THE INFLUENCE OF SALT MARSH MICROBIAL COMMUNITIES ON THE FOUNDATIONAL SPECIES, *SPARTINA ALTERNIFLORA*, IN AN OILED ENVIRONMENT

AN ABSTRACT
SUBMITTED ON THE 1ST DAY OF JULY 2021
TO THE DEPARTMENT OF
ECOLOGY AND EVOLUTIONARY BIOLOGY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
OF THE SCHOOL OF SCIENCE AND ENGINEERING
OF TULANE UNIVERSITY
FOR THE DEGREE
OF
DOCTOR OF PHILOSOPHY

BY

(Stephen K. Formel)

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ABSTRACT

During the Deepwater Horizon (DWH) oil spill in 2010, approximately 0.5 billion liters (3.1 million barrels) of oil were released into the northern Gulf of Mexico during the largest marine oil spill in history . A significant portion of the released oil was weathered into residues by physical, photochemical, and biological processes prior to landing on 1773 km of coastline, including 754 km of marsh shoreline in Louisiana. Researchers endeavored to describe effects of oil residues in the soil on salt marsh organisms and communities. Many studies focused on two pillars of salt marsh ecology: the microbial communities through which a large portion of the salt marsh food web is connected and *Spartina alterniflora*, a foundational species of Gulf Coast salt marshes. In this dissertation I describe how cryptic, or difficult to observe, elements of salt marsh ecology, like microbial communities and plant genetics, respond to oil residues in the environment. Using a suite of field, growth chamber, and greenhouse experiments I show that these microbial communities are difficult to characterize and may respond to other factors more strongly than they do to oil residues. I present evidence that the plant is resilient to oil in the environment, and changes in its microbiome, but exerts a measurable influence on the biodegradation of oil residues and the microbiome in the soil. This dissertation provides a greater understanding of the complexity of the salt marsh response to an oil spill.

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INTRODUCTION

During the Deepwater Horizon (DWH) oil spill in 2010, approximately 0.5 billion liters (3.1 million barrels) of oil were released into the northern Gulf of Mexico during the largest marine oil spill in history (Barbier 2015). A significant portion of the released oil was weathered into residues by physical, photochemical, and biological processes (Matthew et al. 2016) prior to landing on 1773 km of coastline, including 754 km of marsh shoreline in Louisiana (Michel et al. 2013). Researchers endeavored to describe effects of oil residues in the soil on salt marsh organisms and communities (Bernhard et al. 2019; Deis et al. 2017; Fleeger et al. 2015; Hughes et al. 2018; Lin et al. 2016; Lumibao et al. 2018; McCann et al. 2017). Many studies focused on two pillars of salt marsh ecology: the microbial communities through which a large portion of the salt marsh food web is connected (Teal 1962) and *Spartina alterniflora*, a foundational species of Gulf Coast salt marshes. However, an initial wave of research revealed the need to address considerable deficits in understanding of salt marsh responses to the deposition of oil residues.

The response and contributions of microbial communities were difficult to assess, given our lack of knowledge about their compositions before the arrival of oil residues. Characterization of microbial communities and biodegradation processes was further complicated by burial and redistribution of oil residues via sedimentation and wind-wave action. Despite some evidence of post-spill interactions between oil residues and microbial communities in salt marsh soils (reviewed in Atlas et al. (2015)), questions remain about the consistency of microbial community responses and the extent to which differences in the relative abundance of particular microbes corresponds to a relative

reduction of oil in the environment (Atlas and Hazen 2011; Engel et al. 2017; Lin and Mendelssohn 2012).

Spartina alterniflora showed remarkable resilience to deposition of oil residues, recovering almost to reference levels in less than four years (Lin et al. 2016). Yet very little was known about the cryptic contributions of the plant genome to that resilience, or the relative influence of the plant microbiome on the response. In extreme environments, like a salt marsh, where the soil is saline and hypoxic, plants may be especially dependent on their microbiome for resilience to stress and to respond to toxins in the soil. Preliminary research after the DWH oil spill suggested that *S. alterniflora* growing in areas contaminated with oil depended on its microbiome, as evidenced by a corresponding shift in the microbiome to include more taxa with biodegradation potential (Kandalepas et al. 2015).

Another major post-spill goal was to understand the contributions of microbial communities to biodegradation of oil residues. Many studies focused on the role of microbes in biodegradation of oil residues in seawater and coastal soils (Joye et al. 2014), but few efforts considered the possible interactions of plants with the microbial communities. Recent work has demonstrated the phytoremediation potential of *S. alterniflora* (Cavé-Radet et al. 2020; Hong et al. 2015; Su et al. 2016); it suggests that the plant may be harnessed to accelerate decomposition of oil by selecting for oil-degrading microbial communities in the soil in combination with enhanced delivery of oxygen and plant enzymes to the soil.

Optimizing methods for restoration of oiled salt marshes would be especially meaningful in the Gulf of Mexico, where there is a persistently high threat of petroleum

contamination. Studies of restoration techniques tested after the DWH oil spill also supported re-planting of heavily-damaged coastlines as a means to hasten re-vegetation and reduce erosion (Zengel et al. 2015). Research has also shown that it is possible to inoculate grasses with microbial symbionts to enhance plant growth and function in extreme and toxic environments (Li and Zhang 2015; Márquez et al. 2007; Redman et al. 2011; Yuan et al. 2016). Therefore, a favorable strategy for restoration of oiled sites might include planting *S. alterniflora* with a microbiome that has been pre-selected for an oiled environment.

Dissertation Structure

The initial step necessary for understanding how a catastrophe has affected a community is to describe the response of the community to the disturbance, in this case the oiling received by the salt marshes after the DWH oil spill. For this purpose, in chapter one we describe spatial and temporal differences between salt marsh soil fungal communities at two southern Louisiana salt marshes with contrasting oiling histories. Analyses based on high-resolution, unbiased spatial sampling demonstrated that fungal communities did not align with shoreline classification of oiling less than three years after initial oiling, despite observable differences in oil residues and secondary oiling. In chapter two, we follow this work with a growth chamber experiment which attempts to understand the importance of the initial salt marsh conditions in defining the response of two cryptic salt marsh elements: the *S. alterniflora* root transcriptome and the soil microbiome. We show that bacterial communities and the root transcriptome are defined by the soils in which they are growing, far more than they are defined by the oil content

of the soil. These findings highlight the importance of pre-oil spill samples in describing the response of the plant and microbial communities. Finally, after the DWH oil spill it was clear some sort of restoration effort was warranted. Non-intervention was the recommended response to prevent further damage to fragile marshes (Michel et al. 2013), with the expectation that as time passed, microbial degradation would attenuate the effects of oil residues on marsh ecosystems. In chapter three we build on the work of others by demonstrating that the plant presence also enhances biodegradation of oil residues, supporting the argument that, at least in some marshes, re-planting might be a better strategy than non-intervention. We show that the microbiome of the soil and *S. alterniflora* can be manipulated through soil amendments, but that the manipulations do not influence biodegradation rates or plant morphology.

We have endeavored to describe some of the cryptic elements of salt marsh ecology. We emphasize that the microbial communities are difficult to characterize and may respond to other factors more strongly than they do to oil residues. We present evidence that the plant is resilient to oil in the environment, and changes in its microbiome, but exerts a measurable influence on the biodegradation of oil residues and the microbiome in the soil. This dissertation provides a greater understanding of the complexity of the salt marsh response to an oil spill.

CHAPTER 1

SPATIAL AND TEMPORAL COMPARISONS OF SALT MARSH SOIL FUNGAL COMMUNITIES FOLLOWING THE DEEPWATER HORIZON SPILL

INTRODUCTION

The provisional nature of responses to contamination of coastal marsh ecosystems during the 2010 Deepwater Horizon (DWH) oil spill revealed the need to address considerable deficits in understanding of shoreline remediation and recovery. Approximately 0.5 billion liters (3.1 million barrels) of oil were released into the northern Gulf of Mexico during the largest marine oil spill in history (Barbier 2015). A significant portion of the released oil was weathered into residues by physical, photochemical, and biological processes (Matthew et al. 2016) prior to landing on 1773 km of coastline, including 754 km of marsh shoreline in Louisiana (Michel et al. 2013). Non-intervention was the recommended response to prevent further damage to fragile marshes (Michel et al. 2013), with the expectation that as time passed, microbial degradation would attenuate the effects of oil residues on marsh ecosystems. However, characterization of expected progress was complicated by burial and redistribution of oil residues via sedimentation and wind-wave action. Despite some evidence of post-spill interactions between oil residues and microbial communities in salt marsh soils (reviewed in Atlas et al. (2015)) questions remain about the length and consistency of microbial community responses and

the extent to which differences in the relative abundance of particular microbes corresponds to a relative reduction of oil in the environment (Atlas and Hazen 2011; Engel et al. 2017; Lin and Mendelssohn 2012).

Understanding of microbial degradation of oil in coastal marshes following the DWH spill largely derives from work on soil bacterial communities. The focus on bacteria in part reflects longstanding perspectives that hydrocarbon-degrading prokaryotes are a key element to removal of oil from marine and coastal environments (Joye et al. 2016). There is growing recognition, however, that fungi have the potential to respond to and interact with oil residues. While fungi are generally considered to be less efficient metabolizers of hydrocarbons than bacteria, interactions of fungi with bacterial communities can foster stepwise degradation of hydrocarbons (Atlas 1995; Atlas and Hazen 2011; Head et al. 2006; Joye et al. 2016; Joye et al. 2014; Leahy and Colwell 1990; Matthew et al. 2016; Mendelssohn et al. 2012). And, unlike bacteria, fungi also have the potential to transport and disperse hydrocarbons via mycelial networks (Furuno et al. 2012).

Only a few studies have examined soil fungal communities following contamination from the DWH oil spill. Whereas (at least) nine studies have characterized salt marsh soil bacterial communities relative to oiling from the DWH spill (Atlas et al. 2015; Bae et al. 2018; Beazley et al. 2012; Engel et al. 2017; Looper et al. 2013; Mahmoudi et al. 2013; Marton et al. 2015; Rodrigue et al. 2020; Tatariw et al. 2018), to our knowledge, only two studies have thus far profiled salt marsh soil fungal communities in relation to the oil spill (Lumibao et al. 2018; Mahmoudi et al. 2013) This asymmetry in taxonomic focus is well reflected in the availability of data on soil

microbes in archives of research on the DWH spill. For example, only two datasets were returned in a recent search of the Gulf of Mexico Research Initiative Information and Data Cooperative (GRIIDC, Harte Research Institute, Texas A&M University – Corpus Christi) database for "fungi", compared with 171 datasets that were returned from a search for "bacteria". Accordingly, additional in-depth, assessments of salt marsh fungal communities are warranted to better understand the fate and impact of oil in contaminated salt marshes.

Here we present spatial and temporal comparisons of salt marsh soil fungal communities in two southern Louisiana (LA) salt marshes with contrasting histories of contamination from the DWH oil spill. We profiled fungal communities alongside corresponding measurements of polycyclic aromatic hydrocarbons (PAHs) at a heavily oiled, but remediated, site in Bay Jimmy, (henceforth "Heavily Oiled") and a lightly oiled site in Fourchon (henceforth "Lightly Oiled" (Figure 1-1; Michel et al. (2013)) to assess whether and how fungal responses to oiling are distinguishable from natural heterogeneity. Unlike most other studies of microbial responses to oiling, we did not target plots with evident oil residues. Rather, we sampled across transects with the goal of an unbiased characterization of entire marsh shorelines. Furthermore, we assessed both marsh sites during two distinct seasons following a balanced sampling design. Our analyses probed for relationships between PAHs and alpha diversity, beta diversity, and differential abundances of taxa, with a focus on known hydrocarbon-degraders. Notably, we applied multinomial regression models and used ranked differential abundances to characterize microbial responses to the DWH oil spill. Taking these approaches enabled us to sidestep problematic assumptions about sampling and technical biases that are

typical of microbial analyses based on next-generation sequencing (Gloor et al. 2017). This allowed more confident identification of the taxa that are most differentially abundant between marsh sites and seasons, and thus more informed perspectives on the pervasive effects of primary and secondary oiling events, including detectability relative to natural heterogeneity in salt marsh soil fungal communities. We were also able to determine benchmark sampling thresholds necessary to clearly describe salt marsh soil fungal communities and to infer the merits of judgment-based and systematic sampling designs (Edwards 1998; Smith et al. 2017) to capture microbial community responses to patchily distributed oil residues across marsh shorelines.

METHODS

STUDY SITES

Our study focuses on the two sites described by Kandalepas et al. (2015) and Lumibao et al. (2018). Briefly, our Heavily Oiled site in Bay Jimmy, LA (29°26'37.66" N 89°53'14.74" W) is a salt marsh island dominated by *Spartina alterniflora.* After the site was oiled in June 2010, it served as a test site for shoreline remediation (Zengel 2011), though surface and subsurface oil residues persisted across the shoreline, including the area we sampled that was replanted with *Spartina alterniflora* as part of a separate study on marsh restoration (Bernik et al. 2021; Blum et al. 2014). Our Lightly Oiled site, located at Fourchon, LA (29°08'00" N 90° 08' 43" W), is a salt marsh immediately north of Caminada Headlands beach that is dominated by *Spartina alterniflora* and invading *Avicennia germinans*. At the time of the spill, the site remained largely unoiled, although some oil was deposited along specific locations of the shoreline

by storms in May 2010 (Rodrigue et al. 2020). While both sites are located within the Barataria Basin, the Heavily Oiled site tends to be less saline than the Lightly Oiled site according to historical data from nearby Louisiana Coastal Reference Monitoring Sites (CRMS). From 2006 to 2020, monitoring sites near the Heavily Oiled site had a yearly average of 11.4 ppt salinity (sd = 5.2), with a monthly mean salinity of 7.3 ppt (sd = 4.1) in June and 14.8 ppt (sd = 5.6) in December. Monitoring sites near the Lightly Oiled site had a yearly average 16.5 ppt salinity (sd = 4.1) and 15.2 ppt (sd = 4.8) in June and 18.4 ppt (sd = 4.6) in December. However, these conditions should be taken as broad trends in the general area rather than precise site descriptions.

SAMPLING

We collected a total of 89 soil cores, with 41 taken from the Heavily Oiled site and 48 taken from the Lightly Oiled site during the winter (January) and summer (late June/early July) of 2013. At the Heavy Oiled site, sampling locations corresponded to 22 plots (5 x 5 m) established by Bernik et al. (2021) spanning a 350 m southwesterly shoreline. At the Lightly Oiled site, we sampled soil cores from 24 plots (3 x 3 m) in areas dominated by *S. alterniflora*. Plots ran across two 16 m transects, perpendicular to the shoreline and separated by 12 m of oiled shoreline. Note that sampling differs at the two sites due to the physiography of each site and study designs of previous research. Consequently, the area sampled at the Heavily Oiled site was approximately 5 times greater, constituting approximately 25 times more shoreline than the sampled area at the Lightly Oiled marsh. While this difference in sampling area may affect site level estimates of microbial alpha diversity and beta diversity (dispersion) at the two sites, it

would not be expected to affect estimates of plot level compositional differences, including relative increases in hydrocarbon degrader abundance. Soil cores measured 2 cm in diameter by 6 cm deep. Samples were taken from the approximate center of each plot at both sites. Cores were immediately placed on ice and frozen at -20° C within 24 hours of collection.

OIL ANALYSIS

We analyzed 39 cores sampled from the Heavily Oiled site and 43 cores from the Lightly Oiled site for oil content (Table 1-1). The seven remaining samples were not analyzed because of errors in sample management after completion of DNA extractions. We analyzed PAH content in the bottom 2-6 cm of the cores (Table 1-1). Approximately 10 g of soil was used for extraction of crude oil components for each core. Samples were analyzed with gas chromatography-mass spectrometry (GC-MS) following methods detailed by Curtis (2018) with the exception that samples were mixed with diatomaceous earth, instead of sodium sulfate and magnesium sulfate, to remove moisture prior to accelerated solvent extraction. Four groups of alkylated PAHs were quantified (C1-C4 naphthalenes, C1-C4 phenanthrenes, C1-C3 dibenzothiophenes and C1-C3 chrysenes) based on previous studies of crude oil fate in coastal ecosystems (Collins et al. 2020; Curtis 2018; Rodrigue et al. 2020).

statistical analyses of PAHs

Unless otherwise mentioned we conducted all statistical analyses in R, version 4.0.2 (R Core Team 2020). Analyses and figures depended heavily on the *tidyverse* (Wickham et al. 2019), *cowplot* (Wilke 2020), *compositions* (van den Boogaart et al.

2020), *phyloseq* (McMurdie and Holmes 2013), *vegan* (Oksanen et al. 2016), *data.table* (Dowle and Srinivasan 2020), *genefilter* (Gentleman et al. 2020), *ggrepel* (Slowikowski 2020), *ggpolypath* (Sumner 2016), *ggforce* (Pedersen 2020), *rgdal* (Bivand et al. 2020), and *ggsn* (Santos Baquero 2019) packages.

Differences in total PAH abundance between sites and seasons were modeled in the package *brms* (Bürkner 2017; Carpenter et al. 2017). Based on exploratory analysis of the data, and comparisons to models built on gaussian and gamma distributions without transformation of total PAHs, we selected a log-transformation of total PAHs and a skew normal distribution, with the default priors, as the best fitting model:

log(Total PAHs) ~ Site*Season.

We performed a principal components analysis (PCA) to visualize variation in PAH composition of each sample (not shown) and we tested for differences in PAH composition by conducting a PERMANOVA of Aitchison distances. Aitchison distance accounts for the compositional nature of the data through transforming values into centered log-ratios and then taking the Euclidean distance of sample compositions (Brückner and Heethoff 2017; Pawlowsky-Glahn and Egozcue 2006). We then used the *ecodist* and *geosphere* packages to conduct Mantel tests for an autocorrelation of Aitchison distances of PAHs with geographic distance.

MICROBIOME METAGENOMICS

We analyzed microbial content in the top 2 cm of each core as described in Lumibao et al. (2020). Briefly, genomic DNA was extracted with the Mo-Bio Power Soil

Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's protocol and sent to ACGT Inc. (Illinois, USA) for amplification and sequencing on an Illumina NextSeq 500 platform (Illumina Inc., San Diego, CA) Fungi were targeted by sequencing the ITS1 region of the rDNA using primers BITS/B58S3 (Bokulich and Mills 2013).

sequence trimming and quality filtering

ACGT Inc. demultiplexed and trimmed reads for adapters and quality control with Cutadapt 1.14 (Martin 2011) in paired-end mode. After quality filtering, reads were merged and clustered at 97% similarity into operational taxonomic units (OTUs) with QIIME 1.9.1 (Caporaso et al. 2010) using an open reference method with the UCLUST algorithm (Edgar 2010). UNITE 7.1 was used as a reference database for clustering and subsequent taxonomic assignment (Nilsson et al. 2015). To improve understanding of the influence of rare taxa on analyses, we broadly categorized OTUs as "dominant" if they were represented by ≥ 0.1% of the mean total reads in ≥ 5 samples (approximately ¼ of the number of samples taken at each site and timepoint). Otherwise, OTUs were classified as "rare". These thresholds and binary designations were used to gain perspective on the potential influence of rare taxa on ecological metrics and analyses and were not relied on to assess the contributions of rare taxa to microbial community composition. Thus, while we conducted parallel analyses with the full set of OTUs and a set without rare taxa to understand the robustness of our inferences, we hereafter focus on analyses based on the complete set of OTUs. Results of the analyses based on the subset without rare taxa are available upon request. All sequences were deposited in NCBI

GenBank under the BioProject accession PRJNA603629.

statistical analyses of microbial alpha diversity

We estimated the alpha diversity of each site and time point with three methods to circumscribe possible sources of error.

(1) We used a traditional approach in which abundance-weighted alpha diversity is calculated for each sample (plot), and then grouped by site and time point for statistical comparisons. This approach gives a sense of the variation in diversity across a site but not the diversity at the site level. Diversity was calculated with functions from the vegan package (Oksanen et al. 2016).

(2) We estimated abundance-weighted diversity at the site-level by pooling the OTUs of each sample within a site or time point prior to estimating diversity. This approach captures site level richness but may inflate the unevenness of other diversity metrics. Values for the abundance-weighted method should be interpreted as the number of equally abundant species that would yield the equivalent diversity to the observed diversity for that group of samples.

(3) We estimated diversity as OTU incidence (i.e., presence/absence) within and across samples for each site and time point. Here, values should be interpreted as the number of equally frequent species (i.e., occurrences in all samples) needed to yield the equivalent observed diversity of the samples being considered. For example, diversity would be equivalent to richness if the number of samples in the group is equal to one.

Here, site richness is captured as well as the frequency of occurrence across samples. This gives a sense of how evenly taxa are shared by plots across the site.

Diversity estimates for the second and third methods were executed through the R package iNEXT (Chao et al. 2014; Hsieh et al. 2016). Variation was calculated as 95% CI from 1000 bootstraps of a "bootstrap community", with a sample size equal to the total number of reads in the group of samples (Chao and Jost 2015).

Because detection and importance of rare species in hyper-diverse microbial communities can be uncertain, we also calculated diversity with each of these methods for Hill numbers of order q = 0, 1, and 2. The orders estimate the effective number of species when rare species are weighted with the same consideration as species richness (q = 0), Shannon diversity (q =1), and Simpson diversity (q = 2).

We generated rarefaction curves with the third method to estimate the number of soil cores needed to accurately estimate site-level diversity. We constructed 95% confidence intervals (CI) by bootstrapping the estimates 1000 times and considered any two points along the curve to be significantly different at the 5% level if the confidence intervals did not overlap.

Lastly, we used the first method to look for a relationship between the alpha diversity and PAH content of each sample. Based on exploratory analyses, we relied on the package brms to fit untransformed data to "skew normal" distributions with default priors.

statistical analyses of microbial beta diversity

We calculated multivariate pseudo-standard error (MultSE) to estimate the sampling thresholds necessary to characterize community composition according to Bray-Curtis dissimilarity values. MultSE measures variability in the group centroid as a function of the number of samples in the group and can be interpreted like rarefaction curves. The mean variability in the position of the sample centroid is estimated by permutation and the error bars are 95% CI, estimated by bootstrapping. Both estimates were based on 10,000 iterations, implemented with code derived from Anderson and Santana-Garcon (2015) that was optimized for efficiency by Jon Lefcheck (https://github.com/jslefche/multSE).

We assessed whether Bray-Curtis dissimilarity values of community composition differed by site, time, and total PAH abundance with the *adonis* (PERMANOVA) function in the *vegan* package. We performed post-hoc PERMDISP tests to determine whether differences were due to shifts in community heterogeneity or composition (Anderson and Walsh 2013), with corroboration from a non-metric multidimensional scaling ordination (NMDS). We also used the *vegan* package to conduct Mantel tests of autocorrelation between community composition and geographic distance. Additionally, we tested for a linear relationship between community composition and PAH abundance via a distance-based redundancy analysis (dbRDA), with significance determined by the ANOVA-like permutation test implemented in *vegan*. For all of the aforementioned tests, we evaluated the potential influence of relative abundance and composition metric on community composition by running parallel analyses with Jaccard index and Aitchison distance values instead of Bray-Curtis dissimilarity values.

statistical analyses of differentially abundant taxa

We identified differentially abundant taxa with the software Songbird (Morton et al. 2019) using default filtering parameters. Songbird relies on multinomial regression to estimate a log-ratio of taxa (i.e., differentials) within an assemblage as a function of explanatory variables. The transformation liberates comparisons of relative abundances of taxa from the bias of microbial DNA load in each sample and centers the information around zero. The multinomial model avoids the problematic assumptions of independence or normality while addressing zero-inflation, which is a common feature of microbial community datasets. The most extremely ranked OTUs are those that have changed the most, relative to the average taxa, in reference to the explanatory variable. Importantly, a log-ratio of zero does not necessarily indicate that the absolute abundance of the taxon did not change. It only indicates that it did not change relative to the average microbe in the data (Morton et al. 2019). We selected models by visual comparison of model fits and the Q^2 statistic generated by the QIIME2 (Bolyen et al. 2019) implementation of Songbird. Q^2 , which is functionally similar to the measure R^2 used in standard linear regression, is calculated as 1 – (avg. absolute model error / avg. absolute baseline model error). Therefore, a Q^2 value close to 1 denotes a high predictive accuracy of microbial composition by the model, whereas a value close to zero (including negative values) indicates low predictive accuracy and/or overfitting.

Lastly, we conducted a literature review to derive support for determinations of hydrocarbon-degrading taxa, regardless of inferred associations with PAHs. Drawing on several prior reviews (Atlas 1981; Blasi et al. 2016; Cerniglia 1997; da Silva et al. 2003; Furuno et al. 2012; Hashem et al. 2018; Kirk and Gordon 1988; Müncnerová and

Augustin 1994; Prenafeta-Boldú et al. 2018; Prince 2010; Verkley et al. 2004), we compiled a list of fungal genera with species described as being associated with hydrocarbons or having the ability to metabolize hydrocarbons.

RESULTS

OILING HISTORY AND PAH WEATHERING RATIOS

We did not find a difference in PAH abundance or PAH composition in our soil cores between sites or seasons (Figure 1-1, 1-2, Table 1-2, 1-3). Approximately 80% of our samples contained 1-10 µg/g of total PAHs, which is comparable in magnitude to content observed in a coincident survey of other sites oiled during the DWH spill (Turner et al. 2019). The remaining samples contained one to two orders of magnitude more PAHs. We found that higher molecular weight 4-ring C1-C3 chrysenes constituted a relatively small proportion of detected residues compared to other PAHs, especially 2- and 3-ring phenanthrenes, which is suggestive of secondary oiling. Notably, within-site distribution of PAHs did not align with survey-based observations of where oil was most heavily deposited as a consequence of the DWH spill (Figure 1-1C, 1-1D). We expected oil residues at the Heavily Oiled site to progress along a gradient of high to low abundance from the west to east end of the island, but we instead recovered a heterogeneous distribution. At the Lightly Oiled site, we found oil residues further into the marsh than expected considering that deposition was observed at the midpoint of the shoreline. There was no evidence for spatial autocorrelation between Aitchison distance of PAHs in each sample and geographic distance according to a Mantel test.

SEQUENCING AND DIVERSITY

After filtering, we retained a total of 1,326,461 putative fungal reads (mean = 14,904, median = 7923) corresponding to 194 OTUs. Alpha diversity estimates at the plot-level (Method 1) suggested that, on average, a single sample would capture about 1/3 of the site-level richness. Plots did not differ in diversity between site or time point, except for estimates of richness between the sites during the second time point (Figure 1-3A). At the site level (Methods 2 and 3), The Lightly Oiled site was more diverse than the Heavily Oiled (i.e., no overlap of 95% confidence intervals) during the first time point, but only for the second Hill order. However, the difference in diversity between the sites increased at the second time point as alpha diversity at the Lightly Oiled site increased in diversity, relative to the first time point and alpha diversity at the Heavily Oiled site decreased relative to the first time point.

Analyses of the minimum number of samples needed for statistical consistency at Hill order q =2 indicate that all sites and seasons required a minimum of 5-6 samples for diversity estimates to fall within the 95% confidence intervals of the full set of samples. At order q = 1, 8-9 samples were required to reach the same threshold. At order q = 0, 12-15 samples were required to consistently estimate species richness (Figure 1-3B, Table 1-4). Filtering out rare OTUs did not reduce these thresholds. We also found that 17-20 samples were necessary to arrive at Bray-Curtis dissimilarity estimates that were consistent with the maximum number of samples per group according to the MultSE metric (Figure 1-4A, Table 1-8).

We detected patterns of change in the composition of fungal communities by site and season. According to NMDS ordinations and PERMANOVA tests, community

composition differed strongly by site and season based on Bray-Curtis dissimilarity (Table 1-6), Jaccard index and Aitchison distance values (results not shown). Group dispersions only differed (Table 1-6) for sites when measured according to Jaccard index and Aitchison distance values. Site and seasonal differences in community composition are well illustrated by the NMDS ordination of Bray-Curtis dissimilarity values (Figure 1-4B). Despite finding evidence of geographical structure between sites, we did not detect any within-site spatial autocorrelation that was consistent across Bray-Curtis dissimilarity, Jaccard index, and Aitchison distance values (Table 1-7).

DIFFERENTIAL RANKING OF FUNGI

A total of 111 OTUs met the filtering criteria for differential ranking. Of these OTUs, 68% (77 OTUs and 57% of reads) were Ascomycota and 18% of OTUs were Basidiomycota (20 OTUs and 12% of reads). Approximately 13% of OTUs (15 OTUs and 30% of reads) could not be assigned to a phylum. We designated 97 of the 111 OTUs to be "dominant" OTUs, constituting 85% of the Ascomycota OTUs, 95% of the Basidiomycota OTUs, and 86% of the unassigned OTUs. For all three groups, the dominant OTUs represented 99% of the reads of each group.

We considered OTUs to be strongly associated with a site or season when recovered within the extreme deciles of site and season rankings (e.g., Figure 1-5). Eleven dominant OTUs were strongly associated with the Heavily Oiled site and 12 were strongly associated with the Lightly Oiled site. Nine dominant OTUs were associated with winter sampling and 12 were associated with summer sampling. Only one OTU was associated with the Heavily Oiled site and also strongly associated with summer, while

two OTUs were associated with the Lightly Oiled site and summer. No OTUs were strongly associated with a single site and winter sampling.

Members of the Dothideomycetes were strongly associated with both sites whereas OTUs assigned to Sordariomycetes were strongly associated with the Heavily Oiled site (Figure 1-5). Of the 11 OTUs associated with the Heavily Oiled site, 9 could be identified to genus: *Didymella* (2 OTUs), *Buergenerula, Gibberella, Hasegawazyma, Kohlmeyeriopsis, Paraconiothyrium, Rhodotorula, Scedosporium*. Nine of the 12 OTUs associated with the Lightly Oiled site could be assigned to genus: *Teratosphaeria, Aureobasidium, Coniothyrium, Erythrobasidium, Naganishia, Phaeosphaeria, Rhodotorula*. OTUs assigned to Dothideomycetes and Agaricomycetes were associated with both seasons, whereas OTUs assigned to Eurotiomycetes were strongly associated with summer sampling (Figure 1-5) and members of the Sordariomycetes were primarily associated with winter sampling. We were able to assign nine of the 12 OTUs associated with summer sampling to genus, which included: *Aspergillus* (4 OTUs), *Penicillium, Phanerochaeate, Symmetrospora, Paraconiothyrium,* and *Erythrobasidium*. Only four of the nine OTUs associated with winter sampling could be assigned to the following genera: *Exserohilum, Ceriporia, Schizophyllum,* and *Alternaria*.

RELATIONSHIPS BETWEEN OIL ABUNDANCE AND MICROBIAL COMMUNITIES

Linear regression did not support a relationship between total PAH abundance and alpha diversity (Table 1-5). Likewise, dbRDA and PERMANOVA did not detect a relationship between total PAHs and fungal community composition beyond the

influence of site and season (ANOVA-like permutation test on margins, results not shown).

We also found that total PAH abundance added negligible predictive power to Songbird models of differential ranking. A model of *site + season* had a Q^2 of 0.157, and the addition of *total PAHs* reduced the Q^2 to -0.05. Visual inspection of Songbird models indicated overfitting of all models that included PAHs, whether PAHs were included as the abundance of all PAHs, individual classes of PAH, or log-ratios of 2- and 3-ring PAHs to chrysenes.

Although differential ranking did not recover a relationship between differential abundance of OTUs and total PAHs in our data, we recovered literature-based indications of differential associations of known hydrocarbon-degrading taxa with sites or seasons that might be legacies of the oil spill (Figure 1-5). We compiled a list of 196 fungal genera with species described as being associated with hydrocarbons or having the ability to metabolize hydrocarbons (Atlas 1981; Blasi et al. 2016; Cerniglia 1997; da Silva et al. 2003; Furuno et al. 2012; Hashem et al. 2018; Kirk and Gordon 1988; Müncnerová and Augustin 1994; Prenafeta-Boldú et al. 2018; Prince 2010; Verkley et al. 2004). From this list we identified 17 taxa present at our two sites, including: *Acremonium, Alternaria, Aspergillus, Aureobasidium, Candida, Chaetomium, Cladosporium, Colletotrichum, Coniothyrium, Exophiala, Kluyveromyces, Paraconiothyrium, Penicillium, Phanerochaete, Rhodotorula, Scedosporium,* and *Trichoderma.* Of these 17 genera, *Aspergillus, Candida, Paraconiothyrium, Penicillium, Phanerochaete, Rhodotorula* have been shown to interact with one or more of the four PAH classes detected at our study sites.

Differential ranking of hydrocarbon degraders showed that *Rhodotorula lamellibrachiae*, *Paraconiothyrium variabile* and *Scedosporium minutisporum* were more abundant at the Heavily Oiled site than the Lightly Oiled site. On the other hand, *Aureobasidium pullulans*, *Rhodotorula mucilaginosa,* and a *Coniothyrium* sp. were more abundant at the Lightly Oiled site (Figure 1-6A). Only one dominant OTU (an *Alternaria* sp.) fell into the decile strongly associated with winter sampling, while 6 OTUs (representing 4 species) were strongly associated with summer sampling. These included *Aspergillus niger*, *Aspergillus subversicolor*, *Penicillium citrinum, Paraconiothyrium variabile,* and a *Phanerochaete* sp. (Figure 1-6B).

DISCUSSION

Our findings shed further light on how salt marsh soil fungal communities responded to oiling from the DWH oil spill. We detected clear evidence that fungal community composition differed according to site and season three years after the spill. Several fungal genera were associated with seasonality. Notably, neither alpha nor beta diversity could be explained by variation in oiling, though we did recover evidence of seasonal associations of known hydrocarbon degrading taxa, where some genera were relatively more abundant during the summer than winter. Our unbiased approach to sampling demonstrated that the shoreline classifications of oiling did not necessarily reflect the distribution of oil residues on a scale that is meaningful to microbial communities. Additionally, our findings suggest that previous studies have severely under-sampled marsh soils for the purposes of describing differences in alpha and beta diversity of microbial communities.

SAMPLING AND CHARACTERIZATION OF OILING

We found that the oil content of our samples did not differ by site or season (Figure 1-1), which was unexpected given the stark differences in oiling history of our two study sites. Finding comparable oil content despite notable differences in oiling history likely in part reflects secondary transport of oil and differences in the stability of the areas we sampled at each site. At the Heavily Oiled site, we sampled plots that were originally located landward of areas that were subject to initial oil deposition. Over time, however, the distance separating most of the plots from open water declined, due to wind-wave driven shoreline erosion (Bernik et al. 2021). At the Lightly Oiled site, a small strip of oil residues was known to have been deposited on the outer meter of a more stable shoreline. Thus, we can surmise that we captured secondary oiling (i.e., redistribution of lighter PAHs by water or air) of the sampled areas at each site. This inference is supported by the relatively low amounts of chrysenes found in each sample compared to PAHs like phenanthrenes.

We found evidence of persistent patches of oil residue at the Heavily Oiled site although there were no overall differences in PAH content between the two study sites. This was reflected in several of our samples that exhibited relatively high PAH content, comparable in magnitude to levels measured at the site during surveys conducted in 2011 and 2012 (Zengel et al. 2015). Evidence of patchiness at the Heavily Oiled site is consistent with findings from a regional survey of marsh shorelines (Turner et al. 2019) indicating that oil residues were initially patchily distributed but became more evenly redistributed over time. Findings from the summer sampling of our Heavily Oiled site

closely resembles those from samples taken from nearby Bay Batiste in June 2013 by Turner et al. (2019) in terms of time, geography, oiling history, erosion rates (McClenachan et al. 2013; Zengel et al. 2015), and sampling regime. Notably, we detected much greater variability (i.e., patchiness) in the total abundances of PAHs in our samples, some of which may be due to differences in site conditions like the extent of wind-wave action and shoreline remediation (Zengel et al. 2015). It also may reflect differences in the scale of shoreline transects that were sampled at each site; we sampled about one-eighth of the shoreline length that Turner et al. (2019) sampled at Bay Batiste. It is thus possible that both sites exhibited comparable oiling, but that it was better captured via more intensive sampling of a smaller area (i.e., at our study site). Nonetheless, the frequency of residual oil patches was relatively low at both sites, raising the possibility that we characterized the response of microbial communities to conditions adjacent to or between heavily oiled patches.

OILING AND FUNGAL COMMUNITY COMPOSITION

In addition to finding that alpha and beta diversity of soil fungi differed between heavily and lightly oiled salt marshes, we detected evidence of site-specific seasonal effects. We expected to observe higher site-level alpha diversity at the Heavily Oiled site in part because the area sampled was larger than the area sampled at the Lightly Oiled site (Lomolino 2000). We also expected both sites to become more diverse in the summer, reflecting longer days, warmer temperatures, and higher productivity (Dybala et al. 2015; Shimadzu et al. 2013; Tonkin et al. 2017).

Our findings were only partially consistent with expectations. Overall, alpha diversity of fungi was greater in the Heavily Oiled site during the winter. While alpha diversity at the Heavily Oiled site declined from winter to summer, it appears that there was a slight increase in diversity at the Lightly Oiled site. And, while variation in community composition was smaller at the Lightly Oiled site as expected, it did not differ by season (Figure 1-4B). It is important to note that we did not detect relationships between PAHs and fungal diversity using traditional analyses like linear regression and dbRDA. This suggests that any possible influence of PAH abundance on fungal diversity was not measurable three years after the oil spill. An important caveat, however, is that our approach to sampling yielded comparatively few samples with a high abundance of oil, which could have constrained our statistical analyses of relationships with soil fungal communities.

Comparison of our results to those of a related study (Lumibao et al. 2018) suggests that the observed differences in seasonal changes in fungal diversity between our study sites might be a reflection of oiling history. Lumibao et al. (2018) found the Lightly Oiled site to be more diverse than the Heavily Oiled site during the summer of 2016. Additionally, Lumibao et al. (2018) found that fungal diversity was lower at the Heavily Oiled site relative to a nearby reference site, unlike fungal diversity at the Lightly Oiled site, suggesting that differences in seasonal variation in diversity reflects differences in oiling at the two sites. It is important to recognize, however, that some of the observed differences in fungal diversity might also be related to variation in other site-specific factors like vegetative productivity or facilitation of marsh function by invertebrates that were affected by the oil spill (Deis et al. 2017; Fleeger et al. 2019;

McCann et al. 2017). Observed differences in soil fungal diversity might additionally reflect disturbance regimes, as the Heavily Oiled site was more disturbed by the oil spill and subsequent remediation efforts. The Heavily Oiled site is also subject to greater wind-wave action (Bernik et al. 2018; Kandalepas et al. 2015). Further study is thus warranted to better understand the relative importance of oiling versus other factors like disturbance and productivity to gain broader perspectives on drivers of soil fungal diversity.

DIFFERENCES BETWEEN SITES AND SEASONS

Our findings offer further evidence of site-specific variation in fungal community structure (Figure 1-4B). Consistent with our observations, Lumibao et al. (2018) also detected clear differences in community composition between the same study sites, noting that fungal communities are likely shaped by geographically variable drivers such as hydrology and salinity. It is possible, however, that the observed changes in community composition are attributable to differences in biotic factors. For example, the strong association of certain Sordariomycete OTUs with the Heavily Oiled site but not the Lightly Oiled site (Figure 1-5C) might be related to differences in plant litter turnover between the two sites (Benner et al. 1984; Garzoli et al. 2015).

We found some notable departures from the overall pattern of site-specific compositional differentiation. For example, we found Dothideomycetes to be diverse and ubiquitous. While this is consistent with their cosmopolitan nature (Ohm et al. 2012), prior work by Bik et al. (2012) found a higher relative abundance of Dothideomycetes in oiled samples. Differential ranking, which allowed us to determine the consistency of

change across the class rather than examining aggregated differentials, illustrated that OTUs assigned to Dothideomycetes were evenly distributed across the rankings of sites and seasons (Figure 1-5A, 1-5B), indicating that most were fairly common across both sites and seasons. Only certain Dothideomycetes, like *Paraconiothyrium* and *Phaeosphaeria* appear to exhibit meaningful differences in abundance between sites and seasons. As a counterexample, OTUs classified as Agaricomycetes (Figure 1-5D) were strongly associated with one season or the other; none were ubiquitous. While this suggests that extremely ranked OTUs are indicators of local conditions and thus deserving of closer inspection, it is possible that the prevalence of Dothideomycetes changes over time, which is suggested by the earlier timing (i.e., relative to the onset of the DWH oil spill) of the samples collected by both Bik et al. (2012) and Mahmoudi et al. (2013). Some additional clarity might be gained through greater phylogenetic resolution (Joye et al. 2016) and employment of methods like multinomial regression to elucidate OTUs and representative DNA sequences, which can mask differences in community composition.

Far less is known about seasonal variation than spatial variation in soil fungal communities (Averill et al. 2019), especially in salt marshes. Members of the genera strongly associated with one or the other season have been detected as saprotrophs or endophytes in salt marshes worldwide (Calado 2016; Calado et al. 2019; Dini-Andreote et al. 2016; Kim et al. 2014; Mavrodi et al. 2018; Newell 2003; Walker and Campbell 2009). Intriguingly, the genera that ranked as most differentiated during the summer (Figure 1-6B) are phylogenetically linked to hydrocarbon degraders (Prince 2010; Valentín et al. 2006; Verkley et al. 2004). Thus, while the increased seasonal prevalence

of these taxa is not incontrovertible evidence of greater hydrocarbon metabolism, it does suggest that hydrocarbon degraders are dynamic members of salt marsh soil fungal communities.

SAMPLING AND CHARACTERIZATION OF MICROBIOTA

We were able to determine benchmark sampling thresholds necessary to clearly describe variation in soil fungal diversity and composition that can occur across marsh shorelines. We found that estimates of alpha and beta diversity were not stable until at least 5-15 samples were included in analyses, depending on the metric of interest (Figure 1-3, 1-4, Table 1-4). This stands in stark contrast to the design of most prior studies of microbial responses to oil exposure from the DWH spill, which have relied on fewer than three samples to characterize microbial communities at a given site and time point (Atlas et al. 2015; Bae et al. 2018; Beazley et al. 2012; Bik et al. 2012; Engel et al. 2017; Looper et al. 2013; Mahmoudi et al. 2013; Rodrigue et al. 2020; Tatariw et al. 2018). Furthermore, our results indicate that multinomial regression (like most general linear models) is more robust when based on at least 10 replicates per group. This suggests that future work should involve collecting that many or more samples per site to estimate alpha and beta diversity, particularly when multinomial regression is being conducted for identification of differentially abundant taxa.

CONCLUSIONS

Several long-term studies of oil spill outcomes have noted that redistribution of oil (Engel et al. 2017; Kim et al. 2017; Shigenaka 2014) can confound understanding of site contamination and recovery. Our study builds on this idea by demonstrating that local heterogeneity in soil microbial communities can similarly confound measurements of ecological diversity. Thus, steps must be taken to overcome both challenges to clearly describe the relationship between two noisy variables: oil residues and microbial diversity. Our work indicates that this potentially can be achieved through high-resolution structured (i.e., transect or plot-based) spatiotemporal sampling (Engel et al. 2017) that is complemented by targeted sampling of evidently oiled areas (Atlas et al. 2015; Looper et al. 2013; Mahmoudi et al. 2013). As others have recognized (Engel et al. 2017), a combined approach may be especially warranted for salt marshes, where residual effects of oil spills can be particularly difficult to capture and follow over time.

AVAILABILITY OF DATA AND MATERIAL

Data are publicly available through the Gulf of Mexico Research Initiative Information & Data Cooperative (GRIIDC) at https://data.gulfresearchinitiative.org (UDI: R5.x286.000:0013). DNA sequences are stored on NCBI GenBank under the BioProject accession PRJNA603629.

Code availability

Code used in analysis is located at: https://github.com/sformel/S2

CHAPTER 1 FIGURES

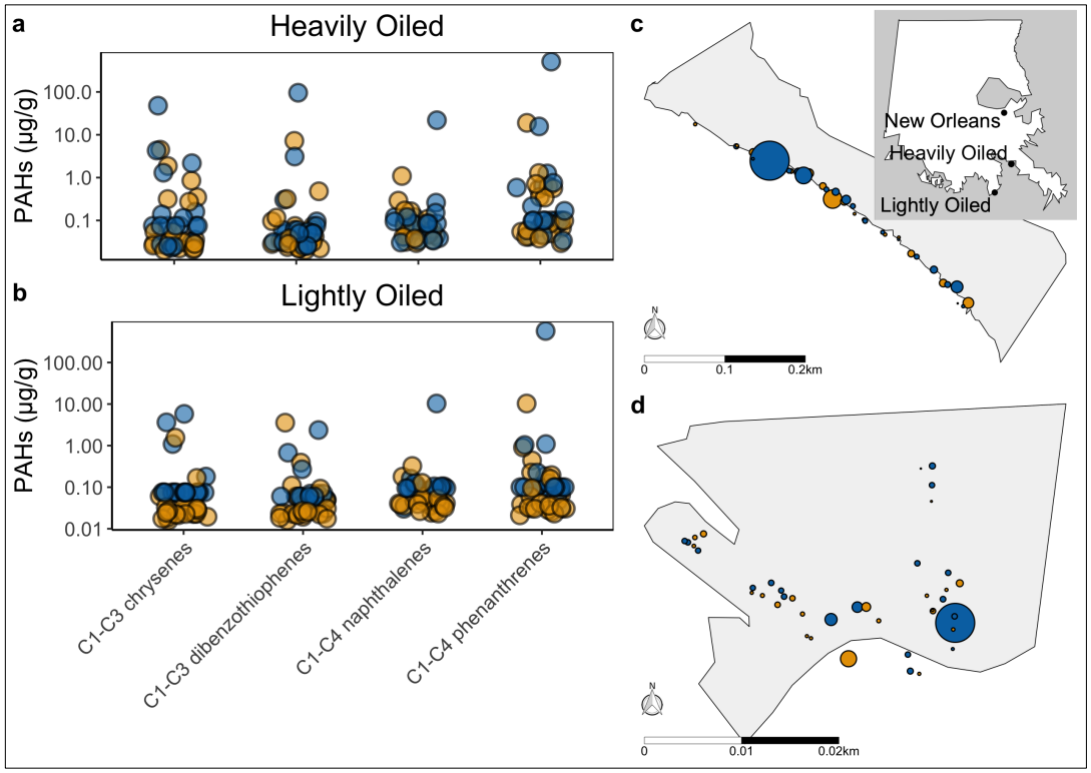

Figure 1-1: Distribution of polycyclic aromatic hydrocarbons (PAHs) within samples and at sites.

(a, b) Abundance of the four groups of alkylated PAHs measured at each site, with the y-axis log-transformed for visual clarity. (c, d) Schematic of the Heavily Oiled site (c) and the Lightly Oiled site (d), where each dot (jittered for clarity) represents a sample, colored by sampling timepoint and sized by the quantity of PAHs present in the core. Inset shows location of sites in southeastern Louisiana relative to New Orleans.

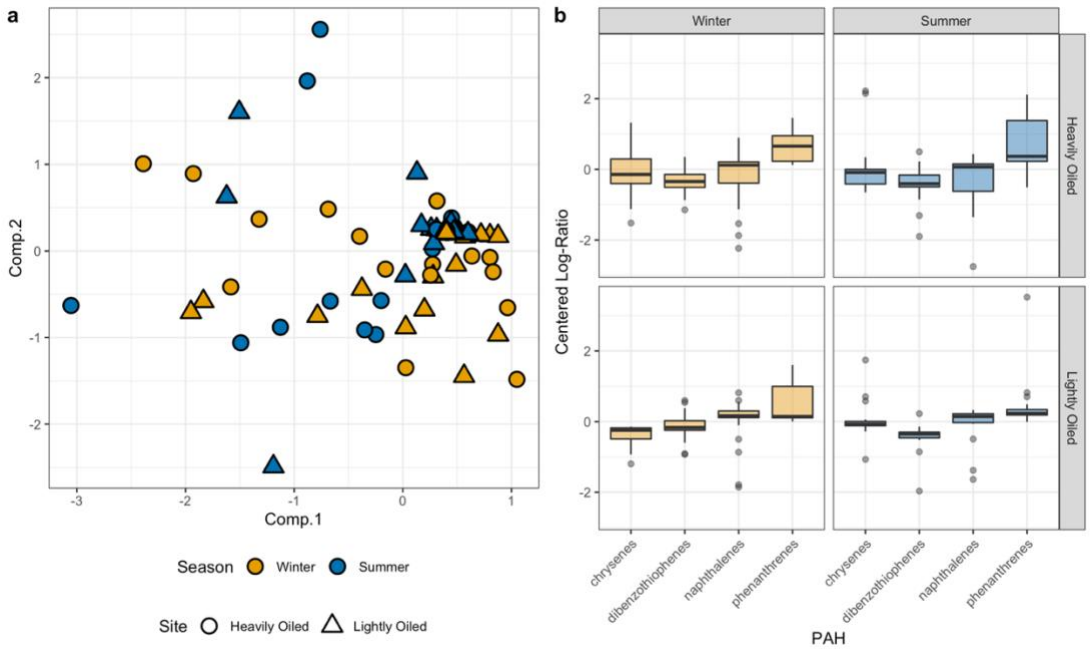

Figure 1-2: Additional analyses of oil chemistry.

(a) Principal component analysis of centered log-ratio transformed abundance (i.e., Aitchison distances) of the four measured PAH classes. (b) Boxplot of transformed values of PAH abundance by site and season.

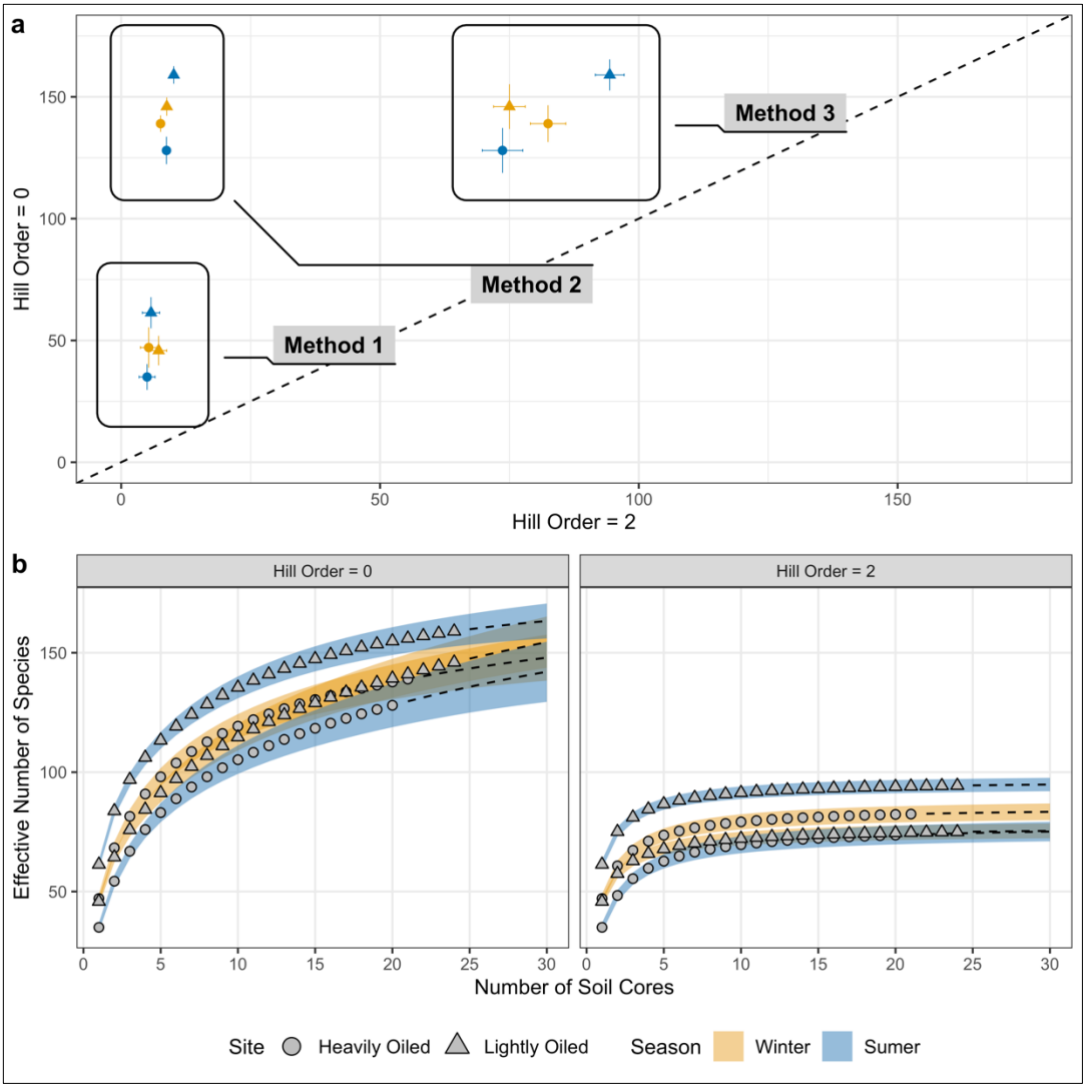

Figure 1-3: Comparisons of fungal alpha diversity for each combination of site and season.

Comparisons of fungal alpha diversity for each combination of site and time point. (a) Alpha diversity as estimated by three different methods. Method 1 is abundance-weighted diversity measured at the plot level. Method 2 is abundance-weighted diversity of the pooled samples from each site and time point. Method 3 represents incidence-based diversity for each site and time point, where estimates are calculated as richness,

weighted by the number of samples in which each OTU appears across the site and time point. The y-axis represents the effective number of species for Hill order = 0 and the x-axis represents the same for Hill order = 2. The identity line represents where perfectly even communities would fall on the plot. The mean diversity of each site and time point is represented by the point, and error bars represent 95% CI generated from 1000 bootstraps. (b) Rarefaction curves for Method 3 alpha diversity as a function of the number of samples included. The effective number of species is shown on the y-axis and the number of soil cores included in each estimate is represented on the x-axis. Error bars are shown by the shaded area around the line and represent 95% CI. The point before the dashed line represents the observed diversity. All points to the left are interpolations of diversity and the dashed points to the right represent estimates extrapolated from the curve.

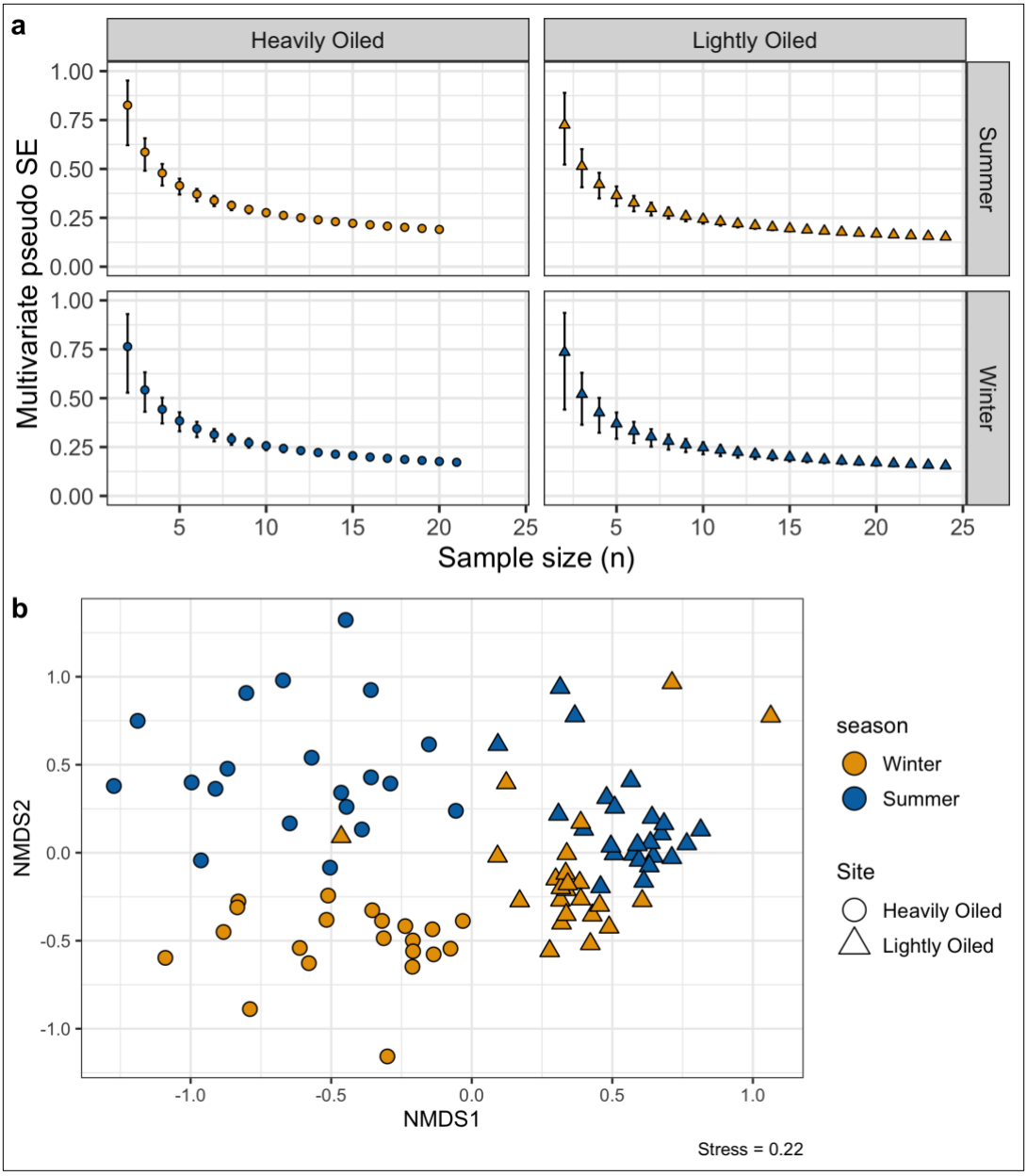

Figure 1-4: Visualizations of differences in community composition.

(a) MultSE estimations for site and season. The x-axis represents the number of samples included in the estimate of MultSE and the y-axis is an estimate of multivariate variation where the symbol is the mean variability in the position of the sample centroid and the error bars are 95% CI. If the CI of two groups of samples do not overlap, then the groups

would be considered statistically different by PERMANOVA by virtue of having different group centroids. (b) NMDS of fungal community composition based on Bray-Curtis dissimilarity values.

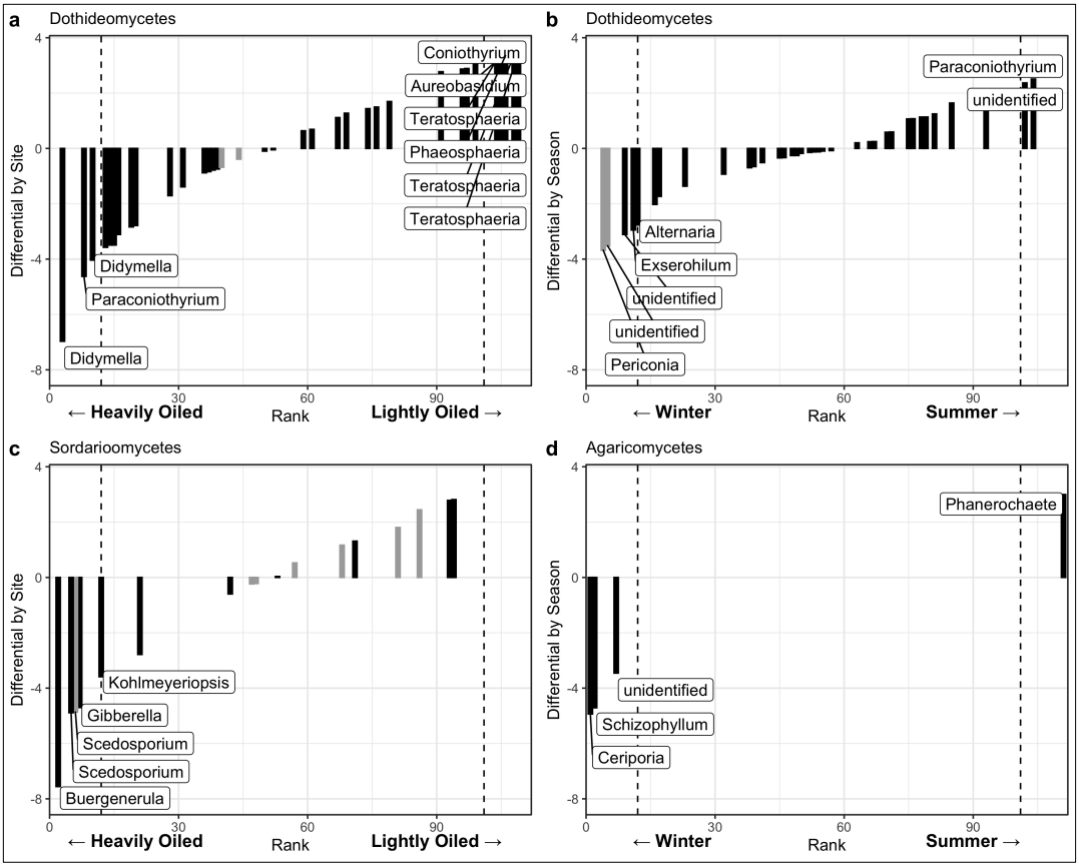

Figure 1-5: Differential ranking analysis with Songbird according to site and season. Rank is on the x-axis and is relative to the differential on the y-axis. Gray bars represent rare taxa and black bars represent dominant taxa. Dotted lines demarcate the lower and upper deciles of the rankings. The genera in the most extreme deciles are labeled with the genus assigned to the OTU. (a, b) Class *Dothideomycetes* is evenly ranked according to site and season. Extreme rankings are particular OTUs that are most abundant at one site or season relative to the average OTU. Middle rankings imply little to no change in relative abundance with respect to the covariate, but do not necessarily signify no change in absolute abundance. (c) Class Sordariomycetes are more abundant at the Heavily Oiled site. (d) Specific genera in class Agaricomycetes are strongly associated with one season

or the other; no OTUs are in the middle rankings.

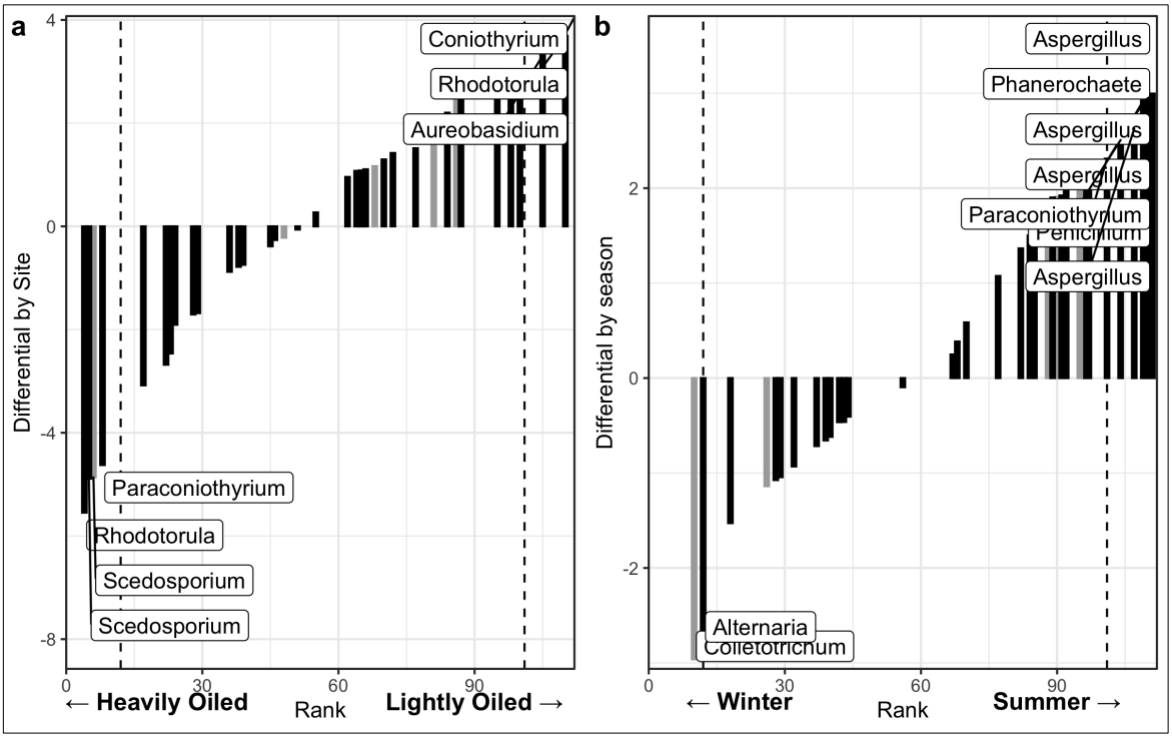

Figure 1-6: Differential ranking analysis with Songbird, highlighting hydrocarbon degrading taxa.

Rank is on the x-axis and is relative to the differential on the y-axis. Gray bars represent rare taxa and black bars represent dominant taxa. Dotted lines demarcate the lower and upper deciles of the rankings. The genera in the most extreme deciles are labeled with the genus assigned to the OTU. (a) OTUs assigned to genera with known hydrocarbon degrading species. Several are associated with each site. (b) Several OTUs classified as yeast that are phylogenetically related to hydrocarbon degraders are relatively more abundant during the summer.

CHAPTER 1 TABLES

Table 1-1: Sampling replicate numbers for sequencing and PAH analysis.

Sampling replicate numbers for sequencing and GC/MS analysis of PAHs. N = total number of samples.

Sampling Date	Site	N	Sequenced	GC/MS Analysis
January 2013	Lightly Oiled	24	24	24
Early July 2013	Lightly Oiled	24	24	19
January 2013	Heavily Oiled	21	21	20
Late June 2013	Heavily Oiled	20	20	19

Table 1-2: Bayesian linear regression of PAHs against site and season.

Results from a Bayesian linear regression of log (Total PAHs) ~ Site*Season. If the estimated distribution for slope (β) crosses zero, it is unlikely the slope of the relationship between Total PAHs and diversity is not zero for a given factor.

	β Estimate	**β Est.Error**	**Lower CI**	**Upper CI**
Intercept	-0.359	0.234	-0.822	0.106
Site	0.111	0.322	-0.528	0.740
Season	-0.274	0.295	-0.875	0.292
Site*Season	-0.489	0.430	-1.333	0.345
Sigma	1.373	0.115	1.167	1.621
Alpha	7.397	1.895	4.289	11.706
LP	-140.212	1.833	-144.635	-137.701

Table 1-3: PERMANOVA of PAH composition.

Results from the PERMANOVA of PAH composition as a function of site and season based on Aitchison distances and 9999 permutations.

Adonis: Aitchison	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
site	1	2.664	2.633	1.846	0.022	0.142
season	1	2.475	2.475	1.715	0.020	0.169
site:season	1	0.742	0.742	0.514	0.006	0.664
Residuals	78	112.532	1.442	0.950		
Total	81	118.413	1.000			

Table 1-4: Fungal diversity as a function of number of soil cores.

Estimates of salt marsh soil fungal diversity based on N soil cores. Effective number of species is an estimate of alpha diversity with regard to q (Hill orders), calculated by the incidence-based iNEXT estimator. 95% confidence intervals are described in columns labeled Lower CI and Upper CI, respectively. Min. samples is the minimum number of samples necessary for the CI to overlap with the CI using the number of replicates in N. Estimate type is either observed (O) or extrapolated (E).

Site	Season	q	Effective Number of Species	Lower CI	Upper CI	N	Min. Samples	Estimate Type
Heavily Oiled	Summer	0	128	118.956	137.044	20	13	O
Heavily Oiled	Winter	0	139	131.702	146.298	21	13	O
Lightly Oiled	Summer	0	159	152.472	165.528	24	15	O
Lightly Oiled	Winter	0	146	136.696	155.304	24	16	O
Heavily Oiled	Summer	2	73.662	69.743	77.581	20	7	O
Heavily Oiled	Winter	2	82.465	79.144	85.786	21	7	O
Lightly Oiled	Summer	2	94.389	91.522	97.257	24	7	O
Lightly Oiled	Winter	2	74.997	71.856	78.137	24	6	O
Heavily Oiled	Summer	0	142.046	129.624	154.469	30	17	E
Heavily Oiled	Winter	0	147.953	138.365	157.54	30	17	E
Lightly Oiled	Summer	0	163.301	155.776	170.827	30	17	E
Lightly Oiled	Winter	0	154.456	143.58	165.331	30	19	E
Heavily Oiled	Summer	2	75.118	71.052	79.184	30	8	E
Heavily Oiled	Winter	2	83.405	80.014	86.796	30	8	E
Lightly Oiled	Summer	2	94.833	91.941	97.725	30	8	E
Lightly Oiled	Winter	2	75.414	72.238	78.59	30	7	E

Table 1-5: Bayesian linear regression of PAHs against diversity.

Results from a Bayesian linear regression of Total PAHs ~ Alpha Diversity for three orders of Hill numbers. If the estimated distribution for slope (β) crosses zero, it is unlikely the slope of the relationship between Total PAHs and diversity is not zero.

Hill Order	Parameter	Estimate	Est.Error	Q2.5	Q97.5
0	β	-0.010	0.020	-5.18E-02	0.029
0	R^2	0.015	0.020	2.04E-05	0.074
1	β	-0.004	0.006	-1.99E-02	0.005
1	R^2	0.017	0.028	1.50E-05	0.106
2	β	-0.002	0.004	-1.22E-02	0.003
2	R^2	0.015	0.025	1.21E-05	0.091

Table 1-6: Community composition as a function of site and season.

(top) Results from the PERMANOVA on community composition as a function of site and season based on Bray-Curtis dissimilarity and 9999 permutations. (middle, bottom) Results from companion tests of ANOVAs of beta dispersion of site and season.

Adonis: Bray-Curtis	**Df**	**SumsOfSqs**	**MeanSqs**	**F.Model**	**R2**	**Pr(>F)**
site	1	3.793	3.792	12.351	0.1166	0.0001
season	1	1.602	1.602	5.217	0.0492	0.0001
site:season	1	1.025	1.024	3.336	0.0315	0.0002
Residuals	85	26.1	0.307	0.802		
Total	88	32.52	1			

Betadisper: Site: BC	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Groups	1	0.029	0.029	2.614	0.109
Residuals	87	0.969	0.011		

Betadisper: season: BC	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Groups	1	0.009	0.009	1.108	0.295
Residuals	87	0.727	0.008		

Table 1-7: Mantel tests of community composition vs oil composition and geographical distance.

(top) Results from a Mantel test for spatial autocorrelation of community composition based on Bray-Curtis dissimilarity values and geographical distance. (bottom) Results from a Mantel test for spatial autocorrelation of oil composition and geography based on Aitchison distance values.

Community composition vs space

Site	Season	mantel.stat(r)	p-value
Lightly Oiled	Summer	0.190	**0.022**
Lightly Oiled	Winter	-0.008	0.932
Heavily Oiled	Summer	-0.022	0.769
Heavily Oiled	Winter	-0.151	0.079

Oil vs space

Site	Season	mantel.stat(r)	p-value
Lightly Oiled	Summer	-0.136	0.189
Lightly Oiled	Winter	-0.120	0.148
Heavily Oiled	Summer	0.0002	0.999
Heavily Oiled	Winter	-0.039	0.737

Table 1-8: Community composition as a function of the number of soil cores.

Minimum number of samples necessary to fall within MultSE confidence intervals of full sample set. All columns described as "Full" represent the values for the entire set of samples for that site and season. The minimum number of samples needed for the subsample to be statistically indiscernible (i.e., overlapping CI) from the full set of samples is in the column labeled "Min. number of samples". The mean represents the group centroid as measured by Bray-Curtis dissimilarity and the confidence intervals (CI) represent a multivariate pseudo-standard error (MultSE).

Group	Mean - Full Set	Lower CI - Full Set	Upper CI - Full Set	Min. number of samples
Heavily Oiled WINTER	0.1719	0.1626	0.1804	17
Heavily Oiled SUMMER	0.1903	0.1822	0.1969	18
Lightly Oiled SUMMER	0.1521	0.1433	0.1599	20
Lightly Oiled WINTER	0.1547	0.1418	0.1655	18

CHAPTER 2

MEASURING THE EFFECTS OF OIL ON THE *SPARTINA ALTERNIFLORA* ROOT TRANSCRIPTOME AND SOIL MICROBIOME

INTRODUCTION

After the Deepwater Horizon (DWH) oil spill in 2010, researchers endeavored to describe effects of oil residues in the soil on salt marsh organisms and communities (Bernhard et al. 2019; Deis et al. 2017; Fleeger et al. 2015; Hughes et al. 2018; Lin et al. 2016; Lumibao et al. 2018; McCann et al. 2017). Many studies focused on two pillars of salt marsh ecology: *Spartina alterniflora*, a foundational species that dominates Gulf Coast salt marshes, and the microbial communities through which a large portion of the salt marsh food web is connected (Teal 1962). Of particular interest was how the plant roots were responding, since they are thought to shape the structure, chemistry, and microbial communities of the soil (Bernik et al. 2021; Lumibao et al. 2020). Likewise, the soil microbial communities garnered special interest as they were suspected of harboring bacteria and fungi that would drive a dynamic community response to the introduction and biodegradation of oil residues in the environment (King et al. 2015). However, generalizing the response of plant roots and soil microbial communities is difficult because the roots and microbes respond dynamically to the biotic and abiotic

conditions of the soil, like hydrology, nutrient content, and plant genetics (Angermeyer et al. 2016; Barreto et al. 2018; Bernhard et al. 2019; Kolton et al. 2020; Lumibao et al. 2020; Reinhold-Hurek et al. 2015). Furthermore, after the DWH oil spill, almost all field studies of salt marshes lacked pre-spill samples, necessitating the use of reference sites, that is unoiled sites, as controls for the introduction of oil (see Kandalepas et al. (2015) for example). Although best efforts are made to pick reasonable reference sites, if the effect of the oil is not strong, it is entirely possible that differences in pre-oil spill conditions at the oiled and reference sites might outweigh the effects of the oil deposition at the oiled site, leading to erroneous conclusions that differences are primarily due to exposure to oil residues. Thus, a basic question remains unanswered: can the response of the plant roots and soil microbial communities be recognized without samples of the pre-oil conditions?

Mesocosm and field experiments have demonstrated that the introduction of oil residues may perturb elements of salt marsh ecology, like *S. alterniflora* morphology, physiology, and microbial community composition (Hughes et al. 2018; Lin and Mendelssohn 2012; Mendelssohn and Lin 2002; Pezeshki et al. 2000; Su et al. 2016). But a key aspect of these experiments is controlling for ecological variation to isolate the effects of the oil. Few experiments have examined how subtle variation in initial soil conditions might drive our interpretation of the salt marsh response to oil. Similarly, few have asked how much variation is acceptable before a site can no longer serve as an acceptable reference.

Such variation might be particularly problematic in cryptic elements of salt marsh ecology, like the *S. alterniflora* root transcriptome and the soil microbial community. A

lack of baseline knowledge about the genetics of the plant and the composition of the soil microbial communities has made it difficult to generalize these ecological responses that are measured through molecular traits. A hexaploid organism like *S. alterniflora* has the potential to produce millions of gene isoforms, and salt marsh soil microbial communities may have thousands to tens-of-thousands of sequence variants. The response of such highly multivariate elements is unlikely to be easily interpreted without knowledge of their state prior to the introduction to oil.

Here, we report a growth chamber experiment in which we examined the variability of the *S. alterniflora* root transcriptome response and the soil microbial community to oil residues in the environment. We manipulated a homogenous salt marsh soil, from a single source, to yield two soils that were slightly different in soil chemistry and microbial community. We then exposed these soils to identical amounts of oil residues to test whether the response of the root transcriptome and soil microbiome depended on initial soil conditions. We tested four specific hypotheses: (1) the root transcriptome responds to oil in the environment with a set of differentially expressed genes that are consistent no matter the initial conditions; (2) the changes in the root response are not detectable in the plant morphology; (3) the response of the soil microbiome to oil residues in the soil diverges with differences in initial soil chemistry and initial soil microbiome composition; and (4) the effect of the oil on the salt marsh soil microbiome can be discerned by identification of the differentially abundant taxa that are common to the oiled treatments of each soil.

METHODS

The experiment consisted of a full-factorial design with two treatments of two levels each: (1) whether soil had been autoclaved or not and (2) the inclusion of weathered oil in the soil or not. For each combination of treatments there were 12 replicates. However, we only sequenced three replicates for root transcriptomics and soil metagenomics because of budgetary limitations. The experiment ran for 4 months in two consecutive phases. The first month tested how treatments affected germination and seedling growth. After one month all the plants, except for the largest, in each mesocosm were removed by clipping them at the soil surface. Then began the second phase in which we examined how the treatments affected plant growth, the root transcriptome, and the soil microbiome after three months.

SEED COLLECTION AND PROCESSING

Seeds were collected in November 2017 from Grand Isle State Park, LA. We collected all seeds from one visibly distinct clump of *S. alterniflora* stems with the goal of growing half-sibling plants and limiting genetic variation. We transported seeds, on ice, to the lab where they were placed in deionized water and stratified at 4 °C until planting five months later. Immediately prior to planting seeds we reduced the microbes associated with the seeds by surface-sterilization. First, seeds were put in a tea strainer and agitated in DI water until no more detritus came off. Following the methods of Kandalepas et al. (2015), and working in a biosafety cabinet, the seeds were then agitated in 95% EtOH for 3 min, 10% bleach (0.525% sodium hypochlorite) for 30 min, then sterile DI water for 10

seconds. The water was drained, and seeds placed in a sterile petri dish in the hood.

SOIL COLLECTION AND PROCESSING

We collected salt marsh soil in May 2018 from Bay Jimmy, LA, USA [Latitude 29.440446, Longitude -89.885863]. The mud was brought back to Tulane University and kept in a cool room overnight. The next morning the mud was sieved through a 6.35 mm (1/4") screen. We stored 50% of the mud at 4 °C for use as "not-autoclaved" soil. To perturb the soil chemistry and microbial community, the other 50% of the mud (48 L) was autoclaved at 121 °C for 60 min, allowed to rest for 6 hours, mixed, autoclaved again and placed at 4 °C. The following morning, approximately 36 hours after the mud was first collected, we assembled and planted the mesocosms.

MESOCOSM DESIGN

Each mesocosm consisted of a 1.9 L (0.5 gallon) glass jar, 14.1 cm (5.57 inches) in diameter and 15.9 cm (6.28 inches) in height, (Burch Bottle and Packaging Inc., Waterford, NY, USA) resting inside a 3.8 L (one gallon) high density polyethylene plastic bucket (Cary Company, Addison, IL, USA). The glass jar had five, 1.9 cm (0.75 inch) drain holes drilled in the bottom to allow water movement. Before mesocosm assembly, the jar was wrapped in tin foil and autoclaved for 20 minutes at 121 °C.

MESOCOSM ASSEMBLY

We assembled mesocosms in a biosafety cabinet to minimize contamination of the experimental space and in alternating order of treatments to avoid effects of batch assembly. Gloves were changed as often as necessary to maintain cleanliness and the hood surface cleaned with 95% EtOH and 10% bleach whenever dirty and between each mesocosm. To begin, we used sterile tools to remove the foil covering the mouth, and drain holes, of the glass jar. Sterile cotton pads were placed inside the jar over drain holes to keep soil from leaking out and the jar placed in the bucket. Lastly, we placed approximately 1800 mL of the appropriate soil into the jar. Twelve seeds were planted in a 4 x 3 grid, using sterile tools, and the mesocosm covered with stretch wrap to prevent contamination. For oil-treated mesocosms, we mixed in 200 ml of oil (approximately 10% oil by volume) into the soil in a sterile container before we placed the soil in the mesocosm. We used heavily weathered oil skimmed off the surface of the ocean during the Deepwater Horizon oil spill (see Acknowledgements).

After assembly, we distributed mesocosms over two tables in four randomized blocks in a walk-in Conviron GR48 growth chamber (Winnipeg, MB, Canada). We filled mesocosms with tap water up to the soil surface and covered the top with a lightly ventilated shrink wrap to reduce inoculation from spore fall. The shrink wrap was removed after two weeks.

GROWTH CHAMBER CONDITIONS

We programmed the growth chamber to mimic ideal growing conditions for southern Louisiana according to the methods of Krauss et al. (1998). Briefly, a Conviron Model GR48 Plant Grow Room was set to 29 °C, full light and 90% humidity from 6am to 8pm

and 27 °C, no light, and 90% humidity for 8pm to 6am. PAR measured about 175 $umol \cdot m^{-2}s^{-1}$ at soil surface and about 580 $umol \cdot m^{-2}s^{-1}$ near the bulbs. We were unable to keep the chamber at the goal of 90% humidity and ran the experiment at approximately 70%.

We topped of mesocosms with tap water daily to keep the soil saturated. We completely drained and refilled the water once per week, simultaneously rotating blocks. After we completed the initial phase of the experiment, we added one gram of 15-9-12 NPK Osmocote plus fertilizer (The Scotts Company LLC Marysville, OH) to each mesocosm and covered the soil with 75 mL of sterile sand to reduce further inoculation from spore fall.

SAMPLING AT TIME ZERO

Immediately following mesocosm assembly, we froze two 50ml tubes of each soil treatment combination at -20 °C. We recorded germination and seedling survival daily for the first month. We then removed every plant, except the largest, by clipping the plant at the base.

WEEKLY PLANT MEASUREMENTS

After thinning the plants, we recorded the number of stems, and counted the number of leaves per stem, every week. We measured stem height to the nearest 0.5 cm from soil surface to the auricle of the topmost unfurled leaf. We measured stem diameter by laying a ruler across the bucket rim and using calipers to record thickness at that height to the

nearest 0.1 mm. Some stems grew out of the drainage holes and in between the experimental space and the plastic bucket. We noted they were growing outside the experimental space but otherwise measured them the same, approximating the soil surface by eye.

HARVESTING MATURE PLANTS

After 13 weeks, we harvested the plants and collected soil samples. We used clean gloves for every mesocosm and cleaned tools and surfaces, with 10% bleach and 95% ethanol, between every mesocosm. As quickly as possible, we removed the plant and soil ball by destroying the glass jar. We collected 50 ml of soil from the root zone, avoiding root destruction, and immediately placed the soil on dry ice until it could be placed at -20 °C later that day. Then healthy, turgid roots, approximately 1 mm in diameter, were collected, rinsed in DI water and frozen in a 2 ml tube in liquid nitrogen. We stored them at -80 °C until RNA extraction. The remaining plant tissue was rinsed until clean and then placed in an oven to dry. Mass was recorded once it had become stable over consecutive days. On average it took about six minutes to collect soil and roots used in downstream ‘omics analyses. We guarded against batch effects of collection by rotating through treatments and blocks as we collected. We split collections over two consecutive days to shorten the total time and limit bias from the circadian rhythms of the plant. In total, each collection day ran from about 2pm to 8pm.

SOIL CHEMISTRY ANALYSIS

Soil was analyzed for basic chemistry including Melich 3 extractable P, K, Ca, Mg, Na, S, Cu, and Zn (Mehlich 1984) and pH, percent C and percent N (McLean 1983) by the Louisiana State University AgCenter (Baton Rouge, LA).

ROOT RNA

extraction

We extracted total root RNA one month after harvesting. Four replicates from each treatment were extracted (one from each block) and we sequenced the best three extractions for each combination of treatments. The range of concentrations was 29.9 ng/ul to 106.56 ng/ul with a mean of 46.34 ng/ul. The range of RIN values was 7.1 to 8.9 with a mean of 8.1. We extracted total RNA root samples using the Qiagen RNeasy Plant Mini kit (Cat #74904, Hilden, Germany) following the standard protocols. Briefly, using sterile technique, 0.2g of frozen root tissue was extracted for each sample. To aid in lysis, we first ground tissue in a mortar and pestle with liquid nitrogen. We soaked mortars and pestles in 10% bleach and autoclaved beforehand to remove relic nucleic acids. All equipment and working areas were thoroughly cleaned to minimize contamination from nucleic acids and nucleases. If a sample did not yield a concentration of at least 10 ng/ μL and RIN > 7 then it was re-extracted.

sequencing

We mailed total extracted RNA on dry ice to Novogene Corp (Sacramento, CA) for stranded RNA-seq at their facility in Tianjin, China. One ug of RNA was used for cDNA

library construction at Novogene using an NEB directional RNA lib prep kit (cat# E7420L, New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol. Libraries were sequenced on an Illumina HiSeq 4000 Platform (Illumina, San Diego, CA, USA) using a paired-end 150 run (2×150 bases). On average, sequencing yielded 48,722,643 reads per sample (SD = 9,381115.3), after quality filtering. On average, 92.8% of bases had a quality score > Q30 (SD = 0.96).

sequence processing and transcriptome assembly

Sequences were trimmed and filtered with Trimmomatic, version 0.38, (Bolger et al. 2014) to remove adapters and for quality control. Reads were de-novo assembled with Trinity, version 2.8.4 (Grabherr et al. 2011; Haas et al. 2013), using default settings. The assembly was assessed by alignment of the raw reads to the assembly with Bowtie2, version 2.3.4.3 (Langmead and Salzberg 2012). The resulting assembly was aligned to the UNIPROT Swiss-Prot database, version 2018_01, (The UniProt Consortium 2016) with BLAST+, version 2.5.0 (Camacho et al. 2009) to check for contamination. We noted any strong homology to sequences from non-plant organisms and we filtered our trimmed sequences to remove contaminants with BBduk, version 38.41 (Bushnell 2014), against reference genomes that were available for the non-plant organisms noted above. We then re-assembled the transcriptome with the same Trinity pipeline described above. Scripts provided by Trinity were used to calculate quality statistics of the assembly, like N50 and Ex90N50. Benchmarking Universal Single-Copy Orthologs (BUSCO) scores were generated with BUSCO, version 3 (Simão et al. 2015) against the embryophyte_odb9 database.

SOIL DNA

extraction

Soil DNA extraction followed the methods of Lumibao et al. (2018). In summary, using sterile technique, 0.25g of soil was extracted for each sample using the Qiagen DNeasy Powersoil kit (Cat #12888, Hilden, Germany) observing the manufacturer's standard protocols. If a sample did not yield a concentration of at least 10 ng/µL then it was re-extracted.

16S library prep and sequencing

We sent soil DNA to Novogene Corp (Sacramento, CA) for sequencing. The NEBNext® Ultra II FS DNA Library Prep Kit for Illumina (cat# E7805L, New England Biolabs, Ipswich, MA, USA) was used for library prep according to the manufacturer's protocol with primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) targeting the V4 region. Libraries were sequenced on a NovaSeq 6000 platform (Illumina, San Diego, CA, USA) using a paired-end 250 bp run. On average, sequencing produced 192,296 reads per sample (SD = 62740.2) after quality filtering. The quality score for 96.02% of bases was > Q30 (SD = 1.3).

16S processing and clustering

Sequences were processed following the DADA2 ITS Pipeline Workflow, version 1.12 (Callahan et al. 2016) in (R Development Core Team 2019). The pipeline also depends on the R packages ShortRead (Morgan et al. 2009), BioStrings (Pagès et al. 2019) , and ggplot2 (Kahle and Wickham 2013). After visual examination, samples were trimmed and filtered with default parameters at base number 235 for both read 1 and read 2. The main DADA2 algorithm with default parameters was then employed to learn the error rates of the sequences and dereplicate them, then infer the correct sequence for each read. Paired-end reads were then merged into amplicon sequence variants (ASVs), chimeras removed and taxonomy assigned against the SILVA132 database (Yilmaz et al. 2014). ASVs assigned to chloroplast or mitochondrial taxonomy were filtered out of the data.

ITS1 library prep and sequencing

Library prep for the ITS1 DNA region followed the methods of Lumibao et al. (2018), a 2-step library prep process using primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC). The multiplexed libraries were sent to the Duke University Sequencing and Genomic Technologies Facility (Durham, North Carolina, USA) for sequencing. 300 bp PE sequences were generated on an Illumina MiSeq platform (Illumina, San Diego, CA, USA). Sequencing yielded an average of 64,546 identifiable PE reads per sample (SD = 7,293) and an average phred score of 32.3 (SD = 0.36).

ITS1 processing and clustering

Sequences were processed following the DADA2 ITS Pipeline Workflow, version 1.8 (Callahan et al. 2016) in (R Development Core Team 2019). The pipeline is extremely similar to the pipeline used for 16S processing, described above. However, a fundamental difference is samples were trimmed and filtered with cutadapt (Martin 2011) based on detection of primer sequences from the first PCR in library prep to account for the inherent variation in length of the ITS1 region among species. Then sequences were trimmed and filtered again after examining quality profiles of the reads. The main DADA2 algorithm with default parameters was then employed to learn the error rates of the sequences and dereplicate them, then infer the correct sequence for each read. Paired-end reads were then merged into ASVs, chimeras removed and taxonomy assigned against the UNITE database (UNITE Community 2019).

SEQUENCE ARCHIVING

All reads are archived in the National Center for Biotechnology Information (NCBI) (Bethesda, MD USA) under BioProject ID PRJNA745438, “Measuring the effects of oil on the Spartina alterniflora root transcriptome and soil microbiome”.

STATISTICS

Statistical Analysis of Germination

We tested the percentage of seeds germinated and median germination time with ANOVAs. A separate examination of the percentage of germinated seeds that survived to the second phase of the experiment required a Kruskal-Wallis test because data could not be transformed to fit assumptions of normality. Median germination time, the time to 50% of maximal germination, was calculated with the *t50* function from the package *germinationmetrics* (Aravind et al. 2020).

Statistical Analysis of Plant Growth

We compared dry plant biomass and plant morphology with two-way, type III ANOVA tests of the autoclaving and oil addition treatments, when the assumptions of the ANOVA could be met. Otherwise, we used Bayesian regression with the *brms* package (Bürkner 2017; Carpenter et al. 2017) choosing the appropriate distribution based on exploratory analysis of the data, and comparisons to models built on gaussian distributions. All plant morphological traits were modeled for measurements taken at the last time point in the experiment. Models for stem height and stem diameter were based on gamma distributions using default priors. Regression of stem count and leave per stem against the treatments used negative binomial distributions with default priors.

Statistical Analyses Software

Unless otherwise mentioned we conducted all statistical analyses in R version 4.0.2 (R Core Team 2020). Analyses and figures depended heavily on the *tidyverse* (Wickham et al. 2019), *cowplot* (Wilke 2020), *compositions* (van den Boogaart et al. 2020), *phyloseq* (McMurdie and Holmes 2013), *vegan* (Oksanen et al. 2016), *data.table* (Dowle and Srinivasan 2020), packages.

Statistical Analyses of Soil Chemistry

We created a biplot to visualize variation in chemical composition of each soil sample and we tested for differences in chemical composition by conducting a PERMANOVA of Aitchison distances using the *adonis* function in the *vegan* package (Oksanen et al. 2016). Aitchison distance accounts for the compositional nature of the data through transforming values into centered log-ratios (clr) and then taking the Euclidean distance of sample compositions (Brückner and Heethoff 2017; Pawlowsky-Glahn and Egozcue 2006). Prior to transformation, we converted percent C and percent N to ppm to make the unit consistent with all other elemental measurements. pH was not clr-transformed prior to calculating Euclidean distance for the sample because it does not represent part of the composition.

Statistical Comparisons of Transcriptome by Treatment

We tested for differences in transcriptome composition by conducting a PERMANOVA of Aitchison distances using the adonis function in the vegan package (Oksanen et al. 2016). This test was visually corroborated with a PCA. Prior to performing the tests, transcripts that totaled fewer than 10 counts across all samples were filtered out. Post-hoc PERMDISP tests determined whether differences were due to shifts in community heterogeneity or composition (Anderson and Walsh 2013), with corroboration from Principle Components Analyses (PCA). Linear relationships between transcriptome composition and soil chemistry were investigated via a redundancy analysis (RDA), with significance determined by the ANOVA-like permutation test implemented in *vegan.* We removed variables with high collinearity with the *vif.cca* function and selected model parameters with the *ordistep* function from.

Statistical Analyses of Differential Expressed Genes

Transcript abundances were estimated with Salmon, version 0.11.4 (Patro et al. 2017). Differential abundance of expression was estimated with the R package, *ALDEx2* (Fernandes et al. 2013) following the same guidelines mentioned above. However, due to limited computing power, we ran the analysis three times, using 500 Monte Carlo samples each and then averaged the results per the recommendations of the *ALDEx2* developers (SF personal communication). Prior to performing the tests, transcripts that totaled fewer than 10 counts across all samples were filtered out.

Functional Gene Annotation

Differentially expressed genes were functionally annotated with TOA (Mora-Márquez et al. 2020), a workflow specifically designed for annotation of non-model plant 'omics assemblies. TOA optimizes annotation by allowing the user to rank the databases used for annotation in a manner that is logical for the study plant. Our annotation workflow used BLAST+ to align transcripts first to the PLAZA Monocots 4.0 database (Van Bel et al. 2017), then the PLAZA Dicots 4.0 database, NCBI RefSeq Plant (O'Leary et al. 2016), followed by the PLAZA Gymnosperms 1.0 database, and finally NCBI nr database. All databases were downloaded on January 9, 2021.

GO Enrichment Analysis

GO terms were compiled from TOA annotation output (go-stats.csv) and singular enrichment analysis (SEA) was conducted in agriGO (Du et al. 2010) using *Oryza sativa* L. ssp. *Japonica* as the species and the Rice TIGR genemodel as the background reference. Significance of term enrichment was determined by a hypergeometric test, and the p-value corrected to the Yekutiele FDR. Terms with an FDR of less than 0.05 were fed into REVIGO (Supek et al. 2011) and redundant terms reduced using *O. sativa* as the species and SimRel as the measure of semantic similarity. Results were plotted in R following the protocol of Bonnot et al. (2019).

Statistical analyses of microbial alpha diversity

We estimated the alpha diversity for each soil sample with the package *phyloseq* (McMurdie and Holmes 2013) and converted results into Hill numbers of order q = 0, 1,

and 2. The orders estimate the effective number of species when rare species are weighted with the same consideration as species richness (q =0), Shannon diversity (q =1), and Simpson diversity (q =2). We tested for statistical differences in diversity with ANOVA tests on the autoclaving, oil, and time treatments.

Statistical Analyses of Microbial Beta Diversity

We assessed whether Aitchison distance values of community composition differed by autoclave and oil treatments with the *adonis* (PERMANOVA) function in the *vegan* package (Oksanen et al. 2016). Post-hoc PERMDISP tests determined whether differences were due to shifts in community heterogeneity or composition (Anderson and Walsh 2013), with corroboration from Principle Components Analyses (PCA). Linear relationships between community composition and soil chemistry were investigated via a redundancy analysis (RDA), with significance determined by the ANOVA-like permutation test implemented in *vegan.* We removed variables with high collinearity with the *vif.cca* function and selected model parameters with the *ordistep* function from.

Statistical Analyses of Differentially Abundant Taxa

We identified differentially abundant taxa with the *ALDEx2* package using the functions described in the original *ALDEX* package (Fernandes et al. 2013), which accounts for the compositional nature of the data. Our low replicate number prevented us from leveraging the statistical tests implemented in *ALDEx2.* Instead, we defined differentially abundant taxa as those in which the variation of abundance between

conditions was at least twice as large as the variation of abundance within a condition, as recommended by the authors of *ALDEX*. Default setting were used in conjunction with 1000 Monte Carlo samples.

RESULTS

SOIL CHEMISTRY

Soil chemistry differed according to the oil addition treatment, autoclaving treatment, and between the beginning and end of the experiment (Figure 2-1, Table 2-1). The oil addition treatment explained 35% of variation in the model, time explained another 28% and the autoclaving treatment an additional 10%. Both treatments interacted significantly with time but not with each other. The addition of oil increased the carbon content of the soil, which correlated strongly with an increase in pH. By the end of the experiment, the unoiled samples had relatively less copper content compared to the oiled samples (Figure 2-2), but relatively more phosphorus, magnesium and sulfur content. The differences, however, were not as stark as the differences in carbon content and pH. The samples from the outset of the experiment were higher in sodium and potassium content, but lower in nitrogen than at the end of the experiment. The autoclaving treatment did not have a large effect within in the pre-experiment samples. But by the end of the experiment, autoclaved versus non-autoclaved samples were significantly different in nutrient composition, primarily due to a relative decrease in sodium (Figure 2-2).

TRANSCRIPTOME ASSEMBLY

Trinity assembled 586,431,947 PE reads, 97.71% of which could be remapped to the transcriptome after assembly. The assembly contained 1,639,719 gene isoforms organized into 1,084,620 'genes' with 47.42% GC content. Average contig length was 646.69 bp and N50 length was 1000 bp, indicating at least half of the bases assembled were found in contigs at least 1000 bp long. A more informative statistic for transcriptomes, which can include many short, lowly expressed contigs, is Ex90N50. Ex90N50 calculates the N50 value on 90% of the total cross-sample normalized expression data, as estimated by Salmon (Patro et al. 2017). For our assembly, Ex90N50 was calculated as 1537 bp, representing 539,644 ‘genes’. Of the 1440 BUSCO groups searched, 1324, or 92% were recovered from our assembly. Of these, 1183 were duplicated BUSCOs and 141 single-copy BUSCOs.

ROOT TRANSCRIPTOME COMPOSITION

The root transcriptome was statistically distinguishable by the autoclave treatment but not the oil addition treatment. There was, however, a statistically detectable interaction of the oil addition and autoclave treatments, apparently due to the strong effect of oil on the soil that had not been autoclaved (Figure 2-3, Table 2-2). Redundancy analyses (RDA) of root transcriptome Aitchison distances and soil chemistry did not explain transcriptome composition better than the autoclaving treatment alone (results not shown).

DIFFERENTIAL GENE EXPRESSION

The soil that was not autoclaved and not oiled contained 1,057,124 gene isoforms compared with 850,006 when oil was added. We identified 6852 gene isoforms as differentially abundant between the oiled and unoiled treatments for the not-autoclaved soil. In contrast, we recovered 826,769 gene isoforms from the autoclaved soil when no oil was added and 810,710 when oil was added. 3319 of the isoforms were identified as differentially abundant between the oiled and unoiled treatments. The autoclaved and not-autoclaved soils shared only 126 isoforms that were differentially abundant with respect to the oil treatment. Of these 126 isoforms, 37 were differentially abundant in opposite directions, 31 less abundant when oil was added and 58 more abundant when oil was added.

FUNCTIONAL ANNOTATION AND GO ENRICHMENT ANALYSIS

The 58 gene isoforms that were differentially more abundant when oil was added could be translated into 65 predicted peptides. Of these, 50 sequences were annotated against the Monocot database, one was annotated against the Dicot database and 14 were unable to be annotated. The 68 gene isoforms that were differentially less abundant predicted 32 peptides from the 31 isoforms. Annotations for 22 of the sequences came from the Monocot database, 2 from the RefSeq plant database, and 8 sequences were unable to be annotated.

GO enrichment analysis recovered 66 significantly enriched terms after redundant terms were removed (Figure 2-4). Terms associated with differentially more abundant

genes included 29 biological processes, 1 cellular component and no terms related to molecular function. Genes that were with differentially less abundant were annotated with 27 Biological Processes, 5 Cellular Components, and 4 Molecular Functions.

SEED GERMINATION AND SURVIVAL

Seeds successfully germinated in all but two replicates. One of those replicates represented autoclaved soil with no oil added, the other replicate was autoclaved soil with oil added. An ANOVA on the percentage of seeds germinated in each replicate found a significant effect of the autoclaving treatment ($F_{1,44} = 4.33$, $p = 0.04$), but not the oil addition treatment ($F_{1,44} = 1.37$, $p = 0.24$). However, the effect of the autoclave treatment was no longer significant after removing the two replicates in which there was no germination. No differences were found in germination rates of the two treatments.

One more replicate for each of the treatments described above had only a single seed germinate and die before the second phase of the experiment began, reducing the total number of replicates for each to 10. A Kruskal-Wallis test on percent of seeds surviving after germination found no difference between treatments (results not shown).

PLANT BIOMASS AND MORPHOLOGY

Dry biomass of the harvested plants differed by treatments for aboveground tissue but not for belowground tissue. Aboveground tissue showed a relative increase in biomass when the soil was autoclaved ($F_{1,40} = 18.74$, $p = 0.00009$) and a relative decrease

for plants to which oil had been added ($F_{1,40} = 4.53$, $p = 0.039$), but no interaction between the two treatments. The ratio of belowground to aboveground tissue did not differ by treatment.

Four plant traits were measured: stem count, stem height, leaves per stem and stem diameter. We found no difference among treatments for the first three traits. However, the oil treatment decreased average stem diameter by 0.86 mm, 95% CI [0.77, 0.94] according to Bayesian regression. Autoclaving the soil had no effect on stem diameter.

MICROBIAL ALPHA DIVERSITY

Prokaryote and fungal alpha diversity did not differ by the autoclave, oil, or time treatments according to ANOVA. But, if the models were split into individual models of each time point, then prokaryote alpha diversity was statistically reduced by the autoclave treatment, at the second Hill order only, at the beginning (ANOVA $F_{1,4} = 29.18$, $p = 0.01$) and end (ANOVA $F_{1,8} = 3.64$, $p = 0.006$) of the experiment. Fungal alpha diversity showed a statistical reduction explained by the autoclave treatment for Hill order of zero (ANOVA $F_{1,8} = 15.89$, $p = 0.02$) and Hill order of one (ANOVA $F_{1,8} = 6.74$, $p = 0.02$) for post-experiment samples only (Figure 2-5).

MICROBIAL BETA DIVERSITY

Prokaryote beta diversity was significantly different by autoclaving treatment, but not oil addition treatment (Figure 2-6, Table 2-3). Fungal communities, however, could not be statistically differentiated by either the autoclave or oil addition treatments (Figure 2-6, Table 2-4). If samples were subset by autoclave treatment level, oiled samples remained statistically indistinguishable. RDA models, constructed by forward variable selection, of microbial community Aitchison distances and soil chemistry did not explain community composition better than the autoclaving treatment alone, for prokaryote communities, or the null model for fungal communities (results not shown).

Differential Abundance of Prokaryotes

Soil that was not autoclaved contained 8038 ASVs in the unoiled samples compared with the 8811 ASVs recovered from the oiled samples. 3730 ASVs were shared between the two groups, but only 118 ASVs were identified as differentially abundant between the oiled and unoiled samples. Autoclaved soil had 7702 ASVs when unoiled, and 8197 ASVs when oil was added. The oiled and unoiled autoclaved soils shared 3482 ASVs, 47 of which were considered differentially abundant. However, only one ASV, a *Spirochaeta* sp., was identified was differentially abundant between the oiled and unoiled samples for both the autoclaved and not-autoclaved soils.

Two soils might not share many ASVs responding to oil, but it's possible that a functional response to oil may be identified at a coarser phylogenetic level, like class or family, if the functionality is linked to phylogeny. Therefore, we searched for taxa that were identified as relatively more abundant in oiled samples for both soils *and* not

relatively less abundant in either soil. Three phyla, Euryarchaeota, Crenarchaeota, and Spirochaetes met these criteria and continued to meet these criteria to the classes of Methanomicrobia, Bathyarchaeia, and Spirochaetia. None of the Bathyarchaeia ASVs could be assigned taxonomy below class, and Methanomicrobia resolved into multiple orders that were not found in both autoclave treatments levels. Spirochaetia resolved into the *Spirochaeta* sp. ASV noted above.

differential abundance of fungi

Soil that was not autoclaved contained 590 ASVs in the unoiled samples compared with the 521 ASVs recovered from the oiled samples. 258 ASVs were shared between the two groups, but only 6 ASVs were identified as differentially abundant between the oiled and unoiled samples. Autoclaved soil had 435 ASVs when unoiled, and 512 ASVs when oil was added. The two groups of autoclaved soils shared 153 ASVs, 3 of which were considered differentially abundant. No ASVs were identified as differentially abundant between the oiled and unoiled samples for both the autoclaved and not-autoclaved soils.

DISCUSSION

We tested four hypotheses in our work and found: (1) the root transcriptome can respond to oil in the environment, but the group of genes that are differentially expressed depend on the soil conditions prior to oil being introduced to the environment; (2) the changes in the root response are not detectable in the belowground biomass but may be

reflected in aboveground biomass or morphology; (3) the soil microbiome did not respond to the addition of oil residues to the soil, but prokaryotes did diverge relative to initial composition; and (4) there is no effect of the oil on the microbiome that can be discerned by identification of the differentially abundant taxa that are shared by the different soils.

SOIL CONDITIONS

We autoclaved the soil to create soils of two different chemical and microbial compositions from a homogeneous mixture of freshly harvested salt marsh soil. The advantage of this approach was a high confidence in the controlled initial compositions of our soils. The disadvantage was that the autoclave is an artificial form of disturbance that may be excessively destructive to soil organic matter and microbes (Berns et al. 2008).

Nevertheless, the oil addition, autoclaving, and time treatments explained approximately three-quarters of the variation in soil chemistry of our samples. However, no interactions of the three treatments were statistically significant, suggesting that the effects of the three treatments on soil chemistry were independent. The pH of the soils did not appear affected by the autoclaving treatment, although the addition of oil did appear to increase the pH of the soil, an effect that was exacerbated over time. In general, pH was slightly lower than the pH of porewater measured in contaminated salt marshes after the DWH oil spill (Natter et al. 2012), probably due to the use of freshwater instead of saltwater. Our intent in designing the experiment was to test for the effect of oil

specifically and avoid interactions that may occur between oil and salinity, so we did not add any salts beyond the fertilizer described above.

Measurements of initial conditions show that soil chemistry of the four combinations of treatments were distinguishable in samples taken immediately after the treatments. The four combinations of treatments continued to diverge over the course of the experiment. Presumably, these trajectories are functions of the soil chemistry interacting with the biotic and abiotic components of the experiment. Importantly, the oil addition treatment was performed after the autoclaving treatment and the oiled soil should therefore be considered a derivative of the autoclaving treatment, not a completely different soil. Despite this caveat, soil chemistries were much more similar according to the oil addition treatment than the autoclaving treatment. This is consistent with the premise of our experiment that oiled soil is recognizably oiled in terms of soil chemistry even if there are other differences in initial soil chemistries.

PLANT RESPONSE

Externally, *S. alterniflora* showed a negligible response to oil residues in the soil, consistent with the experiments reviewed in Pezeshki et al. (2000). More specifically, there were no differences in seed germination or belowground biomass relative to the oil addition or autoclave treatments, consistent with similarly designed experiments (Bergen et al. 2000; Mendelssohn et al. 1995) and the greenhouse work described in Chapter 3 of this dissertation. There were detectable differences in aboveground biomass and a reduction in stem diameter relative to the oil. But these results were subtle and may have only been detectable due to the highly controlled growth chamber environment.

ROOT TRANSCRIPTOME RESPONSE

The root transcriptome responded strongly to the differences in the soil created by autoclaving, but the effect of the oil addition could only be detected in the not-autoclaved soil. Why one solicited a stronger response than the other is unclear, although it may indicate an interaction of the oil with soil chemistry or the microbial community to which the plant is sensitive. The critical point is that the plant can respond to oil through the root genome, but the strength of that response may depend on initial conditions of the soil.

Our transcriptome assembly statistics highlighted the complexity of the *S. alterniflora* genome. We recovered over 1.6 million gene isoforms related to approximately 1 million putative genes. Our BUSCO score suggests we captured the majority of the genome, yet the high number of duplicated BUSCO groups indicate a complex genome, likely due to the hexaploidy of *S. alterniflora* and genetic variation in the experimental plants, which were not clones.

We are the first to report the response of the entire root transcriptome to oil residues in the soil with high-resolution next-gen sequencing methods. However, RamanaRao et al. (2012) inspected expressed sequence tags (ESTs) in *S. alterniflora* roots after exposure to petroleum hydrocarbons. ESTs are a comparatively low-resolution method that cannot describe the entire transcription response of the genome. Despite these differences in methodology, two of the ten ESTs identified and annotated by RamanaRao et al. (2012), were similar in functional annotation with four differentially expressed gene isoforms that we identified. All four of the gene isoforms were included

in the 126 differentially expressed isoforms shared by the autoclaved and no-autoclaved soils, with relatively similar effect sizes in both autoclaved and not-autoclaved soils.

The first, matching GO term 0008536, was annotated by RamanaRao et al. (2012) as a Ran1-binding protein and was found to be downregulated in *S. alterniflora* roots that had been subjected to oil. This corresponded to annotation of two gene isoforms we recovered, although we found them to be upregulated when oil residues were in the soil. Our annotations were based off homology to a Ran GTPase binding protein and a C2H2 zinc finger protein.

The second annotation matched GO term 0003676, for nucleic acid binding, and was described as a downregulated, glycine-rich RNA-binding protein by RamanaRao et al. (2012) when oil was present. The two isoforms we identified were also downregulated in response to oil and were annotated as homologous to an SAP domain-containing protein and a DEAD-box ATP-dependent RNA helicase. Taken together, the matching annotations are evidence for a stress response by the plant which depends on complex transcription and nuclear transport mechanisms (Giri et al. 2013; Han et al. 2020; Nawaz and Kang 2017; Nielsen 2020).However, the contrasting directions of regulation in some of the annotations underscore the potential complexity of the response.

Two other groups examined the genetic response of leaf tissue to oil residues in the soil (Alvarez et al. 2018; Robertson et al. 2017). While this provides an interesting look at how the photosynthesizing tissue is responding to oil in the soil, it ignores the tissue directly in contact with the oil: the roots. Nevertheless, Alvarez et al. (2018) found evidence for a *S. alterniflora* PAH xenome that was quite different from the PAH xenome identified in *Arabidopsis thaliana,* although still containing many of the

multigenic families that have been implicated in the regulation of xenobiotics, like ATP-binding cassette (ABC) transporters (Edwards et al. 2011). In the group of 126 differentially expressed gene isoforms found in both autoclaved and not-autoclaved soils we recovered annotations for four of the six multigenic families examined by Alvarez et al. (2018), corresponding to five gene isoforms. These included upregulation of two putative ABC transporters, one glycosyltransferase, and one glutathione transferase, and downregulation of one putative alpha/beta hydrolase. But we did not annotate any of these 126 gene isoforms as belonging to the cytochrome P450s or malonyltransferase families.

Notably, none of these were described as functionally enriched GO terms, suggesting that these annotations might not represent broad functional changes, but rather changes in singular genes without strong biological significance. However, the common differential expression detected in plants growing in autoclaved and not-autoclaved soils also suggest that the response of that particular gene to the oil residues may be strong enough to supersede the noise of different initial soil conditions and may represent a gene that merits further attention.

MICROBIAL ALPHA DIVERSITY

Microbial alpha diversity was likely decreased by the autoclaving treatment, but not the oil addition. However, as the differences made by the autoclaving treatment were mostly detectable at higher orders of Hill numbers, it might be more appropriate to say the reduction in alpha diversity was explained by an increase in the number of rare species. Furthermore, differences in alpha diversity, particularly at the outset of the

experiment, may have been convoluted by sequencing of relic DNA (Carini et al. 2016). But the reduction was not statistically consistent across Hill orders, suggesting that the ecological significance of the reduction was marginal.

The negligible relationship between alpha diversity and oil is not surprising as some field studies of salt marsh soil microbial diversity have found minimal evidence for a relationship between microbial alpha diversity and oil residues (Looper et al. 2013; Lumibao et al. 2018), while others have found a negative relationship, but only at extremely high concentrations of oil residues (Atlas et al. 2015; Engel et al. 2017).

MICROBIAL COMMUNITY COMPOSITION AND DIFFERENTIAL ABUNDANCE

Prokaryote community composition could be statistically distinguished by the autoclaving treatment but not by the oil addition treatment. We were surprised by this lack of difference, as many studies have shown oil addition to drive a deviation in prokaryote community composition (Gutierrez et al. 2013; Head et al. 2006; Hu et al. 2017; Joye et al. 2016; Joye et al. 2014; Kostka et al. 2011). Regardless, the result that we wish to highlight is that oiled prokaryote communities were more similar to the communities in their parent soils than to other oiled communities. This contrasts with the characterization of soil chemistry and reflects the experimental design. Our results suggest that for prokaryote communities in salt marsh soils, a community that receives oil should be thought of as a derivative of that particular soil and will share more with the parent soil than oiled soil from another salt marsh.

It can be argued that the composition of two oiled soil prokaryote communities might be statistically indistinguishable through community metrics but be recognizable as oiled through relative increases in known oil-degrading taxa (Atlas et al. 2015; Engel et al. 2017; Gutierrez et al. 2016; Gutierrez et al. 2013; Joye et al. 2016; Joye et al. 2014; Mahmoudi et al. 2013; Yang et al. 2016) . However, in our results the prokaryote species scored as differentially abundant with respect to oil, did not overlap between the two soils, with the exception of a *Spirochaeta* sp. Recovering evidence of *Spirochaeta* from two oiled salt marsh soils is reasonable as *Spirochaeta* are common inhabitants of aquatic environments and have been described as key members of prokaryote communities at oceanic hydrocarbon seeps (Joye 2020). We found three classes of prokaryotes, Methanomicrobia, Bathyarchaeia, and Spirochaetia, that were only identified as relatively more abundant in the presence of oil, suggesting a consistency of response at the ASV level for these clades. None of the Proteobacteria, which are known for hydrocarbon degrading abilities (Joye et al. 2016) showed a consistent response by ASVs across the classes, orders, families, or genera within the phylum. That is not to say that these taxa do not respond to hydrocarbons, but rather that they must be examined at a fine phylogenetic resolution if they are to be used as indicators of a community response to the presence of oil. If microbial community responses are subtle enough to only be detected at these fine scales, then an understanding of the pre-spill community composition is necessary to describe these changes.

In contrast to prokaryotes, fungal communities were statistically indistinguishable by the autoclave or oil addition treatment. The minimal response by the fungal communities to oil is consistent with the results of this dissertation and of others

(Mahmoudi et al. 2013). But we were surprised by the minimal effect of the autoclaving treatment. It may be that fungal spores are more resilient to autoclaving than prokaryotes, or that relic fungal DNA may be more resilient to degradation. Another distinct possibility is that the fungal community in a salt marsh soil is most strongly influenced by the environment and rapidly incorporates migrating spores from the air and water. The indifference of the fungal community composition to these perturbations to soil chemistry supports the hypothesis that differences found between oiled and unoiled reference sites may only be evidence of differences driven by the site, not a robust response to the addition of oil to the system.

CONCLUSIONS

In our experiment, two sets of plants exposed to the same oil, growing in the same atmospheric conditions and hydrology showed different genetic responses to slightly different soil conditions. Likewise, two oiled soil prokaryote communities were more different in composition from each other than from their unoiled parent soils, despite similar beginnings. Our work demonstrates that understanding pre-oil conditions may be key for detecting shifts in plant root genetic response and soil microbial communities in an oiled environment. If samples of the pre-oil spill conditions are unavailable, then extra care should be taken when interpreting results from reference sites, which may vary substantially in pre-spill conditions. The more distance between oiled and reference samples, whether geographical or biogeochemical, the less confident we should be that we can identify the effects of the oil via differences in microbial composition of the oiled and reference samples.

CHAPTER 2 FIGURES

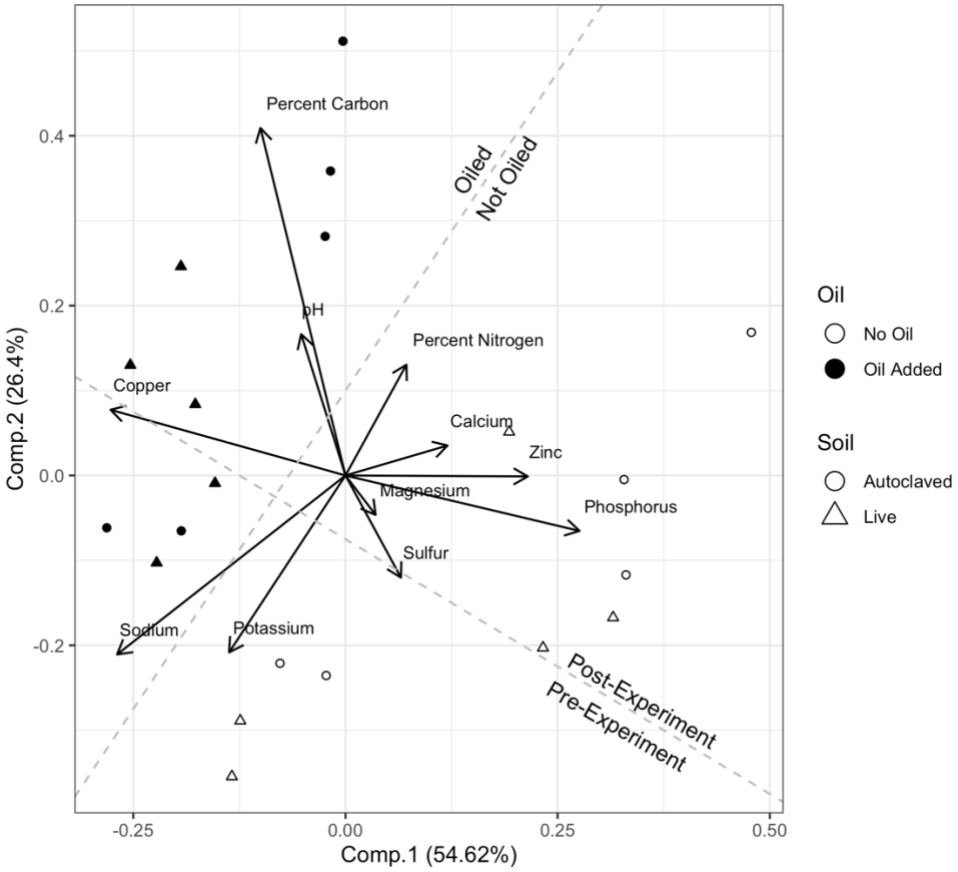

Figure 2-1: Biplot of soil chemistry

Biplot of soil chemistry, shapes represent the autoclaving treatment and colors represent the oil addition treatment. Elemental analyses were clr-transformed prior to performing the PCA. Dashed lines serve as references to delineate the two major explanatory factors: (1) samples that were taken at the beginning of the experiment vs the end of the experiment and (2) samples that had oil added to them or not.

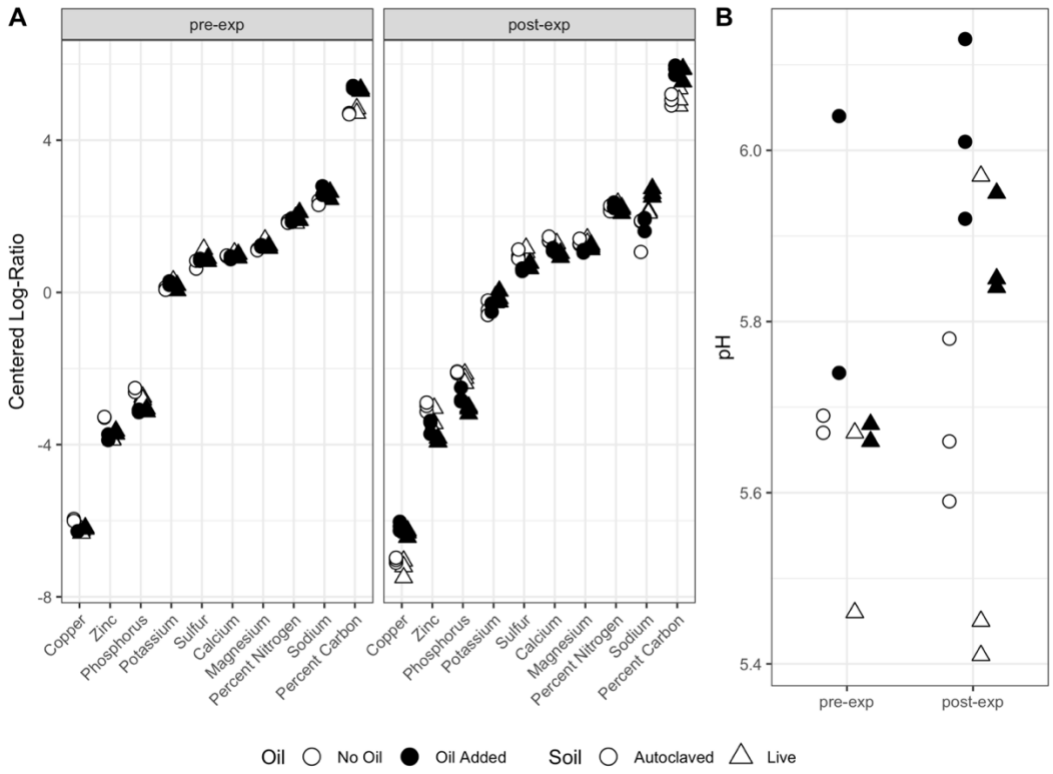

Figure 2-2: Additional comparisons of soil chemistry

Additional comparisons of soil chemistry. Shapes represent the autoclaving treatment and colors represent the oil addition treatment. (A) clr-transformations of all elements analyzed, ordered by median group value from left to right. The geometric mean of all values within a sample is represented by zero on the y-axis. (B) Soil pH of each sample, separated out by sampling time on the x-axis.

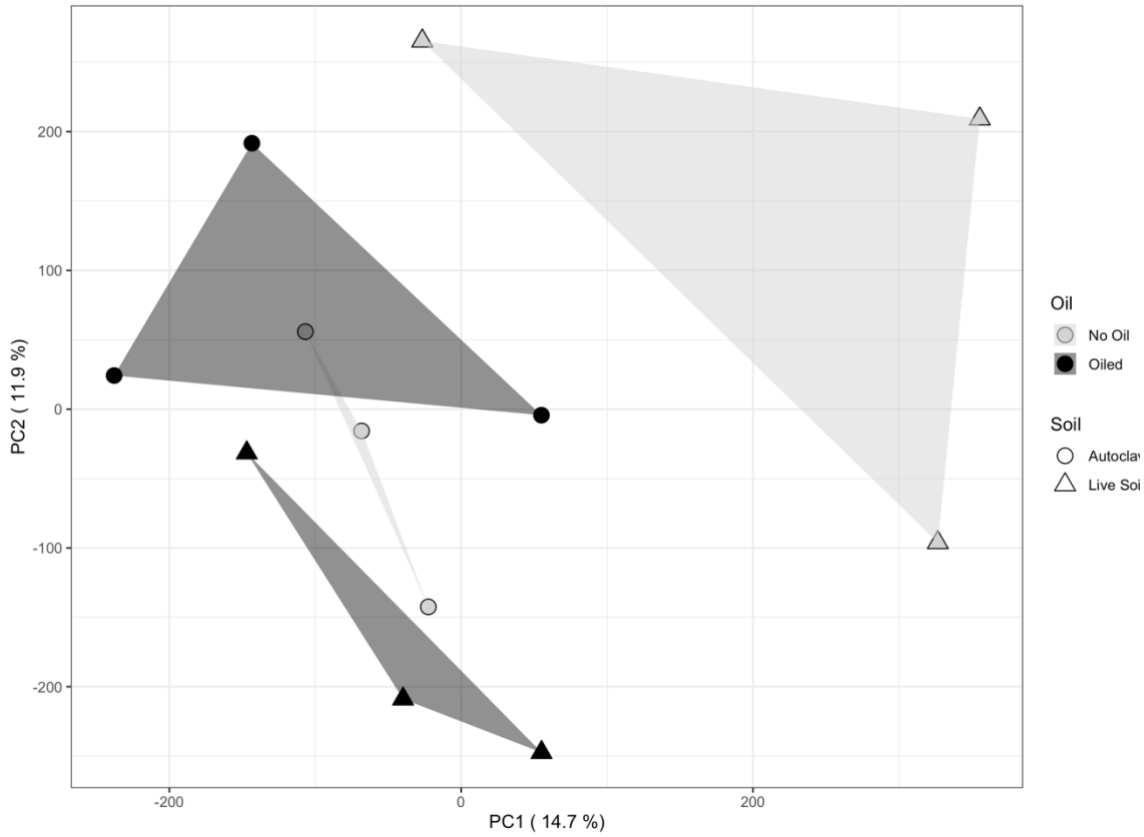

Figure 2-3: PCA of the root transcriptome

PCA of the root transcriptome based on Aitchison distances. The autoclave treatment is represented by shape and oil addition treatments are represented by color.

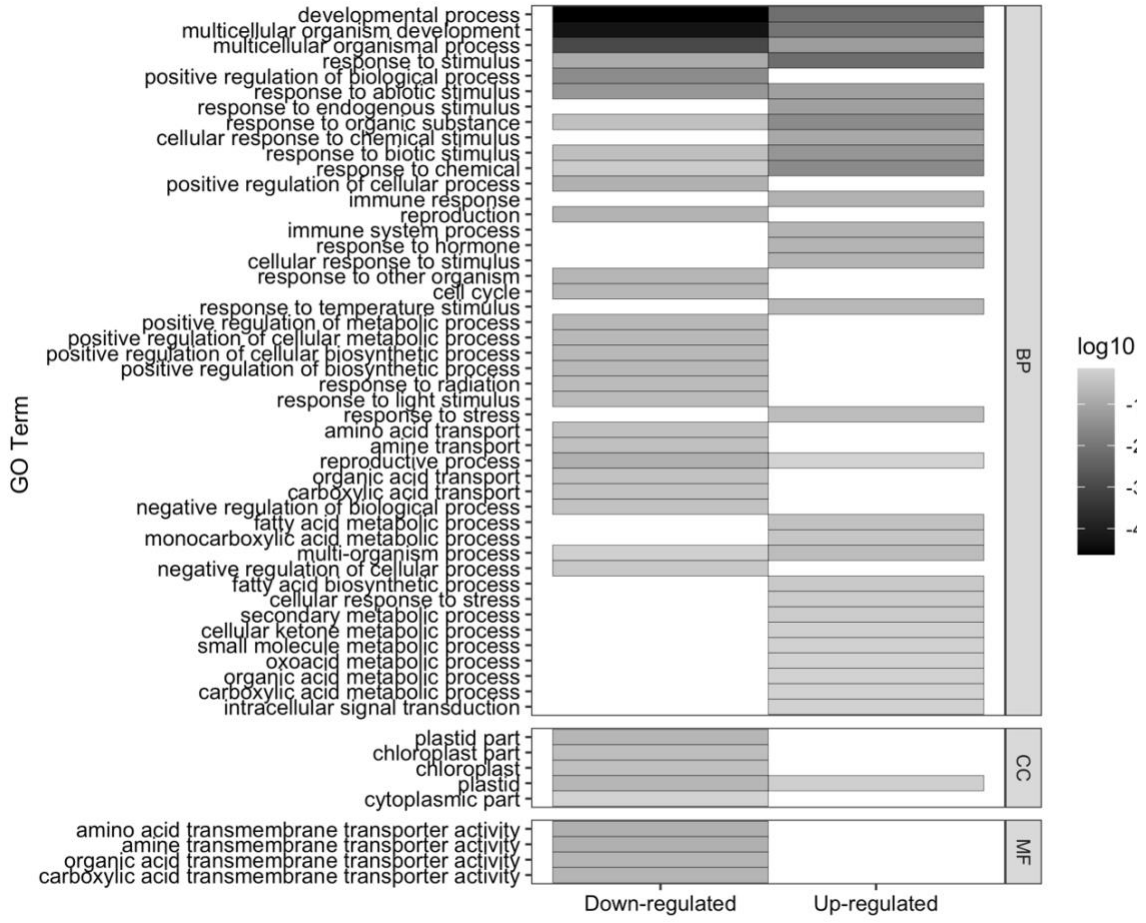

Figure 2-4: Enriched GO terms in the root transcriptome

Enriched GO terms. Terms are organized into three groups along the y-axis, as described on the right-hand side of the plot. BP represents Biological Processes, CC represents Cellular Components, and MF represent Molecular Functions. Descriptions of each GO term are on the left hand side. Cells are colored by the log10 of the p-value, where a darker shade means a smaller p-value. Cells are organized into two columns on the x-axis: values for terms associated with genes that were differentially less abundant (down-regulated) or more abundant (up-regulated).

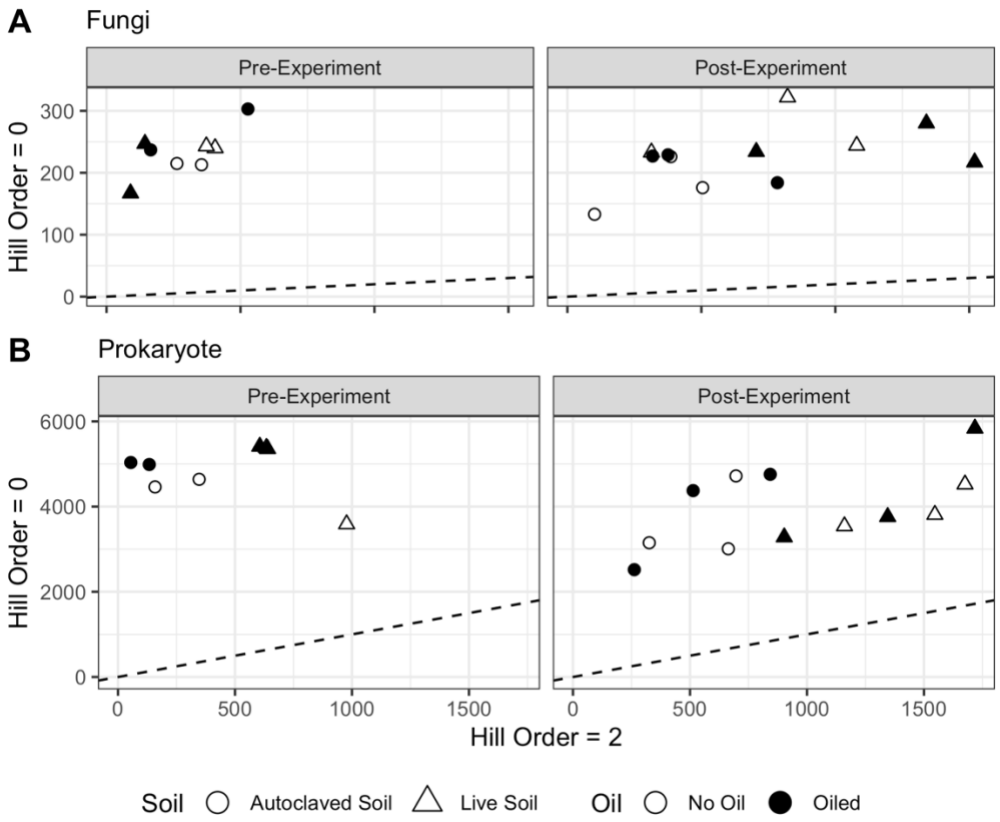

Figure 2-5: Alpha diversity of fungal and prokaryote communities

Alpha diversity of (A) fungal and (B) prokaryote communities. Shapes represent the autoclaving treatment and colors represent the oil addition treatment. The y-axis represents the effective number of species for Hill order = 0 and the x-axis represents the same for Hill order = 2. The identity line represents where perfectly even communities would fall on the plot. Panels are separated by columns into the samples taken at the beginning and end of the experiment.

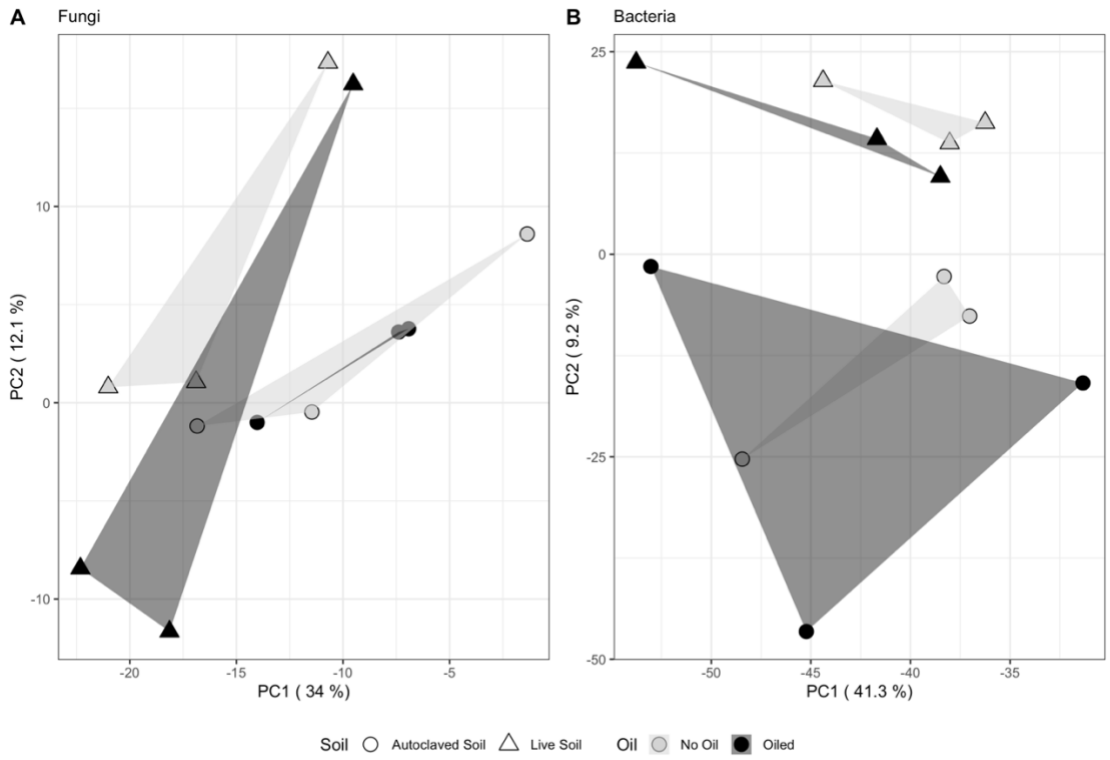

Figure 2-6: Beta diversity of fungal and prokaryote communities

Beta diversity of (A) fungal and (B) prokaryote communities represented by PCA based on Aitchison distances. The autoclave treatment is represented by shape and oil addition treatments are represented by color.

CHAPTER 2 TABLES

Table 2-1: PERMANOVA on soil chemistry.

PERMANOVA on Aitchison distances of soil chemistry. The term soil" represents the autoclave treatment, "oil" represents the addition of oil to the soil, and "time" indicates the differences between samples at the beginning and end of the experiment. Significant results are bolded.

Treatment	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
soil	1	2.0168	2.0168	10.6	0.09882	**0.0001**
oil	1	7.2494	7.2494	38.103	0.35522	**0.0001**
time	1	5.757	5.757	30.259	0.28209	**0.0001**
soil:oil	1	0.2016	0.2016	1.06	0.00988	0.3435
soil:time	1	0.6301	0.6301	3.312	0.03087	**0.0337**
oil:time	1	1.7716	1.7716	9.312	0.08681	**0.0002**
soil:oil:time	1	0.4986	0.4986	2.621	0.02443	0.0663
Residuals	12	2.2831	0.1903	0.11187		
Total	19	20.4082	1			

Table 2-2: PERMANOVA on the root transcriptome.

PERMANOVA on Aitchison distance of transcriptome composition. The term "soil" represents the autoclave treatment and "oil" represents the addition of oil to the soil. Significant results are bolded.

Treatment	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
soil	1	242345	242345	1.2609	0.10799	**0.0339**
oil	1	228977	228977	1.1914	0.10204	0.0696
soil:oil	1	235187	235187	1.2237	0.1048	**0.0457**
Residuals	8	1537590	192199		0.68517	
Total	11	2244099			1	

Table 2-3: PERMANOVA on prokaryote community composition.

PERMANOVA on Aitchison distance of prokaryote community composition. The term "soil" represents the autoclave treatment and "oil" represents the addition of oil to the soil. Significant results are bolded.

Treatment	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
soil	1	4182.6	4182.6	1.53936	0.13258	**0.0004**
oil	1	2912.1	2912.1	1.07175	0.09231	0.1976
soil:oil	1	2715.2	2715.2	0.99929	0.08607	0.3996
Residuals	8	21736.9	2717.1		0.68904	
Total	11	31546.8			1	

Table 2-4: PERMANOVA on fungal community composition.

PERMANOVA on Aitchison distance of fungal community composition. The term “soil” represents the autoclave treatment and “oil” represents the addition of oil to the soil.

Treatment	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
soil	1	581.1	581.09	1.26762	0.11347	0.1199
oil	1	456.5	456.52	0.99586	0.08915	0.4223
soil:oil	1	416.1	416.05	0.90759	0.08125	0.5844
Residuals	8	3667.3	458.41		0.71614	
Total	11	5120.9			1	

CHAPTER 3

THE INFLUENCE OF *SPARTINA ALTERNIFLORA* ON OIL RESIDUES IN THE SOIL AND THE EFFECTS OF SOIL AMENDMENTS ON THE PLANT MICROBIOME

INTRODUCTION

The importance of *Spartina alterniflora* in Gulf Coast salt marsh function and maintenance is well-established, making *S. alterniflora* a natural focal point for understanding salt marsh resilience to the Deepwater Horizon (DWH) oil spill. This foundational grass showed remarkable resilience to deposition of oil residues, recovering almost to reference levels in less than four years (Lin et al. 2016). However, very little is known about the possible contributions of symbiotic plant microbes to this resilience. In extreme environments, like a salt marsh, where the soil is saline and hypoxic, plants may be especially dependent on their microbiome for resilience to stress and to respond to toxins in the soil. Preliminary research after the DWH oil spill suggested that *S. alterniflora* growing in areas contaminated with oil depended on its microbiome, as evidenced by a corresponding shift in the microbiome to include more taxa with biodegradation potential (Kandalepas et al. 2015).

In addition to understanding the recovery of vegetation, a major post-spill goal was understanding biodegradation of oil residues. Many studies focused on the

importance of microbes in biodegradation of oil residues in seawater and coastal soils (Joye et al. 2014), but few efforts considered the possible interactions of plants with the microbial communities. Recent work has demonstrated the phytoremediation potential of *S. alterniflora* (Cavé-Radet et al. 2020; Hong et al. 2015; Mendelssohn and Lin 2002; Su et al. 2016); suggesting that the plant may be harnessed to accelerate decomposition of oil by selecting for oil-degrading microbial communities in the soil in combination with enhanced delivery of oxygen and plant enzymes to the soil.

Optimizing methods for restoration of oiled salt marshes would be especially meaningful in the Gulf of Mexico, where there is a persistently high threat of petroleum contamination. One possibility is enhancing the recovery of *S. alterniflora* through re-planting. Studies of restoration techniques tested after the DWH oil spill supported re-planting of heavily-damaged coastlines as a means to hasten re-vegetation and reduce erosion (Zengel et al. 2015). Other research has shown that it is possible to inoculate grasses with microbial symbionts to enhance plant growth and function in extreme and toxic environments (Li and Zhang 2015; Márquez et al. 2007; Redman et al. 2011; Yuan et al. 2016). Therefore, a favorable strategy for restoration of oiled sites might include planting *S. alterniflora* with a microbiome that has been pre-selected for an oiled environment.

To examine this strategy, we designed a greenhouse experiment that tested three specific hypotheses: (1) The presence of *S. alterniflora* in an oiled environment enhances the degradation of oil residues; (2) the plant microbiome can be manipulated by application of soil inocula to the nascent plant; and (3) in an oiled environment, a soil inoculum that is pre-exposed to oil residues enhances the growth of the plant and

degradation of oil relative to an inoculum that is naïve to oil. We ran the experiment for two years, sampling soil, roots and leaves every six months. Every two months we applied soil inocula from either (1) a salt marsh that had been oiled in the DWH oil spill, or (2) one that was comparatively un-oiled.

We expected the presence of the plant to clearly reduce polycyclic aromatic hydrocarbons (PAHs) in the soil and to correspond to soil microbial communities that were compositionally different than when no plant was present. Moreover, we predicted that we could manipulate the plant microbiome by applying soil inocula to our mesocosms with the expectation that the microbiome of the soil, roots, and leaves would have distinct signatures corresponding to the soil inocula and the presence of oil. Finally, we thought plant growth would be reduced in an oiled environment, but the negative effect would be reduced by inoculating the plant with a microbiome that had previously been optimized to exist with oil residues in the environment.

METHODS

EXPERIMENTAL DESIGN

Three groups of response variables, plant morphology, PAHs, and microbial communities, were measured in response to three combinations of treatments. The experiment was a full factorial design in which each of the three treatments consisted of two levels: (1) the presence or absence of the plant; (2) inoculation of the mesocosms with soil from a marsh that had either been heavily oiled in the DWH oil spill or a marsh that was relatively unoiled by the DWH oil spill; and (3) the addition of 1.6 L of weathered oil to the soil or not. This resulted in 8 treatment combinations, with 10

replicates for each treatment combination (n=80 mesocosms). However, because of financial constraints, only 3 replicates were sequenced for microbial communities and for PAH content. PAH measurements were then augmented with an additional 75 samples from the first and last sampling periods to add clarity to the results. Throughout the experiment care was taken to avoid cross-contamination by cleaning tools with 95% ethanol, 10% bleach, and changing gloves when necessary.

SETUP

Seeds from *Spartina alterniflora* were collected in November 2015 in southern Louisiana and placed at 4 °C to stratify. In February 2016 the microbial community on the surface of the seeds was reduced by subjecting them to a 95% EtOH bath for 3 minutes, then 30%min in 0.825% bleach and rinsed in sterile DI water for 10 seconds. The seeds were then germinated in DI water and transplanted to trays in a Conviron Model GR48 Plant Grow Room (Controlled Environments Ltd., Winnipeg, Canada). The growing substrate was a 1:1 mixture of organic humus and vermiculite that was autoclaved three times at 121 °C for 60 min to reduce the microbial community in the substrate. Seedlings were grown under conditions meant to mimic conditions in southern Louisiana, after the methods of Krauss et al. (1998). Seedlings were watered with deionized water and each tray was given 1 tablespoon of Osmocote fertilizer (14:14:14 NPK). In April 2016 the plantlets were transferred to the greenhouse and allowed to acclimate to greenhouse conditions for about three weeks.

On May 1st the plantlets were planted into pots filled with inoculum soil. The inoculum soil was collected from Bay Jimmy, LA at sites (Figure 3-1) that had been

heavily oiled (29.44464, -89.88959) or relatively unoiled (29.44006, -89.88583) in the DWH oil spill (Zengel et al. 2015). Within 60 hours of collection the soil was sieved through a 1 cm screen, placed in 3.8 L (1 gallon) trade pots and either planted or left unplanted. The pots were randomized in five blocks on an approximate East-West axis across four tables that were oriented approximately North-South. The plants grew in the inoculum for one month and then the entire plant-soil plug was used to construct the mesocosm.

Each mesocosm consisted of a 11.3 L (3 gallon) trade pot nested in a water-filled 18.9 L (5 gallon) bucket (Figure 3-2). The soil/plant plug was placed inside of the 11.3 L (3 gallon) trade pot containing 9.5 L (2.5 gallon) of a 2:3 organic humus to sand mixture. Half of the mesocosms also had 1.6 L of naturally weathered oil mixed into the soil. The oil was skimmed off the surface of the ocean during the Deepwater Horizon oil spill (see acknowledgements). To reduce spore fall germination, 1.9 L (0.5 gallons) of clean sand were placed in an even layer around the top of the plant. Three weeks after mesocosm construction, 8 g of Scott's Osmocote Plus (Marysville, Ohio) was added to the surface and covered with an additional 0.95 L (0.25 gallon) sand layer. The fertilizer is a patterned-release complete nutrient fertilizer containing 15% N, 9% P, and 12% K (releasing at maximum 1.00 g·m-2 nitrate per month). Drip irrigation supplied water to the surface of each pot, and pots were inserted into buckets in order to maintain a high water table. Drainage tubes were installed near the top of each bucket, allowing some flow through. To prevent reactivity to oil, the 11.3 L (3 gallon) trade pots were lined with Teflon bags (P-00113, Welch Fluorocarbon, Inc, Dover, NH, USA). The bottom of each bag was punctured to permit drainage.

MESOCOSM MAINTENANCE

Over the course of the experiment, plants received water from a timer-based drip irrigation, so that some portion of the water would turn over every day. If necessary, during hot summer months the watering was augmented by hand to maintain the height of the water table. Once a month, the mesocosms received 1 L of 5 ppt solution of Instant Ocean salt (Instant Ocean Spectrum Brands, Blacksburg, VA). Every two months mesocosms were augmented with an additional 4 g of fertilizer and 1 L of inoculum, a soil slurry from the same marsh sites used in the original inoculation. Whenever salt built up on the plant leaves, plants were misted to wash salt off the leaves. Unplanted pots were misted for an equal length of time to control for the extra water delivered.

SAMPLE COLLECTION OVERVIEW

We sampled the experiment every 6 months for two years, with a complete harvest of the mesocosms in June 2018. Every sampling period began with measuring plant morphology (details below), followed by harvesting leaves for microbial and CN analysis. Next the mesocosm was sampled for roots and soil as quickly as possible. To avoid resampling the same area, the bucket was divided into quarters and soil and roots sampled from approximately 1”- 8” deep. Fresh gloves were used between every plant, and work done on a large sheet of glass that was laid on the table and cleaned with 10% bleach and 95% ethanol between samples. All tools were also cleaned with bleach and ethanol and, if contaminated with oil, mineral spirits.

The dense root mat made it necessary to use a keyhole saw to cut through the roots. Once the sod was cut, it was removed from the bucket, and placed on the glass, where roots were picked as quickly as possible, and soil placed into a clean beaker.

After sampling, the area was refilled with sand and 1 L of water was added. That area was not resampled for soil or roots in subsequent sampling periods. However, stems that grew into the sand of previously sampled areas were included in subsequent samplings.

PLANT MORPHOLOGY MEASUREMENTS

Four morphological traits were measured for 20 haphazardly selected stems that the collector thought to be representative of the plant in each mesocosm. Care was taken to sample stems across the entire mesocosm. Stem height was recorded by measuring the height, from soil surface to tip of unfurled leaf to the nearest 0.5 cm. Stem diameter was recorded by measuring the diameter of stems ~10 cm above the soil using analog calipers to the nearest 0.1 mm. The number of nodes per stem were also counted for every stem measured. The number of live stems, regardless of size, were counted with a hand-clicker. Stems were counted only once for the initial sampling period because the number of stems per plant was low enough (< 61) that they could be counted confidently. For the subsequent three sampling periods, live stems were counted three times and these values were averaged to obtain the number of live stems.

MICROBIAL SAMPLE COLLECTION AND PROCESSING

Samples were collected using clean gloves. Tools and surfaces were cleaned with 10% bleach and 95% ethanol between every sample.

soil

Approximately 1 L of soil was collected, homogenized in a clean beaker, and distributed to 1.5 ml tubes for microbial analysis, 15 ml tubes for PAH analysis and 50 ml tubes for CN analysis. Soil samples for chemical analysis were placed in refrigeration until analysis. Soil samples for microbial analysis were placed on dry ice within 5 minutes of collection.

leaves

Approximately 5 g of whole, healthy leaves were collected, placed into a clean Ziploc bag and kept in refrigeration. To control for leaf age, the third leaf from the bottom was collected from a given stem. If the third leaf was blemished, then the fourth leaf was collected instead. All subsequent processing occurred within 12 hours of collection. Edges were removed from leaves and leaves chopped into 2x2 mm pieces to prepare them for microbial analysis. Leaves were treated with bleach and ethanol to reduce the presence of microbes on the outside of the tissue. Using sterile technique in a biosafety cabinet and following the methods of Kandalepas et al. (2015), leaf pieces were submerged for 10 seconds in 95% ethanol, 2 minutes in 0.525% sodium hypochlorite (10% bleach) followed by 2 minutes in 70% ethanol and frozen at -20 °C until DNA extraction.

roots

Roots were processed similarly to leaves. A small handful of healthy, but unwashed, roots were collected and placed in refrigeration. Some roots were washed in DI water until 4 g was obtained. The remainder of the root sample was kept in refrigeration until processed for PAH analysis (results not shown). The 4 g of clean roots were chopped into smaller chunks and 2 g set aside for microbial analysis. The remainder was set aside for CN analysis. The 2 g intended for microbial analysis were cut into 5 mm lengths and treated to reduce the external microbes. Root pieces were first submerged for 10 seconds in 70% ethanol, 2 minutes in 2.625% sodium hypochlorite (50% bleach), followed by 3 rinses in sterile DI water and frozen at -20 °C until DNA extraction.

ITS LIBRARY PREP AND SEQUENCING

We followed the methods of Lumibao et al. (2018) to create a metagenome of the fungal ITS1 region. Briefly, the method is a 2-step library preparation using primers ITS1F (CACTCTTTCCCTACACGACGCTCTTCCGATCTCTTGGTCATTTAGAGGAAGTAA) and ITS2 (CACTCTTTCCCTACACGACGCTCTTCCGATCTCTTGGTCATTTAGAGGAAGTAA). After high quality genomic DNA was extracted from tissue, the ITS1 region was amplified in triplicate via PCR. The product of this first PCR is pooled and then indexed with Illumina barcodes using another round of PCR. The PCR products are then pooled in equimolar amounts and sequenced on an Illumina MiSeq platform (Illumina Inc. San

Diego, USA) using 300 bp PE sequencing. Sequencing was carried out by the Duke University Genome Sequencing and Analysis Core Facility (Durham, USA).

processing and clustering

Sequences were processed following the DADA2 ITS Pipeline Workflow, version 1.8 (Callahan et al. 2016) in R version 3.6.1 (R Development Core Team 2019). The pipeline also depends on the R packages ShortRead (Morgan et al. 2009), BioStrings (Pagès et al. 2019) , and ggplot2 (Kahle and Wickham 2013). Briefly, samples were trimmed and filtered with cutadapt (Martin 2011) based on detection of primer sequences from the first PCR in library prep. Then sequences were trimmed and filtered again after examining quality profiles of the reads. The main DADA2 algorithm with default parameters was then employed to learn the error rates of the sequences and dereplicate them, then infer the correct sequence for each read. Paired-end reads were then merged, chimeras remove and taxonomy assigned against the UNITE database (UNITE Community 2019).

16S LIBRARY PREP AND SEQUENCING

Separate leaf, root, and soil tissue was collected to mail (on dry ice) to Duke for 16S analysis. The tissue was processed the same as tissues used in ITS sequencing, except after surface sterilization, leaves and roots were frozen in a CTAB buffer (2 % CTAB, 0.02M EDTA, 0.1M Tris, 1.4M NaCl). Description of the library prep and sequencing processes can be found in Lefèvre et al. (2020).

processing and clustering

Sequences were processed following the DADA2 16S Pipeline Workflow, version 1.12 (Callahan et al. 2016) in R version 3.6.1 (R Development Core Team 2019). The pipeline depends on the same packages cited above. Briefly, samples were trimmed and filtered after examining quality profiles of the reads. The main DADA2 algorithm with default parameters was then employed to learn the error rates of the sequences and dereplicate them, then infer the correct sequence for each read. Paired-end reads were then merged, chimeras remove and taxonomy assigned against the SILVA 132 database (Yilmaz et al. 2014). As a final step, ASVs that were assigned chloroplast or mitochondrial taxonomy were filtered out.

OIL SAMPLE COLLECTION AND PROCESSING

Soil and roots for oil analysis were collected as described above. PAH content was analyzed using gas chromatography/mass spectrometry following the methods of Lumibao et al. (2018) and Curtis (2018).

BIOMASS COLLECTION

In June 2018, the experiment was completely harvested. After leaves were sampled, all stems were clipped, then soil and roots were collected. Plant tissue was dried in an oven at 60 °C until mass was consistent two days in a row.

STATISTICAL ANALYSIS

Unless otherwise mentioned we conducted all statistical analyses in R version 4.0.2 (R Core Team 2020). Analyses and figures depended heavily on the *tidyverse* (Wickham et al. 2019), *cowplot* (Wilke 2020), *compositions* (van den Boogaart et al. 2020), *phyloseq* (McMurdie and Holmes 2013) and *vegan* (Oksanen et al. 2016).

PAHs

We modeled the difference in total PAH abundance between the beginning and end of the experiment with an ANOVA, using log-transformed total PAHs to meet assumptions of normality. Differences in PAH composition were tested with a PERMANOVA, using the *adonis* function in the *vegan* package, and 9999 permutations. Models were based on Aitchison distance (Euclidean distance of center-log ratio (CLR) transformed values) and controlling for between-sampling period variation through the strata argument.

Plant Traits

Differences in plant biomass were modeled using ANOVAs on square-root transformed biomass to meet assumptions of normality. Plant traits were analyzed with Bayesian mixed-effects models (Bürkner 2017; Carpenter et al. 2017) to account for the repeated measures aspect of the experiment and generally followed the structure:

plant trait ~ time + oil addition*inoculum + (1|plantID)

Models were fit to different conditional distributions depending on the model fit after initial exploration of the data. Number of nodes and number of live stems were fit to negative binomial distributions, and stem height and diameter to skew normal

distributions. Results from the model for the stem count and visual inspection of the data led us to test specifically for differences at the final sampling period with a Generalized Linear model (GLM), based on a quasi-Poisson distribution.

Microbial Communities

Alpha diversity of microbial communities was estimated with Shannon's diversity index and tested for statistical differences using GLMs assuming gamma distributions to account for the inability of diversity to be negative. We constructed four models: one testing for differences between tissues, and a model for treatment effects for each of the plant tissues (soil, root, and leaf). Seasonal effects were apparent during exploratory analysis, leading us to group sampling periods into a new variable with two levels: June and November.

We estimated beta diversity via a principal components analysis (PCA) to visualize variation and tested for differences in PAH composition by conducting a PERMANOVA of Aitchison distances. Results were compared with NMDS ordinations and additional PERMANOVAs built on Bray-Curtis and Jaccard Index to probe for sensitivities based on metric and ordination choice.

RESULTS

EFFECT OF PLANT PRESENCE AND INOCULUM ON PAH COMPOSITION

Plant presence affected decomposition of PAHs over the course of the experiment (Figure 3-3), while inoculum treatments did not correspond to any differences in PAH

decomposition. Six months into the experiment no differences in PAH composition could be detected with regard to the plant presence/absence or the soil inocula. However, at the end of the experiment, the total abundance of PAHs was reduced by an order of magnitude (With Plant = 5.96 ug/g ± 2.69 SE ; Without Plant = 57.64 ± 9.36 SE) when a plant was present (ANOVA $F_{1,57} = 14.17$, $p = 0.0003$), and the composition of PAHs was significantly different (PERMANOVA $F = {}_{1,71} = 10.52$, $p = 0.0001$), primarily due to a relative reduction in lighter PAHs compared to chrysenes (Figure 3-3). No differences in PAH content corresponded to the soil inocula (results not shown).

EFFECT OF PAHS AND INOCULUM ON PLANT GROWTH AND MORPHOLOGY

We measured the response of several plant traits and biomass to the oil addition and the inoculum treatments but found few differences. There was no statistical difference in aboveground, or belowground biomass at the end of the experiment for any treatment, nor any difference in the ratio of aboveground to belowground biomass (Figure 3-4). Furthermore, the plants did not differ in stem height, diameter or number of nodes per stem. However, results from a GLM suggest the oil addition did reduce the number of live stems (With Oil = 81.1 stems ± 5.64 SE ; No Oil = 104.7 stems ± 3.68 SE) in the mesocosm (Figure 3-5), although this only manifested for the final sampling period ($F_{1,37} = 10.97$, $p = 0.002$).

MICROBIAL COMMUNITY COMPOSITION AS A FUNCTION OF PLANT COMPARTMENT AND OIL ADDITION

Bacterial Communities

Sequencing yielded 27,935,842 PE reads after quality filtering. Samples had 145,499.2 PE reads on average (SD = 133,843). Despite this large variation in sequencing depth, sequences were of good quality.

Alpha diversity in bacterial communities was significantly higher in soil samples compared with roots or leaves (ANOVA $F_{2,188} = 39.8$, $p < 0.00001$). There were no differences in soil or leaf diversity by any treatment or sampling period, but root communities were less diverse in the November sampling periods compared to the June sampling periods (ANOVA $F_{1,4} = 11.57$, $p = 0.001$).

Bacterial community composition was statistically different between plant compartments (soil, roots, and leaves) (PERMANOVA $F_{2,191} = 5.77$, $p = 0.0001$). Communities in soil samples were statistically different when a plant was present, when oil was added, between the inocula, and the interaction of any two treatments (e.g., plant:oil, oil:inocula, see Table 3-1). Likewise, differences in root community composition were significant for oil addition and inocula treatments (Table 3-2) and showed strong seasonality between November and June (Figure 3-7). These seasonal differences were primarily driven by differences in richness, and a decrease in the relative abundance of Actinobacteria, Bacteroidetes, and Epsilonbacteraeota during November. Bacterial communities in leaves, however, were not significantly different for the main effects of either treatment, although the interaction of the oil addition and soil inoculum was significant (PERMANOVA $F_{1,47} = 1.27$, $p = 0.02$). However, it was difficult to discern which combinations of treatments were driving the significant interaction. Leaf communities also appeared to steadily converge from the beginning of the experiment to the final sampling period (Figure 3-8).

Fungal Communities

Sequencing yielded 15,732,033 PE reads after quality filtering. On average, samples had 66,100 PE reads (SD = 21,494). Alpha diversity in fungal communities was significantly higher in soil samples compared with roots and higher in root samples than leaves (ANOVA $F_{2,188} = 374.3$, $p < 0.00001$). There were no differences in soil, root, or leaf diversity by any treatment or sampling period.

Fungal community composition was statistically different between plant compartments (PERMANOVA $F_{2,191} = 12.98$, $p = 0.0001$). Communities in soil samples were statistically different when a plant was present, between the inocula, and the interaction of the plant and inocula (Figure 3-9, Table 3-4). The interaction appeared to be driven by differences within the not-previously oiled inoculum group and the presence of the plant. No effect was detected from the oil addition. Root community composition was significantly different for oil addition and inocula treatments (Figure 3-10, Table 3-5). Fungal communities in leaves showed difference in community composition corresponding to the inocula and the interaction of inocula with oil addition (Figure 3-11, Table 3-6).

DISCUSSION

An optimal strategy for restoring an oiled salt marsh should maximize foundational plant productivity while simultaneously removing the contaminant – oil residues – from the environment. We highlight three key results that may help inform

such a strategy. First, planting *S. alterniflora* in an oiled environment would likely hasten the decomposition of oil residues. Second, the morphology and productivity of the plant does not appear to react negatively to introduction into an oiled environment. Lastly, the plant's microbiome is dynamic and changes with exposure to different soil microbial communities and seasons, but these changes do not correspond to differences in plant morphology or oil decomposition.

EFFECT OF PLANT PRESENCE AND INOCULUM ON PAH COMPOSITION

Plant presence clearly enhanced the decomposition of PAHs over the course of two years. The patterns we saw in relative decrease in two- and three-ring PAHs to four-ring PAHs (chrysenes) suggest biodegradation of PAHs (Rodrigue et al. 2020). Biodegradation of recalcitrant oil residues in coastal marshes is likely driven by a complex concert of oxygen availability and chemical cues driving changes in microbial communities (Collins et al. 2020; Head et al. 2006; Matthew et al. 2016). The plant may hasten biodegradation of PAHs by enhancing oxygen in the soil (Koop-Jakobsen et al. 2018; Mendelssohn and Lin 2002; Mendelssohn et al. 1981) and/or by delivering enzymes into the soil, which select for soil microbes that degrade oil (Haritash and Kaushik 2009; Mesa-Marin et al. 2019).

It is noteworthy that these differences did not manifest until at least more than six months after the plants were introduced into the soil. This result is at odds with other experiments which have demonstrated hydrocarbon decomposition in shorter periods (Hong et al. 2015; Mendelssohn and Lin 2002; Watts et al. 2006). However, our experiment differed in one key point from many other experiments. Our plants were

started from seed to facilitate the manipulation of the microbiome. This difference also meant that our plants were immature and potentially less productive than if they had been started from rhizomes. The immaturity of the plant may be the explanation for the latency in PAH decomposition.

We had expected the inoculum from the previously oiled marsh to stimulate PAH decomposition by virtue of the microbial community being pre-selected to include taxa that could metabolize PAHs in the environment. There was no difference, however, in PAH decomposition according to the different soil inocula. This null result was consistent with at least two other studies examining the effects of microbial inocula on hydrocarbon degradation (Mendelssohn and Lin 2002; Mesa-Marin et al. 2019). The soil microbial communities showed compositional differences corresponding to the inocula, so the lack of effect was not due to the inefficacy of the treatment. Given the high diversity of the soil microbial communities, it is possible that there may be significant overlap in the functionality of the communities despite measurable differences in their relative compositions, resulting in no difference in the relative rates of PAH decomposition.

EFFECT OF PAHS AND INOCULUM ON PLANT GROWTH AND MORPHOLOGY

S. alterniflora showed surprisingly little response to growing in oiled soil, or from the manipulation of its microbiome. There was no difference in all measured plant traits except for the number of live stems at the end of the experiment. While a reduction in live stem count corresponding to oil exposure is consistent with the work of Hughes et al. (2018), this must be taken with a grain of salt considering the otherwise indifference of the plant to the presence of the oil and the different inocula. Two years is a substantial

amount of time for a highly productive plant, like *S. alterniflora*, to grow in a such a limited space. Many plants were root-bound by the end of the experiment, which may especially explain the lack of difference in biomass. If the plants had maximized their potential in the growing space prior to the two-year harvest, any effects from the treatments would have attenuated by the final harvest. Moreover, our methods results are similar to the work of Mendelssohn and Lin (2002), in which plants were placed into oil soiled, rather than oil being applied to already growing plants. This difference in methodology may be critical to understanding our results; the morphology and biomass of *S. alterniflora* seems to be negatively affected when oil comes into contact with above ground tissues (Hughes et al. 2018; Lin and Mendelssohn 1996; Lin and Mendelssohn 2009; Lin and Mendelssohn 2012), but not when a healthy plant is placed into an oiled environment (Mendelssohn and Lin 2002) .

Prior to our study it was not clear whether shifts in microbial symbionts could be detected in the plant morphological response. Previous work has shown differences in plant morphology (Hughes et al. 2018; Lin and Mendelssohn 2012) and endophyte community composition (Kandalepas et al. 2015) corresponding to oiling, but never the two at the same time. Based on our results, it appears that the microbiome of *S. alterniflora* can detectably shift in composition without influencing the plant morphology significantly. *S. alterniflora* evolved to thrive in a dynamic and extreme environment. It seems possible that the plant may have evolved to optimize morphological stability in response to changes in its microbiome, rather than harnessing the microbiome to mitigate the extreme environment, as has been previously hypothesized about plants living in extreme environments (Rodriguez et al. 2008).

MICROBIAL COMMUNITY COMPOSITION AS A FUNCTION OF PLANT COMPARTMENT AND OIL ADDITION

Microbial communities were able to be manipulated through the application of soil inocula, but evidence also suggested responses to seasonality and the local environment. Our previous work indicated that endophyte communities in *S. alterniflora* shift in response to oiling (Kandalepas et al. 2015), a result that is supported here. However, it is worth noting that soil fungal community composition did not correspond to the oil addition treatment, while roots and leaves did. On the other hand, soil bacterial communities shifted in the soil and roots in response to oil addition but appeared to be primarily responding to the greenhouse environment in the leaves. It is known that PAHs can accumulate in leaf tissues of smooth cordgrass in oiled salt marshes (Kassenga 2017; Mohammad 2014; Sebastian 2019). Therefore, it is possible that the differential response of fungi and bacteria leaf communities to the oil treatment is indicative of the relative sensitivity of those leaf communities to oil.

Bacterial communities in the roots showed strong seasonality that was not seen in the soil, indicating a filtering effect by the plant roots. Importantly, the homogenous communities described in November corresponded to a relative decrease in a few generally abundant phyla: Actinobacteria, Bacteroidetes, and Epsilonbacteraeota. The samples taken in November were taken after the plants had set seed and were becoming dormant. The relative decrease in these phyla was likely a response to the changing plant exudates with the onset of dormancy. It is also entirely possible that the plant was in

some way filtering out taxa that are not beneficial to the plant during winter, however, this hypothesis could not be tested by our experiment.

CONCLUSION

We have demonstrated that the microbiome of *S. alterniflora* is manipulable through soil inoculation, but manipulations did not change plant morphology or relative decomposition of PAHs in the soil. However, decomposition of PAHs did correspond to the presence of the plant, highlighting another potential role of the plant in restoration beyond re-vegetation and erosion reduction.

CHAPTER 3 FIGURES

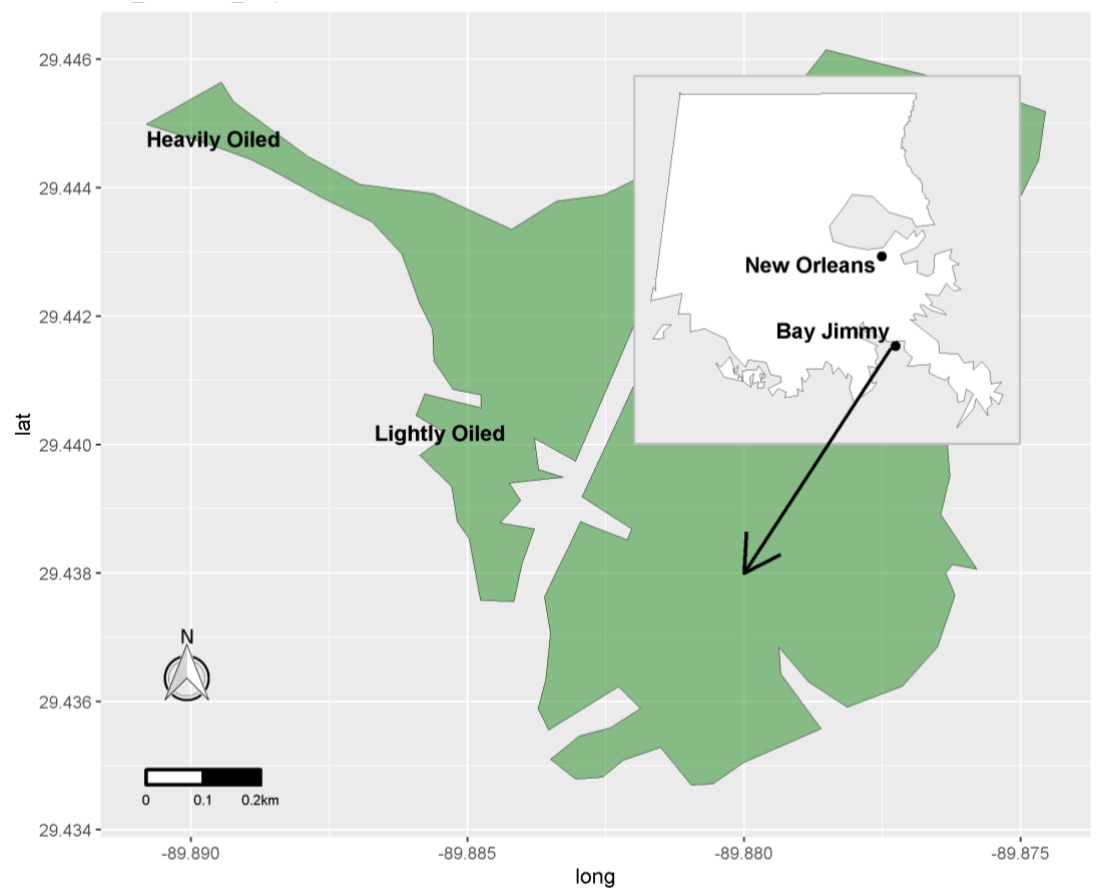

Figure 3-1: Sites of inoculum collection.

Inoculum was collected at two sites on the infamous “Porkchop Island” in Bay Jimmy, LA. The western end of the island was heavily oiled by the DWH oil spill, while the eastern end remained comparatively unoiled.

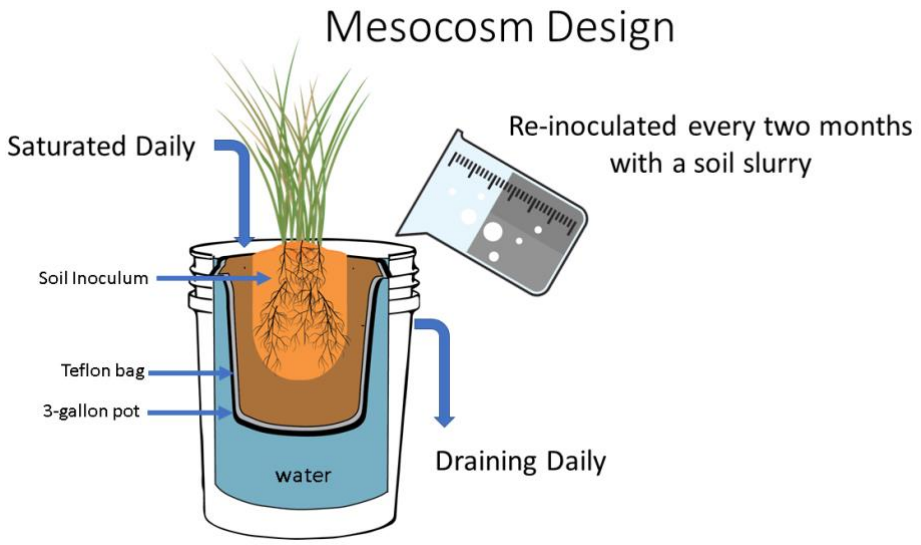

Figure 3-2: Mesocosm design.

Plants were grown the soil inoculum (orange) to acquire a microbiome from the inoculum. The plug was then placed in a 3-gallon trade pot that was lined with a Teflon bag. The trade pot sat in a 5-gallon bucket, through which water was cycled daily. Every two months more inoculum was harvested from the field sites and applied as a liter of soil slurry to the soil surface.

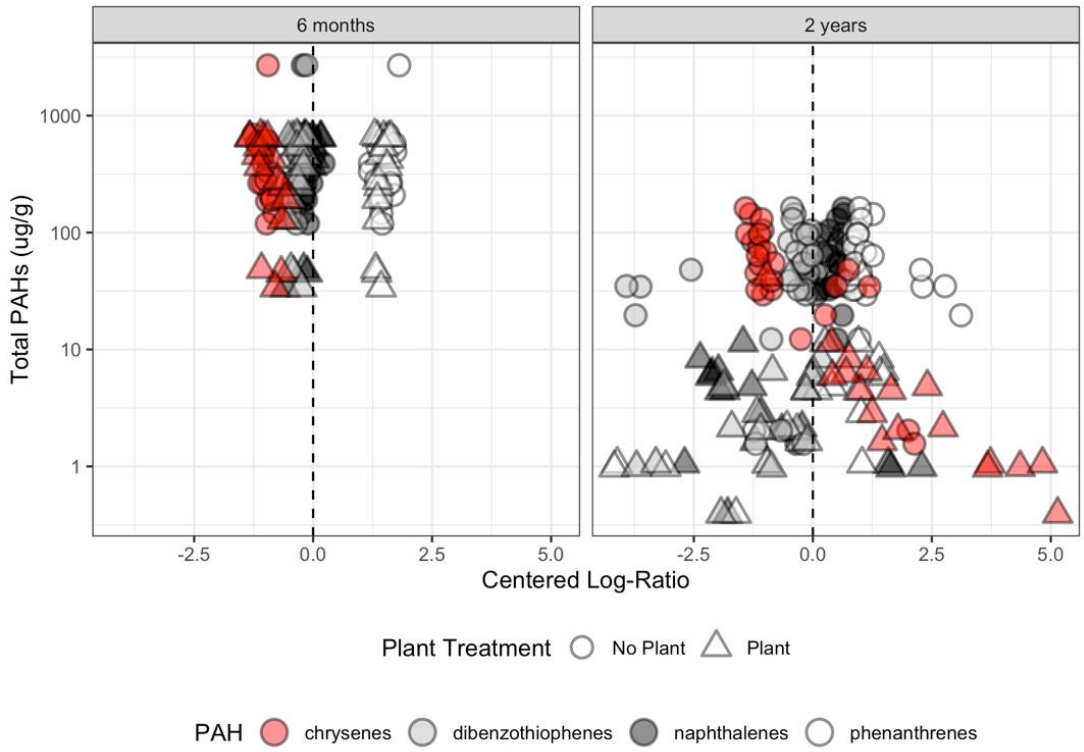

Figure 3-3: Relative abundances of PAHs.

Relative abundances of PAHs 6 months into the experiment (left panel) and at the end of the experiment (right panel). The x-axis represents the centered log-ratio of the PAHs, where the dashed line represents the geometric mean of the sample. Points to the left of the dashed line represent PAHs that were relatively less abundant than average in a sample and point to the right of the dashed line represent PAHs that were relatively more abundant than average. The y-axis represents the total target PAHs measured for a sample. Target PAHs included one 2-ring PAH, two types of 3-ring PAHs (colored in white and gray) and one type of 4-ring PAH (colored red). Triangles represent soil samples from mesocosms in which a plant was growing, circles represent soil from mesocosms with no plant.

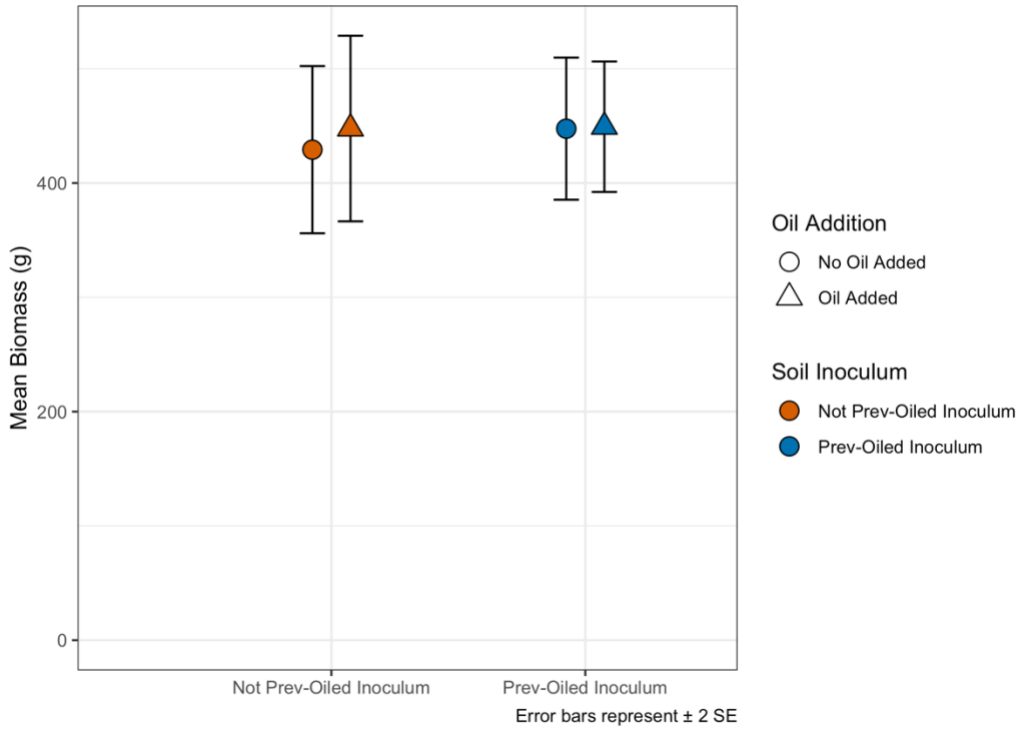

Figure 3-4: Mean plant biomass at the end of the experiment

Triangles represent plants growing in mesocosms to which oil was added, circles are plants to which oil was not added. Soil inocula are represented by color, and a separated on the x-axis for visual clarity.

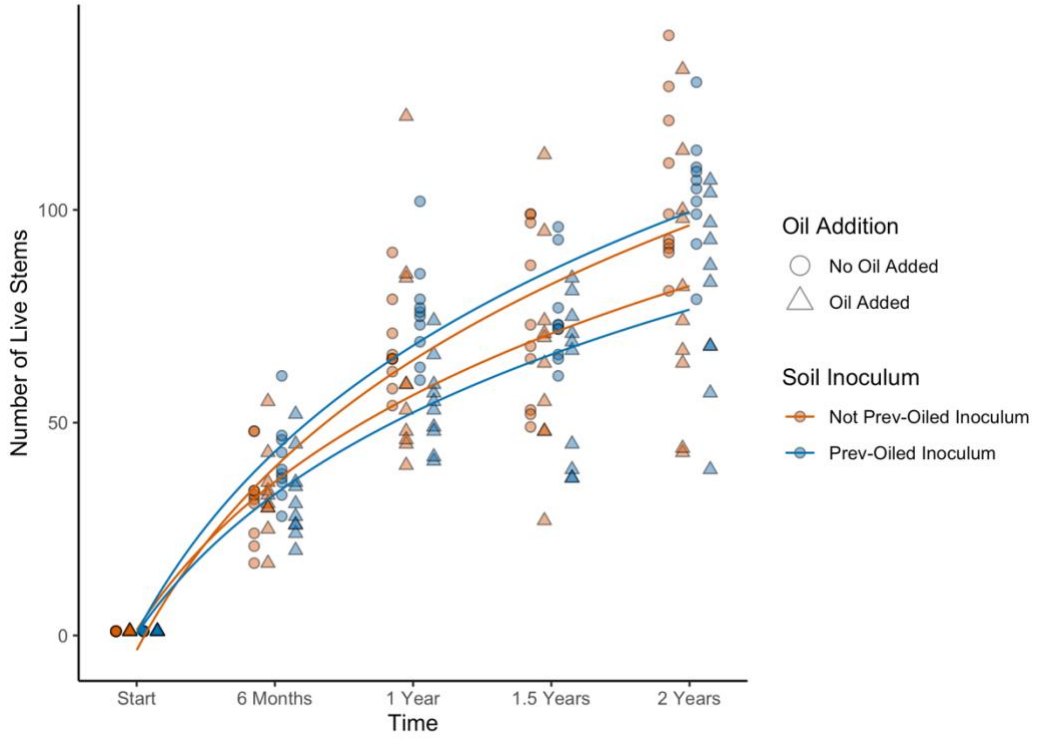

Figure 3-5: Live stem count over the course of the experiment

Triangles represent plants growing in mesocosms to which oil was added, circles are plants to which oil was not added. Soil inocula are represented by color. Curves are logarithmic curves (y ~ log(x)) fitted with the "glm" method in the geom_smooth function from ggplot. By the end of the experiment, stem count had separated into two groups: those in which no oil was added (top two lines) and those in which oil was added (bottom two lines).

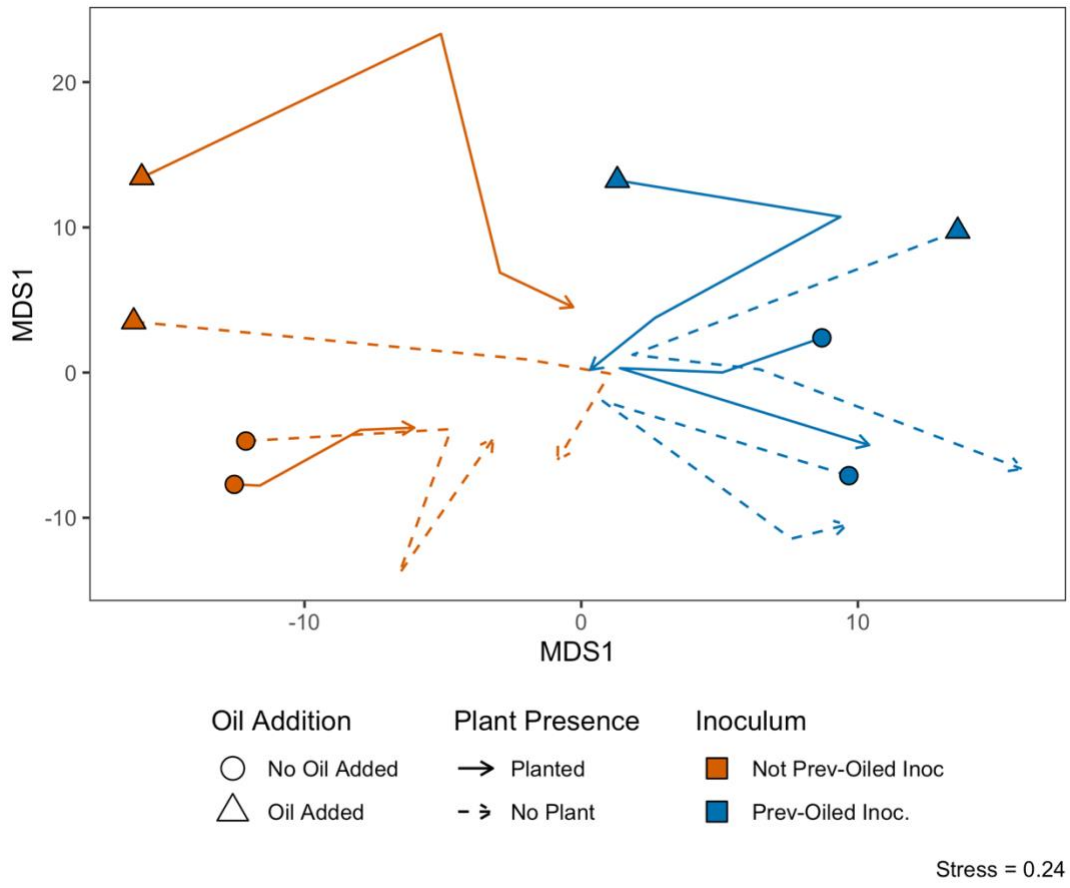

Figure 3-6: NMDS of prokaryote community composition in soil

NMDS of prokaryote community composition in soil, measured by Aitchison distance. The oil addition treatment is represented by shape, the plant presence treatment by line-type, and the inoculum treatment by color. Each line represents the progression of community composition through time for a combination of treatments. Each point on the line represents the centroid for three replicates at a time point. The first time point, six months into the experiment is represented by a circle of triangle. The last time point is represented by an arrowhead.

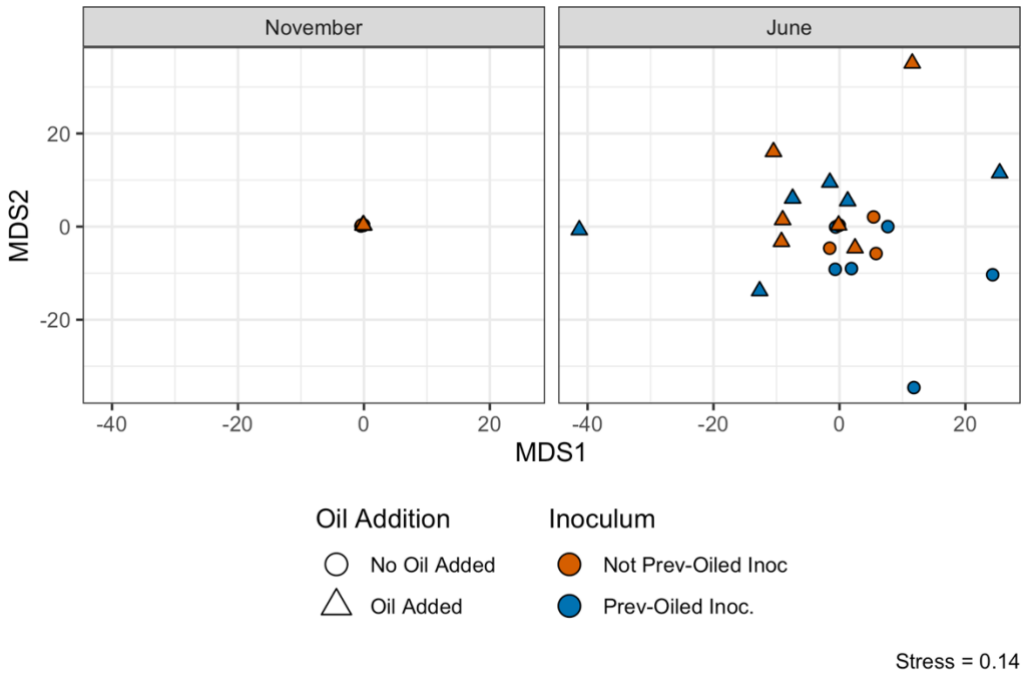

Figure 3-7: NMDS of prokaryote community composition in roots

NMDS of prokaryote community composition in roots, measured by Aitchison distance. The oil addition treatment is represented by shape and the inoculum treatment by color. Panels represent two sampling periods taken one year apart, to visualize the seasonal effects. The November panel represents samples taken 6 months, and 1.5 years into the experiment. The June sample represents samples taken 1 year and 2 years into the experiment.

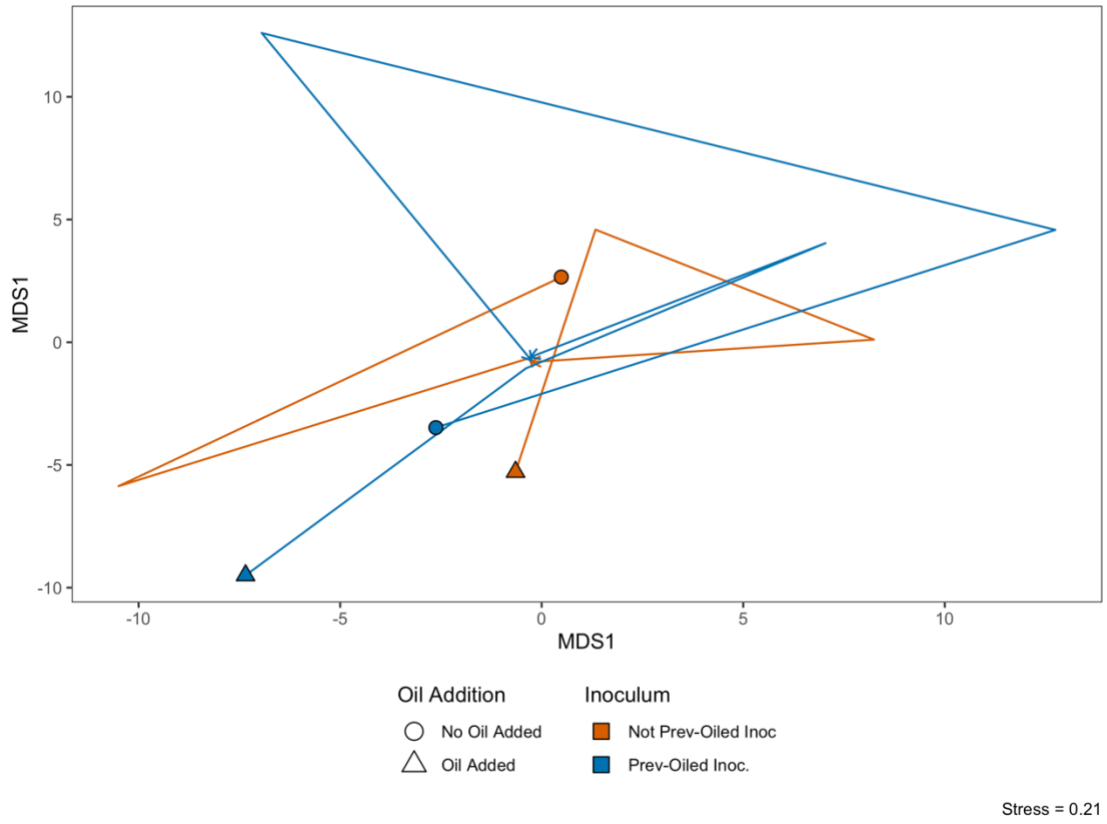

Figure 3-8: NMDS of prokaryote community composition in leaves

NMDS of prokaryote community composition in leaves, measured by Aitchison distance. The oil addition treatment is represented by shape and the inoculum treatment by color. Each line represents the progression of community composition through time for a combination of treatments. Each point on the line represents the centroid for three replicates at a time point. The first time point, six months into the experiment is represented by a circle of triangle. The last time point is represented by an arrowhead.

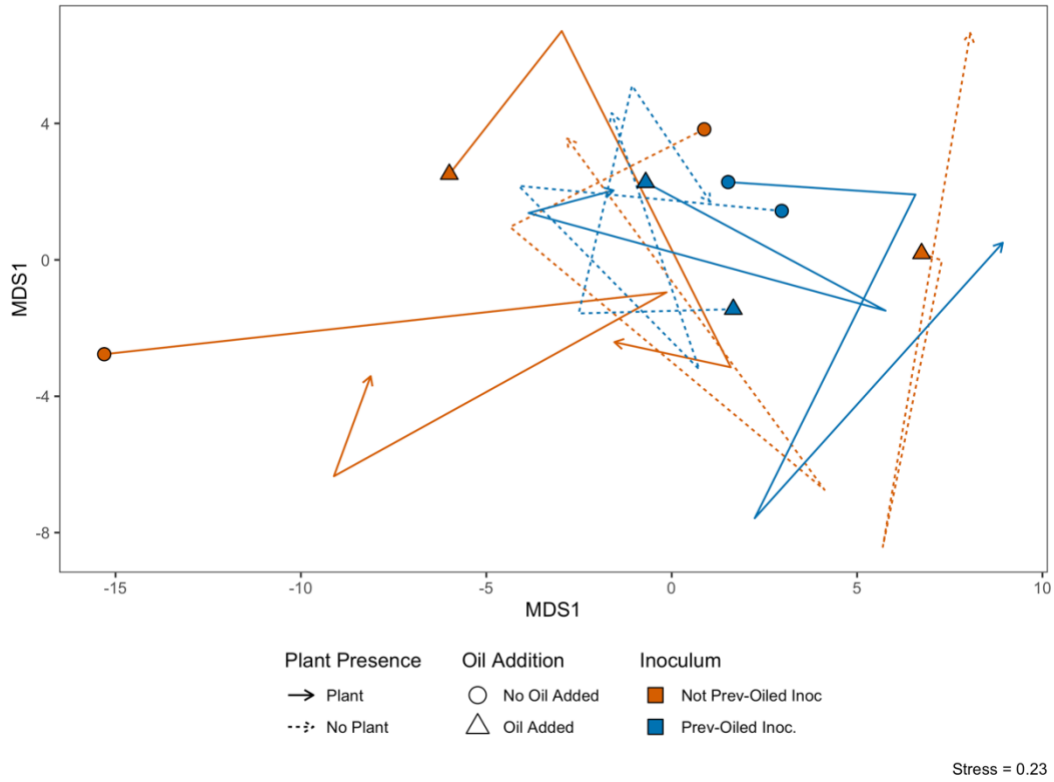

Figure 3-9: NMDS of fungal community composition in soil

NMDS of fungal community composition in soil, measured by Aitchison distance. The oil addition treatment is represented by shape, the plant presence treatment by line-type, and the inoculum treatment by color. Each line represents the progression of community composition through time for a combination of treatments. Each point on the line represents the centroid for three replicates at a time point. The first time point, six months into the experiment is represented by a circle of triangle. The last time point is represented by an arrowhead.

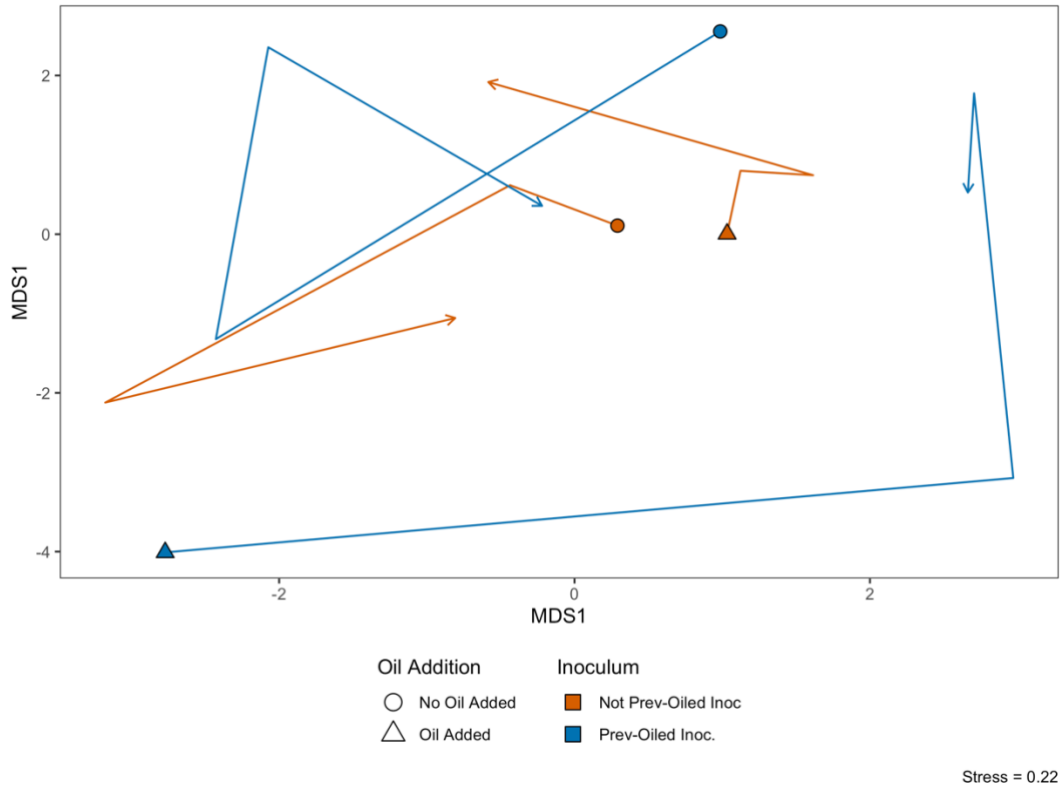

Figure 3-10: NMDS of fungal community composition in roots

NMDS of fungal community composition in roots, measured by Aitchison distance. The oil addition treatment is represented by shape and the inoculum treatment by color. Each line represents the progression of community composition through time for a combination of treatments. Each point on the line represents the centroid for three replicates at a time point. The first time point, six months into the experiment is represented by a circle of triangle. The last time point is represented by an arrowhead.

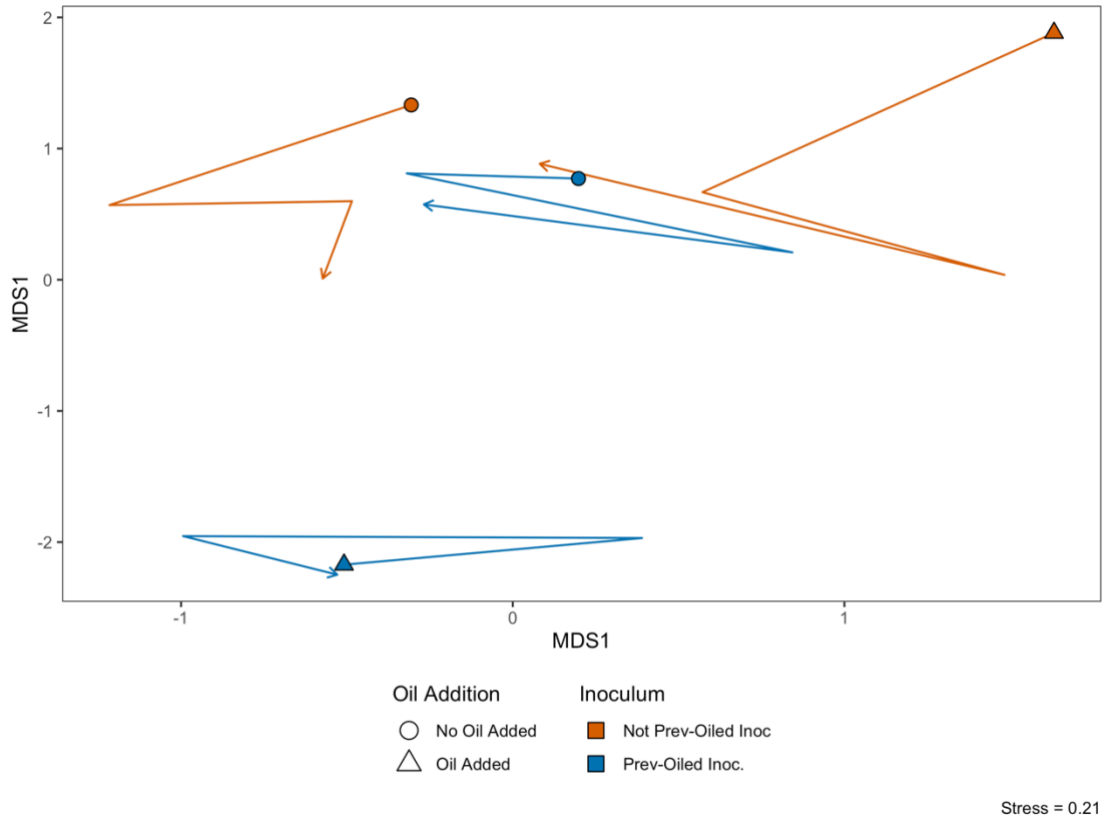

Figure 3-11: NMDS of fungal community composition in leaves

NMDS of fungal community composition in leaves, measured by Aitchison distance. The oil addition treatment is represented by shape and the inoculum treatment by color. Each line represents the progression of community composition through time for a combination of treatments. Each point on the line represents the centroid for three replicates at a time point. The first time point, six months into the experiment is represented by a circle of triangle. The last time point is represented by an arrowhead.

CHAPTER 3 TABLES

Table 3-1: PERMANOVA on soil prokaryote community composition

Results from PERMANOVA on soil prokaryote community composition, as measured by Aitchison distance. The term "plant_trt" refers to the presence/absence of a plant, "orig_soil" refers to the inoculum treatment, and "oil_added" represents the addition of oil or not. Significant results are in bold.

Treatment	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
plant_trt	1	2074	2073.6	2.8889	0.0275	**0.0001**
orig_soil	1	3627	3627.1	5.0531	0.0481	**0.0001**
oil_added	1	2383	2383.4	3.3205	0.03161	**0.0001**
plant_trt:orig_soil	1	936	935.7	1.3036	0.01241	**0.0237**
plant_trt:oil_added	1	1151	1150.5	1.6029	0.01526	**0.0027**
orig_soil:oil_added	1	1216	1216.2	1.6943	0.01613	**0.0012**
plant_trt:orig_soil:oil_added	1	849	849.2	1.183	0.01126	0.071
Residuals	88	63166	717.8	0.83773		
Total	95	75401	1			

Table 3-2: PERMANOVA on root prokaryote community composition

Results from PERMANOVA on root prokaryote community composition, as measured by Aitchison distance. The term "orig_soil" refers to the inoculum treatment, and "oil_added" represents the addition of oil or not. Significant results are in bold.

Treatment	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
orig_soil	1	777.1	777.09	1.3478	0.02831	**0.0003**
oil_added	1	706.5	706.49	1.2253	0.02574	**0.0036**
orig_soil:oil_added	1	597	596.99	1.0354	0.02175	0.0785
Residuals	44	25368.7	576.56	0.9242		
Total	47	27449.3	1			

Table 3-3: PERMANOVA on leaf prokaryote community composition

Results from PERMANOVA on leaf prokaryote community composition, as measured by Aitchison distance. The term "orig_soil" refers to the inoculum treatment, and "oil_added" represents the addition of oil or not. Significant results are in bold.

Treatment	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
orig_soil	1	401	401.02	0.95315	0.02011	0.5375
oil_added	1	492.7	492.69	1.17102	0.02471	0.0648
orig_soil:oil_added	1	535.5	535.47	1.27269	0.02685	**0.0234**
Residuals	44	18512.3	420.73	0.92833		
Total	47	19941.5	1			

Table 3-4: PERMANOVA on soil fungal community composition

Results from PERMANOVA on soil fungal community composition, as measured by Aitchison distance. The term "plant_trt" refers to the presence/absence of a plant, "orig_soil" refers to the inoculum treatment, and "oil_added" represents the addition of oil or not. Significant results are in bold.

Treatment	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
plant_trt	1	669	669.49	1.4558	0.01495	**0.0075**
orig_soil	1	899	898.64	1.95408	0.02006	**0.0001**
oil_added	1	508	507.89	1.10441	0.01134	0.1772
plant_trt:orig_soil	1	775	774.94	1.68511	0.0173	**0.0018**
plant_trt:oil_added	1	514	514.33	1.11841	0.01148	0.1612
orig_soil:oil_added	1	549	549.36	1.19459	0.01226	0.085
plant_trt:orig_soil:oil_added	1	411	411.39	0.89456	0.00918	0.7179
Residuals	88	40469	459.88	0.90343		
Total	95	44795	1			

Table 3-5: PERMANOVA on root fungal community composition

Results from PERMANOVA on root fungal community composition, as measured by Aitchison distance. The term "orig_soil" refers to the inoculum treatment, and "oil_added" represents the addition of oil or not. Significant results are in bold.

Treatment	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
orig_soil	1	197.1	197.05	1.4893	0.03089	**0.0088**
oil_added	1	190.6	190.6	1.4405	0.02988	**0.0152**
orig_soil:oil_added	1	169.8	169.83	1.2836	0.02662	0.0532
Residuals	44	5821.6	132.31	0.91261		
Total	47	6379.1	1			

Table 3-6: PERMANOVA on leaf fungal community composition

Results from PERMANOVA on leaf fungal community composition, as measured by Aitchison distance. The term "orig_soil" refers to the inoculum treatment, and "oil_added" represents the addition of oil or not. Significant results are in bold.

Treatment	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
orig_soil	1	57.96	57.956	2.2277	0.04492	**0.0012**
oil_added	1	32.66	32.662	1.2555	0.02532	0.144
orig_soil:oil_added	1	54.86	54.858	2.1086	0.04252	**0.0034**
Residuals	44	1144.7	26.016	0.88724		
Total	47	1290.18	1			

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