Salt Marsh Bacterial Community Response to the Deepwater Horizon Oil Spill

by

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Thesis submitted in partial fulfillment of
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ABSTRACT

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investigations of the interactions between plant-associated bacteria and the biodegradation of oil spill at other places with a similar ecosystem.

Contents

The original crude oil leaked during the DWH oil spill was the light Louisiana sweet crude oil, which contained a complex mixture of hydrocarbons (Liu et al. 2012). The major oil hydrocarbons contain saturates (e.g., linear, branched, and cyclic alkanes), aromatics (e.g., benzene and PAHs), resins, and asphaltenes (Joye et al., 2016). One study demonstrated that the original crude oil leaked contained approximately 74% saturated hydrocarbons, 16% aromatic hydrocarbons, and 10% polar hydrocarbons (Reddy, 2012). The estimated hydrocarbons contained 15% n-alkanes, 26% branched alkanes, 16% cycloalkanes, 9% alkylbenzenes and indenes, and 3.9% polycyclic aromatic hydrocarbons (PAHs) by weight (Reddy, 2012). Though PAHs only took up a small proportion of the crude oil, they received more attention as PAHs can be both more toxic and bioavailable to animals and plants (Atlas and Hazen, 2011). The low-molecular weight PAHs such as naphthalene, phenanthrene and fluorene were the dominant PAHs in the crude oil. Meanwhile, chrysene, which has a larger molecular weight, was a minor component in the original crude oil (Liu et al., 2012). Different physical and chemical properties of the crude oil components could lead to different ways of transportation and fate as a result of the differences in the weathering processes, such as evaporation, emulsification, dissolution, photo-oxidation and biodegradation (Transportation Research Board and National Research Council, 2003; Liu et al. 2012).

A large portion of the surface oil was transported to the coastal habitats such as beaches and marshes by tides or waves. Consequently, the transported oil contaminants accumulated on the surface or even buried under the sediments of the coastal ecosystems, causing catastrophic and unpredictable impacts on the ecosystems in the Gulf of Mexico coastal regions (Allan et al., 2012).

1.2 Ecological and Economic Impacts in the Gulf of Mexico

The concerns of the public for the devastating impacts of the DWH oil spill on the ecosystems were significant as the coastal habitats of the Gulf of Mexico provide valuable ecological services to the local population by providing food and nursery habitat for a wide variety of fish, birds, crustaceans, plants, other marine species, as well as their associated microbial communities. The ecosystems of the Gulf of Mexico, such as marshes and wetlands, also provide protection for the shorelines from erosion (NOAA-final programmatic). Other valuable ecosystem services that the salt marshes provide, dominated by vegetation such as Spartina alterniflora, are nitrogen cycling and removal that can prevent environmental problems such as eutrophication and shoreline erosion resistance (Tatariw et al., 2018; Schutte et al., 2020; Deis et al., 2019). Salt marshes also have a great capacity to degrade oil (Cagle et al., 2020). However, the DWH oil spill harmed the growth of salt marshes by reducing the photosynthesis, transpiration, or affecting the stem and shoot developments (Lin and Mendelssohn, 2012). Shifts in the

salt marsh vegetation could lead to the shifts in the sediment bacterial community composition, which might lead to further damage to the salt marshes due to possible losses of beneficial symbionts, such as nitrogen-fixing bacteria (Silliman et al., 2012; Kandalepas et al., 2015). For example, for *Spartina alterniflora*, the dominant species in the salt marsh environment, the soil microbial community around it could be changed if the release of root exudates changes, the release of root-associated litter changes, or the release of oxygen from the root, which can change the redox potential, changes (Cagle et al., 2020). Some bacterial endophytes, such as *Bacillus pumilus* and *Pseudomona putida* are known to be capable of promoting the growth of plants, contributing to the degradation of oil hydrocarbons that would increase the environmental stress, and protecting the roots and leaves from the pathogens (Kandalepas et al., 2015; Zheng et al., 2018).

Finally, the Gulf of Mexico coastal habitats also provide over $10 billion per year in revenues by supporting recreation and tourism industries, as well as and commercial fisheries (NOAA-final programmatic; Silliman et al. 2012), and ecological damage could lead to significant economic impact to the region.

1.3 Oil-Degrading Bacteria

Crude oil contains a range of organic chemicals that can cause shifts in the abundance, diversity and community composition of bacteria through factors including

toxic impacts or providing usable carbon substrates for use as energy and carbon sources for growth (Nie et al., 2009).

Biodegradation played an important role in removing the DWH oil spill not only from the water bodies of Gulf of Mexico, but also from the sediments of the deep-sea to coastal beach (King et al., 2015). Though the oil hydrocarbon-degrading bacteria might only make up a small proportion of the microbial community in the marine environments, they are ubiquitous as oil hydrocarbons regularly escape into marine environments from underground reservoirs and seeps (Atlas and Hazen, 2011). While most bacterial oil hydrocarbon degradation occurs under aerobic conditions, some degradation pathways occur under anaerobic conditions, though at a slower reaction rate (Kimes et al. 2014). Some oil hydrocarbon degrading bacteria can mineralize PAHs into carbon dioxide (CO_2), water (H_2O), and biomass completely with dioxygenase and other enzymes (Atlas and Hazen, 2011). Meanwhile, concentrations of elements, such as iron (Fe), nitrate (NO_3-) and phosphate (PO_4^{3-}) can limit the growth of oil hydrocarbon-degrading bacteria, and consequently limit the oil biodegradation rates (Atlas and Hazen, 2011).

In response to the DWH oil spill, multiple oil hydrocarbon-degrading bacteria taxa were enriched rapidly within the deep-water column. For example, early in the oil spill event, the members of the family Oceanospirillaceae , Colwellia genus and

Cycloclasticus genus were dominant in the deep-sea plume, likely because these bacteria have previously been shown to breakdown propane, ethane, and butane (Hazen et al., 2010; Valentine et al., 2010). Two months later, in response to the change in the availability of the oil hydrocarbons, such as the relatively decreased levels of saturated hydrocarbons and the relatively increased levels of aromatic hydrocarbons, the dominant taxa of the bacterial communities changed, causing succession of the dominant members of the bacterial community (Kimes et al., 2014; Hazen et al., 2016). Bacteria such as *Colwellia* and *Cycloclasticus* became dominant. *Colwellia* species could degrade propane, ethane and butane, while *Cycloclasticus* could degrade benzene, toluene, ethylbenzene, xylenes, and some PAHs (Kimes et al., 2014; Hazen et al., 2016). After the well was capped, previously undetected bacteria that can degrade methane, such as *Methylococcaceae*, *Methylophaga*, and *Methylophilaceae*, were increased in abundance (Redmond and Valentine, 2012). Other degraders which could degrade oil hydrocarbons that had a higher molecular weight, such as Flavobacteria, *Rhodobacteraceae*, and *Alteromonadaceae*, also became dominant after the well was capped since the microbial communities were shifted in response to the degradation of spilled crude oil (Hazen et al., 2016). Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes are phyla that are known to contain a great variety of oil-degrading bacteria (Engel et al., 2017; King et al., 2015). There are also over 100 genera of bacterial

genera, such as *Pseudomonas*, *Alcanivorax*, *Altermonas*, *Cycloclasticus*, and *Fusibacter*, that have been identified to contain oil-degrading species (Wang et al., 2020).

1.4 Microbiome Research

In recent years, Next-Generation Sequencing (NGS) technologies have become widespread and the preferable technology for studying complex microbial communities as they have lower cost, lower error rates, generate larger data sets, and enable the characterization of microbial communities that might not be cultivable but viable (Zhang et al., 2018; Chikere et al., 2018). The main principles in NGS involve direct extraction of the biomolecules, such as DNA and RNA, and then sequencing of the biomolecules in a massively parallel fashion (Zhang et al., 2018; Chikere et al., 2018). NGS technologies have been applied to study microbial responses such as the shifts in the bacterial community structure to oil spills in different environments or oil history, such as marine and coastal environments, primarily by studying the 16S rRNA gene. For example, a 454 pyrotag sequencing approach was used to sequence the 16S rRNA genes of the bacterial community from the deep-sea water contaminated with DWH oil spill, and an increase in the abundance of *Colwelliaceae* and Oceanospirillales was observed (Baelum et al., 2012). Later, more NGS technologies, such as Illumina sequencing and Ion Torrent, were used to identify oil-hydrocarbon degrading microbial taxa, such as Oceanospirillales and Proteobacteria, to reveal the most significant changes in response to oil spill, and to

characterize other organisms that might have a functional role in biodegradation (Mason et al., 2012).

1.5 Previous Studies

Since the DWH oil spill blowout, there have been a number of studies investigating the impacts of the oil spill on the microbial communities at different sites along the Gulf of Mexico, including Bay Jimmy and Fort Fourchon, the sites investigated in this work (Kimes et al., 2014; King et al., 2015). By analyzing the changes in the alpha and beta diversity, the community composition, and the association between the fungal communities of leaf, root and soil, Lumibao et al. found that the fungal communities of different sample types (leaf, root and soil) had different responses to oil spill. The leaf fungal community changed significantly in its community composition, and the diversity was lowered significantly, but fungal abundance did not change in response to oil contamination (Lumibao et al., 2018). In contrast, the root fungal communities experienced significant reduction in abundance but the community composition and diversity did not change significantly in response to oil spill. The soil fungal community did not change significantly in either diversity or abundance, while the community composition was different. Lumibao et al. concluded that although the oil spill contributed to the differences in the diversity, abundance and composition of the fungal

communities, the types of the samples (leaf, root, soil) and the sites seemed to contribute more significantly to the changes (Lumibao et al., 2018).

1.6 Objectives

In this study, we examined the long-term impacts of the Deepwater Horizon (DWH) oil spill on the bacterial communities in the oil-polluted soils and associated with plant tissues (leaf and root) of Spartina alterniflora at Bay Jimmy and Fort Fourchon in Louisiana nine years after the spill occurred. The objectives of this study included: (1) to test if the bacterial communities of each sample type had significant differences in the bacterial diversity, abundance, or community composition in response to oil spills at different locations; (2) to test if there was any correlation between sample types (Leaf vs. Root; Leaf vs. Soil; Soil vs. Root) in response to the oil spill; and (3) to identify the most differential abundant taxa that may have implications on persisting oil degradation.

2. Materials and Methods

2.1 Sampling, DNA Extraction, PCR Amplification and 16S rRNA Sequencing

Sequencing data were acquired using the 300 bp paired-end Illumina MiSeq platform. The prokaryotic primers, 819F and 1115Rmod, that target the V5-V6 region of the 16S rRNA gene were used to amplify the bacterial communities. The procedures of performing sampling, DNA extraction, and PCR application conditions were as described previously (Lumibao et al., 2018). There were 130 samples in total, including 40 soil samples, 40 leaf samples and 50 root samples collected from Bay Jimmy and Fort Fourchon in southeastern Louisiana (Fig. 1). Though both Bay Jimmy and Fort Fourchon were contaminated by the DWH oil spill, Bay Jimmy was shown to have a greater oil deposition than Fort Fourchon (Lumibao et al., 2018). At each site, one transect of soil and plant samples was collected from an oil-impacted area, while another transect was collected from a non-oiled area. The leaf and root samples were collected from Spartina alterniflora since this species was among the dominant vegetation at both sites. The soil samples were collected around the root of S. alterniflora and all samples were processed as described in Lumibao et al., 2018.

Figure 1: Map of Sample Collection Sites. Adapted from Lumibao et al., 2018.

2.2 Data Analysis in R

2.2.1 Bioinformatic Analysis

The demultiplexed raw sequencing reads are available publicly in the Sequence Read Archive database (SRA) through the National Center for Biotechnology Information (NCBI) under project number PRJNA597075 (Accession number: from SAMN13662979 to SAMN13662990). The reads generated by the Illumina Miseq platform were first examined for primers and adapters with FastQC (version 0.11.8; Andrew, 2010). The adapters were removed by cutadapt (version 3.3; Martin, 2011). The rest of the statistical analysis was performed in R (version 3.9.2; R Core Team, 2019).

The amplicon sequences were processed using the R package "DADA2" (version 1.12; Callahan et al. 2016), which includes quality filtering, primer trimming, denoising, paired reads merging, and chimera removal. The trimming parameters were as follows: based on the Phred Quality scores, the truncation length (truncLen) was set to 250 bases for the forward reads and 230 bases for the reverse reads. The maximum expected errors (maxEE) parameter was set to 5 for both forward and reverse reads. The rest of the parameters were set as default. After inferring the amplicon sequence variants (ASVs), the merged sequences were used to construct the ASVs table, and then the chimeric sequences were identified and removed (Callahan et al., 2016). After running quality control through the DADA2 pipeline, 15,262,353 sequence reads and 66,002 AVS were

recovered. Sample SBN4S44, which was a soil sample collected from the non-oiled area of Bay Jimmy, was removed from the rest of the analysis because only 8 reads were left.

Taxonomies were assigned by using the Ribosomal Database Project (RDP) naive Bayesian classifier method, which is implemented in the DADA2 package, and the SILVA v132 rRNA reference database (Wang et al., 2007; Quast et al., 2013; Callahan et al. 2016). The ASVs sequence alignment and a maximum likelihood phylogenetic tree construction were done by using PASTA package with default parameters in R (Mirarab et al., 2015; Balaban et al., 2019). A phyloseq object was built for further statistical analysis by combining the ASV table, assigned taxonomy, the built phylogenetic tree, and metadata with the R package "phyloseq" (version 1.30.0; McMurdie and Holmes, 2013).

The ASVs that were either unassigned to a bacterial phylum (23,244 of the ASVs), or assigned to chloroplasts, mitochondria, and archaea (1,132 of ASVs) were removed. 12,905,625 sequence reads distributed across 41,626 ASVs were left. The final dataset contained 129 samples in total, including 39 soil samples, 40 leaf samples, and 50 root samples. Before performing statistical analysis of the bacterial community, the remaining rare taxa were filtered by setting a prevalence threshold that required ASVs to be found in a least five samples, and an abundance threshold that required ASVs to be found with at least 5 sequences per sample (Callahan et al., 2016).

2.2.2 Statistical Analysis

The statistical analyses were separately performed for different sample types (soil, root, and leaf) under each oil spill history. The sequence reads for each ASV were normalized proportionally by dividing the relative abundance of the sequence reads of each ASV by the sum of all sequence reads in the corresponding sample. The normalization was performed using the "transform_sample_counts" function in the "phyloseq" package (version 1.30.0; McMurdie and Holmes, 2014). The relative abundances at family level were plotted by using the "plot_bar" function from the "ggplot2" package (version 3.3.3; Wickman, 2016).

2.2.2.1 Alpha Diversity

Alpha-diversity, or the diversity within a single sample or ecosystem, was measured by the richness and evenness of the sample. By using the constructed ASVs table, Chao1 richness estimator was used to measure richness, which describes the number of species or ASVs in that environment, and the Shannon diversity index was calculated to measure evenness, or the distribution of species (Chao and Chiu 2001; Li et al. 2011). A low Shannon index value indicates that one or few species dominate, while a high value indicates the individuals distribute across all species relatively equally (Callahan et al., 2016; William et al., 2019). These two indexes were calculated by using the "estimate_richness" function in the "phyloseq" package (version 1.30.0; McMurdie and Holmes, 2014). The Wilcoxon rank sum test and global ANOVA tests were

performed by using the "stat_compare_means" function from the "ggpubr" package to test if there was a significant difference in the Shannon index and Chao1 index of the bacterial communities between sample types (soil, leaf, root), locations (Bay Jimmy and Fort Fourchon), and oil history (oiled and non-oiled) (version 0.4.0; Kassambaram, 2018). The analysis of variance (ANOVA) was also performed to test for the differences in bacterial richness and diversity by using the "aov" function in the R "vegan" package (version 2.5-7; Oksanen et al., 2008). The ANOVA was followed by the Tukey Honest Significant Difference post hoc test to determine which variables or interactions between variables (Bay Jimmy vs. Fort Fourchon; Oiled vs. Non-oiled; Leaf vs. Root vs. Soil) contributed to the most significant differences to either the Chao1 index or Shannon diversity index (version 2.5-7; Oksanen et al., 2008).

The Spearman rank correlation test was performed based on the Shannon diversity index to test if there was any diversity correlation in response to oil spill between sample types (Leaf vs. Soil; Leaf vs. Root; Soil vs. Root). The test was done by using the "cor.test" function in the "microbiome" package (version 1.8.0; Lahti and Shetty, 2017).

2.2.2.2 Beta Diversity

To test for dissimilarity in the bacterial community composition between samples from different sites and oil history, non-metric multidimensional scaling plots (NMDS) were constructed by using the Bray-Curtis dissimilarity matrix calculated from

the normalized ASVs table (Callahan et al., 2015; Bray and Curtis, 1957). The function "ordinate" in the "phyloseq" package was used to calculate the distance (version 1.30.0; McMurdie and Holmes, 2014). The 95% confidence intervals ellipses were added to the NMDS plot by using the "stat_ellipse" function in "ggplot2" package (version 3.3.3; Wickman, 2016). Samples that have a smaller dissimilarity values in their bacterial community composition tend to cluster, as compared to the other samples that have a greater dissimilar value (Zane et al., 2017). Site, oil history and sample types were also tested to decide which contributed variations to the observed shifts in the bacterial community composition.

To test if the variations in the bacterial community composition could be explained significantly by oil history or site, permutational multivariate analysis of variance (PERMANOVA) statistics were performed by using the "adonis" function in the "vegan" package to quantify the differences (version 2.5-7; Oksanen et al., 2008). PERMANOVA tests were done through 9999 permutations. The homogeneity of dispersions test was performed by using "betadisper" function to see if there was a significant difference in dispersion that might affect the significance of the PERMANOVA p-value (version 2.5-7; Oksanen et al., 2008)

The Mantel test (Pearson method) was performed by using the Bray-Curtis dissimilarity index to test if there was any significant correlation between sample types (Leaf vs. Root; Leaf vs. Soil; Soil vs. Root) at each site in response to the oil spill. The test

was done by using the "mantel" function in the "vegan" package (version 2.5-7; Oksanen et al., 2008). Canonical correspondence analysis (CCA) was performed to analyze if environmental variables (pH or total PAH concentration) drove the shifts in the soil bacterial community composition by using function "ordinate" in the "phyloseq" package and the "ordiArrowMul" function in the "vegan" package. The significance of the constraints was tested by using "anova" function (version 2.5-7; Oksanen et al., 2008; version 1.30.0; McMurdie and Holmes, 2014).

2.2.2.2 Difference Abundance Analysis

Linear discriminant analysis (LDA) effect size (LEfSe) was used to identify each sample type's potential oil indicator taxa whose relative abundance changed significantly in response to oil spill at each site (Segata et al., 2011). LEfSe analysis was performed based on Kruskal-Wallis test (p-value ≤ 0.05), and the significantly different taxa were ranked by the effect size analysis and represent discriminative features with an LDA score >2.0 (Segata et al., 2011).

DESeq2 package in R was used to identify which ASVs were differentially abundant between groups by using the ASVs table to estimate the variance-mean dependence in raw counts (version 1.26.0; Love et al., 2014). The differential abundance was tested based on the negative binomial Wald test. P-values were adjusted by using Benjamini Hochberg false discovery rate (FDR) correction for multiple comparisons. The threshold was set as p-value ≤ 0.01 to prevent false-positives. The differential abundance

of the ASVs were considered to be significant if the adjusted p-value was less than 0.01, and if the log2FoldChange of the abundance was greater than 2 (McMurdie and Holmes 2014; Love et al., 2014).

3. Results

3.1 Overall Bacterial Community Response

After filtering, 8323 ASVs and 11,277,556 sequence reads from 129 samples were yielded, which accounted for 87.3% of the total number of sequencing reads in the dataset. The mean reads of the soil, leaf, and root samples were 193,751, 83.5, and 74,359 respectively. The samples included 39 soil samples, 40 leaf samples, and 50 root samples. The sequences were assigned to 55 phyla of which the top 10 most abundant across all samples (most raw sequence reads) were: Proteobacteria (50.7%), Bacteroidetes (10.2%), Firmicutes (7.6%), Chloroflexi (4.9%), Epsilonbacteraeota (4.9%), Spirochaetes (4.2%), Planctomycetes (4.0%), Acidobacteria (3.0%), Gemmatimonadetes (1.7%), and Actinobacteria (1.4%). The top 10 most abundant families across all samples were: Lachnospiraceae (6.6%), Desulfobacteraceae (5.5%), Desulfovibrionaceae (4.2%), Desulfobulbaceae (3.6%), Thiovulaceae (3.1%), Desulfarculaceae (2.8%), Spirochaetaceae (2.2%), Marinilabiliaceae (2.0%), Prolixibacteraceae (1.7%), and Thermoanaerobaculaceae (1.6%) (Table. 1). Our data revealed differences in overall abundance as represented by read counts between the bacterial communities of different sample types (leaf, soil, root) from

different sites (Bay Jimmy and Fort Fourchon) with different oil history (oiled and non-oiled) (Fig. 2).

Table 1: Total reads counts and relative abundance of the TOP 10 most abundant taxa at Phylum and Family level.

The diversity of the overall bacterial communities was significantly different according to the three-way ANOVA results across the sample types (Leaf, Root and Soil), but did not differ significantly within sites or oil history (Sample types: $F_{2,117}$=998.588, p-value < 0.001; Sites: $F_{1,117}$=0.325, p-value = 0.570; Oil: $F_{1,117}$=0.012 p-value = 0.913). The subscripts of "F" represent the degree of freedom and the total degree of freedom. Based on the Tukey's Honest significance test of the ANOVA results that compared the diversity pairwise (Leaf vs. Root; Leaf vs. Soil; Soil vs. Root), the diversity between each pair was significant since all p-adj values were less than 0.05. The abundance of bacterial communities was significantly different according to sample types and sites. But they did not differ significantly according to oiling (Sample types: $F_{2,107}$=230.413, p-value < 0.001; Sites: $F_{1,107}$=28.731, p-value = < 0.001; Oil: $F_{1,107}$=0, p-value =1). The Tukey's Honest significance test of the ANOVA results that compared the abundance pairwise (Leaf vs. Root; Leaf vs. Soil; Soil vs. Root) also showed that the abundance between each pair was significant since all p-adj values were less than 0.05.

The bacterial community of each sample type varied in diversity and abundance, regardless of oiling and site (Global ANOVA p-value < 0.01). Regardless of sample types and oiling, there were no significant differences in diversity and abundance between Bay Jimmy and Fort Fourchon (ANOVA: Global p-value (Chao1) = 0.64; ANOVA: Global p-value (Shannon) = 0.89). There was also no significant difference in diversity and abundance between oiled and non-oiled samples, regardless of sample types and

locations (ANOVA: Global p-value (Chao1) = 0.72; ANOVA: Global p-value (Shannon) = 0.81) (Fig. 3A). The bacterial communities of soil samples were the most abundant and diverse as they had the greatest means of Chao1 and Shannon indices, while leaf samples had the lowest mean abundance and richness values (Soil: mean_chao1 = 2314.6±1135.9, mean_shannon = 6.7±0.5; Leaf: median_Chao1 = 4.4±2.0, mean_shannon = 1.1±0.5; Root: mean_chao1 = 311.3±119.0, mean_shannon = 3.8±0.7) (Fig. 3B).

Figure 2: Bacterial community composition. Relative abundance of the top 20 most abundant taxa at the family level. Sequences not classified into any known group were designated as "NA". Abbreviations indicate the sampling site (B = Bay Jimmy, F = Fort Fourchon) and the historic exposure to oil (N = not exposed, O = oil-exposed).

Figure 3: Box plots of by Chao1 richness index and Shannon diversity index of bacterial communities across sample type (soil, leaf, root) at Bay Jimmy and Fort Fourchon. Boxes represent the interquartile range (IQR) between the first and the third quartiles. The interior horizontal black line represents the median value.

The differences in the overall bacterial community composition were tested by PERMANOVA. The sample types, sites and oil history all accounted for significant differences between samples (R^2 sample type = 0.34698, p-value = 0.001; R^2 sites = 0.06478, p-value = 0.001; R^2 oil history = 0.00821, p-value = 0.013). Sample types accounted for the greatest variance (34.7%) to the community composition, while oil history accounted for the least (0.8%). An NMDS plot showed strong clustering based on sample types, suggesting that the community compositions are distinct from one another (Fig. 4A). However, the clustering overlapped when examining the impacts of oiling on the shifts of community composition, suggesting that the oiled samples might have similar community composition to non-oiled samples in general (Fig. 4B).

Figure 4: Non-metric dimensional scaling (NMDS) plots of bacterial communities associated with the sample types, sites and oil history by calculating Bray-Curtis dissimilarity. Symbols indicate the sampling site (B = Bay Jimmy, F = Fort Fourchon), sample types (S = Soil, L = Leaf, R = Root), and oil history (N = Non-oiled, O = Oiled).

3.2 Soil Bacterial Community Response

The top 5 most abundant families of the soil samples at Bay Jimmy under oiled conditions were: Thiovulaceae (7.5%), Desulfobulbaceae (4.4%), Nitrosomonadaceae (3.7%),

Desulfarculaceae (2.5%), and *Desulfobacteraceae* (2.3%). The top 5 most abundant families of the soil samples at Bay Jimmy under non-oiled conditions were: *Desulfobulbaceae* (6.0%), *Nitrosomonadaceae* (4.4%), *Desulfobacteraceae* (3.1%), *Desulfarculaceae* (3.0%), and *Calditrichaceae* (2.0%). The top 5 most abundant families of the soil samples at Fort Fourchon under oiled conditions were: *Desulfobacteraceae* (4.6%), *Desulfarculaceae* (4.0%), *Desulfobulbaceae* (3.3%), *Thiovulaceae* (3.1%), and *Thermoanaerobaculaceae* (3.1%). The top 5 most abundant families of the soil samples at Fort Fourchon under non-oiled conditions were: *Desulfobacteraceae* (5.9%), *Desulfobulbaceae* (4.1%), *Desulfarculaceae* (3.5%), *Thiovulaceae* (3.3%), and *Flavobacteriaceae* (2.8%) (Fig. 2)

There was no observed significant change in the soil bacterial community richness and diversity from oiled and non-oiled environments at both Bay Jimmy and Fort Fourchon (Bay Jimmy: p-value (Chao1) = 1; Bay Jimmy, p-value (Shannon) = 0.5; Fort Fourchon: p-value (Chao1) = 0.97; Fort Fourchon: p-value (Shannon) = 1) (Fig. 3C; Fig. 3F).

The PERMANOVA results suggested that the main factor that contributed to the bacterial community composition changes of the soil samples was the site where the samples were collected as location accounted for 43.2% of the variance in distance (R^2= 0.43161, p value = 0.0001). Meanwhile, oil history only accounted for 4.3% of the variance observed (R^2= 0.04269, p value = 0.0200). The NMDS showed a stronger clustering between location (PERMANOVA $p < 0.05$). By showing the relatively weaker clustering

between oil history, the NMDS plot indicated a separation between oiled samples and non-oiled samples at Bay Jimmy, which might indicate that the bacterial community composition with oil exposure was dissimilar to the bacterial community composition without oil exposure (Fig. 4C). For Fort Fourchon, the separation between oiled samples and non-oiled samples was not as distinct as Bay Jimmy, which might indicate a more similar, though still significantly different, bacterial community composition (Fig. 4C). Though oil history did not significantly change the abundance and diversity of the soil bacterial community, the community composition could still significantly change due to the shifts in the relative abundance of the most dominant taxa between the oiled location and the non-oiled location. ANOVA was also performed and found that the total PAH concentration did not significantly contribute to the changes in the diversity or abundance of the soil bacterial community at each site (ANOVA p-value > 0.05).

A CCA analysis was performed to find relationships between the environmental variables (pH and total PAH concentration) and the sites, and between phyla. It was performed for only the soil samples due to the lack of total PAH concentration data for the root and leaf samples. For the relationship between phyla and environmental variables, pH and total PAH concentration together could explain 6.1% of the total sample variance in the bacterial community differentiation. However, this relationship was not significant (ANOVA: Global p-value = 0.287; pH p-value = 0.904; Total PAH concentrations p-value = 0.106) (Fig. 5A). For the variation relationship between sites

and environmental variables, pH and total PAHs concentration together could explain 8.4 % of the total sample variance in the bacterial community differentiation calculated in CCA which was significant, and the total PAH concentrations contributed significant variation in this comparison (ANOVA: Global p-value = 0.042; pH p-value = 0.573; Total PAHs concentration p-value = 0.015) (Fig. 5B). This CCA indicated that the total PAH concentration was the factor linked to the differences in the relative abundance of the bacterial community across the soil samples from both locations with the length of the gray arrow indicating the strength of correlation. From Figure 5, the total PAH concentrations showed a slightly positive correlation with the bacterial community at Bay Jimmy. This observation might be linked to the fact that Bay Jimmy had been more heavily oiled than Fort Fourchon (Lumibao et al., 2018). At Bay Jimmy, the average total PAH concentrations across the oiled soil samples was 15.6 μg/g and the average pH was 7.148, while for the non-oiled samples, it was 0.8 μg/g and the pH was 7.177. At Fort Fourchon, the average total PAH concentrations across the oiled soil samples much lower at 0.82 μg/g, similar to the values of the unoiled samples at Bay Jimmy. The Fort Fourchon soil pH at oiled sites was 6.887. For the non-oiled samples, the average PAH concentration was 1.1 μg/g with a pH of 6.956.

Figure 5: Canonical correspondence analysis (CCA) plot. The Each axis explains a percentage, indicated in the parentheses, of variability. The arrow lines represent the environmental variables, and the distance between the environmental variables and the phylum/site indicated the strength of correlation. (A): The points with different colors represent different phyla. (B): The points with different colors represent different phyla.

3.3 Leaf Bacterial Community Response

The top 5 most abundant families of the leaf samples at Bay Jimmy under oiled condition were: Burkholderiaceae (36.6%), Bacillaceae (17.6%), Thiotrichaceae (8.7%), Staphylococcaceae (7.9%), and Marinomonadaceae (7.9%). The top 5 most abundant families of the leaf samples at Bay Jimmy under non-oiled condition were: Burkholderiaceae (43.4%), Bacillaceae (18.8%), Thiotrichaceae (10.5%), Rhodocyclaceae (4.6%), and Rhodobacteraceae (1.7%). The top 5 most abundant families of the leaf samples at Fort Fourchon under oiled condition were: Bacillaceae (16.6%), Burkholderiaceae (9.9%), Thiotrichaceae (9.5%), Lentimicrobiaceae (7.4%), and Rhodocyclaceae (6.0%). The top 5 most abundant families of the leaf samples at Fort Fourchon under non-oiled condition were: Bacillaceae (18.7%), Thiotrichaceae (14.5%), Burkholderiaceae (7.8%), Rhodocyclaceae (4.5%), and Pseudomonadaceae (4.4%) (Fig. 2).

The diversity and abundance of the leaf bacterial communities did not change significantly in response to oil history at Bay Jimmy (p-value Chao1 = 0.49; p-value Shannon = 0.28). The diversity of the leaf samples collected at Fort Fourchon also did not have significant differences, but the abundance changed significantly as the oiled samples had a greater mean_Chao1 value than the non-oiled samples (Fort Fourchon: p-value (Chao1) = 0.09; Fort Fourchon: p-value (Shannon) = 0.24; Non-oiled: Mean_Chao1 = 3±1.6; Oiled: Mean_Chao1 = 4.5±2.5) (Fig. 3D; Fig. 3G).

The PERMANOVA results showed that the only factor that contributed significantly to the bacterial community composition changes of the leaf samples was the site. Location accounted 24.5% of the variance in distance ($R^2 = 0.24460$, p value = 0.0001). The NMDS plot showed a strong clustering between site, while there was no distinct clustering of samples based on oil history (Fig. 4D).

3.4 Root Bacterial Community Response

The top 5 most abundant families of the root samples at Bay Jimmy under oiled condition were: Lachnospiraceae (36.6%), Desulfovibrionaceae (17.6%), Desulfobacteraceae (8.7%), Rhizobiaceae (7.9%), and Marinilabiliaceae (7.9%). The top 5 most abundant families of the root samples at Bay Jimmy under non-oiled condition were: Kineosporiaceae (43.4%), Desulfovibrionaceae (18.8%), Thiovulaceae (10.5%), Rhodocyclaceae (4.6%), and Lachnospiraceae (1.7%). The top 5 most abundant families of the root samples at Fort Fourchon under oiled condition were: Lachnospiraceae (16.6%), Desulfovibrionaceae (9.9%), Marinilabiliaceae (9.5%), Desulfobacteraceae (7.4%), and Moduliflexaceae (6.0%). The top 5 most abundant families of the root samples at Fort Fourchon under non-oiled condition were: Lachnospiraceae (18.7%), Desulfovibrionaceae (14.5%), Desulfobacteraceae (7.8%), Marinilabiliaceae (4.5%), and Moduliflexaceae (4.4%) (Fig. 2).

There were no significant changes in the soil bacterial community richness and diversity from oiled and non-oiled environments at either Bay Jimmy or Fort Fourchon (Bay Jimmy: p-value (Chao1) = 0.77; Bay Jimmy, p-value (Shannon) = 0.55; Fort

Fourchon: p-value (Chao1) = 0.59; Fort Fourchon: p-value (Shannon) = 0.45) (Fig. 3E; Fig. 3H).

Based on PERMANOVA, Location contributed most significantly to the bacterial community composition changes of the root samples as the site accounted for 23.1% of the variance in distance (R^2 = 0.23122, p value = 0.0001). Oil history accounted for 3.4% of the variance (R^2 = 0.03389, p value = 0.0152). The NMDS plot also showed a stronger clustering between sites, while the samples also clustered between oiled samples and non-oiled samples within the same site (Fig. 4E).

3.5 Correlation Between Different Bacterial Communities

There was no significant pairwise correlation between the diversity (Shannon diversity index) of sample types (Soil vs. Root; Soil vs. Leaf; Leaf vs. Root) at each site under each of the oil conditions since the p-values obtained from the Spearman's rank correlation test were all greater than 0.05. There was no significant relationship between sample types (Soil vs. Root; Soil vs. Leaf; Leaf vs. Root) at Bay Jimmy, regardless of oil history (Mantel $p > 0.05$). The same was observed for the samples at Fort Fourchon, except for the soil samples and the root samples at Fort Fourchon under non-oiled condition which were significantly different (Mantel r = 0.3668, p-value = 0.017). The Bray-Curtis dissimilarity matrix of the soil samples at Fort Fourchon under non-oiled condition had a correlation with the Bray-Curtis dissimilarity matrix of root samples at Fort Fourchon under non-oiled condition. In other words, under the non-oiled condition

at Fort Fourchon, when samples had an increased dissimilarity in the soil community composition, there was a significant correlation that the samples also became more dissimilar in the root community composition. For those samples with a p-value > 0.05, bacterial composition did not necessarily change in response to the compositional changes in the corresponding sample types.

3.6 Differential Abundance

The ASVs that had a significant differential abundance in response to oil history were identified by performing DESeq2. An ASV was considered to be enriched under the non-oiled condition if the log2fold < -2 and p-value < 0.01. An ASV was considered to be enriched under the oiled condition if the log2fold > 2 and p-value < 0.01 (Love et al., 2014). After comparing the ASVs enriched in oiled condition with the ASVs enriched in the non-oiled condition of the soil samples collected at Bay Jimmy, 52 ASVs were found to be significantly enriched under oiled conditions, while 4 ASVs were enriched significantly under non-oiled conditions (Fig. 6A). As for root samples collected at Bay Jimmy, 11 ASVs were found to be significantly enriched under oiled conditions, while 11 ASVs were significantly enriched under non-oiled conditions (Fig. 6B). For soil samples collected at Fort Fourchon, 204 ASVs were significantly enriched under oiled condition. 50 ASVs were significantly enriched under non-oiled condition (Fig. 6C). As for the Fort Fourchon root samples, 4 ASVs were significantly enriched under oiled condition. 2 ASVs were significantly enriched under non-oiled condition (Fig. 6D). Leaf samples

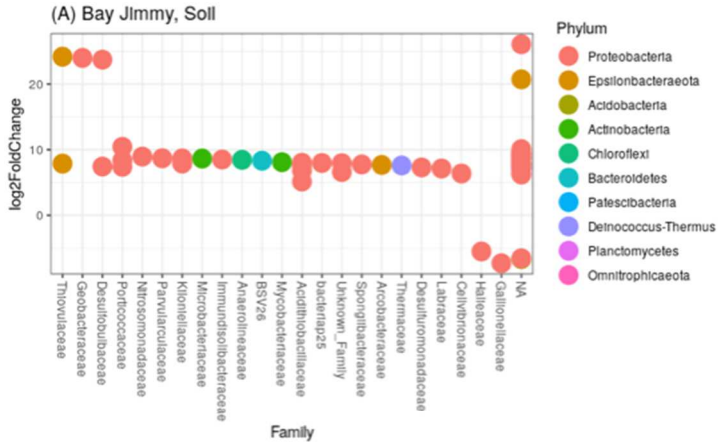
collected at both sites failed to yield ASVs enriched under either an oiled or non-oiled condition, which was also supported by PERMANOVA that oil did not contribute significantly to the variation of the bacterial community (p-value > 0.05). The ASVs with non-assigned family level were extracted from the ASVs table and close relatives were determined using BLAST on NCBI against the nr database. The most significantly enriched ASVs under non-oiled conditions at Bay Jimmy were phylogenetically linked to the families *Gallionellaceae*, Cellvibrionales and possibly *Vicinamibacteraceae* (94% identity) and *Competibacteraceae* (95% identity). The top 5 most significantly enriched soil bacteria enriched under oiled conditions at Bay Jimmy *included Thiovulaceae*, *Geobacteraceae*, *Desulfobulbaceae*, and possibly *Thioalkalibacteraceae* (95% identity) and *Hydrogenimonas* (94% identity).

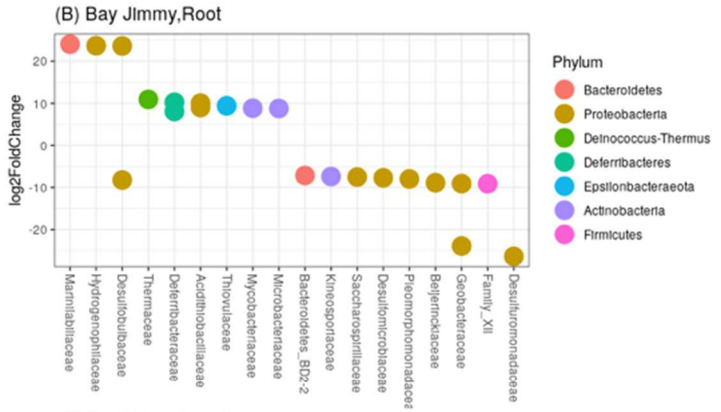
The most significantly different ASVs of root bacteria in abundance enriched under non-oiled conditions at Bay Jimmy were linked to families *Desulfuromonadaceae*, *Geobacteraceae*, *Clostridiales_Family_XII*, *Geobacteraceae* and *Beijerinckiaceae*. The top 5 most significantly different ASVs of root bacteria in abundance enriched under oiled conditions at Bay Jimmy were linked to families *Marinilabiliaceae*, *Hydrogenophilaceae*, *Desulfobulbaceae*, *Thermaceae* and *Deferribacteraceae*.

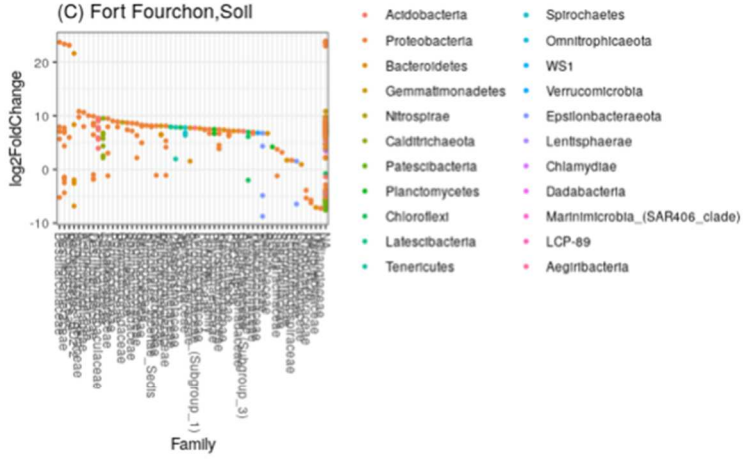
For Fort Fourchon, the top 5 most significantly different ASVs of soil bacteria in abundance enriched under the non-oiled condition were linked to families *Thiovulaceae*, *Haliangiaceae*, *Deferribacteraceae* (91% identity) and *Marinilabiliaceae* possibly. One of the

top 5 ASVs was unassigned and no organisms with high percentage identity (> 90%) could be found by BLAST on NCBI but the corresponding possible family was *Syntrophomonadaceae* (85% identity). The most significantly differential abundant ASVs of soil bacteria enriched under oiled conditions at Fort Fourchon were linked to families *Desulfarculaceae* and the other potential organisms were *Holophagaceae* (95% identity), *Chromatiaceae* (99% identity), and *Thermaceae* (93% identity).

The most significantly differential abundant ASVs of root bacteria enriched under non-oiled conditions at Fort Fourchon were linked to *Desulfobacteraceae* and *Lachnospiraceae*. The top 5 most significantly differential abundant families of root bacteria enriched under oiled conditions at Bay Jimmy were *Lachnospiraceae*, *Desulfuromonadaceae*, and possibly *Geobacteraceae* (92% identity), and *Rhodospirillaceae* (95% identity).


(A) Bay Jimmy, Soil
log2FoldChange
Family
Phylum
Proteobacteria
Epsilonbacteraeota
Acidobacteria
Actinobacteria
Chloroflexi
Bacteroidetes
Patescibacteria
Deinococcus-Thermus
Planctomycetes
Omnitrophicaeota


(B) Bay Jimmy,Root
log2FoldChange
Family
Phylum
Bacteroidetes
Proteobacteria
Deinococcus-Thermus
Deferribacteres
Epsilonbacteraeota
Actinobacteria
Firmicutes


(C) Fort Fourchon,Soil
log2FoldChange
Family
Acidobacteria
Proteobacteria
Bacteroidetes
Gemmatimonadetes
Nitrospirae
Calditrichaeota
Patescibacteria
Planctomycetes
Chloroflexi
Latescibacteria
Tenericutes
Spirochaetes
Omnitrophicaeota
WS1
Verrucomicrobia
Epsilonbacteraeota
Lentisphaerae
Chlamydiae
Dadabacteria
Marinimicrobia_(SAR406_clade)
LCP-89
Aegiribacteria

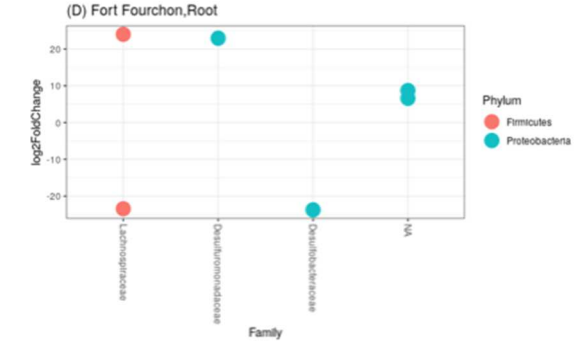

(D) Fort Fourchon,Root
log2FoldChange
Family
Phylum
Firmicutes
Proteobacteria

Figure 6: DESeq2 differential abundance analysis plots. Colored dots represent phylum.

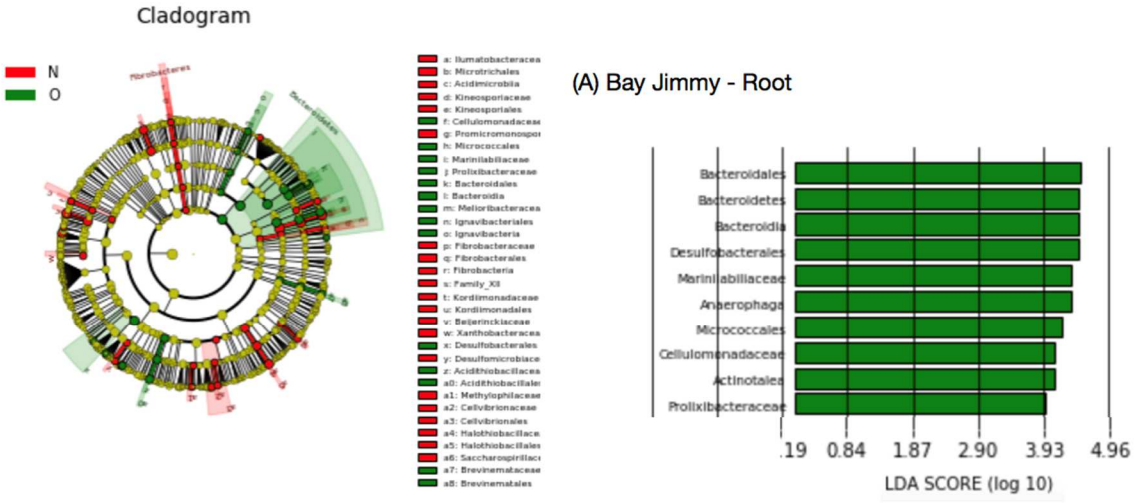
As for the LEfSe analysis, the potential bacterial indicators of oiled and non-oiled conditions were identified by detecting the significant changes in the abundance for taxa at each site (LDA score > 2; p-value ≤ 0.05). For the root samples at Bay Jimmy, 64 unique bacterial taxa (from phylum to genus) with significantly differential abundance were detected, of which 24 unique taxa were detected in the oiled samples and 40 unique taxa were detected in the non-oiled samples (Fig. 7A). The soil samples at Bay Jimmy had a larger number of differential unique taxa where 53 taxa were identified in the oiled samples and 74 unique taxa in the non-oiled samples (Fig. 7B). The LEfSe analysis also revealed that root samples collected at Fort Fourchon had 31 unique taxa with differential abundance in the oiled samples, and 16 unique taxa in the non-oiled samples (Fig. 7C). For the soil samples at Fort Fourchon, 94 enriched unique taxa were detected in the oiled samples, and 50 unique taxa were detected in the non-oiled samples. In general, a total of 202 unique taxa with differential abundance were detected in the oiled samples, and a total of 180 taxa were detected in the non-oiled samples (Fig. 7D). There was no LEfSe results reported for the leaf samples collected at both sites since there was no taxa with significantly different abundance detected.

The top 5 most enriched taxa (LDA score >4) that were most significantly different in abundance in the root sample under oiled environment at Bay Jimmy were: Bacteroidales (Order), Bacterodetes (Phylum), Bacteroidia (Class), Desulfobacterales

(Order), *Marinilabiliaceae* (Family). For the oiled soil samples at Bay Jimmy bacteria were: *Acidithiobacillaceae* (Family), Acidithiobacillales (Order), KCM_B_112 (Family), *Geobacteraceae* (Family), Actinobacteria (phylum). For the oiled root samples at Fort Fourchon: *Campylobacter* (Genus), Campylobacterales (Order), Epsilonbacteraeota (Phylum), Gammaproteobacteria (Class), *Sneathiella* (Genus). For the oiled soil samples at Fort Fourchon the bacteria were: Alphaproteobacteria (Phylum), Betaproteobacteriales (Class), *Nitrosomonadaceae* (Family), *MND1* (Genus), *Xanthobacteraceae* (Family).

The total 202 unique taxa, which were enriched under the oiled condition at both sites, 16 of them belonged to 13 phylum: Acidobacteria (17 taxa), Actinobacteria (10), Bacteroidetes (23), Chloroflexi (1), Entotheonellaeota (5), Epsilonbacteraeota (4), Firmicutes (6), Omnitrophicaeota (5), Planctomycetes (2), Proteobacteria (116), Spirochaetes (3), Tenericutes (8), Verrucomicrobia (2). At the family level for these 202 unique taxa, there were 68 unique assigned taxa and 56 unique unassigned taxa. The taxa with known family assignment included *Acidithiobacillaceae* (8 taxa), *Acanthopleuribacteraceae* (4), *Desulfobulbaceae* (4), *Immundisolibacteraceae* (4), *Kiloniellaceae* (4), *Labraceae* (4), *Melioribacteraceae* (4), *Nitrosomonadaceae* (4), *Porticoccaceae* (4), and *Solibacteraceae*_Subgroup3 (4). The rest of the known families were: *Acidobacteriaceae*_Subgroup_1 (3 taxa), *Burkholderiaceae* (3), *Nitrosococcaceae* (3), *Xanthobacteraceae* (3), *Acetobacteraceae* (2), *Acholeplasmataceae* (2), *Acidiferrobacteraceae* (2),

Balneolaceae (2), *Brevinemataceae* (2), *Cellulomonadaceae* (2), *Cellvibrionaceae* (2), *Clostridiaceae_1* (2), *Desulfuromonadaceae* (2), *Dongiaceae* (2), *Ectothiorhodospiraceae* (2), *Entotheonellaceae* (2), *Flavobacteriaceae* (2), *Geminicoccaceae* (2), *Izimaplasmataceae* (2), *Lachnospiraceae* (2), *Lachnospiraceae* (2), *Marinilabiliaceae* (2), *Mariprofundaceae* (2), *Methyloligellaceae* (2), *Microbacteriaceae* (2), *Mycobacteriaceae* (2), *Omnitrophaceae* (2), *Parvibaculaceae* (2), *Prolixibacteraceae* (2), *Pseudoalteromonadaceae* (2), *Rhizobiales*_Incertae_Sedis (2), *Rhodanobacteraceae* (2), *Rhodomicrobiaceae* (2), *Rhodopirillaceae* (2), *Rikenellaceae* (2), *Sphingomonadaceae* (2), *Spongiibacteraceae* (2), and *Thiohalorhabdaceae* (2). The rest of family all contained one unique taxa (Fig. 8).


Cladogram
N
O
(A) Bay Jimmy - Root
Bacteroidales
Bacteroidetes
Bacteroidia
Desulfobacterales
Marinilabiliaceae
Anaerophaga
Micrococcales
Cellulomonadaceae
Actinotales
Prolixibacteraceae
.19
0.84
1.87
2.90
3.93
4.96
LDA SCORE (log 10)

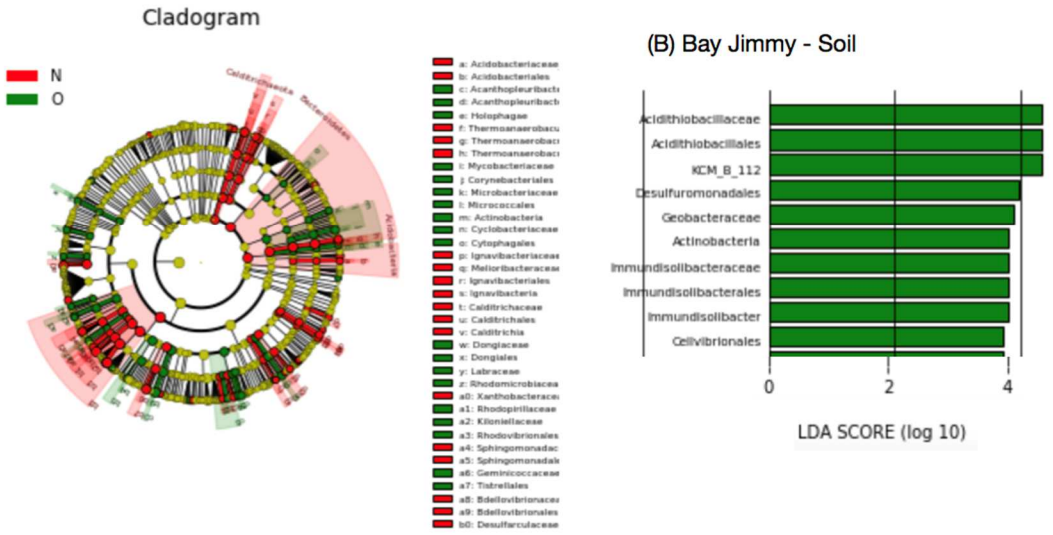
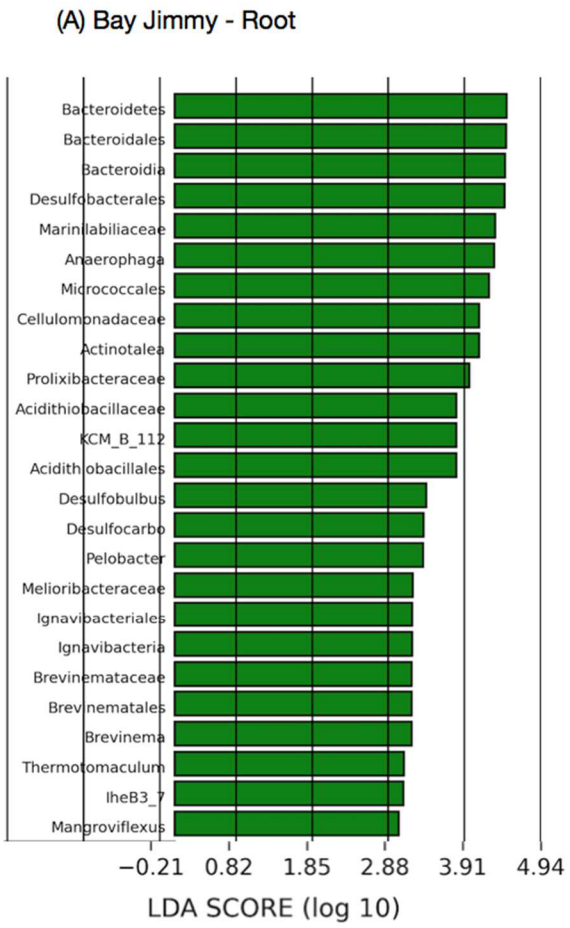
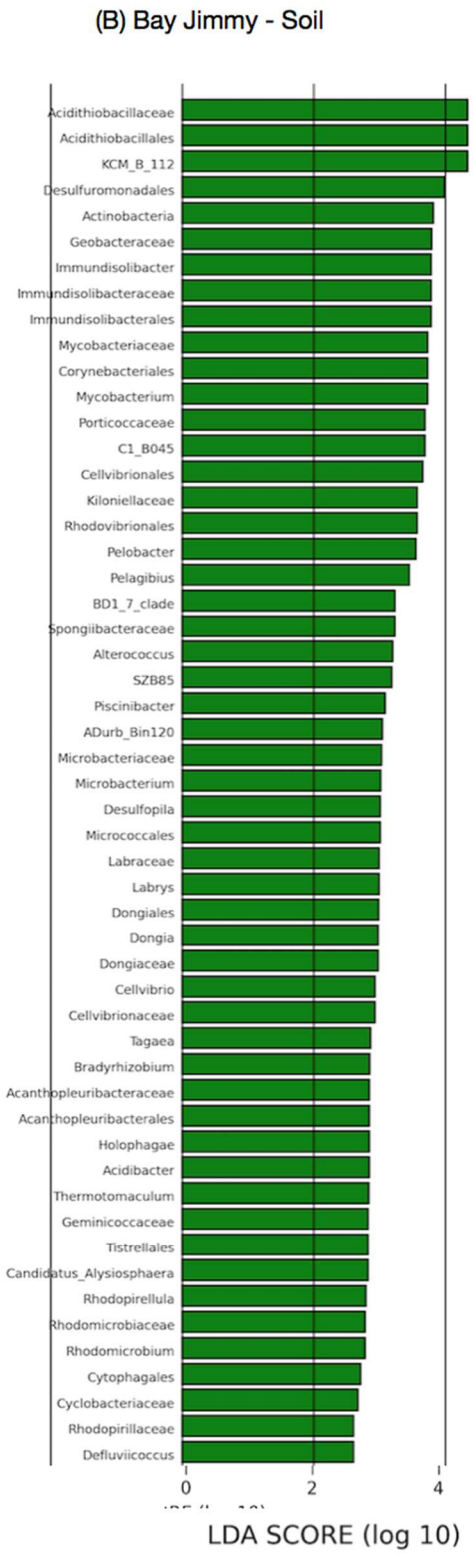
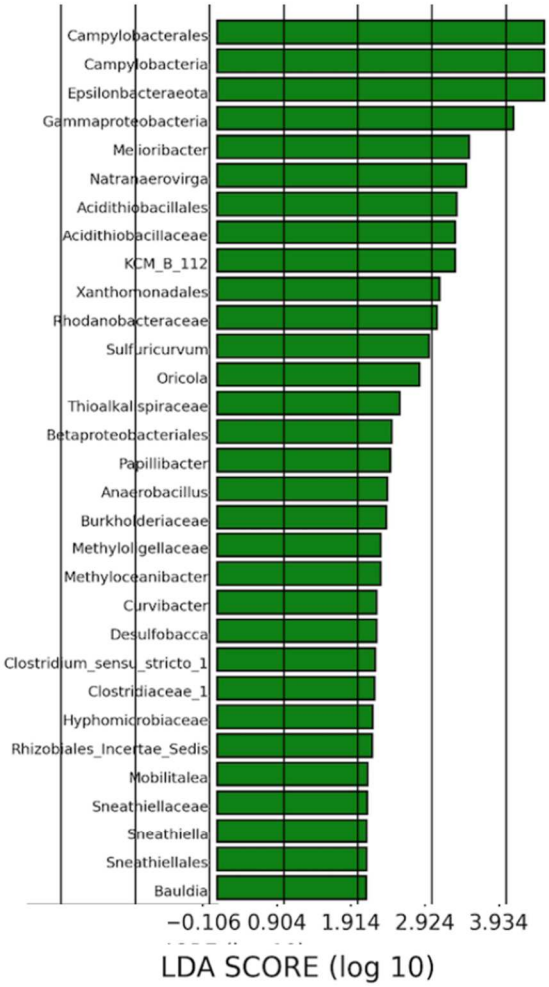

Cladogram
N
O
(B) Bay Jimmy - Soil
Acidithiobacillaceae
Acidithiobacillales
KCM_B_112
Desulfuromonadales
Geobacteraceae
Actinobacteria
Immundisolibacteraceae
Immundisolibacterales
Immundisolibacter
Cellvibrionales
0
2
4
LDA SCORE (log 10)

Figure 7: LEfSe analysis of taxa of sample types (soil and root). Taxa with enriched differential abundance under oiled condition were shown with green. Taxa with enriched differential abundance under non-oiled condition were shown with red. Only the TOP 10 taxa that enriched in the oiled environment, based on LDA score ranking, were shown on profiles on the right. The cladograms indicated the levels of taxonomic classification. The circles from inside-out represented taxonomic assignments from Phylum to Genus. Each point represented a taxa. Taxa that was not differentially different in abundance was marked yellow.


(A) Bay Jimmy - Root
Bacteroidetes
Bacteroidales
Bacteroidia
Desulfobacterales
Marinilabiliaceae
Anaerophaga
Micrococcales
Cellulomonadaceae
Actinotalea
Prolixibacteraceae
Acidithiobacillaceae
KCM_B_112
Acidithiobacillales
Desulfobulbus
Desulfocarbo
Pelobacter
Melioribacteraceae
Ignavibacteriales
Ignavibacteria
Brevinemataceae
Brevinematales
Brevinema
Thermotomaculum
lheB3_7
Mangroviflexus
−0.21
0.82
1.85
2.88
3.91
4.94
LDA SCORE (log 10)


(B) Bay Jimmy - Soil
Acidithiobacillaceae
Acidithiobacillales
KCM_B_112
Desulfuromonadales
Actinobacteria
Geobacteraceae
Immundisolibacter
Immundisolibacteraceae
Immundisolibacterales
Mycobacteriaceae
Corynebacteriales
Mycobacterium
Porticoccaceae
C1_B045
Cellvibrionales
Kiloniellaceae
Rhodovibrionales
Pelobacter
Pelagibius
BD1_7_clade
Spongiibacteraceae
Alterococcus
SZB85
Piscinibacter
ADurb_Bin120
Microbacteriaceae
Microbacterium
Desulfopila
Micrococcales
Labraceae
Labrys
Dongiales
Dongia
Dongiaceae
Cellvibrio
Cellvibrionaceae
Tagaea
Bradyrhizobium
Acanthopleuribacteraceae
Acanthopleuribacterales
Holophagae
Acidibacter
Thermotomaculum
Geminicoccaceae
Tistrellales
Candidatus_Alysiosphaera
Rhodopirellula
Rhodomicrobiaceae
Rhodomicrobium
Cytophagales
Cyclobacteriaceae
Rhodopirillaceae
Defluviicoccus
0
2
4
LDA SCORE (log 10)

(C) Fort Fourchon - Root



(D) Fort Fourchon - Soil

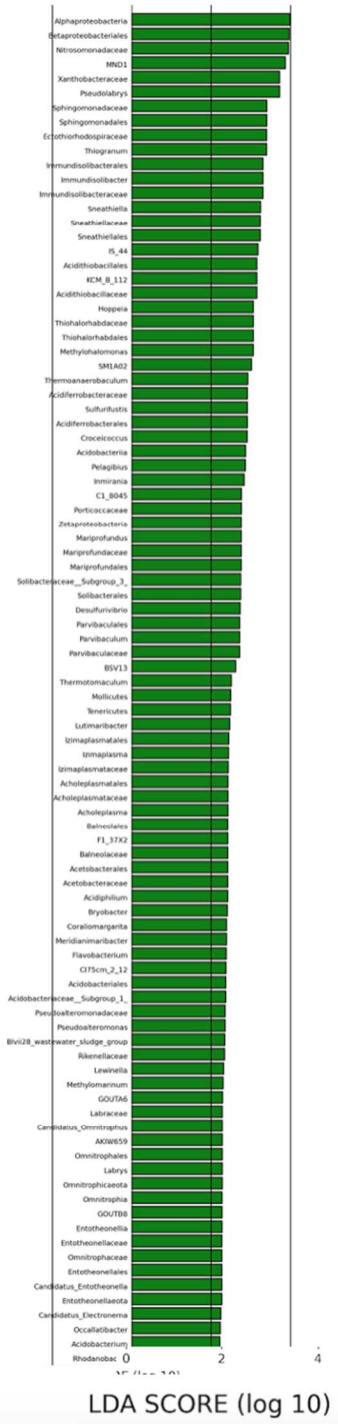


Figure 8: LEfSe analysis with the LDA scores. The green color indicated the bacterial communities which were enriched under oiled condition.

4. Discussion

The aim of this study was to investigate the long-term impacts of oil spill on the leaf, soil and root bacterial communities at Bay Jimmy and Fort Fourchon nine years after the DWH blowout. We found that the bacterial abundance and diversity did not vary significantly under oiled conditions compared to non-oiled conditions over the long term. This result complemented the many studies which have reported a short but strong response of the bacterial community to oil contamination during and shortly after the DWH oil spill event (Kimes et al., 2014; Hazen et al., 2010). These transient effects may be the result of selective advantages on the hydrocarbon-degrading bacteria due to their capabilities of utilizing hydrocarbons for cell growth and energy source, especially aliphatic and aromatic hydrocarbons that have lower molecular weight (Kimes et al., 2014; Hazen et al., 2010). For example, the abundance of one hydrocarbon-degrading bacterial species, Alcanivorax, was found to have a ten-fold increase in the oil-contaminated areas, and made up 10% of the corresponding microbial community by July 2010 (Kimes et al., 2014; Kostka et al., 2011). However, by September 2010, the abundance of this hydrocarbon-degrading bacteria species was less than 1% of the microbial community. Others have also demonstrated that the relative abundance of a hydrocarbon-degrading bacteria decreased as the hydrocarbon levels decreased (Kimes

et al., 2014; Kostka et al., 2011). Additional studies found that the diversity of bacterial communities under the oiled conditions increased at the beginning of the DWH oil spill, but returned back to the level of communities of non-oiled sites as the oil concentration levels decreased over time. This reduction was attributed to oil degradation as measured by the changes in natural organic matter (Atlas et al., 2011; Engel et al. 2017).

In this study, the richness and diversity indices (Shannon and Chao1) of the oiled samples were similar to those of the non-oiled samples within each sample type at each location. This finding suggests the possibility that the accumulated chemical contaminants at the oiled sites of Bay Jimmy and Fort Fourchon have gone through extensive physical, chemical, and biological degradation and that the readily bioavailable hydrocarbons have decreased significantly in concentration. Consequently, the oiled and non-oiled sites did not have a significant difference in the overall concentration of PAHs, with the majority of oil contaminants have been transformed into chemicals that might be less toxic and less-bioavailable to the bacterial communities (Turner et al., 2019; Liu et al., 2012). As a result, the richness and evenness of bacterial communities were not significantly different between oiled and non-oiled sites, as they have potentially recovered from the oil spill after 9 years (Bernhard et al., 2019).

Nonetheless, this study showed that many of the most abundant (top 5) families of bacteria at Bay Jimmy and Fort Fourchon contain putative oil-degrading microbes regardless of oil history, such as members *Desulfobacteraceae*, *Desulfobulbaceae*, and

Desulfovibrionaceae. Each of these families contains strains of anaerobic sulfate-reducing bacteria that have oil-hydrocarbon-degrading capabilities (Matturro et al., 2017; Kümmel et al., 2015; Ellie et al., 2013). This finding might suggest the on-going biodegradation of oil contaminants that are heavier in molecular weight and have been retained in the anaerobic salt marsh sediment, which might contribute to the prevalence of similar oil-degrading bacterial taxa observed at each site (Turner et al., 2019; Silliman et al., 2012). For example, at Bay Jimmy five years after the DWH oil spill, the total hydrocarbon concentration could still be higher than the pre-spill total PAHs concentrations even though almost all *n*-C9-C20 alkanes and two-and three-ring PAHs were already lost (Passow and Overton, 2021).

The presence of some sulfate-reducing bacteria, which might also degrade oil hydrocarbons, at the non-oiled sites might indicate the ubiquity of those bacteria in the coastal and marine environments, such as marine sediment and wetland, since these environments can have a relatively large amounts of sulfate present (Zouch et al., 2017; Cui et al., 2017). The presence of the sulfate-reducing bacterial communities as well as other oil-degrading bacteria, such as members of *Burkholderiaceae* and *Rhodocyclaceae*, might indicate the possibility that there was a natural leakage of oil into the environments (Singleton et al., 2012; Bacosa et al., 2017; Atlas and Hazen, 2011).

Though there was no significant difference in the diversity and abundance of leaf samples between oiled condition and non-oiled conditions overall, leaf samples had the

lowest diversity and abundance among the sample types, regardless of oil history and sites. This observation may be due to a pre-selection of leaf bacterial communities from the prior accumulation of low molecular weight PAHs which could have been previously deposited from the air in different parts of the leaf, such as surface, cuticles and tissue (Kandalepas et al. 2015; Mohammed et al., 2015). As a result, as compared to other plant tissue, the overall diversity and abundance of the leaf bacterial communities may have decreased over time since only few taxa that could metabolize PAHs and their derivatives in response to oil contamination could survive (Kandalepas et al. 2015; Lumibao et al., 2018). The relatively low abundance and diversity of the leaf bacterial communities might continue to be explained by the dry deposition of the airborne PAHs, such as PAHs from the anthropogenic combustion sources (Liang et al. 2017). The low number of sequences recovered from the collected leaf samples might also explain the relatively low abundance and diversity as the bacterial community of leaf might not be as abundant and diverse as soil samples or root samples, regardless of oil contamination, due to plant physiology, or the biotic and abiotic conditions the bacterial communities might experience (Kandalepas et al. 2015; Fitzpatrick and Schneider, 2020; Bodenhausen et al. 2013).

The overall bacterial community composition was significantly variable according to sample types, sites, and oil history, while sample types contributed the greatest variance to the overall bacterial community composition dissimilarity. These

results indicate that for each sample type (Leaf vs. Leaf; Soil vs. Soil; Root vs. Root) between sites, the most abundant taxa of bacterial communities were similar to each other, regardless of oil history. For example, for all soil samples, *Desulfobulbaceae*, *Desulfarculaceae*, and *Desulfobacteraceae* were dominant at each site regardless of the specific oil history (i.e. Non-oiled Bay Jimmy, oiled-Bay jimmy, Non-oiled Fort Fourchon, Oiled Fort Fourchon), though they varied in relative abundance, which might be the result of different selection process due to other site-specific environmental variables (Potts et al., 2019). Proteobacteria, Bacteroidetes and Firmicutes accounted for 68.5% of the relative abundance across all samples.

Though there was no significant change in the abundance and diversity of the bacterial communities in response to oil history, oiling accounted for 0.82% variation in the overall bacterial community composition shifts. The shifts in the root and soil community composition were detected to be significant in response to oiled and non-oiled environment. However, for leaf samples, oiling did not drive the shifts in community composition as none of the bacterial taxa were significantly different between oiled and non-oiled sites. These observations might suggest that the varied composition of bacteria community of different sample types might yield different response to oil contamination since the bacterial communities inhabited in leaf, root, and soil were different. Different bacteria are likely to exhibit different sensitivity to oil contamination. For example, denitrifiers have been shown to be more sensitive to oil

spill than nitrifiers because nitrifiers do not rely on the presence of organic carbon due to their chemoautotrophic classification, while denitrifiers do rely on organic carbon as heterotrophs (Bernhard et al., 2019; Schutte et al., 2020). Similarly, two sulfur-oxidizing families, *Thiovulaceae* and *Desulfobulbaceae*, were among the most dominant taxa in the oiled soil samples at Bay Jimmy, while *Burkholderiaceae* (contains PAH-degraders) and *Staphylococcaceae* (contains PAH-degraders) were among the most abundant ones in the oiled leaf samples, and *Lachnospiraceae* (oil-degrader) and *Desulfovibrionaceae* (sulfur-oxidizing) in the oiled root samples (Li et al., 2018; Kim et al., 1995; Bernhard et al., 2019; Bacosa et al., 2017; Arun et al., 2010).

The bacterial community composition of each sample type was significantly different between Bay Jimmy and Fort Fourchon. Bay Jimmy has been reported to have higher PAHs residue levels than Fort Fourchon, and these two sites might have different site-specific abiotic and biotic conditions that would contribute to the shifts of community composition (Lumibao et al., 2018). For example, the oil hydrocarbons accumulated at Bay Jimmy and Fort Fourchon may be different as those hydrocarbons could have different degrees of weathering before and when they reached the shore of Bay Jimmy and Fort Fourchon potentially due to physical and or chemical degradation processes (Kimes et al., 2014; Allan et., 2016).

5. Conclusion

In this study, the 16S rRNA sequences of the bacterial communities of the leaf, root, and soil samples collected at Bay Jimmy and Fort Fourchon were analyzed to characterize the response of bacterial community to oil contamination 10 years after the Deepwater Horizon blowout. No significant difference in the abundance and diversity of the bacterial community of each sample type between sites and oil history was detected. However, the soil and root community compositions differed significantly between Bay Jimmy and Fort Fourchon, as well as between oiled and non-oiled environments, though the leaf community composition only varied significantly between sites. This study did not characterize the relationship between the bacterial community changes and the interactions with the salt marsh. This study also did not define how site-dependent environmental variables besides the total PAH concentration may have contributed to the shifts in the bacterial community. This study, however, did provide an update on the long-term impacts of oil contamination on the response of the bacterial communities in the salt marsh ecosystem, which may be useful for estimating the long-term bacterial community response to oil spill at other places.

Future studies that combine the quantification of the changes in the soil, root, and leaf bacterial communities with the examination of the oil concentrations in salt marsh sediments 11 years after the DWH oil spill occurred might contribute to the explanation of the similarities in both diversity and abundance of the bacterial

community. Examining the relationship between the oil-hydrocarbons concentrations of the leaf of *Spartina alterniflora* and the shifts in the foliar bacterial community might provide information on the current foliar bacterial community status, which might contribute to the restoration of the ecosystem. Studies that investigate the relationship between the current vegetation states of the salt marshes, such as *Spartina alterniflora*, and the plant-associated bacterial communities might help to characterize the long-term impacts on the recovery of the salt marshes, as well as the recovery of the salt marsh ecosystem.

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