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Spatial and temporal comparisons of salt marsh soil fungal communities following the deepwater horizon spill

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Abstract The unprecedented size of the deepwater horizon oil spill and scope of the subsequent response elicited intense and sustained interest in microbial responses to oiling, especially in salt marshes, which have featured prominently in debates about best practices to prevent and remediate oiling of vulnerable ecosystems. A number of studies have examined salt marsh soil microbial communities following the spill, but most have primarily concentrated on prokaryotes. The extent to which oiling elicited shifts in fungal diversity and community composition remains unclear. Here we present spatial and temporal comparisons of salt marsh soil fungal communities at two southern Louisiana salt marshes with contrasting oiling histories. We profiled fungal communities in 2013 alongside corresponding measurements of polycyclic aromatic hydrocarbons to assess whether and how responses to oiling are distinguishable from natural heterogeneity. 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

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residues and secondary oiling. Notably, extensive sampling allowed delineation of benchmark sampling thresholds and illustrated the value of using ranked differentials of relative abundance to characterize soil fungal communities. Our findings highlight the need for combining high-resolution sampling with judgment-based and systematic sampling approaches to accurately capture responses of salt marsh soil fungal community to oiling.

Keywords Salt marsh · Fungi · Microbiome · Oil · Rhizosphere · *Spartina*

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. Though some evidence has been found of post-spill interactions between oil residues and microbial communities in salt marsh soils [reviewed in Atlas et al. (2015)], characterization of expected progress was complicated by burial and redistribution of oil residues via sedimentation and wind-wave action. Consequently, questions remain about microbial responses to oiling and the fate of oil in the environment (Atlas and Hazen 2011; Lin and Mendelssohn 2012; Engel et al. 2017).

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 with bacterial communities can foster stepwise degradation of hydrocarbons (Leahy and Colwell 1990; Atlas 1995; Head et al. 2006; Atlas and Hazen 2011; Mendelssohn et al. 2012; Joye et al. 2014, 2016; Matthew et al. 2016). 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 of salt marshes from the DWH oil spill. Whereas (at least) eight studies have characterized salt marsh soil bacterial communities relative to oiling from the Deepwater Horizon spill (Beazley et al. 2012; Looper et al. 2013; Mahmoudi et al. 2013; Atlas et al. 2015; Marton et al. 2015; Engel et al. 2017; Bae et al. 2018; Tatariw et al. 2018), to our knowledge, only two studies have thus far profiled marsh soil fungal communities in relation to the oil spill (Mahmoudi et al. 2013; Lumibao et al. 2018). 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 soil fungal communities are warranted to better understand the fate and effects of oil in contaminated salt marshes.

Here we present spatial and temporal comparisons of 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 and a lightly oiled site in Fourchon, [Fig. 1; Michel et al. (2013)] to assess whether and how fungal responses to oiling are distinguishable from natural heterogeneity. We hypothesized that the differential oiling of the two sites results in distinct

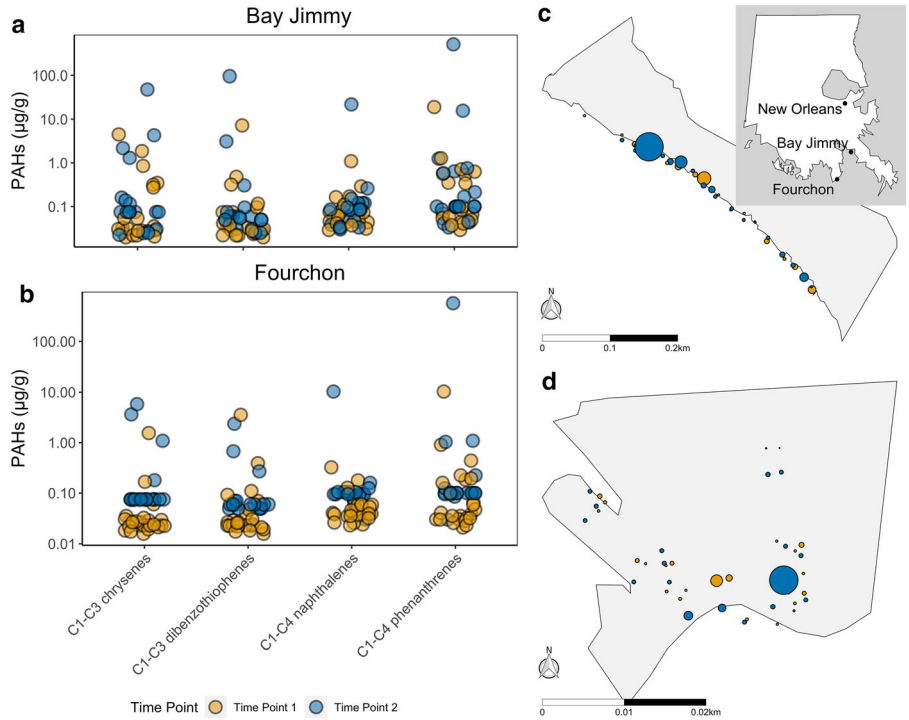

Fig. 1 Distribution of PAHs within samples and at sites. **a** and **b** Abundance of the four classes of PAHs measured at each site, with the y-axis log-transformed for visual clarity. **c** (Bay Jimmy) and **d** (Fourchon) Schematic of each marsh site, where each dot (jittered for clarity) represents a sample, colored by sampling time point and sized by the quantity of PAHs present in the core. Inset shows location of sites in southeastern Louisiana relative to New Orleans

fungal communities that correspond to the relative abundance of local PAHs. 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 time points following a balanced sampling design. Our analyses probed for relationships between PAHs and alpha diversity, beta diversity, and differential abundances of fungal 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), allowing for more confident identification of taxa that are most differentially abundant between marsh sites and time points, and thus providing more informed perspectives on the pervasive effects of primary and secondary oiling events, including detectability relative to natural biotic heterogeneity. 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 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 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. The plots we sampled were replanted with *Spartina alterniflora* as part of a separate study on marsh restoration (Blum et al. 2014; Bernik et al. 2021; Zengel et al. 2021). Our other site, located at Fourchon, LA (29°08′00″ N 90° 08′ 43″ W), is a salt marsh immediately north of Caminada Headlands beach, which is dominated by *Spartina alterniflora* and invading *Avicennia germinans*. At the time of the spill, the Fourchon site remained largely unoiled, although small amounts of oil were deposited along specific locations of the shoreline by storms in June 2010 (Rodrigue et al. 2020). While both sites are located within the Barataria Basin, the Bay Jimmy site tends to be less saline than the Fourchon site according to historical data from nearby Louisiana coastal reference monitoring sites (CRMS). From 2006 to 2020, CRMS sites near the Bay Jimmy site annually averaged 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. CRMS sites near the Fourchon site averaged 16.5 ppt salinity (sd = 4.1) annually, 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 Bay Jimmy site and 48 taken from the Fourchon site. Samples were taken at two time points. Cores were taken during the winter (January) and also during the summer (late June/early July) of 2013 (Online Resource: Table S1). Sampling locations at Bay Jimmy corresponded to 22 plots (5 × 5 m) established by Bernik (2015) spanning a 350 m southwesterly shoreline. At the Fourchon site, we took soil cores from 24 plots (3 × 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 differed at the two sites due to the physiography of each site and the design of prior studies. Consequently, the area sampled at the Bay Jimmy site was approximately 5 times greater, constituting approximately 25 times more shoreline than the sampled area at the Fourchon site. While this difference in sampling area may affect site level estimates of fungal alpha diversity and beta diversity (dispersion) at the two sites, it would not be expected to affect estimates at the plot level. 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 h of collection.

Oil analysis

We analyzed 39 cores sampled from the Bay Jimmy site and 43 cores from the Fourchon site for oil content (Online Resource 1: Table S1). 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 (Online Resource 1: Table S1). 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 classes of alkylated PAHs were quantified (C1-C4 naphthalenes, C1-C4 phenanthrenes, C1-C3 dibenzothiophenes and C1-C4 chrysenes) based on prior studies of crude oil fate in coastal ecosystems (Curtis 2018; Collins et al. 2020; Rodrigue et al. 2020).

Fungal 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 $\geq 0.1\%$ of the mean total reads in ≥ 5 samples (approximately ¼ of the number of samples taken at each site and timepoint). Any OTUs that did not meet these criteria 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. Here we present analyses based on the full set of OTUs because parallel analyses, based on a subset of the data in which the rare taxa were not included, did not change our interpretations. However, we did use the dominant and rare categories to highlight key results in the presentation of our analysis of differentially abundant taxa. The analyses excluding the rare taxa are available upon request. All sequences were deposited in NCBI GenBank under the BioProject accession PRJNA603629.

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 time points were modeled in the package *brms* package (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) $\sim$ Site $\times$ Time point.

We performed a principal components analysis (PCA) to visualize variation in PAH composition of each sample (Online Resource 1: Figure S1) and we tested for differences in PAH composition by conducting a PERMANOVA of Aitchison distances. Aitchison distances account for the compositional nature of the data through transforming values into centered log-ratios and then taking the Euclidean distance of sample compositions (Pawlowsky-Glahn and Egozcue 2006; Brückner and Heethoff 2017). We then used the *ecodist* and *geosphere* packages to conduct Mantel tests for an autocorrelation of Aitchison distances of PAHs with geographic distance.

Statistical analyses of fungal alpha diversity

We estimated the alpha diversity of each site and time point with three approaches to circumscribe possible sources of error. For all three approaches we estimated alpha diversity with richness, the Shannon index (Shannon 1948), and the Simpson index (Simpson 1949). We transformed these indices into Hill numbers, or effective number of species, of orders zero, one and two. Hill order of zero is equivalent to richness, Hill order of one is calculated as the exponential of the Shannon index, and Hill order of two is calculated as the reciprocal of the Simpson index. These transformations represent diversity more intuitively by describing alpha diversity as the number of equally-common species required to give a particular value of an index (Jost 2006).

First, we estimated alpha diversity at the plot level 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. Richness, the Shannon index, and Simpson index were calculated with functions from the *vegan* package (Oksanen et al. 2016) and transformed as described above. We aslo used these estimates to look for a relationship between the alpha diversity and PAH content of each sample using linear regression. Based on exploratory analyses, we relied on the package *brms* to fit untransformed data to "skew normal" distributions with default priors. Second, we estimated 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. Estimates of Hill numbers were calculated with 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 2012). Lastly, we estimated diversity as site-level diversity, based on incidence in plots. Here, estimates are calculated as the site richness, weighted by the number of plots in which each OTU appears across the site and time point. For example, diversity would be equivalent to richness if the number of samples in the group is equal to one. Thus, site richness is captured as well as the frequency of occurrence across samples and gives a sense of how evenly taxa are shared by plots across the site. As described above, estimates of Hill numbers were calculated with the R package *iNEXT* (Chao et al. 2014; Hsieh et al. 2016) and 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. We also generated rarefaction curves to estimate the number of soil cores needed to accurately estimate site-level diversity with this approach and considered any two points along the curve to be significantly different at the 5% level if the confidence intervals did not overlap (Chao et al. 2014).

Statistical analyses of fungal beta diversity

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. Post-hoc PERMDISP tests were used 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, Fig. 3) and Mantel tests were conducted in *vegan* to examine potential autocorrelation between community composition and geographic distance. Variation in Bray–Curtis dissimilarity values for each site was estimated as multivariate pseudo-standard error (MultSE) to determine how sampling effort influenced estimates of community dissimilarity. 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). Finally, 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 metrics 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. We considered OTUs to be strongly associated with a site or time point when recovered within the extreme deciles of site and time point rankings. 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. We augmented our interpretation with 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; Kirk and Gordon 1988; Müncnerová and Augustin 1994; Cerniglia 1997; da Silva et al. 2003; Verkley et al. 2004; Prince 2010; Furuno et al. 2012; Blasi et al. 2016; Hashem et al. 2018; Prenafeta-Boldú et al. 2018), 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 time points (Fig. 1 and Online Resource 1: Tables S2, S3, Fig. S1). 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-C4 chrysenes constituted a relatively small proportion of detected residues compared to other PAHs, especially 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 (Fig. 1C, D). We expected oil residues at the Bay Jimmy 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 Fourchon 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 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 (Fig. 2). At the site level (Methods 2 and 3), Fourchon was more diverse than Bay Jimmy (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 Fourchon increased in diversity, relative to the first time point and alpha diversity at Bay Jimmy decreased relative to the first time point.

Analyses of the minimum number of samples needed for statistical consistency at Hill order q = 2 indicated that all sites and time points required a minimum of 5–6 samples for alpha diversity estimates to fall within the 95% confidence intervals. 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 (Fig. 2B, Online Resource 1: Table S4). 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 (Fig. 3A, Online Resource 1: Table S8).

We detected patterns of change in the composition of fungal communities by site and time point. According to NMDS ordinations and PERMANOVA tests, community composition differed strongly by site and time point based on Bray–Curtis dissimilarity (Online Resource 1: Table S6), Jaccard index and Aitchison distance values (results not shown). Group dispersions only differed (Online Resource 1: Table S6) for sites when measured according to Jaccard index and Aitchison distance values. Site and time point differences in community composition were well illustrated by the NMDS ordination of

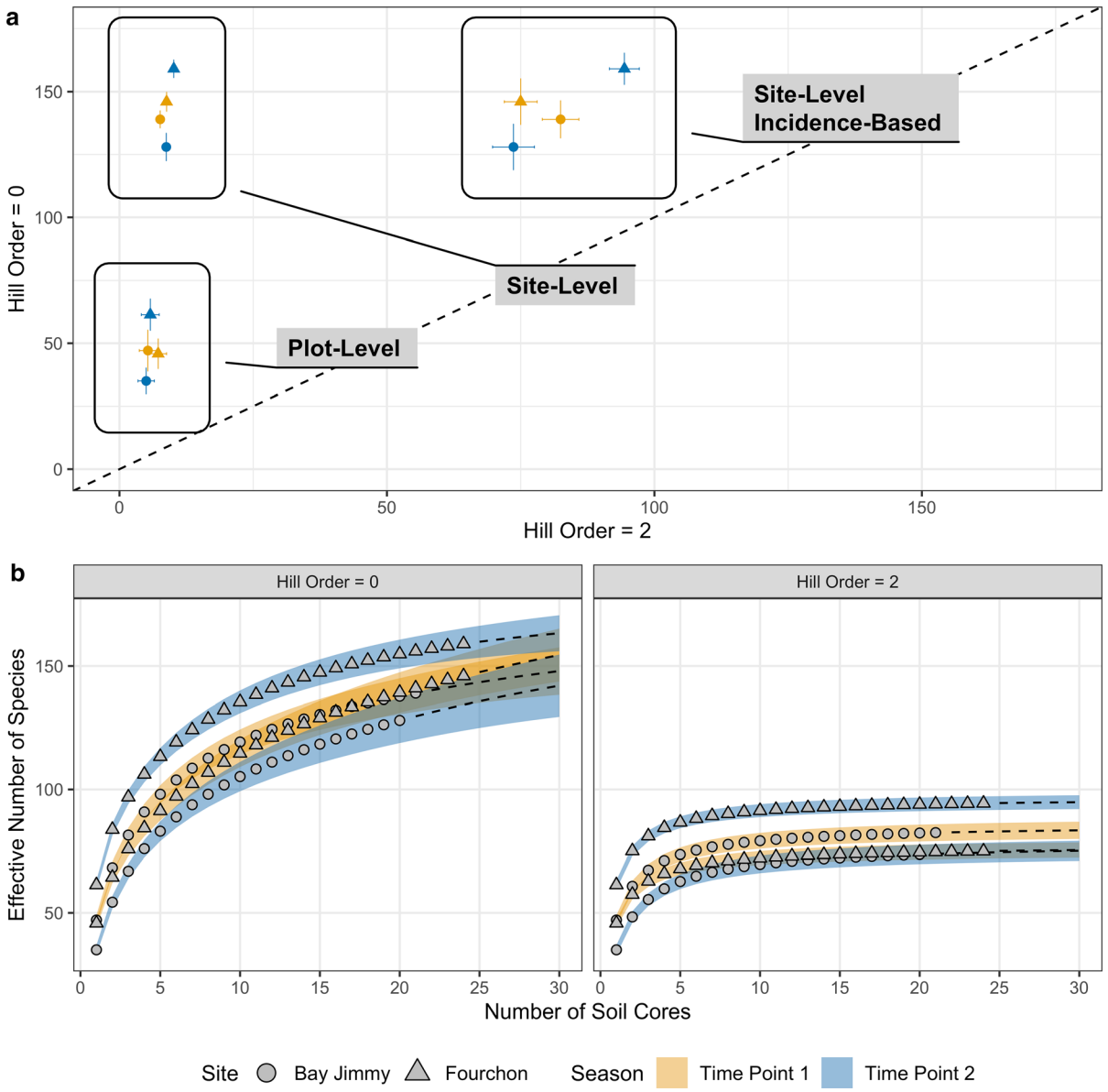

Fig. 2 Comparisons of fungal alpha diversity for each combination of site and time point. **a** Alpha diversity as estimated at the plot-level, site-level, and an incidence-based diversity, where estimates are calculated as the site richness, weighted by the number of plots in which each OTU appears 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 incidence-based 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

Bray–Curtis dissimilarity values (Fig. 3B). 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 (Online Resource 1: Table S7).

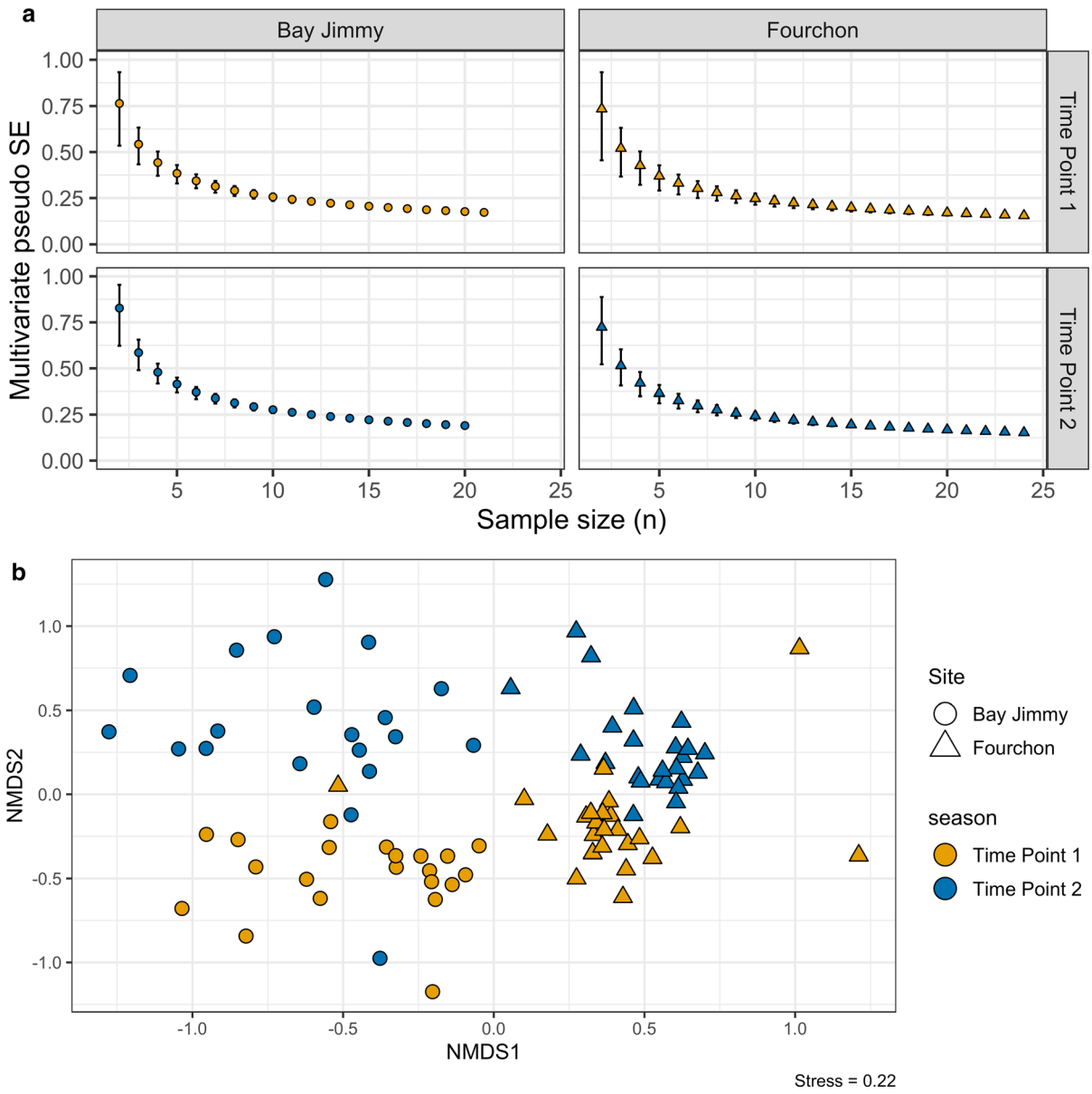

Fig. 3 Visualizations of differences in community composition. **a** MultSE estimations for site and time point. The x-axis represents the number of samples included in the estimate of MultSE and the y-axis is an estimate of multivariate variation where the symbol is the mean variability in the position 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

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 (20 OTUs and 12% of reads) were Basidiomycota. 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 time point when recovered within the extreme deciles of site and time point rankings (Fig. 4, Online Resource 3, Online Resource 2: Figs. 4, 5). Of all the dominant OTUs, 11 were strongly associated with the Bay Jimmy site and 12 were strongly associated with the Fourchon site. We found that nine dominant OTUs were associated with the first time point and 12 were associated with the second time point. Only one OTU was associated with the Bay Jimmy site and strongly associated with the second time point, while two OTUs were associated with the Fourchon site and the second time point. No OTUs were strongly associated with a single site and the first time point.

Some classes of fungi showed strong associations with sites and times points. OTUs assigned to Dothideomycetes and Agaricomycetes were associated with both time points, whereas OTUs assigned to Eurotiomycetes were strongly associated with the second time point (Fig. 4) and members of the Sordariomycetes were primarily associated with the first time point. Members of the Dothideomycetes were strongly associated with both sites whereas OTUs assigned to Sordariomycetes were strongly

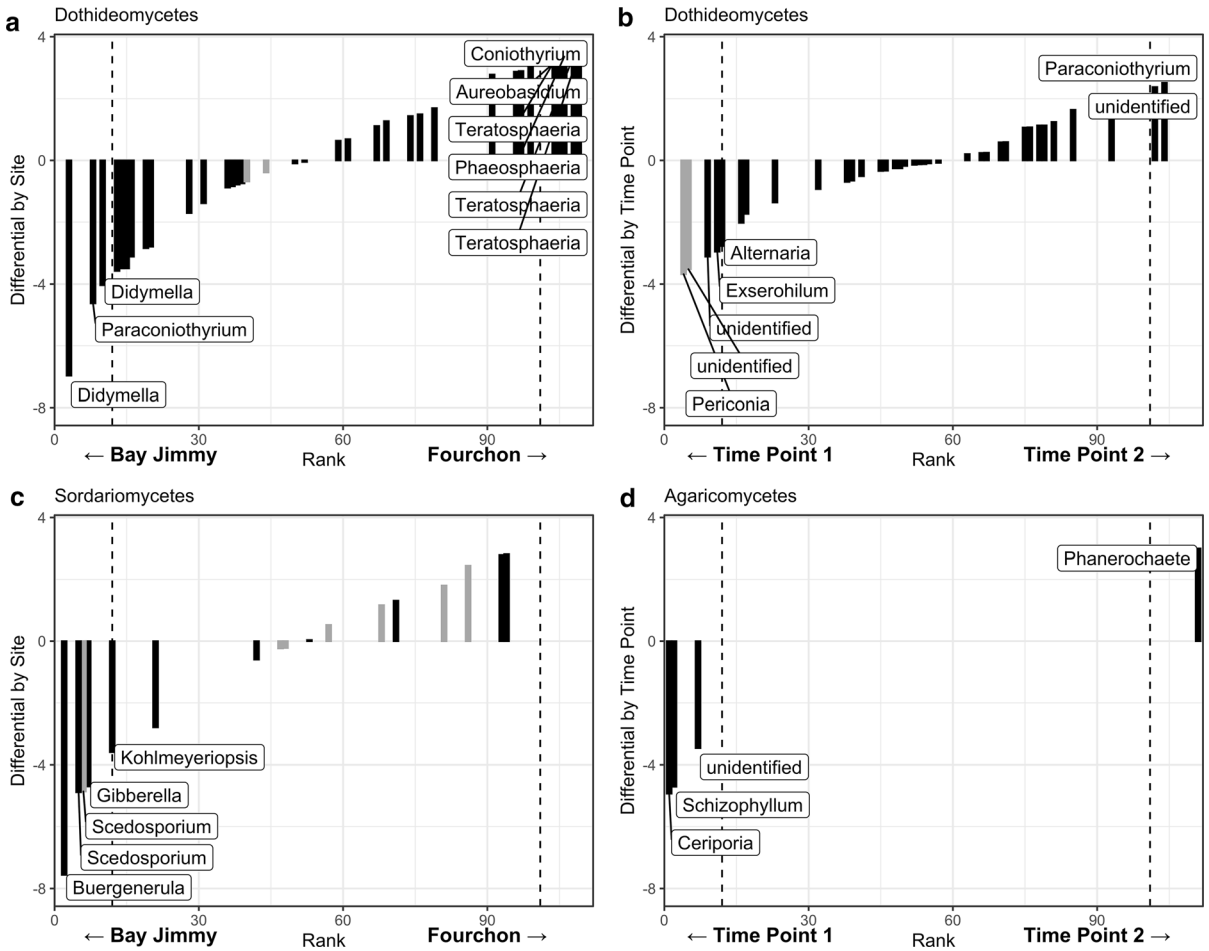

Fig. 4 Differential ranking analysis with Songbird according to site and time point. Rank is on the x-axis and is relative to the differential on the y-axis. Gray bars represent rare taxa and black bars represent dominant taxa. Dotted lines demarcate the lower and upper deciles of the rankings. The genera in the most extreme deciles are labeled with the genus assigned to the OTU. **a** and **b** class *Dothideomycetes* is evenly ranked according to site and time point. Extreme rankings are OTUs that are most abundant at one site or time point 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 Bay Jimmy site. **d** Specific genera in class Agaricomycetes are strongly associated with one time point or the other; no OTUs are in the middle rankings

associated with the Bay Jimmy site (Fig. 4). Of the 11 OTUs associated with the Bay Jimmy site, nine could be identified to genus: *Didymella* (2 OTUs), *Buergenerula, Gibberella, Hasegawazyma, Kohlmeyeriopsis, Paraconiothyrium, Rhodotorula, Scedosporium*. We were able to assign nine of the 12 OTUs associated with the Fourchon site to genus: *Teratosphaeria, Aureobasidium, Coniothyrium, Erythrobasidium, Naganishia, Phaeosphaeria, Rhodotorula* (Online Resource 3: Table S9). We were able to assign nine of the 12 OTUs associated with the second time point to genus, which included: *Aspergillus* (four OTUs), *Penicillium, Phanerochaeate, Symmetrospora, Paraconiothyrium,* and *Erythrobasidium.* Only four of the nine OTUs associated with the first time point could be assigned to the following genera: *Exserohilum, Ceriporia, Schizophyllum,* and *Alternaria*.

Relationships between oil abundance and fungal communities

Linear regression did not support a relationship between total PAH abundance and alpha diversity (Online Resource 1: Table S5). Likewise, dbRDA and PERMANOVA did not detect a relationship between total PAHs and fungal community composition beyond the influence of site and time point (ANOVA-like permutation test on margins, Online resource 1: Table S6).

We also found that total PAH abundance added negligible predictive power to Songbird models of differential ranking. A model of *site + time point* 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 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 investigated known hydrocarbon-degrading taxa, based on literature, to observe any differential associations of these taxa with sites or time points. Such associations might be indicative of the oil spill legacy (Fig. 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; Kirk and Gordon 1988; Müncnerová and Augustin 1994; Cerniglia 1997; da Silva et al. 2003; Verkley et al. 2004; Prince 2010; Furuno et al. 2012; Blasi et al. 2016; Hashem et al. 2018; Prenafeta-Boldú et al. 2018). 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 Bay Jimmy site than the Fourchon site. On the other hand, *Aureobasidium pullulans*, *Rhodotorula mucilaginosa,* and a *Coniothyrium* sp. were more abundant at the Fourchon site (Fig. 5A). Only one dominant OTU (an *Alternaria* sp.) fell into the decile strongly associated with the first time point, while six OTUs (representing four species) were strongly associated with the second time point. These included *Aspergillus niger*, *Aspergillus subversicolor*, *Penicillium citrinum, Paraconiothyrium variabile,* and a *Phanerochaete* sp. (Fig. 5B).

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 time point three years following the spill. Several fungal genera were associated with one of the two time points. Notably, neither alpha nor beta diversity could be explained by variation in oiling, though we did recover evidence of known hydrocarbon degrading taxa genera being relatively more abundant during the second time point (summer of 2013). 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 soil fungal communities. Additionally, our findings suggest that previous studies have severely under-sampled marsh soils for the purposes of

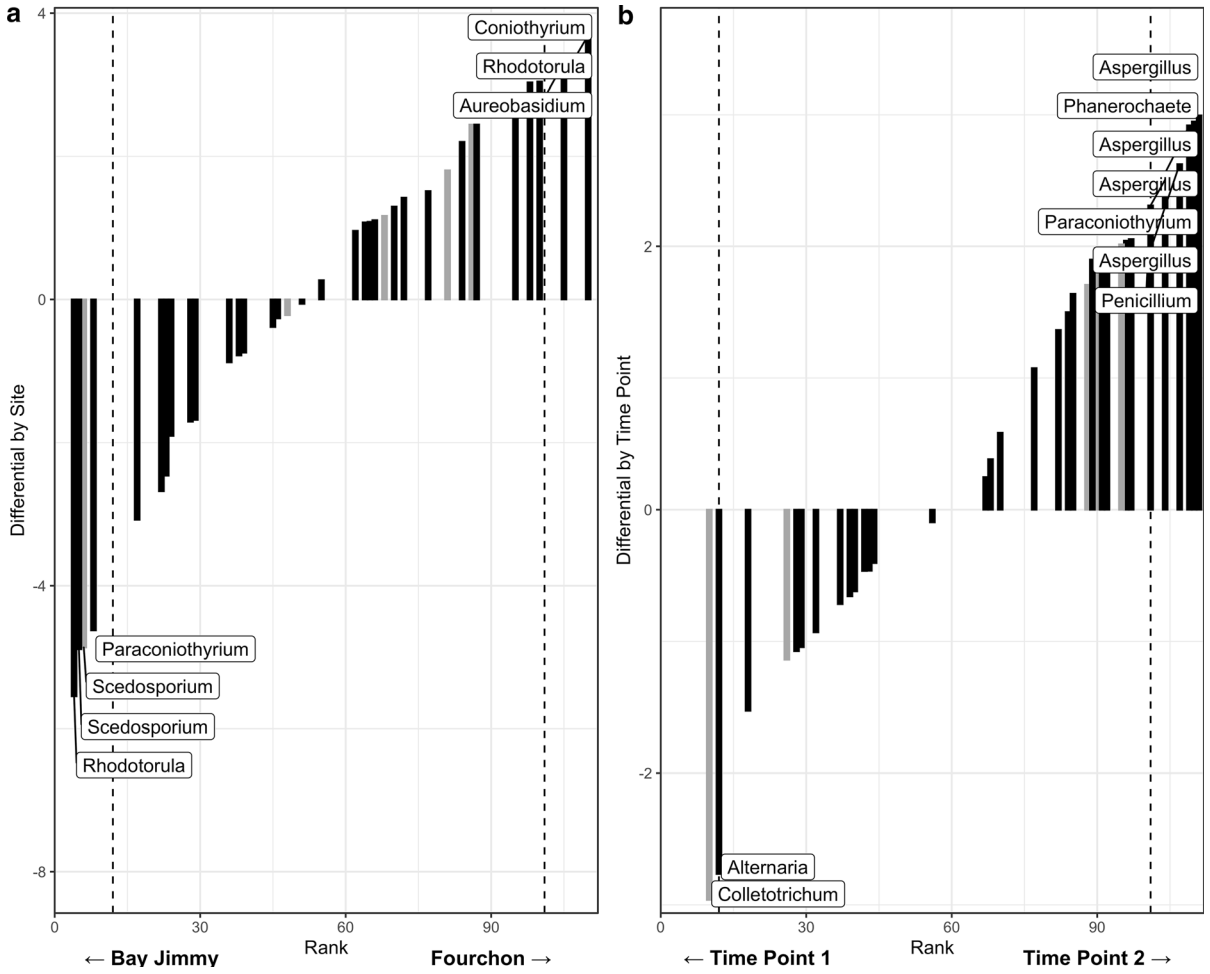

Fig. 5 Differential ranking analysis with Songbird, highlighting hydrocarbon degrading taxa. Rank is on the x-axis and is relative to the differential on the y-axis. Gray bars represent rare taxa and black bars represent dominant taxa. Dotted lines demarcate the lower and upper deciles of the rankings. 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 second time point

describing differences in alpha and beta diversity of fungal communities. Given that soil prokaryote communities are frequently estimated to be even more diverse than the fungal communities examined here (Thompson et al. 2017), it is possible that similar sampling thresholds may also apply to studies of prokaryote communities.

Sampling and characterization of oiling

We found that the oil content of our samples did not differ by site or time point (Fig. 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 Bay Jimmy 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 Fourchon 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 the PAHs we measured in

historically "unoiled" areas are representative of secondary oiling (i.e., redistribution of oil residues by water or air) of the sampled areas at each site. Other possibilities are that replanting the Bay Jimmy sites hastened degradation of the oil residues, as demonstrated in the experiments of Mendelssohn and Lin (2002), and/or extensive microbial biodegradation had already occurred at the Bay Jimmy site. If this is the case, the indistinguishable PAH compositions of the two sites may be representative of background levels of PAH pollution, as southern Louisiana has long been subject to local and regional oil spills. (Colten et al. 2012).

Although there were no overall differences in PAH content between the two study sites, we found evidence of persistent patches of oil residue at the Bay Jimmy site. 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 Bay Jimmy 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 second sampling time point of our Bay Jimmy site closely resembles those from samples taken at 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. The greater variability in our samples 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. Oil residues at both sites may be equally patchy but capturing this quality may require 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 fungal 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 the Bay Jimmy and Fourchon salt marshes, we detected evidence of site-specific temporal effects. We expected to observe higher site-level alpha diversity at the Bay Jimmy site, in part because the area sampled was larger than the area sampled at the Fourchon site (Lomolino 2000). We also expected both sites to become more diverse at the second time point, reflecting longer days, warmer temperatures, and higher marsh productivity (Shimadzu et al. 2013; Dybala et al. 2015; Tonkin et al. 2017). Our findings were only partially consistent with expectations. There were negligible differences in alpha diversity between sites at the first sampling time point. However, the Fourchon site did become more diverse at the second time point compared to the first time point and compared to Bay Jimmy. This was due to a relative increase in diversity at the Fourchon site, relative to time point 1, and a relative decrease in diversity at the Bay Jimmy site relative to time point 1. It is interesting to compare our results to those of a related study (Lumibao et al. 2018) of the same sites in 2016. Lumibao et al. (2018) found the Fourchon site to be more diverse than the Bay Jimmy site during the summer months, consistent with our findings. The similarity supports the notion that this pattern is driven by site-specific conditions, but our results did not find a relationship between oil residues and differences in diversity, making it unlikely that oiling history is the main driver of differences.

Despite temporal changes in alpha diversity, both sites exhibited consistent signatures of variability in community composition at both time points, with the Fourchon site generally being the less variable of the two. (Fig. 3B, Online Resource: Table S6). 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. This apparent lack of relationship may also be the result of replanting at the Bay Jimmy site, which potentially

changed the trajectory of both oil degradation, (Mendelssohn and Lin 2002) and the development of fungal communities, (Cagle et al. 2020) relative to other heavily oiled sites. Finally, our decision to sample PAHs and fungi in different portions of the cores may make correlation analyses unsolid but does not preclude the possibility that the two are related. We are not the first to compare oil residues and microbial communities from two close, but different, spaces (Engel et al. 2017). But in spite of this design flaw but we feel that the results are still worth consideration.

Differences between sites and time points

Our findings offer further evidence of site-specific variation in fungal community structure (Fig. 3B). 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. We did find some notable patterns related to site-specific community composition. For example, we found Dothideomycetes to be diverse and ubiquitous according to differential ranking. This is consistent with their cosmopolitan nature (Ohm et al. 2012), but it is worth noting that certain Dothideomycetes, like *Paraconiothyrium* and *Phaeosphaeria* did show strong associations with sites and time points. On the other hand, members of the Agaricomycetes were only associated with a specific time point, illustrating that a different class of fungi as more likely to contribute to variation in the community composition of salt marsh soil fungal communities.

In general, members of the genera we ranked as strongly associated with one or the other time point have been detected as saprotrophs or endophytes in salt marshes worldwide (Newell 2003; Walker and Campbell 2009; Kim et al. 2014; Calado 2016; Dini-Andreote et al. 2016; Mavrodi et al. 2018; Calado et al. 2019). Intriguingly, the genera that ranked as most differentiated during the second time point (Fig. 5B) are phylogenetically linked to hydrocarbon degraders (Verkley et al. 2004; Valentín et al. 2006; Prince 2010). While such associations might be indicative of the oil spill legacy, the increased temporal prevalence of these taxa should not be viewed as clear evidence of hydrocarbon driven changes in community composition. It does suggest, however, that hydrocarbon degraders are dynamic members of salt marsh soil fungal communities and may be more relatively abundant during the warmer summer months.

Sampling and characterization of soil fungi and other 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 (Fig. 2, Online Resource 1: Table S4). 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 (Beazley et al. 2012; Bik et al. 2012; Looper et al. 2013; Mahmoudi et al. 2013; Atlas et al. 2015; Engel et al. 2017; Bae et al. 2018; Tatariw et al. 2018). Furthermore, our results indicate that multinomial regression (like most general linear models) can be a valuable tool for characterizing microbial communities. It is more robust, however, when based on at least 10 replicates per group. This suggests that future work should involve collecting 10 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 (Shigenaka 2014; Engel et al. 2017; Kim et al. 2017) can confound understanding of site contamination and recovery. Our study builds on this idea by demonstrating that local heterogeneity in soil fungal 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 high-resolution structured (i.e., transect or plot-based) spatiotemporal sampling (Engel et al. 2017) should complement targeted sampling of evidently oiled areas (Looper et al. 2013;

Mahmoudi et al. 2013; Atlas et al. 2015) to fully capture the heterogeneity of oiling and microbial community dynamics. 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 to follow over time.

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Data availability Data are publicly available through the Gulf of Mexico Research Initiative Information & Data Cooperative (GRIIDC) at https://data.gulfresearchinitiative.org (UDI: R5. × 286.000:0013). 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_MG.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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