/analyzed during this study are available in the GenBank repository under the Bioproject accession number PRJNA592287.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

CBC conceived, designed and carried out the work; **CBC, MT and RA** were involved in data analysis and interpretation of data; **CBC, MT and RA** drafted the manuscript and all three authors approved the submitted version.

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Table

Table 1. Physicochemical analysis of the oil pollutes soil prior to remediation

Parameter	0-0.5m	1.0m	1.5m	2.0m	DPR Intervention Value
ETPH (mg/kg)	6231	4836	9112	7273	5000
pH	6.92	-	6.56	-	-
Arsenic (mg/kg)	11.8	-	10.8	-	55
Barium (mg/kg)	204	-	129	-	625
Cadmium (mg/kg)	<2.00	-	<2.00	-	12
Cobalt (mg/kg)	16.4	-	28.8	-	240
Copper (mg/kg)	20.1	-	16.3	-	190
Lead (mg/kg)	22.4	-	14.8	-	530
Mercury (mg/kg)	<1.0	-	<1.0	-	10
Nickel (mg/kg)	35.5	-	36.2	-	210
Total Chromium (mg/kg)	172	-	61.3	-	380
Zinc (mg/kg)	60.4	-	89.1	-	720

* - (Not measured)

Figures

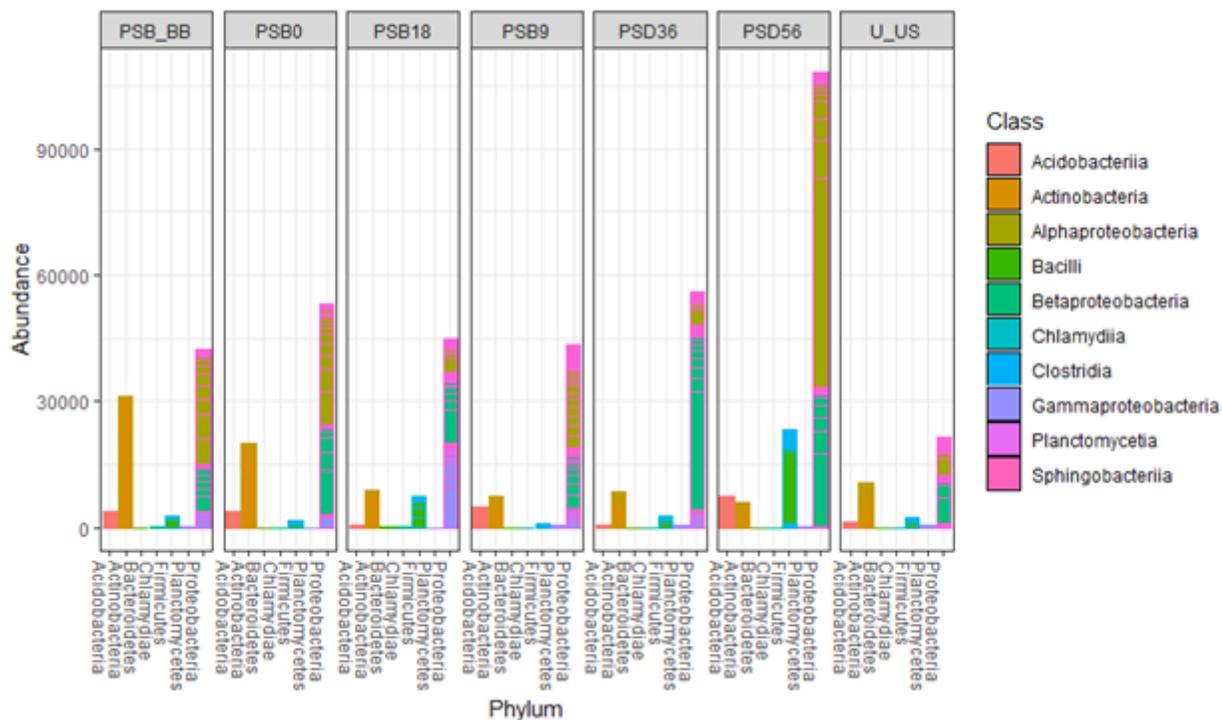


Figure 1

Representation of bacterial abundance across the samples (pristine soil, polluted soils and soils analysed during landfarming) at the phylum level. The bars are coloured according to the 10 most abundant classes that make-up each phylum.

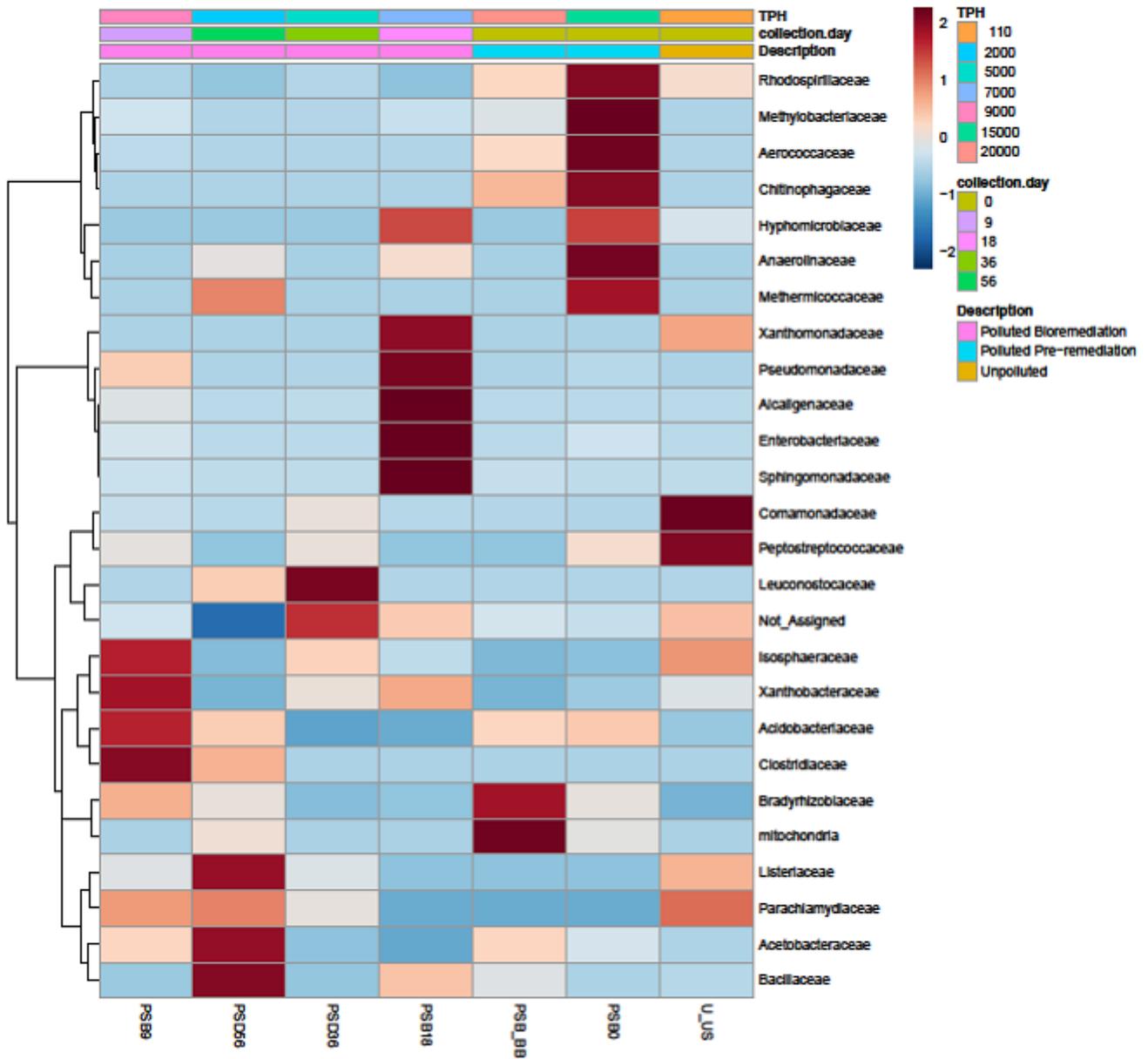


Figure 2

Abundance of the bacterial community at the family level to explore visible changes in the community structure prior to landfarming and during remediation. * PSB_BB = Polluted soil prior to landfarming * PSB0 = Day 0 * UUS = Unpolluted soil * PSB9 = Day 09 * PSB18 = Day 18 * PSD56 = Day 56 * PSD36 = Day 36

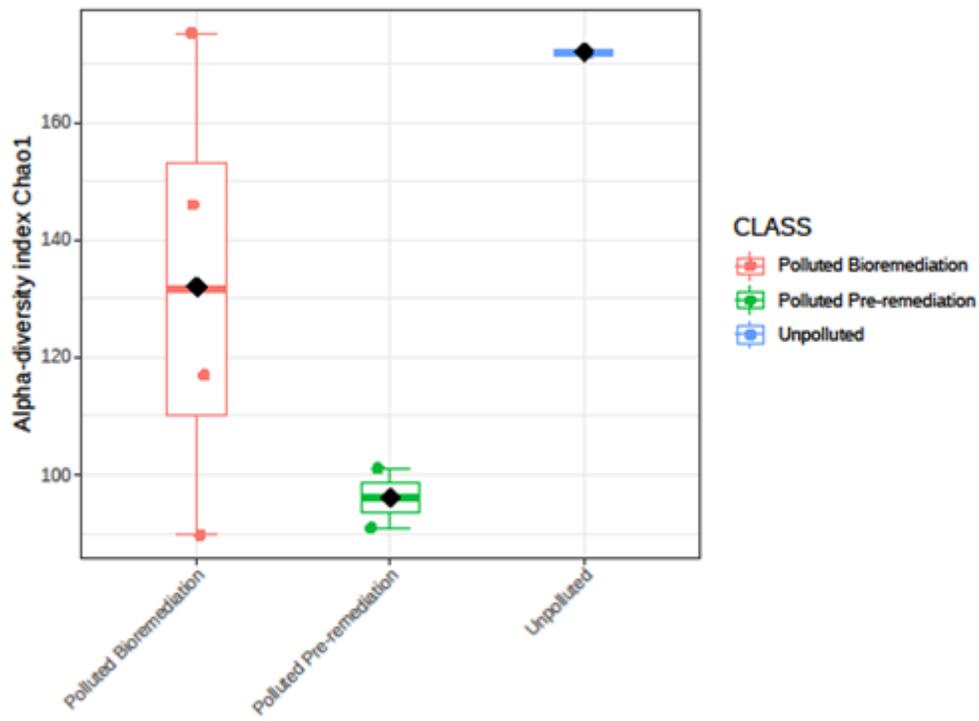


Figure 3

Alpha diversity analysis to visually explore the impact of hydrocarbon stress on the microbial diversity. The box plot are coloured according to pollution status.

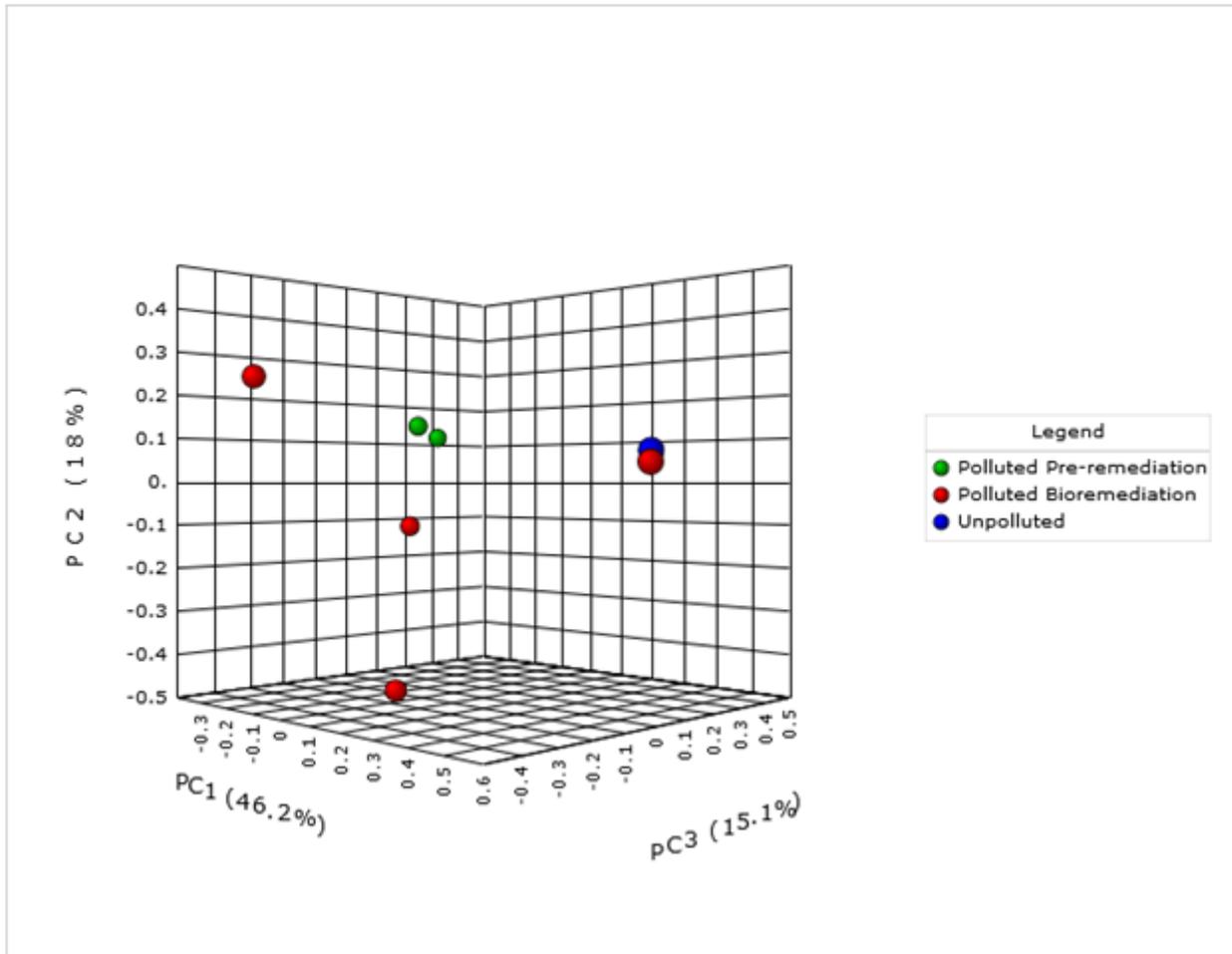
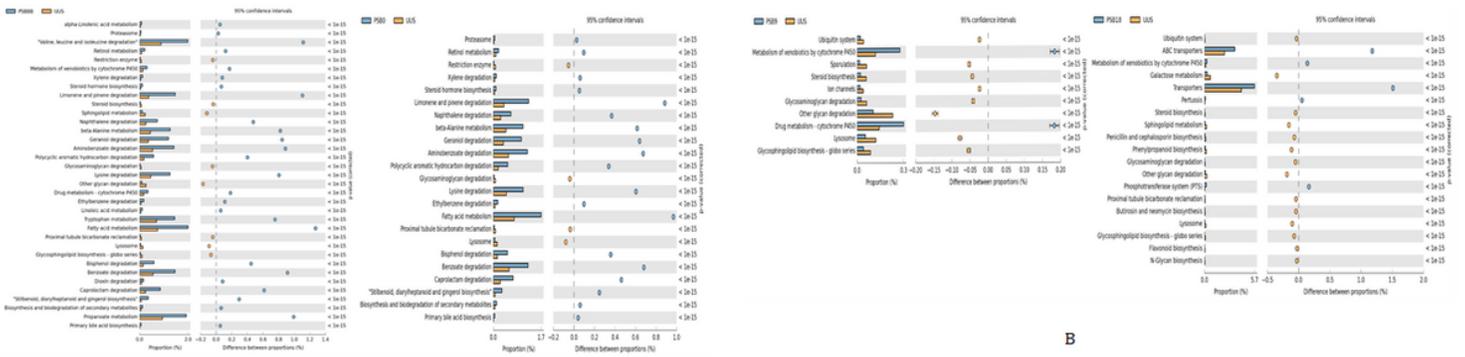


Figure 4

Beta diversity analysis using Bray-cutis distance for all the samples obtained prior to and during remediation.

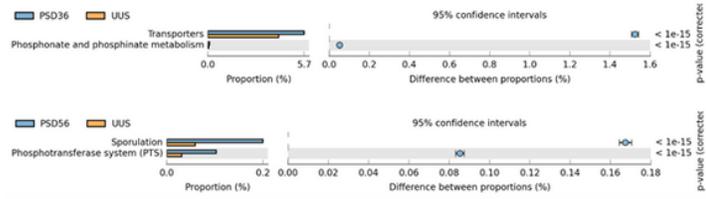
Figure 5 was not provided by the author.

Figure 5



A

B



C

Figure 6

Fig. 6a. Significantly differential functional pathways between the pristine soil, the baseline soil prior to landfarming and the day 0 soil sample when remediation commenced. * PSBBB = Polluted soil prior to landfarming * PSB0 = Day 0 * UUS = Unpolluted soil. Fig. 6b. Significantly differential functional pathways between the pristine soil, the day 09 soil and day 18 soil samples. * PSB9 = Day 09 * PSB18 = Day 18. Fig. 6c. Significantly differential functional pathways between the pristine soil, the day 36 soil and day 56 soil samples. * PSD56 = Day 56 * PSD36 = Day 36