Alterations of Endophytic Microbial Community Function in *Spartina alterniflora* as a Result of Crude Oil Exposure

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Thesis submitted in partial fulfillment of
the requirements for the degree of
Master of Science in the Department of
Civil and Environmental Engineering in the Graduate School
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ABSTRACT

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Abstract

The 2010 Deepwater Horizon disaster remains one of the largest oil spills in history. This event caused significant damage to coastal ecosystems, the full extent of which has yet to be fully determined. Crude oil contains both toxic substances that are detrimental to microbes and compounds that may be used as food and energy resources by some microbial species. As a result, oil spills have the potential to cause significant shifts in microbial communities. In this study, we assessed the impact of oil contamination on the function of endophytic microbial communities associated with saltmarsh cordgrass (*Spartina alterniflora*). Soil samples were collected from two locations in coastal Louisiana, USA: one severely affected by contamination from the Deepwater Horizon oil spill and one relatively unaffected location. *Spartina alterniflora* seedlings were grown in both soil samples under greenhouse conditions, and GeoChip 5.0 was used to evaluate the endophytic microbial metatranscriptome shifts in response to host oil exposure. Microbial functional shifts were detected in functional categories related to carbon cycling, virulence, metal homeostasis, organic remediation, and phosphorus utilization. These findings show that host oil exposure elicits multiple changes in metabolic response from their endophytic microbial communities, producing effects that may have the potential to impact host plant fitness.

Dedication

To Hazel, without whom I would not have made it this far. To my family and friends for their unwavering support, and to my wonderful professors and mentors for their much-needed guidance.

Contents

Abstract iv

List of Figures vii

Acknowledgements viii

1. Introduction 1

2. Materials and Methods 3

2.1 Soil sample collection and germination of plant specimens 3

2.2 Soil collection and initial transplanting methods 3

2.3 Mesocosm setup 5

2.4. Sample collection and processing 7

2.5 RNA extraction and GeoChip 5.0S analysis 8

2.6 Computational analysis 8

3. Results 10

4. Conclusion 13

Appendix A 17

References 18

List of Figures

Figure 1. Locations of soil sample collection. 5

Figure 2. Mesocosm construction and maintenance. 7

Figure 3. Heatmaps of the GeoChip functional categories that displayed either visual clustering or low p-values as identified by adonis test. 12

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1. Introduction

Between 4 and 5 million barrels of oil were estimated to have escaped into the Gulf of Mexico following the catastrophic explosion on April 20, 2010 and subsequent sinking of the *Deepwater Horizon* (DWH) drilling unit (McNutt et al. 2011; U.S. Coast Guard 2011). The unprecedented scale of the spill marked the DWH disaster as the largest offshore oil spill in U.S. history. Remedial actions involved the application of chemical dispersants, controlled *in situ* burns, and physical removal by skimming (US Coast Guard 2011). Such efforts were extensive but did not prevent oil contamination from reaching the coastline of Louisiana, where nearly 2,100 km of coastline were affected.

The resulting exposure of both marine and terrestrial ecosystems to crude oil residue was catastrophic. Thousands of shorebirds, turtles, and individuals of other marine and coastal species were recorded to have been directly killed by oiling (Deepwater Horizon Natural Resource Damage Assessment Trustees 2016). Beyond those species visibly affected by oiling, thousands of kilometers of coastal soils were contaminated with toxic compounds. Several studies have determined the composition of the crude oil released by the DWH spill included polycyclic aromatic hydrocarbons (PAHs), low molecular weight hydrocarbons, and trace amounts of metals such as As, Pb, Co, Ni, Zn, Fe, and Cr, representing a significant toxicological hazard to exposed organisms (Groudeva et al. 2001; Liu et al. 2012).

Unlike animal species, floral and microbial communities lack the ability to relocate from contaminated sites. Marine microbial communities have shown significant shifts in response to crude oil contamination, including enrichment of those species capable of degrading hydrocarbons (Doyle et al. 2018). While such species may be capable of utilizing hydrocarbons as an energy source, detrimental effects to microbes from exposure to toxic metals has been

reported for decades (Bååth 1989; Giller et al. 1998). Furthermore, resulting overgrowth of hydrocarbon degraders may lead to proportionate suppression of other microbial species. Community shifts may lead to far-reaching ecological disturbances should those outcompeted species be beneficial to more complex organisms. Such effects are particularly concerning in cases of microbe-plant interactions due to the importance microbial symbionts can represent to host plant health (Brundrett 2004; Liu et al. 2017).

Microbial species residing within plant tissues may be beneficial, parasitic, or have little to no effect on their host plant. Endophytes are those microbial species which do not damage plant tissue, and neither elicit host defense response nor form membrane-bound or intracellular structures seen in endosymbiotic species (Reinhold-Hurek and Hurek 2011). They have been observed to increase host resistance to both pathogens and abiotic stressors, indicating endophytic communities play a key role in maintaining host health (Khare et al. 2018). Further study of endophytes and their effects on host plants may yield organic methods of disease control, ecosystem conservation, and increased agricultural production.

Plant tissues serve as the external environment for endophytic communities. Host plant responses to biotic and abiotic stressors, therefore, have the potential to induce shifts in these communities. The DWH spill contaminated thousands of kilometers of coastal soils, affecting countless plants and their associated endophytes and representing a significant abiotic environmental stressor. The purpose of this study was to assess whether or not shifts occurred in endophytic community function arising from host exposure to DWH oil spill contamination, and if such shifts were present, which functional gene categories were affected. Saltmarsh cordgrass (*Spartina alterniflora*) was selected as a model organism as it is a coastal marsh grass endemic to the regions of Louisiana contaminated by the DWH spill, where it often comprises the bulk of marshland vegetation (Edwards and Mills 2005).

2. Materials and Methods

2.1 Soil sample collection and germination of plant specimens

Seeds of wild *S. alterniflora* were collected in southern Louisiana in November 2015 and cold stratified at 4 °C for three months. Stratified seeds were surface sterilized via subsequent immersion baths of 95% ethyl alcohol for 3 min and 0.825% NaOCl for 30 min before being rinsed in sterile deionized water for 10 s. The seeds were germinated in sterile deionized water and transplanted into trays containing the prepared substrate (equal volumes of organic humus and vermiculite which was autoclaved three times at 121 °C for 60 min). Planted trays were placed into a Conviron Model GR48 Plant Grow Room (Controlled Environments Ltd., Winnipeg, Canada) and grown under lighting and moisture conditions designed to replicate the climate of southern Louisiana (Krauss et al. 1998). Seedlings were fertilized with approximately 9 g of Scott's Osmocote® (Marysville, Ohio) fertilizer (14:14:14 NPK) and watered with deionized water. After approximately 1 month, plant trays were relocated to a greenhouse environment and allowed to acclimate for 3 weeks. Air temperature and humidity within the greenhouse were monitored with an iButton, model DS1923 (Maxim Integrated, San Jose, CA, USA), and soil temperature was monitored with an iButton, model DS1922L. Greenhouse air temperature averaged 4 °C higher per month than those reported at the New Orleans Airport (MSY), while experimental pot soil temperatures averaged <1 °C cooler. Average greenhouse monthly humidity was 67.5%.

2.2 Soil collection and initial transplanting methods

Soil samples were collected from two locations within Bay Jimmy, LA, (Fig. 1) from a site reported to have been relatively unaffected by oil contamination (29.44006° N, 89.88583° W) and a site reported to have been heavily impacted (29.44464° N, 89.88959° W) by the DWH oil

spill (Zengel et al. 2015). Each soil sample was sieved with a 1.27 cm screen constructed from steel hardware cloth (Blue Hawk, Largo, FL) and used to fill 3.8 L plastic nursery trade pots. On May 1, 2016, acclimatized *S. alterniflora* greenhouse specimens were transplanted into the prepared trade pots no later than 60 h after the soil samples were collected. Pot placement in the greenhouse was randomized; pots were divided into five sections oriented approximately East-West on four tables oriented approximately North-South. Plants were grown in the Bay Jimmy soil for 1 month before being transplanted into 19 L mesocosms.

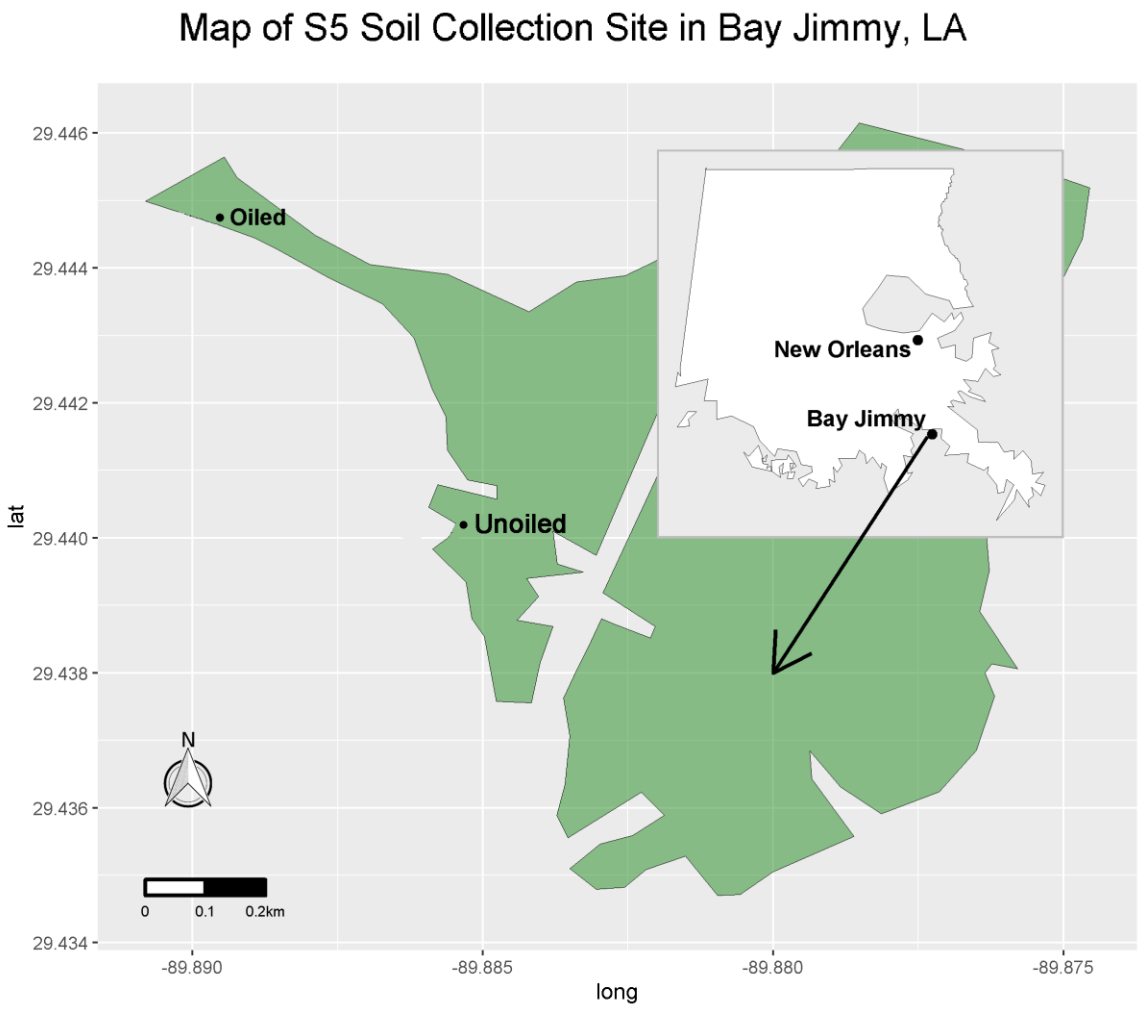


Figure 1. Locations of soil sample collection. The shape of the landmass allowed for little oil contamination to reach the "unoiled" site despite close proximity. Crude oil residue was still visible at the "oiled" site at time of sample collection.

2.3 Mesocosm setup

Mesocosms were constructed to mimic estuarine conditions (Fig. 2). Each consisted of a 11.4 L plastic trade pot nested within a water-filled 19 L plastic bucket with a drainage tube installed near the top. The interiors of the trade pots were lined with Teflon bags (P-00113, Welch Fluorocarbon, Inc, Dover, NH, USA) to prevent reaction between the pot and the experimental conditions. The bottom of each bag was punctured to allow drainage. The 11.4 L trade pots were

filled with 7.6 L of a growth substrate consisting of 2:3 organic humus to sand (Quikrete® Play Sand, Quikrete, Atlanta, GA). Naturally weathered oil originally skimmed from the ocean during the DWH spill was obtained from the Gulf of Mexico Research Initiative (GoMRI). After plant specimens were transplanted into the mesocosms, half of each experimental cohort was amended with 1.6 L of weathered oil. and 1.9 L of sand were then added, in an even layer, to the top of each 11.4 L pot to reduce the likelihood of airborne contamination. Three weeks after being transplanted into the mesocosms, plants were fertilized with 8 g of Scott's Osmocote® Plus (Marysville, Ohio) and each pot was covered with an additional 0.95 L of sand. Plants were irrigated on top of each pot with a timer-controlled drip irrigation system. The irrigation system was tested to confirm uniform flow to each pot, and the frequency of irrigation was adjusted as necessary to maintain moisture levels in the mesocosm. To simulate brackish water influx, each mesocosm was watered with an additional 1 L of 5 g L-1 Instant Ocean salt (Instant Ocean Spectrum Brands, Blacksburg, VA) solution once per month. Mesocosms were maintained through the addition of 4 g of fertilizer and 1 L of a soil slurry obtained from the original Bay Jimmy sites every two months. The soil slurry was sieved in the same manner as the initial soil samples and diluted to a concentration of approximately 1:10 with water from the same tap as the irrigation system before being applied to the mesocosms. Plants were occasionally misted with water to prevent salt buildup on the leaves.

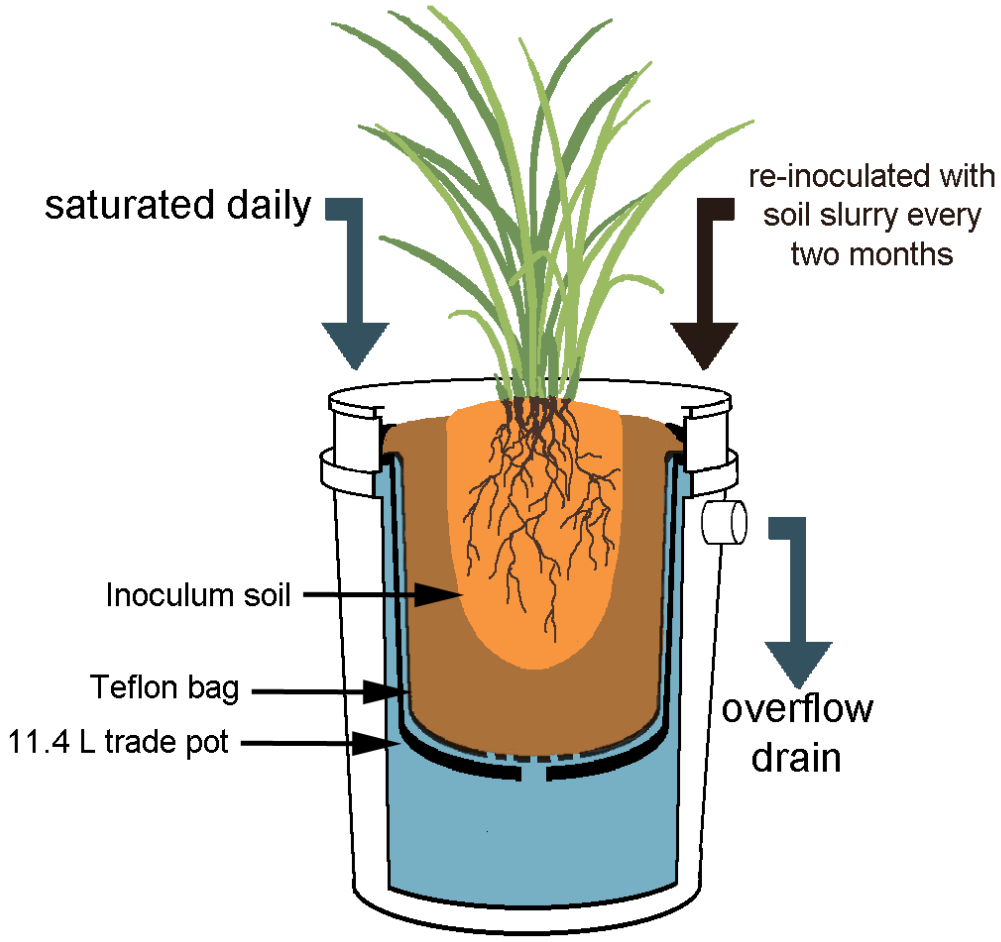


Figure 2. Mesocosm construction and maintenance.

2.4. Sample collection and processing

Samples for GeoChip 5.0 analysis were collected in June 2018, approximately two years after the start of the experiment. Leaves were inspected for overall health, and approximately 5 grams of both whole leaves and root pieces were collected from three plants per treatment group, for a total of twelve plants. Samples were immediately sealed in Ziploc bags and refrigerated. The edges of each leaf were removed, and the remaining portion was chopped into approximately 3 cm pieces using a sterilized blade. All downstream processing was conducted in a biosafety cabinet using sterile techniques. Leaf samples were submerged in subsequent baths of 95 %

ethanol for 10 s, 0.525 % sodium hypochlorite for 2 minutes, and 70% ethanol for 2 minutes in order to reduce microbial contamination on leaf exteriors (Kandalepas et al. 2015). Root samples were submerged in subsequent baths of 70 % ethanol for 10 seconds and 2.6 % sodium hypochlorite for 2 minutes before being rinsed three times in sterile deionized water. Disinfected samples were then frozen in CTAB buffer (2 % CTAB, 0.02M EDTA, 0.1M Tris, 1.4M NaCl) at -20 °C until RNA extraction. All processing was completed within 12 hours of initial collection.

2.5 RNA extraction and GeoChip 5.0S analysis

Both leaf and root samples were pulverized in liquid nitrogen, and RNA was extracted using the QIAGEN RNEasy PowerSoil Total RNA kit (Qiagen, Germantown, MD) according to manufacturer's instructions. Extracted RNA was immediately converted into cDNA using a High Capacity cDNA Reverse Transcription Kit (ThermoFisher, Waltham, MA) according to manufacturer's instructions and sent to Glomics, Inc. (Norman, OK) for analysis via GeoChip 5.0. Hybridization was carried out at 68 °C for 22 h using hybridization buffer (Agilent, New Castle, DE) and 10 % formamide. Reads were scanned and quantified as described in Cong et al. (2015).

2.6 Computational analysis

GeoChip data received from Glomics were normalized using the OU Microarray Data Manager (http://ieg.ou.edu/microarray/). Only the reads with a signal-to-noise ratio greater than 2.0 were retained for further downstream analyses. Resulting GeoChip read data were subdivided into nine major functional group categories: carbon cycling, metal homeostasis, nitrogen cycling, organic remediation, phosphorus, secondary metabolism, sulfur, virulence, or other. Heatmaps were produced for each functional category by averaging the signal intensities of all test condition replicates per gene target. Linear mixed models were used to look for main effects and

interactions between factors (oil exposure and tissue type), with one sample t-tests used to determine significant differences between oil exposure and controls and 2-tailed t-tests used to determine significant differences between clusters identified from heatmaps (Fig. 3). Each functional group was transformed to a Bray-Curtis dissimilarity index, visualized using nonmetric multidimensional scaling (NMDS) (Appendix A), and statistical significance was assessed using the envfit and adonis functions (permutational distance-based multivariate analysis of variance (PERMANOVA)). The Bray-Curtis dissimilarity index was chosen because it has been found to be one of the least vulnerable indices to several types of errors (Schroeder and Jenkins 2018). The multivariate homogeneity of groups dispersions test (betadisper) was calculated and used to run a pairwise comparison between sample conditions (permutest). Functional gene categories displaying a low p-value ($p < 0.1$) from adonis were considered noteworthy and treatment effects per subcategory of these functional categories were analyzed via one sample t-test. Heatmaps were produced in R 4.0.0 statistical software (R Core Team 2020), and Bray-Curtis, NMDS, envfit, adonis, betadisper, and permutest were calculated using vegan package (version 2.5-6) in R 4.0.0 statistical software (Oksanen et al. 2019). Gene target diversity metrics were calculated using the OU Microarray Data Manager GeoChip data analysis pipeline.

3. Results

Heatmaps displayed visual clustering between samples of each tissue type (leaf or root) and treatment (oiled or not) for secondary metabolism, carbon, and virulence functional categories (Fig. 3). For microbial gene targets in the secondary metabolism category, oil exposure was not found to exert an effect on the expression of either rhodopsin or chlorophyll-related genes (Fig. 3A). However, the difference in microbial gene expression for the chlorophyll subcategory was found to differ significantly ($p = 0.02$) between tissue types, with leaf tissue having higher expression. The expression of microbial functional genes related to methane metabolism were found to increase significantly ($p = 0.04$) in root samples exposed to oil, however, there was no such effect observed in leaves (Fig. 3B). Oil exposure was associated with a statistically significant ($p = 0.01$) increase in microbial expression of genes in the antibiotic resistance subcategory for root samples, but not leaves (Fig. 3C). Two statistically significant ($p=0.001$) clusters were observed in the degradation subcategory, but neither oil exposure nor tissue type could be identified as the determining factor (Fig 3C).

Oil exposure was associated with significant shifts in the phosphorus category, though these shifts were restricted to leaf samples and involved only the subcategories of phytic acid hydrolysis ($p = 0.0001$), which was lower in samples exposed to oil, and polyphosphate synthesis ($p = 0.05$), which saw higher levels of expression in samples exposed to oil (Fig. 3D). For metal homeostasis, oil exposure was associated with a significant increase in microbial expression of genes in the arsenic detoxification ($p = 0.004$) subcategory, and decreases in expression of genes in the silicon biosynthesis ($p = 0.03$) and silicon transport ($p = 0.001$) subcategories in leaf samples. Root samples displayed significant decreases in microbial gene expression in the chromium detoxification ($p = 0.001$) and mercury transport ($p = 0.0002$) subcategories, and

significant increases in expression in the subcategories of silicon transport ($p = 0.03$), mercury detoxification ($p = 0.04$), and tellurium detoxification ($p = 0.03$) (Fig. 3E). Shifts associated with oil exposure in the organic remediation functional category for leaf tissue samples included an increased expression level in the subcategories of chlorinated solvents ($p = 0.003$) and herbicide-related compounds ($p = 0.01$), and decreased expression levels in pesticide-related compounds ($p = 0.0003$). Root tissue samples displayed decreased microbial gene expression levels in the subcategories of aromatics ($p = 0.01$) and herbicide-related compounds ($p = 0.001$), and an increased expression level in the subcategory of other hydrocarbons ($p = 0.01$) (Fig. 3F).

Betadisper, envfit, and permutest analyses did not indicate statistically significant results for any functional categories. Shannon diversity values for all samples ranged from 8.94 to 9.90 ($\sigma = 0.25$) and Simpson diversity values ranged from 0.09 to 0.24 ($\sigma = 0.05$). Functional gene targets assigned to the bacterial genera *Pseudomonas* and *Streptomyces* were the most frequently identified across all samples.

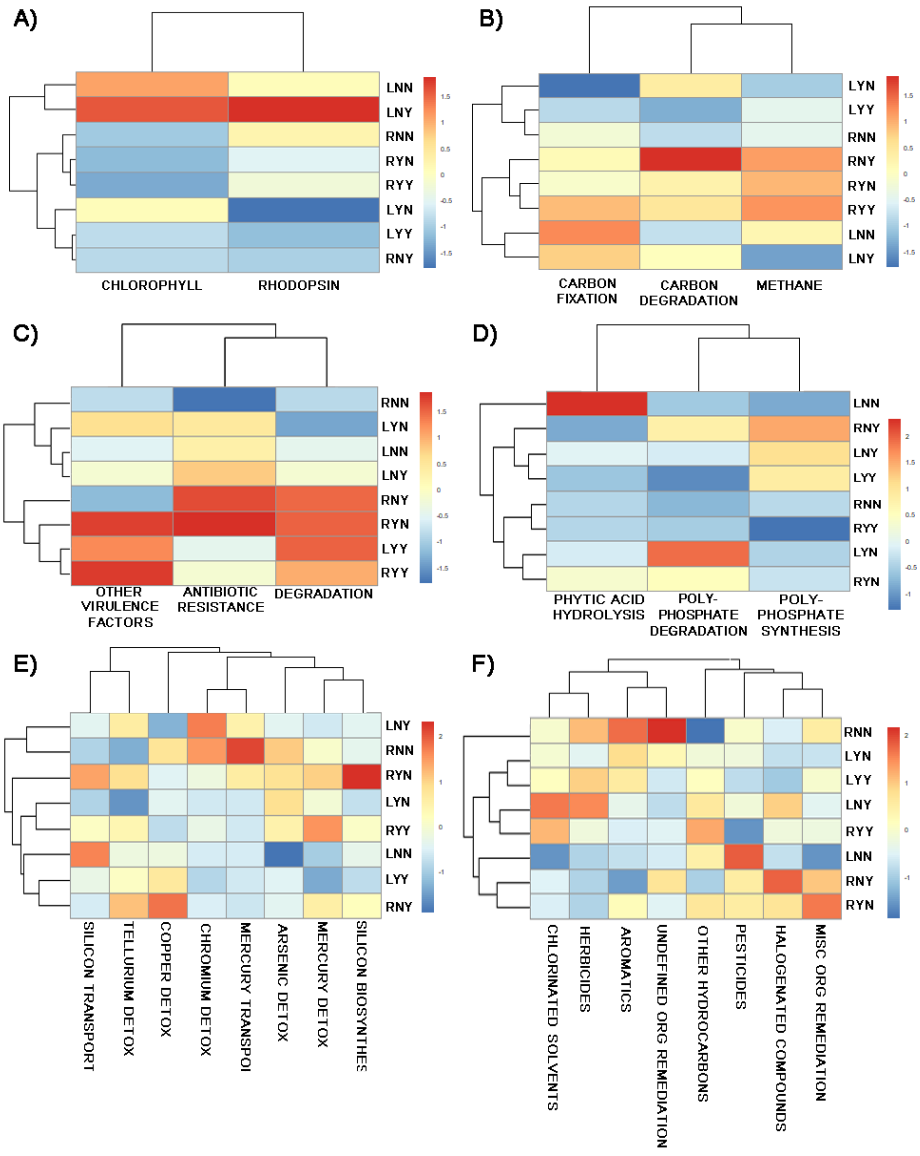


Figure 3. Heatmaps of the GeoChip functional categories that displayed either visual clustering or low p-values as identified by adonis test. A) secondary metabolism, B) carbon, C) virulence, D) phosphate, E) metal homeostasis, and F) organic remediation functional gene categories. Columns delineate gene function subcategories. Row names correspond to experimental groups as follows: the first letter denotes tissue type (L = leaf, R = root), the second letter denotes sample collection location (Y = historically contaminated, N = historically unaffected by oil), and the third letter denotes whether or not crude oil residue was added (Y = oil residue added, N = no oil residue added). Subcategories which displayed statistical significance included: A) chlorophyll, B) methane, C) degradation, D) polyphosphate synthesis and phytic acid hydrolysis, E) all metal homeostasis subcategories aside from copper detoxification, and F) aromatics, chlorinated solvents, herbicide-related compounds, pesticide-related compounds, and other hydrocarbons.

4. Conclusion

Because endophytic communities are reliant on their host plant for survival, they must respond to changes in an internal environment governed by host health and responses to environmental stimuli and stressors. Previous studies have shown that the presence or absence of toxicants such as PAHs and heavy metals can elicit biochemical responses from plants; such alterations to the internal chemistry of the host plant represent a change in habitat to which endophytic communities must adapt (Maliszewska-Kordybach and Smreczak 2000; Sethy and Ghosh 2013; Seneviratne et al. 2017; Udo and Fayemi 1975).

The results of this study support the hypothesis that crude oil contamination and subsequent uptake by plants can affect their endophyte communities. Clustering was observed in heatmaps between both samples with the same soil inoculant collection location and samples obtained from the same tissue type, suggesting similar trends in microbial gene expression between these experimental cohorts. This was confirmed through t-test analyses of the subcategories in question. For example, the expression of genes related to chlorophyll were expected to differ between leaf and root tissue types. Leaves, which are exposed to light, had a high expression level, while roots, which would normally not be exposed to sunlight, had low expression levels. In fact, only two subcategories (herbicide-related compounds and silicon transport) displayed significant shifts in both tissue types. All other significant shifts in microbial gene expression were restricted to either root or leaf tissue. These findings are in line with other studies, as it has been previously demonstrated that microbial communities can differ vastly between different organ systems and tissue types of their host organism (Ugarelli et al. 2019; Wallace et al. 2018; Wei and Ashman 2018).

Crude oil has been demonstrated to contain heavy metals, and as such is capable of contaminating areas affected by spills with significantly elevated concentrations (Liu et al. 2012;

Osuji and Onojake 2004). The uptake of such metals into plant tissue from contaminated sediment is well-documented, to the extent that hyperaccumulator species play key roles in phytoremediation (Leitenmaier and Küpper 2013). The GeoChip 5.0 array contains microbial gene targets for arsenic, chromium, copper, mercury, and tellurium detoxification. Of these, significant shifts were detected in the expression of arsenic, chromium, mercury, and tellurium detoxification genes, suggesting that the environmental concentration of one or more of these metals may have been altered by the presence of crude oil residue. We suspect that increased exposure of the host plants to crude oil residue resulted in increased uptake of heavy metals, which in turn may have induced endophyte cellular defenses against metal toxicity.

GeoChip 5.0 gene targets in the organic remediation category include genes associated with the degradation of PAHs, including genes specifically identified with the degradation of benzene, toluene, ethylbenzene, and xylene, chlorinated aromatics, and multiple others. Crude oil on average is comprised of between 0.2 and 7% PAH by volume, most of which are low molecular weight 2-ring or 3-ring molecules (Neff et al. 2005). The Macondo well falls on the higher end of this range, with a PAH concentration estimated at 3.9% (Reddy et al. 2012; Tidwell et al. 2016). Previous studies have observed conflicting trends of increasing or decreasing toxicity as PAHs increase in molecular weight and number of rings, suggesting that factors beyond PAH concentration, such as associated microbial community composition, soil chemistry, and plant species involved, contribute to detrimental effects on plant growth (Baek et al. 2004; Henner et al. 1999). Low molecular weight PAHs are generally more volatile, water soluble, and easier for microorganisms to degrade than high molecular weight PAHs, and as such are removed from the environment much faster (Ghosal et al. 2016). PAHs have been found to infiltrate plant tissue, both via translocation following root uptake and through their stomata as a result of atmospheric deposition (Lin et al. 2017). Therefore, the shifts observed in this functional category strongly

suggest the uptake of PAHs by the plants in our study, and suggest that the plant's endophyte community may provide the host some protection against PAH exposure.

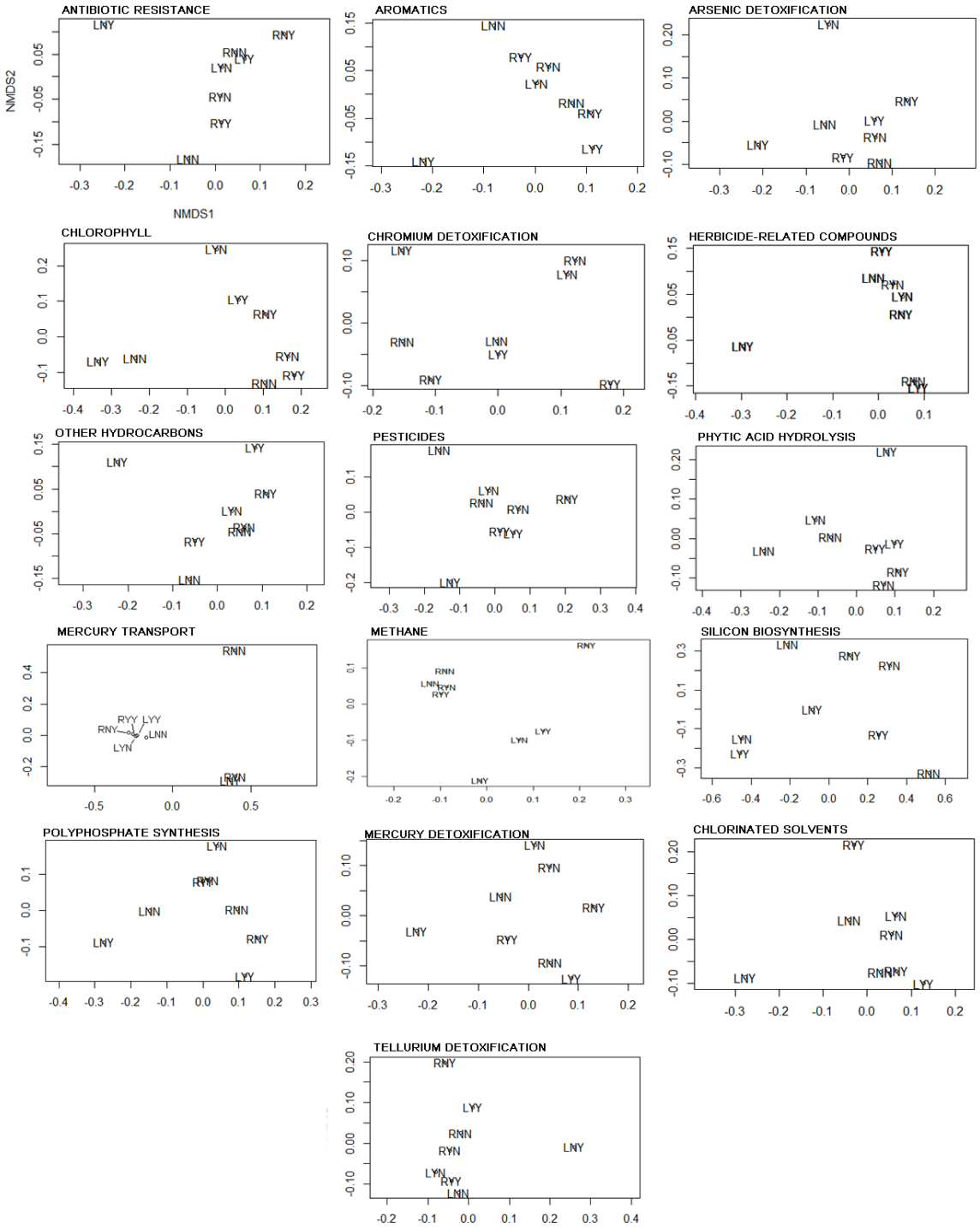
PAHs have previously been shown to decrease phosphorus transport from soil in plants reliant on symbiotic mycorrhizal fungi, but multiple studies have shown *S. alterniflora* to be resistant to forming mycorrhizal associations (Calonne et al. 2014; Hoefnagels et al. 1993; McHugh and Dighton 2004). Alternatively, the availability of nutrients such as phosphorus is often a limiting factor in the degradation of hydrocarbons by known degraders (Cunliffe and Kertesz 2006; Vyas and Dave 2010). It is therefore possible that the activation of degradation pathways required increased uptake of phosphorus by exposed endophytes, resulting in the observed shifts in phosphorus-related gene expression as endophytes responded to a decrease in available phosphorus and/or an increase in metabolic demand.

The results of this study suggest that endophytic community function is impacted by and responds to host exposure to crude oil residue. Subsequent alterations in expression of functional genes related to secondary metabolism, carbon, phosphorus, virulence, metal homeostasis, and organic remediation suggest a change in the internal chemistry of exposed *S. alterniflora*. This may be due to biochemical shifts within host tissue as a response to compounds present within the crude oil residue, or a direct adaptation by the microbial communities to crude oil compounds taken up by host tissues. Despite these shifts, diversity was not observed to change significantly across treatments. This may be due in part to the fact that the GeoChip 5.0 array is not designed to focus on identifying community composition. Further study is necessary to determine such changes, as well as those related to community density and spatial distribution throughout host tissue that may be spurred by similar environmental alterations.

The interaction between host plant development and plant-associated microbial communities is a developing research area with much importance to the broad field of

phytoremediation (Redfern and Gunsch 2016). Herein, efforts were made to ensure seedlings for this study were germinated in sterile conditions. Nonetheless, while the seeds themselves may have contained endophytes that were unaffected by external sterilization, the potential lack of endophytic and symbiotic microbial communities may have affected host seedling development and led to alterations in mature host immune response that shifted experimental communities away from those that would have been observed in natural ecosystems. Thus, subsequent studies may benefit from an earlier exposure of host seedlings to inoculant substrate or the incorporation of field trials in order to more firmly establish target microbial associations. Sampling more replicates per treatment may also be of benefit, and serve to clarify smaller scale shifts potentially masked by the sensitivity of the GeoChip microarray. Finally, the bimonthly additions of a soil amendment from sample locations allowed for experimental soil communities to track the seasonal variation of natural systems potentially absent in greenhouse conditions, but such exposures may have contributed to some of the variation in the GeoChip results. Clearly, research gaps remain to fully untangle the exact relationships between external environment, host plant function, and endophyte community health. However, further research into the influence of endophytic communities on the health and resiliency of their host plants holds great potential for advances in agriculture, the conservation of natural ecosystems, and phytoremediation.

Appendix A


ANTIBIOTIC RESISTANCE
AROMATICS
ARSENIC DETOXIFICATION
CHLOROPHYLL
CHROMIUM DETOXIFICATION
HERBICIDE-RELATED COMPOUNDS
OTHER HYDROCARBONS
PESTICIDES
PHYTIC ACID HYDROLYSIS
MERCURY TRANSPORT
METHANE
SILICON BIOSYNTHESIS
POLYPHOSPHATE SYNTHESIS
MERCURY DETOXIFICATION
CHLORINATED SOLVENTS
TELLURIUM DETOXIFICATION
NMDS1
NMDS2

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