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Salt Marsh Bacterial Communities before and after the *Deepwater Horizon* Oil Spill

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ABSTRACT Coastal salt marshes along the northern Gulf of Mexico shoreline received varied types and amounts of weathered oil residues after the 2010 *Deepwater Horizon* oil spill. At the time, predicting how marsh bacterial communities would respond and/or recover to oiling and other environmental stressors was difficult because baseline information on community composition and dynamics was generally unavailable. Here, we evaluated marsh vegetation, physicochemistry, flooding frequency, hydrocarbon chemistry, and subtidal sediment bacterial communities from 16S rRNA gene surveys at 11 sites in southern Louisiana before the oil spill and resampled the same marshes three to four times over 38 months after the spill. Calculated hydrocarbon biomarker indices indicated that oil replaced native natural organic matter (NOM) originating from *Spartina alterniflora* and marine phytoplankton in the marshes between May 2010 and September 2010. At all the studied marshes, the major class- and order-level shifts among the phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* occurred within these first 4 months, but another community shift occurred at the time of peak oiling in 2011. Two years later, hydrocarbon levels decreased and bacterial communities became more diverse, being dominated by *Alphaproteobacteria* (*Rhizobiales*), *Chloroflexi* (*Dehalococcoidia*), and *Planctomycetes*. Compositional changes through time could be explained by NOM source differences, perhaps due to vegetation changes, as well as marsh flooding and salinity excursions linked to freshwater diversions. These findings indicate that persistent hydrocarbon exposure alone did not explain long-term community shifts.

IMPORTANCE Significant deterioration of coastal salt marshes in Louisiana has been linked to natural and anthropogenic stressors that can adversely affect how ecosystems function. Although microorganisms carry out and regulate most biogeochemical reactions, the diversity of bacterial communities in coastal marshes is poorly known, with limited investigation of potential changes in bacterial communities in response to various environmental stressors. The *Deepwater Horizon* oil spill provided an unprecedented opportunity to study the long-term effects of an oil spill on microbial systems in marshes. Compared to previous studies, the significance of our research stems from (i) a broader geographic range of studied marshes, (ii) an extended time frame of data collection that includes prespill conditions, (iii) a more accurate procedure using biomarker indices to understand oiling, and (iv) an examination of other potential stressors linked to *in situ* environmental changes, aside from oil exposure.

KEYWORDS *Deepwater Horizon*, Gulf of Mexico, PAHs, bacterial diversity, *n*-alkanes, organic matter, sediment

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Coastal salt marsh ecosystems are environmentally sensitive and subject to both marine and terrestrial environmental stressors (1), with stressors defined as conditions or parameters in excess of their normal range and variability that may adversely affect how communities function (2, 3). Significant deterioration of salt marshes along the Louisiana coastline has been linked to natural stressors (in many cases exacerbated by anthropogenic activities), such as hurricanes, sea-level rise, subsidence, and diminishing sediment supply (4–8) and to direct anthropogenic stressors from canal development, introduction of invasive species, pervasive nutrient pulses, pollutant accumulation, and freshwater flooding due to river diversions (9–11), as well as from accidents such as chemical and oil spills (12 and 13–16). The *Deepwater Horizon* (DWH) explosion and Macondo Prospect well blowout in the Mississippi Canyon Block 252 (MC252) on 20 April 2010 released an estimated 4.9 million barrels of Louisiana sweet crude oil into the Gulf of Mexico (17). Shoreline Cleanup Assessment Techniques (SCAT) teams surveyed 7,058 km of northern Gulf coastline, finding that 1,773 km had trace to heavy oiling (18) (Fig. 1). Marshes comprised approximately 45% of the surveyed coastline, with roughly 95% of the oiled marshes being in Louisiana (19).

Insights into ecosystem resistance, resilience, and recovery can be gained following a disturbance like an oil spill by monitoring changes in biodiversity and community structure through time, including at the microbial level (16, 20–22). However, although microorganisms are known to regulate marsh biogeochemical reactions (6, 23, 24), predicting whether and how marsh microbial communities would respond to the DWH oil spill was a challenge because diversity had been understudied (8, 16, 25, 26), and almost nothing was known about community functional redundancy that could enhance response and resistance (20, 21, 27–30). Initially, some DWH spill researchers proposed a swift microbial response (16, 17) because microbes have the capacity to degrade constituent carbon compounds in oil (31–35) and earlier nutrient enrichment, metal exposure, and oiling experiments provided evidence that marsh communities could withstand low levels of disturbance from an oil spill (25, 36). Short-duration studies based on research conducted during one sampling time or from $<$6 to 9 months of sampling events in 2010 and 2011 confirmed that the relative abundances and species richness for bacterial communities exposed to weathered oil residues changed through time and recognized a greater diversity among known hydrocarbon-degrading bacteria as the amount of petroleum hydrocarbon concentrations increased (37–40). Although a cascading series of long-term biogeochemical consequences, due to the microbial response, was anticipated for the marsh ecosystem (41), some of the earlier studies suggested a faster-than-expected recovery (16, 42). This assessment was likely premature because high concentrations of weathered oil and oil residues, trapped by salt marsh vegetation, lingered for more than a year after the spill and were expected to persist under anoxic conditions for potentially decades (43–46). Therefore, the long-term effect of the spill on marsh microbial communities, and the implications for coastal ecosystems overall, still remain unclear because long-term studies of marsh microbial communities have been needed (47, 48).

Our research was motivated by the need to understand the long-term consequences of oiling on marsh bacterial community compositions, and our investigation differed from earlier DHW studies in several ways. Although some marshes in the northern Barataria Bay were previously examined following the DWH spill (40, 42), we expanded the geographic extent of study across three different regions of southern Louisiana to include salt marshes in Breton Sound and Terrebonne Bay, which have not been intensely studied (49). We also collected data before any of the marshes were oiled in May 2010 and evaluated bacterial communities in the context of natural, preexisting environmental gradients that could also be considered natural stressors, including those caused by inundation frequency, salinity, temperature, and redox fluctuations, as well as vegetation changes, without the potential effects of specific normal (*n*)-alkane and/or polycyclic aromatic hydrocarbon (PAH) compounds introduced from the oil spill. We sampled the same marshes up to 38 months after the spill

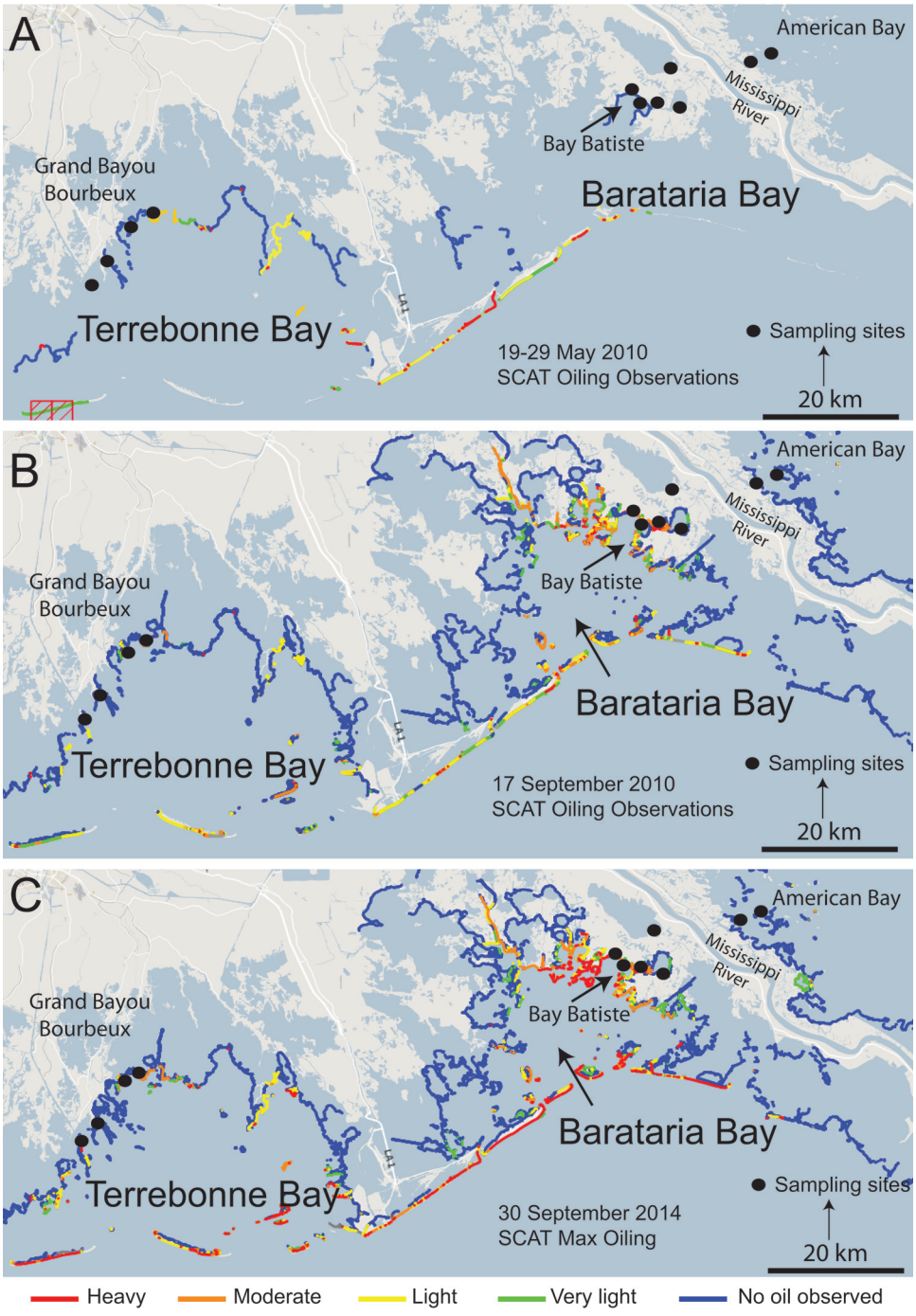

FIG 1 Maps from Shoreline Cleanup and Assessment Technique (SCAT) observation data from the *Deepwater Horizon* Response and the Natural Resource Damage Assessment, available from the Environmental Response Management Application (ERMA) online mapping tool (https://erma.noaa.gov/gulfofmexico/erma.html#) (117), with base maps adapted from OpenStreetMap under a Creative Commons license. (A) Cumulative SCAT oiling shoreline observations from 19 May 2010 through 29 May 2010, which bracketed the first sampling time in this study. (B) Cumulative SCAT oiling shoreline observations from 17 September 2010, after the second sampling time in this study when new sites were added. (C) Cumulative SCAT observations done 30 September 2014 show maximum shoreline oiling (120).

and tracked changes among the types and amounts of organic carbon (C) sources from petroleum or natural (i.e., native) organic matter (NOM), including vascular plants and marine phytoplankton, more accurately by using calculated refractory biomarker indices of *n*-alkane and PAH compounds.

RESULTS AND DISCUSSION

Vegetation changes due to flooding history and oiling. One goal of our study was to understand how the natural environmental gradients occurring before and after the DWH oil spill influenced bacterial community compositions. A marsh's inundation history, and particularly salinity fluctuations, can affect oxygen and nutrient availability, herbivory rates, sedimentation, and plant composition and productivity, which could all lead to changes in the microbial system, without any confounding oiling effects (50–52). To supplement our field data (see also Table S1 in the supplemental material), we used data reported in the Coastwide Reference Monitoring System stations (http://www.lacoast.gov/crms2/Home.aspx) to evaluate potential relationships from 2007 to 2014 among some of the natural environmental factors related to inundation, daily water level, salinity, and temperature. The results from analyzing this 7-year data set indicated that the American Bay marshes nearest river diversions were experiencing more frequent periods of inundation before the oil spill (Fig. 2B). According to pairwise comparisons of data from the sampled marshes grouped by time, the measured inland water depth at 10 m also differed significantly over time (nonparametric permutational multivariate analysis of variation [NPMANOVA] $F = 6.59$, $P = 0.0012$) (see Table S2a in the supplemental material), with marshes in all the regions except Bay Batiste being flooded more frequently over the course of the study. During the time of the oil spill, water salinity differed significantly between September 2010 and June 2011 and between June and September 2011 (see Table S2b in the supplemental material). The difference in the first time period was likely because river diversions in southern Louisiana were opened to full capacity from late April through October 2010 in an attempt to inhibit oil penetration inland (53, 54), even though freshwater flooding negatively impacts fisheries and oyster abundances (55).

We originally sampled only *Spartina alterniflora* marshes, but the plant diversity at some marshes increased over time to include *Spartina patens*, *Juncus roemerianus*, *Distichlis spicata*, and *Aster* spp. (see the additional data and Table S3 in the supplemental material). Vegetation die-off was not observed at the marshes sampled, unlike marshes in other studies (see, for example, reference 42). The *S. alterniflora* canopy height was used as a rough proxy for plant health and productivity in the absence of stem density information for all of the marshes sampled through time (see, for example, reference 56). Despite modest increases due to seasonal growth at some locations, *S. alterniflora* height significantly decreased at 6 of the 11 marshes ($F = 3.76$, $P = 0.012$) (Table 1 and Fig. 2C; see Table S2c in the supplemental material). Plant height changes did not link to temperature, salinity, or inundation history, but negatively correlated with inland *n*-alkane concentrations (Fig. 2D; Spearman's rho -0.47, $P = 0.0016$) and PAH concentrations (Fig. 2E; rho $= -0.50$, $P = 0.001$), which also differed significantly over time. The highest hydrocarbon concentrations were in September 2011, which was more than 1 year after the spill (Fig. 2D and E; see also Tables S2d and S2e in the supplemental material). The negative effects of oiling on *S. alterniflora* aboveground biomass and productivity are known (57), but the expectation was that the vegetation would recover postspill (16, 42). Instead, our results showed significant plant height reduction, which match the results from other long-term, postspill studies indicating oil exposure diminished vegetation health and productivity (58, 59) and contributed to accelerated shoreline retreat (60, 61).

Changes in the sources of organic C and weathered oil residues. The organic C contributions from vegetation, phytoplankton, and petroleum at each of the marshes over time were determined from calculated refractory biomarker indices for the *n*-alkanes (see Table S4 in the supplemental material). The higher concentrations of long-chain homologues (nC_{24} to nC_{35}) with an odd-to-even C number preference reflect biogenic contributions, which were predominately from higher plant epicuticular leaf waxes (62, 63). The odd-to-even C number preference, and dominance of longer-chain compounds for samples collected in May 2010, indicated that plants primarily contributed to marsh NOM, with a secondary input from autochthonous

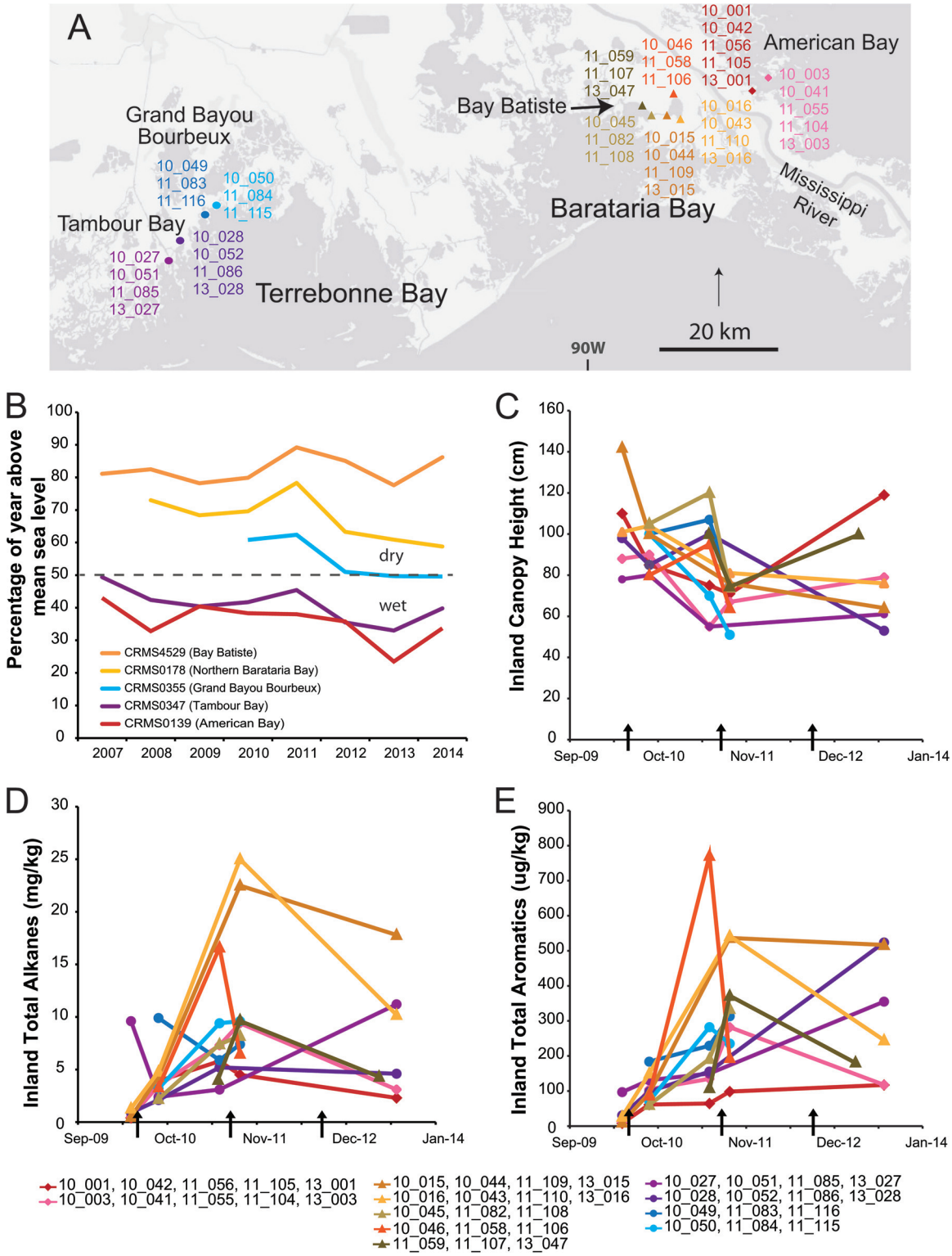

FIG 2 (A) Location map for salt marsh sampling from 2010-2013, with the base map adapted from Open Street Maps and visualized by ArcGIS Online (Esri). Sampled marsh locations correspond to the year and sample number listed on Table 1, without the "ELM" prefix, and are color-coded in panels A, C, D, and E. (B) The percentage of time during a year that the marsh surface is above mean sea level or, conversely, the amount of time a marsh surface is flooded, according to Coastwide Reference Monitoring System (CRMS) station data (http://www.lacoast.gov/crms2/Home.aspx). Stations are color-coded to match the closest sampled marsh. Marshes in American Bay and Terrebonne Bay are flooded more frequently than marshes in Barataria Bay. (C) Canopy height of *Spartina alterniflora* at 10 m inland for each marsh over time. (D and E) Total *n*-alkane concentrations (D) and aromatic compound concentrations (E) for each of the marshes over time. The black arrows for panels C to E refer to major meteorological events during the sampling, including Topical Storm Bonnie (25 July 2010), Tropical Storm Lee (4 September 2011), and Hurricane Isaac (29 August 2012).

TABLE 1 Summary of marsh vegetation and physicochemistry, organized by location and sampling events[a]

Geographic location and site no.	Sampling time	Water temp (°C)	Salinity (psu)	Inland at 10 m				Offshore		Edge at 1 m		SCAT 2010 and 2014 max oiling categories
				Canopy ht (cm)	Water depth (cm)	Total *n*-alkanes (mg kg^{-1})	Total aromatics (μg kg^{-1})	Sediment % H_2O	Sediment % Org C	Total *n*-alkanes (mg kg^{-1})	Total aromatics (μg kg^{-1})	
American Bay												
ELM-10-001	May 2010	29.7	2.69	110	5	0.4	8.0	55.4	6.3	NM	NM	No oil
ELM-10-042	Sept 2010	31.3	4.22	85	3	3.9	61.4	48.2	5.8	0.8	21.2	
ELM-11-056	June 2011	28.7	13.93	75	0	5.8	64.3	62.9	9.0	2.7	58.8	
ELM-11-105	Sept 2011	29.3	9.6	71	0	4.5	98.4	66.2	14.0	3.0	110.1	
ELM-13-001	Aug 2013	31.8	6.12	119	0*	2.3	117.4	52.9	7.56	NM	NM	No oil
ELM-10-003	May 2010	30.3	3.31	88	3	0.5	10.9	80.1	30.2	NM	NM	No oil
ELM-10-041	Sept 2010	30.3	5.13	90	1	3.5	105.0	73.5	20.9	1.0	28.6	
ELM-11-055	June 2011	28.3	19.01	55	0	7.3	134.8	69.5	20.5	6.3	62.48	
ELM-11-104	Sept 2011	29.2	9.0	67	0	9.4	282.3	77.0	26.4	8.1	211.1	
ELM-13-003	Aug 2013	31.5	5.36	79	0*	3.1	117.6	82.8	26.1	NM	NM	No oil
Bay Sanbois, Northern Barataria Bay												
ELM-10-015	May 2010	31.8	8.77	142	13.5	0.5	10.2	89.2	68.7	NM	NM	No obs.
ELM-10-044	Sept 2010	30.8	8.55	100	2.5	4.3	93.6	82.8	34.8	1.8	52.4	
ELM-11-109	Sept 2011	29.5	9.9	76	0	22.5	536.8	88.7	65.6	15.7	545.5	
ELM-13-015	Aug 2013	31.0	9.41	64	0*	17.8	516.7	91.9	67.9	NM	NM	Moderate
ELM-10-016	May 2010	32.1	8.53	101	1	1.3	26.8	82.9	34.0	NM	NM	No obs.
ELM-10-043	Sept 2010	29.9	8.47	104	0	4.8	150.0	79.4	30.9	2.0	70.4	
ELM-11-110	Sept 2011	30.6	9.8	81	0	25.0	542.2	90.6	54.3	25.6	707.3	
ELM-13-016	Aug 2013	30.7	9.33	76	0*	10.2	244.9	76.4	32.3	NM	NM	No oil
Tambour Bay, Terrebonne Bay												
ELM-10-027	May 2010	28.1	16.16	78	7	9.6	96.9	60.2	10.9	NM	NM	No obs.
ELM-10-051	Sept 2010	30.2	16.27	80	0	2.4	131.0	47.1	5.5	2.0	394.0	
ELM-11-085	June 2011	27.2	15.64	55	0	3.1	149.8	63.9	13.3	3.3	85.5	
ELM-13-027	Aug 2013	30.3	20.46	61	0*	11.2	355.0	31.9	3.11	NM	NM	No oil
ELM-10-028	May 2010	28.8	15.76	98	16	0.9	30.5	49.3	14.2	NM	NM	No obs.
ELM-10-052	Sept 2010	30.6	13.57	85	0	2.1	98.4	57.5	10.2	0.8	230.0	
ELM-11-086	June 2011	27.7	15.24	100	2	5.2	155.6	52.2	8.2	2.1	315.2	
ELM-13-028	Aug 2013	30.4	17.85	53	0*	4.6	524.0	55.9	10.0	NM	NM	No oil
Grand Bayou Bourbeux, Terrebonne Bay												
ELM-10-049	Sept 2010	32.5	10.65	100	0	9.9	184.0	82.6	29.8	2.3	114.0	No oil
ELM-11-083	June 2011	27.4	14.48	107	0	5.9	229.3	79.3	37.9	13.5	169.0	
ELM-11-116	Sept 2011	28.8	13.8	75	0	7.4	313.4	84.8	29.5	27.1	699.1	Moderate
ELM-10-050	Sept 2010	33.6	11.72	100	0	3.2	71.2	56.4	53.5	30.1	882.0	Light
ELM-11-084	June 2011	28.3	15.83	70	1	9.4	282.3	41.8	6.6	6.7	714.0	
ELM-11-115	Sept 2011	29.0	14.12	51	8.75	9.6	235.2	79.3	19.5	15.0	269.2	Moderate
Bay Batiste, Northern Barataria Bay												
ELM-10-046	Sept 2010	30.8	7.39	80	0	78.1	24.9	5.0	614.0	3.3	87.4	No obs.
ELM-11-058	June 2011	28.7	13.23	95	0.5	78.2	17.4	4.7	104.4	16.6	771.0	
ELM-11-106	Sept 2011	26.2	9.6	64	0	77.5	17.4	7.9	195.9	6.5	194.32	No oil
ELM-10-045	Sept 2010	31.1	8.64	105	0.5	69.2	17.0	1.0	34.8	2.2	60.5	No obs.
ELM-11-082	June 2011	29.5	12.93	120	10	80.8	28.7	12.2	148.8	7.4	191.7	
ELM-11-108	Sept 2011	29.5	10.2	74	0	74.7	19.5	8.5	219.5	8.2	334.5	Heavy
ELM-11-059	June 2011	28.9	13.16	100	0	52.0	34.2	227.8	8,880.3	4.0	106.8	No obs.
ELM-11-107	Sept 2011	27.3	10.3	73	0	75.3	16.0	7.0	574.6	9.5	360.9	
ELM-13-047	May 2013	30.2	8.1	100*	0	82.5	26.9	4.3	153.5	NM	NM	Heavy

[a]The total *n*-alkane and total aromatic hydrocarbon concentrations are reported for 10 m inland and 1 m from the marsh edge (from Turner et al. [46]). Sediment water and organic C content were measured from offshore sediments that correspond to microbiological samples. Shoreline oiling categories from SCAT data, ranked as no oil to heavy oiling, are included, observed 19 to 29 May 2010 and from maximum cumulative oiling by 30 September 2014 at the sampled marshes (120) (Fig. 1). *, estimated. NM, not measured. No obs., no SCAT observations were made for May 2010.

sources. The nC_{29} homologue dominated, and the average proportion of wax (indicated by the %wax values) was ~81% (range, 70.5 to 97.4%), which corroborated previous findings from pristine salt marshes that plant wax contributes from 80 to 87% (64) to > 90% (62) of the NOM. The shorter chain homologues (nC_{12} to nC_{23}) without an odd-to-even C number preference have been associated with petroleum (62, 64, 65). One of the samples collected in May 2010 in Bay Sansbois marshes was dominated by nC_{18} and had carbon preference index (CPI) values close to 1, although the SCAT maps did not indicate any visually apparent oiling at the site at that time.

The concentration of total *n*-alkanes increased at all sites by September 2010 (Fig. 2D), including at the American Bay and Tambour Bay marshes where the SCAT maps did not indicate any oiling over time (Fig. 1C and Table 1). For about half of the marshes sampled, the nC_{18} became the peak compound (see Table S4 in the supplemental

material), although the concentrations were not significantly different from those in the samples collected in May 2010 (see Table S2d in the supplemental material). One explanation for the increase in *n*-alkane concentrations could be that plant-sourced *n*-alkanes can accumulate seasonally, perhaps because of resistance to microbial degradation (66). However, if the higher *n*-alkane concentrations were due solely to plant maturation and limited decomposition throughout the year, or particularly during and after the summer growing season, then the hydrocarbon distribution patterns and relative abundances of specific *n*-alkanes (e.g., nC_{29}) should have remained relatively unchanged over time (67). By June 2011, however, *n*-alkane concentrations differed significantly from values in May 2010, with the proportion of wax contributions decreasing, the CPI values dropping to ~1, and the Pr/Phy ratios decreasing from values indicative of terrestrial NOM to values indicative of fresh petroleum deposited under anoxic conditions (63) (see Tables S2d and S4 in the supplemental material). Higher abundances of low-molecular-weight (LMW) *n*-alkanes (e.g., nC_{10} to nC_{19}) peaked in September 2011 for most sites, at up to 25.0 mg kg^{-1} (see Table S4 in the supplemental material). Moreover, half of the marshes had *n*-alkane biomarkers denoting oil contamination, despite the lower concentrations due to the effects of abiotic weathering and biodegradation over time (44).

Most of the earlier DWH studies did not consider how the heterogeneous, weathered oil residues could linger in marsh sediments for years (32, 43) or become redistributed by storms years after the main oiling. We detected oil up to 100 m into the marshes by late 2011 (46), which contradicted earlier studies that claimed oil was restricted to shoreline edges (42, 58). The remobilization and burial of oil likely occurred after Tropical Storm Lee in 2011 and also after Hurricane Isaac in 2012 (Fig. 2D and E) (45, 46). Hydrocarbon concentrations peaked in late 2011 for some Barataria Bay marshes that were along the storm path. None of the sampled marshes in this study, therefore, were free of petroleum hydrocarbons, although the SCAT mapping of cumulative oiling (ending in 2014) indicated that there was no apparent oiling at some locations (Fig. 1C and Table 1). The inconsistency is understandable, because the SCAT mapping is based on observations of oil along shorelines, rather than measuring hydrocarbon compounds from collected soils and sediments. Therefore, even if the marshes did not look contaminated by oil, especially for the light oiling category, the oil may have been incorporated into the inland soils and subtidal sediments but not visually observed.

The total *n*-alkane concentrations in August 2013 increased in the Terrebonne Bay marshes but decreased in the Barataria Bay marshes (Fig. 2D). The biomarker calculations in both regions suggested that the organic C contributions were from vegetation and not oil, although some LMW *n*-alkanes were still detected at low concentrations, particularly at the Terrebonne Bay sites (see Table S4 in the supplemental material). The CPI values were also significantly different in 2013 compared to in 2010 and 2011 (see Table S2f in the supplemental material), but the Pr/Phy ratios increased and were comparable to May 2010 prespill ratios, thereby suggesting alkane degradation (63, 68). Evidence of biodegradation was noted by others (38, 40, 69, 70). By June 2013, the concentrations of *n*-alkanes and PAHs were 1 and 5%, respectively, of early 2011 concentrations, which confirmed that petroleum hydrocarbons were still present in the marshes, but at lower levels likely due to sediment sequestration, weathering, and degradation (68).

Petrogenic and pyrogenic (i.e., from combustion of grass, wood, or coal) sources were determined from the sediments and soils using the PAH compound concentrations and molecular ratios. Total PAH concentrations remained elevated through 2013 compared to prespill values at most marshes, and the Flu, Phe, and Chry concentrations were higher in 2011 than in 2010 (Fig. 2E; see Tables S5 and S2e in the supplemental material). We expected that the PAH compound concentrations would generally decrease as the molecular weight increased because the half-lives for LMW PAH compounds, which have higher volatility and enhanced bioavailability, range from ~10 to 120 days, and the half-lives for high-molecular-weight (HMW) compounds, like BaP,

range from 200 to >1,400 days (71). We also expected that biodegradation should have decreased PAH levels from the time of initial oiling (35, 37, 72). However, the concentration of PAH compounds increased over time, and the molecular ratios of key PAHs indicated that the source(s) also changed (see Fig. S1A and B in the supplemental material). Specifically, the Phe0/An values, which can suggest a petrogenic source if over ~15, significantly differed for all the sampled marshes ($F = 8.44$, $P = 0.0001$) (see Table S2g in the supplemental material), and by 2013 these values indicated a pyrogenic source. Further, the pyrogenic index (PI) values (see Fig. S1C and D in the supplemental material) also revealed a shift from petrogenic to pyrogenic PAHs at most of the marshes (62, 73). For inland samples, pyrogenic PAHs correlated with higher CPI values from *n*-alkanes, which were indicative of, and confirmed, the return to plant wax contributions (see Fig. S1E and F and Table S4 in the supplemental material).

Collectively, the weathered oil residues were geographically widespread throughout southern Louisiana, and the highest concentrations of oil residues in the marshes we sampled occurred from June through September 2011, despite previous reports from near Grand Isle, Louisiana, claiming that peak oiling occurred before March 2011 (74), or that peak oiling occurred in northern Barataria Bay marshes only months after the spill (40, 42). In addition, our analyses indicated that the petrogenic hydrocarbon contributions to marshes decreased after September 2011 when marsh vegetation and 4- to 6-ring HMW PAHs dominated NOM. The increased PAH levels, up to 542.2 μg kg^{-1}, may be due to preferential sorption onto the plant-dominated NOM and anoxic sediments over time (75), which would also manifest as a shift from petrogenic to pyrogenic PAHs. Although the highest PAH concentrations, in total or as individual PAH compounds, were below the concentrations thought to induce toxic effects (76), this PAH source transition likely impacted the microbial communities because petrogenic PAHs are generally more bioavailable than pyrogenic compounds (32). However, the impact of PAHs on marsh sediment microbial communities, as well as to marsh plants and the ecosystem at large, are still being realized (see, for example, references 35, 77, 78).

None of the marshes in our long-term study could be considered true reference sites, as they had been exposed to oil residues. However, because some marshes, like those in American Bay or Terrebonne Bay, had lower cumulative hydrocarbon concentrations over time than marshes in Barataria Bay, this provided a low- to high-oiling gradient from which we could analyze the microbiology data. Incidentally, the widespread distribution of oil throughout Louisiana marshes over time may have compromised the use of reference sites in other marsh microbiology studies after the DWH oil spill. For instance, Mahmoudi et al. (40) used sample locations of Silliman et al. (42) in northern Barataria Bay and considered reference sites to be unimpacted by the DWH spill. However, their references sites had an average concentration of total *n*-alkanes of 24.5 mg kg^{-1} ($n = 6$) and an average total PAH compound concentration of 983 μg kg^{-1} ($n = 6$), values which exceed all but four of our marsh edge samples for *n*-alkanes and all but one of our sampled marsh edges for PAH concentrations for any sampling time (see Table S5 in the supplemental material). These values make it difficult to understand how these microbiology results could reflect reference, or unimpacted, conditions. Moreover, other studies, such as that of Atlas et al. (69), have reported TPH values that are not easily comparable to our data because of methodological differences, but the TPH values in 2011 and 2013 were orders of magnitude higher than the combined total concentrations for *n*-alkanes and PAHs at our sites. Again, the microbial diversity results at those marsh locations, which were apparently heavily oiled, are difficult to evaluate as being reference sites that would have been at all comparable to communities prior to the disturbance.

Bacterial community shifts linked to a changing marsh environment. Of the 997,649 16S rRNA gene amplicons retrieved, there were 436,106 screened, trimmed, and chimera-removed amplicons used to evaluate bacterial community compositional changes (see Table S6 in the supplemental material). The taxonomic compositions

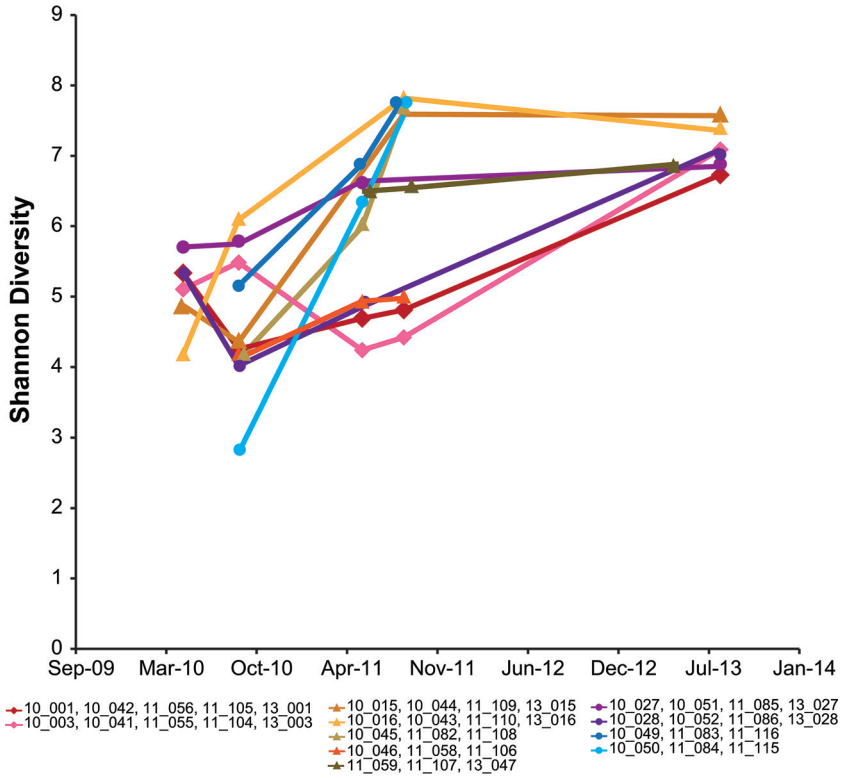

FIG 3 Shannon diversity index values per sampled marsh through time. Refer to Fig. 2 for sample locations. Plotted data are color-coded for each marsh location on Fig. 2A, with the sample numbers listed for each site from Table 1 without the "ELM" prefix.

compared by sample depth were not statistically different ($P > 0.05$) (see Table S7 in the supplemental material), and so the relative abundances were combined for each sampling event (see Table S8 in the supplemental material). The bacterial community compositions did not significantly differ by geographic region but did significantly differ over time from 2010 to 2013 ($F = 9.437$, $P = 0.00001$) (see Table S8 in the supplemental material) and became more diverse based on H′ values (Fig. 3 and 4; see also Table S6 and S9 in the supplemental material). The calculated Bray-Curtis dissimilarity index (BCDI) values indicated that the May 2010 and September 2010 communities were compositionally more similar to each other, with BCDI values averaging 0.74. The average H′ values decreased between May 2010 (5.09, $n = 6$) and September 2010 (4.61, $n = 10$), and diversity increased from June 2011 (5.67, $n = 9$) to September 2011 (6.58, $n = 9$) and then decreased again in May to August 2013 (7.08, $n = 7$). The community compositions were significantly different between September 2010 and June 2011 ($F = 5.411$, $P = 0.006$), and the lower BCDI values indicated that communities at each of the marshes had become more dissimilar, with values averaging 0.69. The communities became more distinct between September 2011 and August 2013 ($F = 7.719$, $P = 0.0003$), with the BCDI values averaging 0.62, perhaps because diversity increased through time. Interestingly, the average BCDI value for only marshes sampled in August 2013 was 0.84, which was the highest value of all the comparisons and suggested that the 2013 communities were more similar to each other compositionally than to the other communities during any of the previous sampling times. Of all the comparisons, however, the most significant compositional differences were between May 2010 and August 2013 ($F = 17.44$, $P = 0.00001$), which confirmed that pre- and postspill communities were different.

An increase in diversity following a stress or disturbance has been reported for a number of other ecosystems (21, 27), if the habitat was not destroyed faster than organisms could adapt or recover. Other microbiological studies after the DWH oil spill

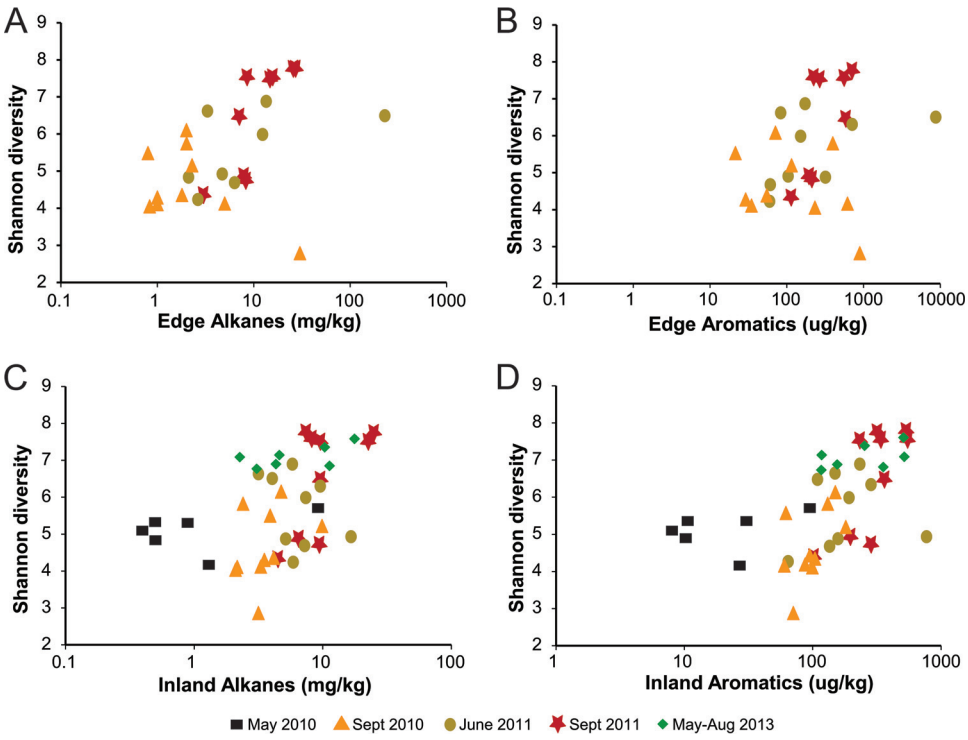

FIG 4 Comparisons between concentrations of total *n*-alkanes and aromatics and Shannon diversity values over time for marsh edge measurements (A and B) and 10-m-inland measurements (C and D), with May 2010 data (inland hydrocarbon only) being solids squares and August 2013 (inland hydrocarbon only) being closed green diamonds.

also note higher diversity among marsh communities over time (37, 69). The influx of degradable organic C from hydrocarbons may have contributed to a higher bacterial diversity (33, 79), as long as the effects of toxicity or overselection of groups capable of degrading hydrocarbons were minimized (33, 80, 81). Minimizing overselection of hydrocarbon degraders was not possible, and so it may be that the heavy oiling levels measured in the Atlas et al. (69) study, and even those of the Mahmoudi et al. (40) study, caused toxicity because, in contrast to our findings, the lowest H′ values in the Atlas et al. (69) study corresponded to exceptionally high TPH levels, whereby even their highest H′ values correlated to lower TPH concentrations that were still greater than nearly all of our measured hydrocarbon values.

The *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and/or *Chloroflexi* phyla in the marshes sampled in this study made up more than 70% of the total bacterial communities for all the marshes over time (Table 2; see also Fig. S2 and Table S9 in the supplemental material). Several key community shifts were apparent, as evidenced from the BCDI values (Fig. 5). First, the dominant taxonomic groups at the phylum level changed between May 2010 and September 2010 from *Proteobacteria* (Fig. 5A), predominately *Gammaproteobacteria*, to *Firmicutes* (Fig. 5B and Table 2; see also Fig. S2 in the supplemental material). The relative abundance of *Firmicutes* negatively correlated with the concentrations of *n*-alkanes (rho = −0.56, $P = 0.0001$) and PAHs (rho = −0.33, $P = 0.003$), and positively correlated to the lower Phe0/An values indicative of pyrogenic PAH sources (rho = +0.5, $P = 0.0009$). *Firmicutes* were dominated by *Clostridiales* in May 2010 (49% of all *Firmicutes*), but the representation of *Bacillales* increased as the overall proportion of *Firmicutes* decreased by May 2011 when relative abundances of *Thermoanaerobacterales* increased (Table 2, Fig. 5B). The *Bacillales* are known to be hydrocarbon-degraders (82–84) and *Thermoanaerobacterales* include taxa capable of cellulolytic metabolism (85) or syntrophic acetate oxidation with hydrogenotrophic methanogens (86). This compositional shift could be associated with changes in the

TABLE 2 Top five highest average relative abundances for major phyla and ranking of relative abundances for proteobacterial classes for each sampling time

	Relative abundance[a]									
	May 2010 (n = 6)		Sept 2010 (n = 10)		June 2011 (n = 9)		Sept 2011 (n = 9)		August 2013 (n = 7)	
Type	Phylum or class	%	Phylum or class	%	Phylum or class	%	Phylum or class	%	Phylum or class	%
Phyla	*Proteobacteria*	49.6	*Firmicutes*	43.0	*Proteobacteria*	35.2	*Proteobacteria*	40.3	*Proteobacteria*	57.0
	Firmicutes	24.6	*Proteobacteria*	34.6	*Firmicutes*	27.0	*Chloroflexi*	18.3	*Chloroflexi*	9.8
	Bacteroidetes	9.9	*Actinobacteria*	8.0	*Bacteroidetes*	12.4	*Firmicutes*	13.2	*Cyanobacteria*	7.8
	Actinobacteria	4.4	*Bacteroidetes*	4.9	*Actinobacteria*	4.8	*Bacteroidetes*	7.1	*Planctomycetes*	7.2
	Chloroflexi	2.9	*Acidobacteria*	2.9	*Chloroflexi*	7.0	*Planctomycetes*	4.7	*Bacteroidetes*	7.0
	Acidobacteria	2.5	*Planctomycetes*	1.3	*Acidobacteria*	3.4	*Actinobacteria*	3.5	*Actinobacteria*	4.4
Proteobacterial classes	*Gammaproteobacteria*	60.6	*Gammaproteobacteria*	65.9	*Gammaproteobacteria*	38.9	*Alphaproteobacteria*	32.9	*Alphaproteobacteria*	58.7
	Alphaproteobacteria	20.0	*Alphaproteobacteria*	10.9	*Alphaproteobacteria*	25.3	*Gammaproteobacteria*	29.0	*Gammaproteobacteria*	23.0
	Betaproteobacteria	18.8	*Deltaproteobacteria*	9.5	*Deltaproteobacteria*	18.1	*Deltaproteobacteria*	26.2	*Deltaproteobacteria*	11.7
	Deltaproteobacteria	11.5	*Betaproteobacteria*	7.5	*Betaproteobacteria*	9.1	*Betaproteobacteria*	6.5	*Betaproteobacteria*	4.6
	Epsilonproteobacteria	3.7	*Epsilonproteobacteria*	6.3	*Epsilonproteobacteria*	8.8	*Epsilonproteobacteria*	5.3	*Epsilonproteobacteria*	1.8

[a]The numbers of marsh locations per sampling time are indicated in parentheses.

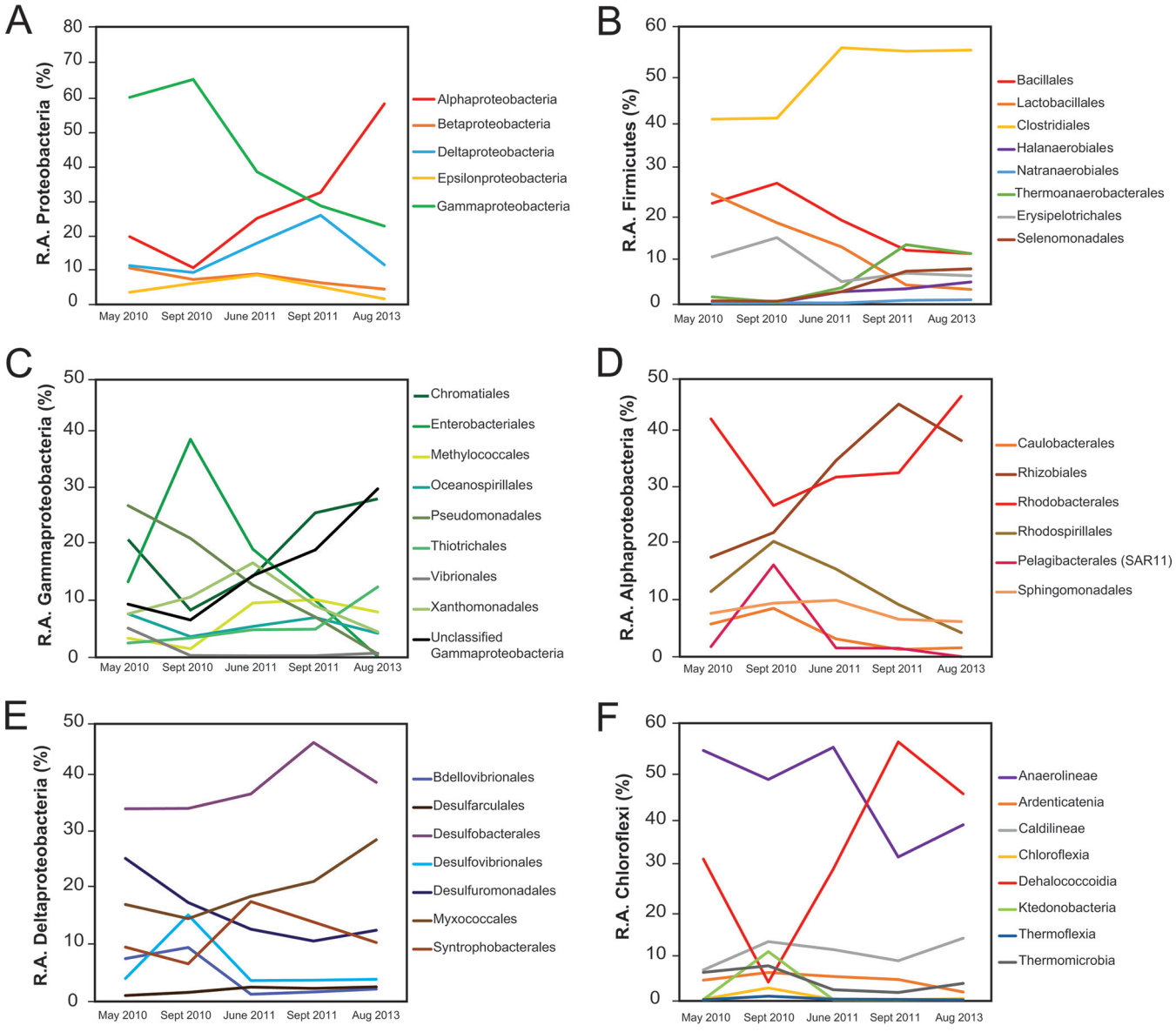

FIG 5 Average changes in relative abundances (R.A.) for all of the sampled marshes at specific times for the classes within the *Proteobacteria* phylum (A), orders within the *Firmicutes* phylum (B), orders within the *Gammaproteobacteria* class (C), orders within the *Alphaproteobacteria* class (D), orders within the *Deltaproteobacteria* class (E), and classes within the *Chloroflexi* phylum (F). The lines shown on the graphs that connect each of the sampling times are only meant to guide the eye between comparisons and do not imply a continuum of data over the 38 months of study.

availability and type of organic C sources, from plant-derived NOM to oil-derived organic C and then back to plant-derived NOM or to a change in the sediment redox status or the salinity of water flooding the marshes.

Another major taxonomic shift occurred after September 2010 among the proportions of gamma-, alpha-, and deltaproteobacterial classes (Fig. 5C to E). For the *Gammaproteobacteria* (Fig. 5C), the proportion of *Pseudomonadales* (represented by *Pseudomonas* and *Alkanindiges* spp., known hydrocarbon degraders) in May 2010 was higher than the other orders until September 2010 when the proportion of *Xanthomonadales* increased and *Enterobacteriales* spiked. The *Enterobacteriales* were predominately represented by *Serratia*, *Citrobacter*, *Klebsiella*, and *Raoultella*, all genera previously associated with hydrocarbon degradation (83, 87, 88). Other groups, however, decreased, such as the *Rhodobacterales* that are known to be oil- and PAH degraders during the DWH oil spill and elsewhere (89–93) and have been implicated in DWH oil

degradation in other coastal habitats (40, 94, 95). Our results differ from those of Mahmoudi et al. (40), who detected more *Rhodobacterales* in marshes 5 months after the spill from northern Barataria Bay. Other known hydrocarbon-degraders retrieved in high abundances during the spill in the open ocean (e.g., *Oceanospirillales* and *Alteromonadales*) (90, 92, 96, 97) were encountered in much lower abundances in the marsh sediments that we analyzed.

Through September 2011, the proportions of *Gammaproteobacteria* decreased as hydrocarbon concentrations increased (rho = −0.44, $P = 0.004$ for inland *n*-alkanes; rho = −0.49, $P = 0.001$ for inland PAHs), despite relative increases during this time for some orders of known oil degraders (i.e., *Chromatiales*, *Methylococcales*, etc.) (33, 93, 98). Moreover, the proportion of *Alphaproteobacteria* surpassed *Gammaproteobacteria*, as the deltaproteobacterial diversity increased to over 26% of the *Proteobacteria* (Table 2 and Fig. 5A). The proportion of *Alphaproteobacteria* positively correlated to *n*-alkane (rho = +0.3, $P = 0.05$) and PAH levels (rho = +0.51, $P = 0.0007$), and specifically to pyrogenically sourced PAHs and a return to plant-derived NOM for the marsh sediments. The alphaproteobacterial representation also shifted, with a decrease in Rhodobacterales (represented by *Oceanicola*, *Paracoccus*, *Roseicyclus*, and *Loktanella*) and an increase in *Rhizobiales* (represented by C_1 metabolizers *Methylosinus*, *Hyphomicrobium*, and *Methylovirgula* spp.) (Fig. 5D). The proportion of *Deltaproteobacteria*, dominated by *Desulfobacterales* and increasing to the highest relative abundances in September 2011 (Fig. 5E), also positively correlated to the concentrations of *n*-alkane (rho = +0.47, $P = 0.002$) and aromatics (rho = +0.47, $P = 0.001$). These prevalent groups of sulfate-reducers are considered major hydrocarbon degraders (32). Sulfate reduction, in general, has been linked to high rates of MC252 oil degradation in marsh sediments (34, 37, 69, 99).

By August 2013, the *Rhodobacterales* replaced *Rhizobiales*, and the *Gammaproteobacteria* were predominately associated with putative sulfur cycling, including the unclassified genera *Thiohalobacter*, *Thioprofundum*, and *Sedimenticola* and the *Chromatiales* genera *Ectothiorhodosinus* and *Thiohalospira*. The shift to more sulfur and sulfide oxidizers at shallow sediment depths through time likely reflects the increased abundances of putative sulfate reducers belonging to the *Deltaproteobacteria*, specifically associated with the timing of higher petroleum-derived hydrocarbon concentrations in the sediments but also to a potential shift in the redox state of the sediments because of greater water depths over the marshes.

Another notable change in phylum-level representation was between September 2011 and August 2013, when the *Chloroflexi* represented by the *Anaerolineae* and *Dehalococcoidia* classes became the second most abundant phylum after *Proteobacteria* (Table 2 and Fig. 5F). The *Chloroflexi* are associated with natural hydrocarbon seeps (100), anaerobic pipelines (101), and oil contaminated experiments (81, 102, 103). The relative abundances of *Chloroflexi* positively correlated to the concentration of total inland *n*-alkanes (rho + 0.46, $P = 0.002$), to total aromatics (rho = +0.66, $P < 0.0001$) and, interestingly, to the presence of *J. roemerianus* (rho = +0.31, $P = 0.04$). However, the *Chloroflexi* were also negatively correlated to the Phe0/An values, which are indicative of petrogenic hydrocarbon sources (rho −0.63, $P < 0.0001$), suggesting an affinity to pyrogenic PAH compounds. The relative abundances of *Dehalococcoidia* peaked in September 2011 and surpassed the abundance of *Anaerolineae* (Fig. 5F). Most *Dehalococcoidia* are associated with dehalogenation (104, 105), but uncultured groups have been retrieved from, if not considered dominant in, shallow marine sediments associated with sulfur cycling and organic C oxidation, specifically of aromatic compounds (106), which corroborates the noted shift among the major gammaproteobacterial groups associated with sulfur metabolism.

Salt marshes are dynamic, heterogeneous systems that are affected by daily, seasonal, and annual processes that affect microbial community structure and composition (24, 29, 51, 107). Therefore, we used nonmetric multidimensional scaling (NMDS) analysis to explain community compositional changes through time as a function of the different environmental parameters that were statistically different or that correlated to

bacterial community membership, including salinity, temperature (as a proxy for season), inland water depth, inland canopy height, the presence of *J. roemerianus* and *D. spicata* plants, inland concentrations of *n*-alkanes and aromatics, and CPI and Phe0/An values indicative of petrogenic oil sources. Marsh communities clustered more closely in NMDS space by time and not region (Fig. 6A). Changes in the ordination of each marsh community through time, based on the prevalent changes in the bacterial community composition (Fig. 6A), also followed similar trajectories through time (Fig. 6B to G). Marshes with the highest measured hydrocarbon concentrations (e.g., Bay Batiste, Terrebonne Bay; Fig. 6B to F) had bacterial communities separated by the most distance across NMDS space (e.g., from the far left side to the far upper-right corner), particularly between September 2010 (Fig. 6A, triangles) and September 2011 (Fig. 6A, stars). These distances represented the greatest changes in organic C concentrations at those marshes and shifts from oil- to plant-derived NOM. Those marsh communities also had higher relative abundances of *Chloroflexi* and *Deltaproteobacteria* in September 2011 compared to the other marshes (Fig. 6A). Moreover, the NMDS trajectory for salinity was similar to that of inland concentrations of *n*-alkanes and aromatics (Fig. 6A), thereby potentially confounding interpretations of whether salinity or oiling could be used to explain community shifts through time. However, marshes with the greatest salinity excursions (American Bay, Fig. 6G) also had lower hydrocarbon concentrations (Table 1) and experienced an increase in *J. roemerianus* vegetation; the ordination shifts across NMDS space from June to September 2011 were accounted for by these dimensions. By August 2013 (Fig. 6A, diamonds), all marsh communities clustered together in NMDS space, shifting from the September 2011 positions along dimensions of vegetation, CPI and Phe0/An values, and *n*-alkane and PAH concentrations. The 2013 community ordinations were proximal to the May 2010 communities, despite the compositions being significantly dissimilar to each other. The ordination of 2010 and 2013 marsh communities in NMDS space likely reflected the influence by organic C sourced from vegetation and not oil residues.

Concluding perspectives. Unraveling the long-term consequences of the DWH oil spill on marsh ecosystems is difficult because of inadequate baseline information on community composition and dynamics prior to the disturbance, particularly at the microbial level (47, 48). Our research focused on understanding the long-term effects of oiling on marsh bacterial communities by simultaneously assessing how natural environmental gradients that could be considered natural stressors influenced community compositions. The prespill samples, collected before oiling in May 2010 from across a broad geographic area throughout southern Louisiana, provided unprecedented insight into the baseline composition of sediment bacterial communities that were influenced by plant-sourced NOM, salinity, and inundation history without any confounding influences of oil.

In contrast, other studies of the DWH oil spill started within months of the spill, or after peak oiling by as much as one to 2 years (37, 42, 69, 99). Comparing our findings with results from those earlier studies indicates that the DWH oil spill appeared to cause a rapid response among bacterial communities from salt marshes (37, 40, 42), the open ocean (90, 96, 97, 108–110), and sandy beaches (94, 95, 111–114). Initially, we observed that marsh bacterial communities were dominated by *Proteobacteria*, which were replaced by *Firmicutes* only 4 months later. Within the proteobacterial classes, such as the *Gammaproteobacteria* and *Deltaproteobacteria*, order-level shifts, particularly among putative hydrocarbon-degrading taxa, correlated with changes in the concentrations of petroleum hydrocarbons and salinity reductions due to freshwater diversions affecting each marsh location. By 1 year after the spill, none of the sampled marshes were free of oil and overall bacterial diversity was higher. Several distinct taxonomic shifts corresponded to changes in plant height and type, salinity, and periods of inundation, as well as the relative contributions of petrogenic versus pyrogenic hydrocarbons introduced into the marsh sediments. Despite the specific environmental conditions at each of the marshes sampled, bacterial communities in August 2013 were more compositionally similar to

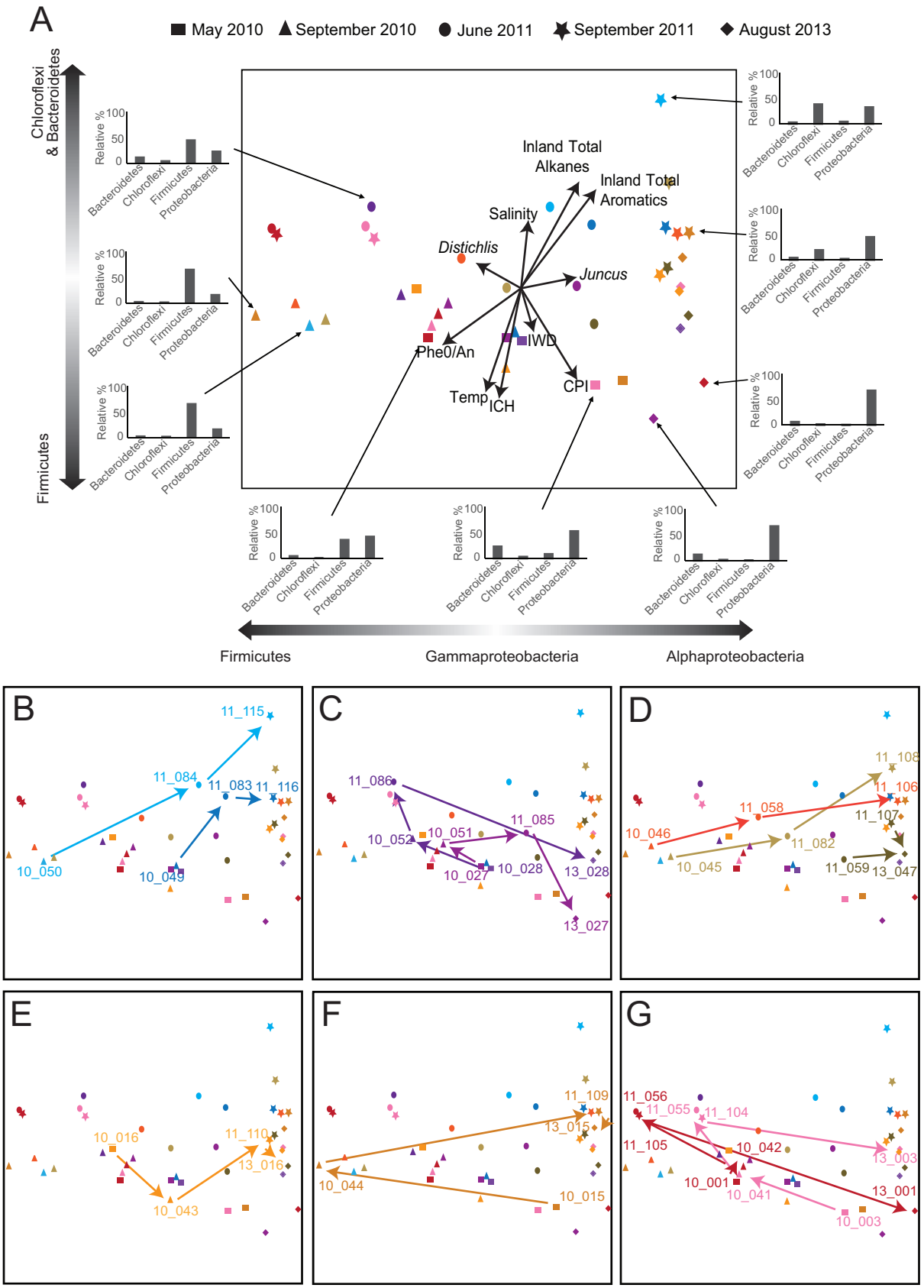

FIG 6 (A) NMDS plot of bacterial diversity (color-coded by site number from Fig. 1A) against 10 environmental dimensions (vectors) having statistical significance based on NPMANOVA tests ($P < 0.05$). Sampling times are plotted with distinct shapes for each of the sampling times.

(Continued on next page)

each other than to any other time period sampled. Furthermore, these communities were significantly different than the prespill, baseline communities.

Unlike earlier studies of salt marshes (40, 69), the open ocean (97, 109, 110), and beaches (114), we did not find evidence of bacterial communities returning, or recovering, to a prespill community of non-oil degraders within months to a year after the spill. The difference is most likely caused by the brief sample collection intervals used in those earlier studies, which could have inaccurately addressed the potential effects of persistent oil residues in the anoxic sediments and soils on the bacterial communities (44, 45), as the degradation of *n*-alkanes and PAHs would take years to decades, respectively, to return to pre-DWH conditions (46). The 2013 results of our study also indicate that recovery had not occurred because the postspill community compositions were significantly different from prespill compositions. Without a clear indication that recovery is possible or will happen, more research is needed. In particular, as our results have shown, future evaluations will need to control for environmental changes that likely also induce bacterial community compositional shifts. In addition, continued monitoring and future (meta)'omics approaches should uncover whether the expected functional diversity changes at the base of the marsh ecosystem affected the overall, long-term biogeochemical cycling and food web dynamics.

MATERIALS AND METHODS

Selection of marshes for sampling and sampling timeline. Because the oil spill was an uncontrolled event, with the timing and location of oiling uncertain, we originally sampled over a broad area of southern Louisiana for a descriptive survey to detect patterns of change in the marshes (115) as a comparative mensurative experiment (*sensu*, 116). Site selection and distribution are described by Turner et al. (46). Briefly, sites were originally chosen based on SCAT mapping of detected weathered oil residues. These maps were available through the Environmental Response Management Application (ERMA) online mapping tool (https://erma.noaa.gov/gulfofmexico/erma.html#) (117) from the Office of Response and Restoration (OR&R) at National Oceanic Atmospheric Administration (NOAA) (Fig. 1A). We also based sampling on the OR&R trajectory maps for surface oil plume migration. Our first sampling of *Spartina alterniflora*-dominated marshes from Breton Sound (American Bay), northern Barataria Bay (including Bay Sansbois), and Terrebonne Bay occurred before visual oiling was recorded at those sites (Fig. 1A; see also Table S1 in the supplemental material) (45, 46). Soils and sediments were sampled for hydrocarbon concentrations, vegetation type and coverage, and faunal diversity at 10 m inland of the seaward edge of each marsh, and a subset of the marshes were chosen to examine sediment bacterial diversity from 1 m seaward of the marsh edge, including sites (at the time) that appeared to have limited oiling and could potentially be considered reference sites (*sensu lato* 118). SCAT data indicated more widespread contamination in northern Barataria Bay compared to other bays by September 2010 (Fig. 1B), and additional microbiology sampling sites were added in this region (see Table S1 in the supplemental material). As the spill continued, highly weathered oil, mousse, and tarballs visibly impacted shorelines throughout the Gulf in 2011 (44). As such, not all original sites could be resampled, primarily due to potential exposure of the team and boats to toxic concentrations of oil on the marshes, but also because of weather conditions (e.g., thunderstorms and high waves) or due to parish, state, or federal response, cleanup, remediation, and restoration efforts. A few sites had to be moved (less than 100 m away) because of landowner permission or problems with site accessibility during low tides.

Over the 38-month period of study, 11 marsh locations were sampled at least three or four times each for hydrocarbon concentrations, vegetation type and coverage, and microbiology (Fig. 2A; see also Table S1 in the supplemental material). The sampling frequency deliberately bracketed *S. alterniflora* seasonal growth, with highest biomass generally in September before inflorescence (119), and the periodic sampling also minimized impact to marsh ecosystems under stress from oiling. Moreover, the tiered sampling approach, with a subset of locations used for microbial characterization but a larger number of locations used for hydrocarbon assessment, was similar to the scheme used by Atlas et al. (69), although our study began prior to oiling and included more sampling events. Specifically, in the Atlas et al. study (69), which also did not sample during 2012, more than half of the marshes were only sampled twice, once in 2011 and once in 2013. The broader geographic extent of the sampled marshes in our

FIG 6 Legend (Continued)
Bar graphs around NMDS plot are for representative communities and are used to show compositional changes for dominant phyla and classes from the sediment samples over time. Changes in diversity according to a Bray-Curtis distance matrix are represented on the two NMDS axes visualized in two dimensions. Differences in sample ordination correspond to the vector of influence for environmental parameters. The stress for the data set is 0.09, suggesting the NMDS adequately represents true distances in multidimensional space. (B to G) Trajectories displaying changes in NMDS ordination of marsh sediment bacterial communities at each marsh, with arrows guiding the eye from sampling event to sampling event. Symbols correspond to sampling time and symbols are color-coded for sampling locations on Fig. 2A. All of the data from plot A are represented on these plots, but only specific changes across NMDS space are noted for marshes from similar geographic areas, such as plots B and C from Terrebonne Bay, plots D, E, and F from northern Barataria Bay, and plot G from American Bay.

study also captured a wider range of oiling than in the previous studies. Cumulative SCAT observations through the end of 2014 indicated that the marshes we sampled in northern Barataria Bay received heavy to moderate oiling, whereas the marshes at American Bay and in Terrebonne Bay received no to moderate oiling (Fig. 1C) (120).

Sediment characterization, hydrocarbon analyses, and refractory biomarker indices. Subtidal sediment samples for geochemical characterization and microbiology were collected using a WaterMark push core sediment sampler with 20-cm long polycarbonate barrels. Cores were taken from 1 to 2 m seaward of the marsh edge at the sediment-water interface. Variation in sampling distance from the vegetated edge was caused by irregularities due to terracing or marsh undercutting. Sediment from 0 to 1 cm (labeled "A" depth) and 1 to 2 cm (labeled "B" depth) depths were sectioned aseptically in the boat into separate sterile Whirl-Pak bags. The samples were kept on ice in the field, frozen within 12 to 24 h of collection, and kept frozen until analysis. The thawed homogenized aliquots for each depth were separated in the laboratory to measure total organic water and C content using the loss-on-ignition method (94). The marsh soil samples for petroleum hydrocarbon analyses used for comparison came from the top 5 cm of marsh soil at 10 m inland from the marsh edge, as well as from 1 m inland from the edge (45, 46). Table 1 summarizes the sampling times and basic marsh field data and sediment analyses. All sediment data for each marsh sample collected are archived in the Gulf of Mexico Research Initiative Information and Data Cooperative (GRIIDC) database (https://doi.org/10.7266/N7Q23X55).

The target *n*-alkanes and eight parent PAH compounds and their associated alkyl homologs were quantified using gas chromatography-mass spectroscopy operated in the selective ion mode, and compositional matches to MC252 oil were done from qualitative comparisons of specific compound ratios (45, 46, 60). All petroleum hydrocarbon data collected for previously published studies are archived in the GRIIDC database (https://doi.org/10.7266/N7Z60KZR), with only those data used in this study provided as Table S5 in the supplemental material.

Earlier studies following the DWH event report total petroleum hydrocarbon (TPH) concentrations or bulk concentrations of *n*-alkanes or PAH compounds (see, for example, 37, 40, and 69). However, TPH measurements, bulk PAHs, and especially bulk *n*-alkane concentrations, capture non-petroleum hydrocarbon source contributions, such as from terrestrial plant leaf epicuticular waxes (62, 67, 121). Therefore, although total *n*-alkane concentrations were determined by summing all measured compounds from the marsh sediment samples (nC_{10} to nC_{35}), distinction among the different size and size ranges of *n*-alkanes was also done because specific NOM sources are linked to these sizes and size ranges. For example, nC_{14} to nC_{20} can be attributed to microbial biosynthesis (122, 123). For marine phytoplankton (including phototrophic and nonphototrophic bacteria), nC_{17} is the dominant homologue, whereas nC_{15} or nC_{17} are the dominant homologues for algae, and zooplankton have bimodal nC_{18} and nC_{24} peak dominance (122, 124, 125). nC_{29} is the dominant homologue of *S. alterniflora* epicuticular plant wax (62). Moreover, because *n*-alkanes are stable and can persist for decades or longer under anoxic conditions, various refractory biomarker indices calculated from the concentrations of specific *n*-alkanes provide information about the nature of changes that may have affected *n*-alkane source distribution through time (see the supplemental material). The carbon preference index (CPI), average chain length, natural *n*-alkanes ratio, percentage of plant wax, and terrestrial-aquatic ratio calculations (see also the supplemental material) provided more accurate assessments of *n*-alkane signatures than if considering only TPH and bulk concentrations alone (63, 126).

The concentrations and molecular ratios of PAH compounds were also compared using previously described approaches (62, 63, 127). Crude oil has, in general, higher concentrations of alkylated PAH homologues compared to parent compounds. Naphthalenes, fluorenes, phenanthrenes (Phe), anthracene (An), and alkalyated chrysenes are commonly sourced from petroleum, and HMW compounds such as the parent pyrene, fluoranthrene, chrysene (Chry), benzo(*b*)fluoranthene, benz(*a*)pyrene (BaP), dibenz(*a*,*h*)anthracene, and benzo(*ghi*)perylene originate primarily from pyrogenic sources or because of the enhanced weathering of LMW compounds in oils (32). The ratios for parent Phe0 to An and fluoranthene (Fl) to pyrene (Py) were calculated, and the [Fl/(Fl + Py)] values were compared to the pyrogenic index (PI) (73) (see also the supplemental material).

Microbiological sample collection. Bacterial diversity was determined from 16S rRNA gene sequences that were retrieved from the top 0 to 1 cm and 1 to 2 cm of subtidal marsh edge sediments. The nucleic acids were extracted using the modified approaches of Somerville et al. (128), Zhou et al. (129), Mitchell and Takacs-Vesbach (130), and Engel and Gupta (94). Triplicate analyses of up to 2 g of thawed sediment were aseptically mixed with fresh sucrose lysis buffer (SLB [pH 8.0]; 50 mM Tris-HCl [pH 8.0], 40 mM Na-EDTA, 0.5 M sucrose) and 1 mg ml^{-1} lysozyme. The lysates were vortexed harshly for 5 min and then placed in a 37°C water bath for 1 h. Each of the cooled sediment lysates were mixed in triplicate with a solution of (final concentration) 5% sodium dodecyl sulfate, 2.5 mg/ml proteinase K, and 0.1% cetyltrimethylammonium bromide (CTAB) made with SLB. This procedure yielded nine extractions per sample. The lysates were incubated at 55°C for up to 5 h at 100 rpm and then cooled, and the supernatant was transferred into 1.5 M ammonium acetate (final concentration) before mixing and centrifugation. The supernatant was transferred to a 100% cold isopropanol solution before incubation on ice for up to 4 h. The nucleic acids were pelleted by centrifugation and washed twice with 70% molecular grade ethanol before eluting in Tris-EDTA (pH 8.0) buffer and storage at −20°C.

After nucleic acid extraction, aliquots were homogenized per sample for tag-encoded GS FLX+ amplicon pyrosequencing using Titanium technologies (Roche 454 Life Sciences, Branford, CT). Sequencing was done by the Research and Testing Laboratories in Lubbock, Texas, for the May 2010 to May 2011 samples, and by the Molecular Research LP in Shallowater, Texas, for the September 2011 and August 2013 samples. Negative and positive controls were used throughout these procedures, from the initial

DNA extractions to sequencing. The samples were purified to remove PCR inhibitors and trace humic substances, and the V1-V3 region of bacterial 16S rRNA genes was amplified using the primers 28F (5′-GAGTTTGATCNTGGCTCAG-3′) and 519R (5′-GTNTTACNGCGGCKGCTG-3′) (131, 132).

The raw amplicons generated for this study were submitted to the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/) under BioProject PRJNA232457 and are also archived at GRIIDC (https://doi.org/10.7266/N7D21VH8). Summaries of the raw data are provided as Table S6 in the supplemental material. The taxonomic classifications are included at the phylum level (see Table S9A in the supplemental material) and at the class and order levels (see Table S9B in the supplemental material) and are archived at GRIIDC (https://doi.org/10.7266/N7C8278T).

Amplicon processing, clustering, and taxonomic assignments. The amplicons were sorted according to barcodes, and the raw sequence reads were checked for quality, which included eliminating poor quality reads (<20) or those that were <200 bp long before alignment in the Ribosomal Database Project (RDP) Pipeline (133) using the RDP Infernal Aligner. Chimera were removed using UCHIME with USEARCH (134). Nonbacterial rRNA sequences were also removed. A distance matrix was created using the mcClust complete-linkage clustering algorithm in the Functional Gene pipeline (135) at 0 to 4% distances, and estimators of operational-taxonomic-unit richness were calculated using the RDP pipeline, including Chao1 and Shannon diversity (H′) indices (see, for example, reference 136). The taxonomic assignments from phylum to genus levels were made for all curated amplicons per sample at an 80% confidence level directly by the RDP Classifier. Values with <80% confidence assignments were considered unclassified reads.

Comparative statistical analyses. The abundance counts for each of the binned taxonomic units were adjusted for amplicon library size, and the presence/absence data were used to construct a Bray-Curtis similarity matrix among sampling times for each of the marshes using the program PAST (v2.14) (137). The composite of the May 2010 bacterial communities and environmental conditions were considered the baseline conditions from which all other data were compared. We used nonparametric permutational multivariate analysis of variation (NPMANOVA) calculations (99,999 permutations of group membership) to test for the significance of relative taxonomic abundances among samples through time (138, 139). The F statistics and Bonferroni-corrected *P* values tested for significant differences in the means of environmental variables over time. A Spearman's rank correlation test, reported as rho coefficients, evaluated environmental variables and abundances of specific taxonomic groups. The statistical significance for all tests was defined by *P* values set at alpha = 0.05, but values from 0.05 to 0.1 were recorded to identify potentially weaker relationships. Nonmetric multidimensional scaling (NMDS) analyses, based on the approach by Taquchi and Oono (140), were computed using a Bray-Curtis distance matrix of abundance data, and marsh environmental variables, including hydrocarbon data, were $\log_{10}(x + 1)$ transformed (except pH) (see, for example, reference 139). The ordination stress indicator was set at <0.2.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00784-17.

SUPPLEMENTAL FILE 1, PDF file, 2.0 MB.

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A.S.E. performed field work and sample processing, data analysis, archival, and interpretation, complied figures and tables, wrote the paper, and obtained funding for the project (2011 to the present). C.L. performed sample processing and data analysis and interpretation as part of his M.S. thesis at Louisiana State University. A.T.P. contributed to sample processing, data analysis and archival, and writing the paper. L.C.A. obtained original funding (2010-2011), performed field work and data interpretation, and edited the paper. R.E.T. obtained funding for the original project (2010 to 2011),

performed field work, data analysis and interpretation, and edited the paper. E.B.O. performed hydrocarbon analysis and data interpretation. All authors read and approved the manuscript.

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