

Oil Biodegradation and Oil-Degrading Microbial Populations in Marsh Sediments Impacted by Oil from the Deepwater Horizon Well Blowout

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Supporting Information

ABSTRACT: To study hydrocarbon biodegradation in marsh sediments impacted by Macondo oil from the *Deepwater Horizon* well blowout, we collected sediment cores 18–36 months after the accident at the marshes in Bay Jimmy (Upper Barataria Bay), Louisiana, United States. The highest concentrations of oil were found in the top 2 cm of sediment nearest the waterline at the shorelines known to have been heavily oiled. Although petroleum hydrocarbons were detectable, Macondo oil could not be identified below 8 cm in 19 of the 20 surveyed sites. At the one site where oil was detected below 8 cm, concentrations were low. Residual Macondo oil was already highly weathered at the start of the study, and the concentrations of individual saturated hydrocarbons and polycyclic aromatic hydrocarbons continued to decrease over the course of the study due to biodegradation. *Desulfococcus oleivorans*, *Marinobacter hydrocarbonoclasticus*, *Mycobacterium vanbaalenii*, and related mycobacteria were the most abundant oil-degrading microorganisms detected in the top 2 cm at the oiled sites. Relative populations of these taxa declined as oil concentrations declined. The diversity of the microbial community was low at heavily oiled sites compared to that of the unoiled reference sites. As oil concentrations decreased over time, microbial diversity increased and approached the diversity levels of the reference sites. These trends show that the oil continues to be biodegraded, and microbial diversity continues to increase, indicating ongoing overall ecological recovery.



INTRODUCTION

The *Deepwater Horizon* (DWH) exploratory well blowout on April 10, 2010 resulted in a tragic loss of life and the release of Macondo oil and gas into the Gulf of Mexico until July 15, 2010, when the well was capped. Some of the oil components dissolved; some of the oil was chemically and physically dispersed and moved away from shore as fine droplets in the deep water (1000–1500 m¹); and some of the oil formed larger, more buoyant droplets that surfaced and moved shoreward, in some cases contaminating coastal marshes and sand beaches.^{1–3} Although initial studies focused on the oil and gas entrained in the water column rather than on impacted shorelines^{1,4–5} the longer-term consequences concern the fate of the oil that contaminated sediments, including oil-contaminated marshes.

Immediately after the oil reached beaches and marshes, shoreline cleanup and assessment techniques (SCAT) team observations were used to estimate the extent of shoreline oiling.

Initially 1773 km of shoreline were reported to be oiled (44.9% in marshes); one year later, oil remained on 847 km, and two years later it remained on 687 km, although at much lesser degrees of oiling.³ Greater oiling of the shorelines would have occurred had the oil not been dispersed.

Within three years, the extent of oiled shoreline had been reduced from an initial 1773 km (44.9% of which were oiled marshes) to 632 km (74% of which were classified as trace, e.g., less than 1% oiled).⁶ SCAT surveys conducted in March 2014 showed that the extent of the remaining oil contaminations had been greatly reduced; very few marsh segments were still considered to be heavily oiled, and almost all of the previously

Received: February 2, 2015

Revised: May 29, 2015

Accepted: June 19, 2015

Published: June 19, 2015



oil-impacted sites showed no observable oil.⁷ The extent of heavily oiled shorelines had declined by 87.8% in year 1, 95.9% by year 2, and 96.2% by year 3.⁶ The decline of heavily oiled marsh shorelines was presumably due to natural attenuation processes (biodegradation and other weathering processes) because marsh cleanup was limited to 1–2% of the heavily oiled marshes.⁶

Even before impacting the shoreline, Macondo oil had already undergone weathering, having lost the lower-molecular-weight components.^{8–12} Silliman et al.¹³ found that oil coverage declined rapidly with distance from the shoreline in coastal Louisiana marshes, reaching less than 50% coverage by 8.2 m from the marsh edge. They reported that after 18 months, oil-impacted marsh sediments had hydrocarbon concentrations comparable to those of nonimpacted reference sites.

Several studies examined the microbial communities in oil-impacted shorelines with respect to the hydrocarbon biodegradation potential. Kostka et al.¹⁴ identified 14 genera of hydrocarbon utilizing bacteria from oiled beach sands in Florida. These were primarily Gammaproteobacteria, including known oil degraders (*Alcanivorax*, *Marinobacter*, *Pseudomonas*, and *Acinetobacter*). Lamendella et al.¹⁵ reported that highly contaminated sand beaches at Grand Isle, Louisiana, United States, had higher abundances of Alphaproteobacteria and Gammaproteobacteria than unoiled beaches, and a *Marinobacter* species isolated from this beach was capable of degrading hydrocarbons.

Kappell¹⁶ found that the relative abundance of functional genes involved in oil degradation pathways, including the pathways for the degradation of polycyclic aromatic hydrocarbons (PAHs), were greater in an oiled Alabama sand beach than in a comparable unoiled Florida sand beach, suggesting that biodegradation was ongoing. Using mesocosms, they showed that the microbial community of the oiled sand beach had a high capacity for biodegradation of low-molecular-weight PAHs, fluorene, and naphthalene, but not for the high-molecular-weight nonpetroleum hydrocarbon benzo[a]pyrene. Known PAH degraders and genera frequently associated with hydrocarbon degradation (including representatives of Flavobacteria (*Flavobacterium* and *Sediminicola*), Alphaproteobacteria (*Thalassospira*), and Gammaproteobacteria (*Cycloclasticus*, *Marinobacter*, *Halomonas*, and *Pseudomonas*)) represented a major portion of the microbial community.

Liu and Liu¹⁷ examined the microbial populations in oil mousse collected from the leaves of *Spartina alterniflora*. They found that *Vibrio* (Gammaproteobacteria) represented 57% of the microbial community, suggesting that this indigenous genus is particularly responsive to the oil contamination in salt marshes.

Mahmoudi et al.¹⁸ observed a higher relative abundance of Alphaproteobacteria, and in particular enrichment of the bacterial groups Rhodobacterales and Sphingomonadales, in impacted salt marsh sediments 5 months after oil intrusion. After 13 months, hydrocarbon concentrations had decreased, and the taxonomic composition of the impacted and the reference sites was similar. The implication of this finding was that as oil hydrocarbons were biodegraded, the microbial population indicated ecological recovery as populations shifted from oil-degrading bacteria such as Sphingomonadales¹⁹ and Rhodobacterales¹⁴ back to those that were not oil degraders.

Similarly, Looper et al.²⁰ identified 17 bacterial genera known for their capacity to degrade hydrocarbons, including *Mycobacterium*, *Novosphingobium*, *Parvibaculum*, *Pseudomonas*, and *Sphingomonas*, in contaminated shoreline sediments. They concluded that the distinct wetland microbial communities responded to

the influx of Macondo oil with decreased diversity and the concurrent enrichment of oil-degrading species.

Beazley et al.⁸ reported that the relative abundance of phyla containing previously described hydrocarbon-degrading bacteria (Proteobacteria, Bacteroidetes, and Actinobacteria) was high in June 2010 in hydrocarbon-contaminated sediments of an Alabama marsh but decreased to background levels by September 2010 once hydrocarbons were below detection limits. They also observed that the functional genes involved in hydrocarbon degradation were enriched in hydrocarbon-contaminated sediments and then declined significantly once hydrocarbon concentrations decreased.

Koo et al.^{21,22} found that in some cases, microbial diversity actually increased when fresh Macondo oil was added to mesocosms prepared from disturbed sediments collected from restored Gulf of Mexico coastal areas. Bacterial populations shifted rapidly, over 1 to 4 weeks, towards members of the taxonomic groups that are capable of surviving in a Macondo-oil-contaminated environment. Tenericutes were detected shortly after treatment; members of this phylum have previously been shown to be stimulated in oil-impacted salt marshes.⁸ Proteobacteria, e.g. *Marinobacter* spp., became the dominant population; Firmicutes and Bacteroidetes also had elevated abundances in oil-treated microcosms. The rapid proliferation of hydrocarbonoclastic bacteria under aerobic conditions in this experiment suggests their potential involvement in the degradation of spilled oil along the Gulf of Mexico shoreline, had the oil made landfall prior to weathering.

Boopathy et al.,²³ using sediment collected at a Louisiana marsh site containing Macondo oil, showed that hydrocarbons were degraded under sulfate-reducing anaerobic conditions. Similarly, Natter et al.²⁴ found that sulfate-reducing bacteria were enriched in sediments impacted by Macondo oil relative to nonoiled sediments.

In a study performed on salt marshes in Bay Jimmy, Louisiana, Perry²⁵ found evidence for anaerobic microbial activities in Deepwater Horizon oil-spill-impacted salt marsh sediments; specifically, the expression of the *bssA* gene, involved in anaerobic hydrocarbon degradation, peaked in winter and was highest at more highly oil-impacted sites. Measurements of H₂S concentrations in a moderately impacted marsh site in Mississippi found up to 231 mg/L, indicating that the microbial community was likely dominated by sulfate-reducing bacteria that might be involved in anaerobic hydrocarbon degradation.²⁶ These studies are especially relevant given that coastal Gulf of Mexico marshes are generally anaerobic.

Taken together, these reports indicate that most of the oil from the April 2010 DWH blowout that reached the shorelines did not move far inland, and that although the microbial population structure was affected, the concentrations of hydrocarbons decreased on impacted shorelines in the months following the oiling of marsh sediments, presumably due to microbial biodegradation.

The current study provides a long-term evaluation of the fate of the residual oil in coastal marshes, which King et al. indicated was much-needed.²⁷ The current study examined both the oil-degrading microbial populations and the weathering of the oil in the surface and subsurface sediments of heavily oiled marshes where oil persisted beyond 18 months following the well blowout. It is the only study that has looked at both the microbes, including anaerobic oil degraders, and the detailed oil chemical changes in heavily oiled marshes over an extended 3 year period following the Deepwater Horizon accident.

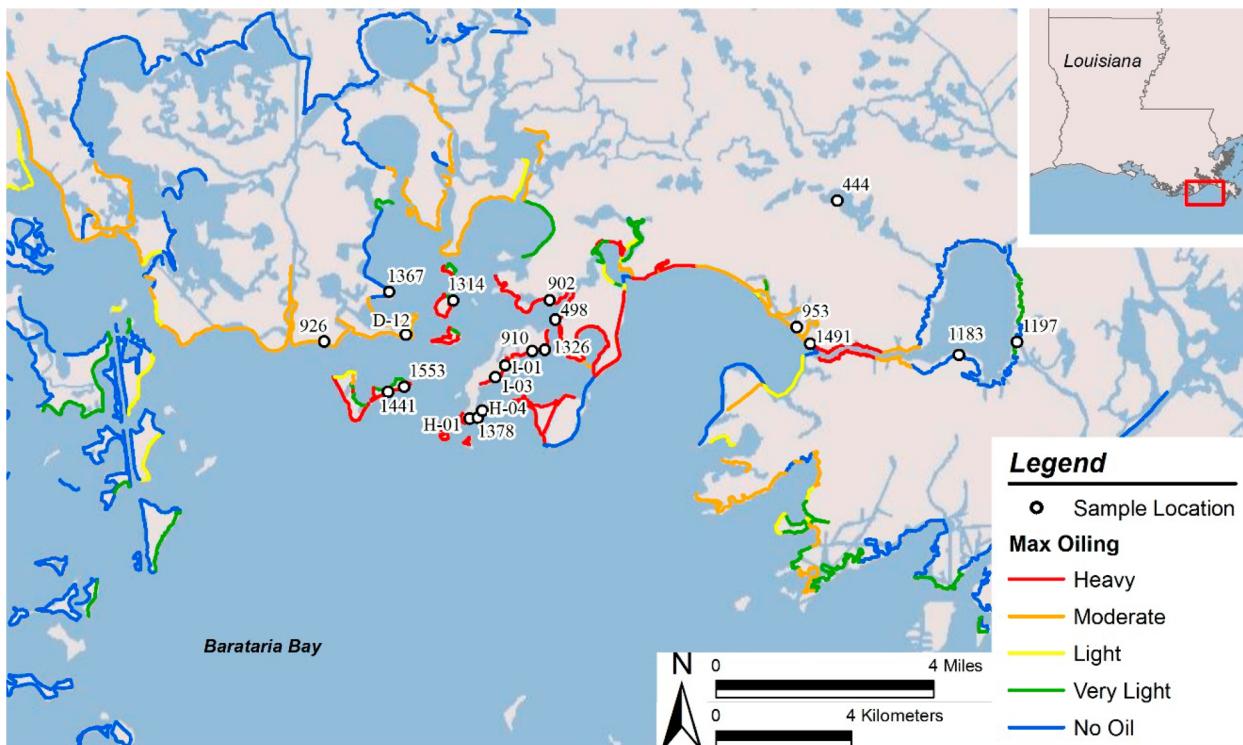


Figure 1. Locations of sampling sites in Bay Jimmy (Upper Barataria Bay, Louisiana) and SCAT oiling levels as determined in Spring 2010.

EXPERIMENTAL SECTION

Samples. Marsh sediment samples were collected at 20 site locations in Bay Jimmy within Barataria Bay in Louisiana (Figure 1) between 2011 and 2013. Sites included both mixed vegetation and predominantly *Spartina* marshes. Site selection was biased towards sites that SCAT teams had reported to be heavily oiled by the *DWH* oil release so as to be able to follow the biodegradation of the oil over an extended time period.⁷ Whenever possible, cores were taken to include visible surface oil. Some moderately oiled, lightly oiled, and unoiled (reference) sites were also included for comparison (Table S1 in the Supporting Information). Sampling occurred in the fall of 2011, the spring of 2012, and the fall of 2013, but not all sites were sampled on each occasion. Redox potentials were measured at 1 and 10 cm using an Acumet AP71 meter (Fisher Scientific). Sample cores were collected using Plexiglas corers (10 cm diameter × 30 cm length). The cores were frozen and subsequently extruded in the laboratory and then cut into 0–2, 2–4, 4–8, and 8–12 cm sections for hydrocarbon and microbial analyses. Sectioned samples were stored frozen at –20 °C prior to extraction. Once extracted, DNA for microbial analysis was stored at –80 °C.

Analytical Chemistry and Chemical Fingerprinting. Core samples were extracted and analyzed for total petroleum hydrocarbon (TPH), saturated hydrocarbons (SHCs), PAHs, and petroleum biomarkers. Samples from the 2013 campaign were also analyzed for dipropylene glycol *n*-butyl ether (DPnB), a marker for the oil dispersant used during the spill response. Details of the extraction and analytical methods (including descriptions of the quality control samples and the use of surrogate standards, internal standards, and other QC samples) are presented in the Supporting Information.

The GC–FID chromatograms, and in some cases the petroleum biomarker data, were used to identify the extent to

which hydrocarbon material measured in sediment cores could be attributed to Macondo oil. The GC–FID chromatograms provided a qualitative means for the source attribution of hydrocarbon material: chromatograms were dominated by a weathered oil signature, a background signature, or a combination of both (Figures S1A–C in the Supporting Information). GC–MS analyses were used for quantitative estimates of the total PAH (TPAH) as well as losses to specific PAH components. For samples that exhibited a weathered oil signature or a combination of weathered oil and background, a quantitative GC–MS-based investigation of the source of hydrocarbons was achieved by comparing selected diagnostic petroleum biomarker ratios in the analytical sample to those in the concomitantly analyzed Macondo control oil. Petroleum biomarker ratios to 17 α (H),21 β (H)-hopane (C30-hopane) were selected following Faskness et al.,²⁸ taking into account the consideration pointed out in Aepli et al.,²⁹ where the authors demonstrated that homohopanes and triaromatic steranes can be degraded in the years following an oil spill. If a linear fit of the relationship produced a slope approaching 1 and the coefficient of determination (r^2) of the regression was >0.9 , then the source of the hydrocarbons was determined to be Macondo oil. If a linear fit produced a slope approaching 1 but r^2 was 0.8–0.9, then the nature of the hydrocarbon materials was a mix of Macondo oil and background. Samples that exhibited primarily a background signature were considered background and were not subject to the petroleum biomarker analysis.

The percent loss of TPAH was determined for samples identified as containing Macondo oil on the basis of the mass balance of the concentration of TPAH normalized to the concentrations of the conserved biomarker C30 hopane:

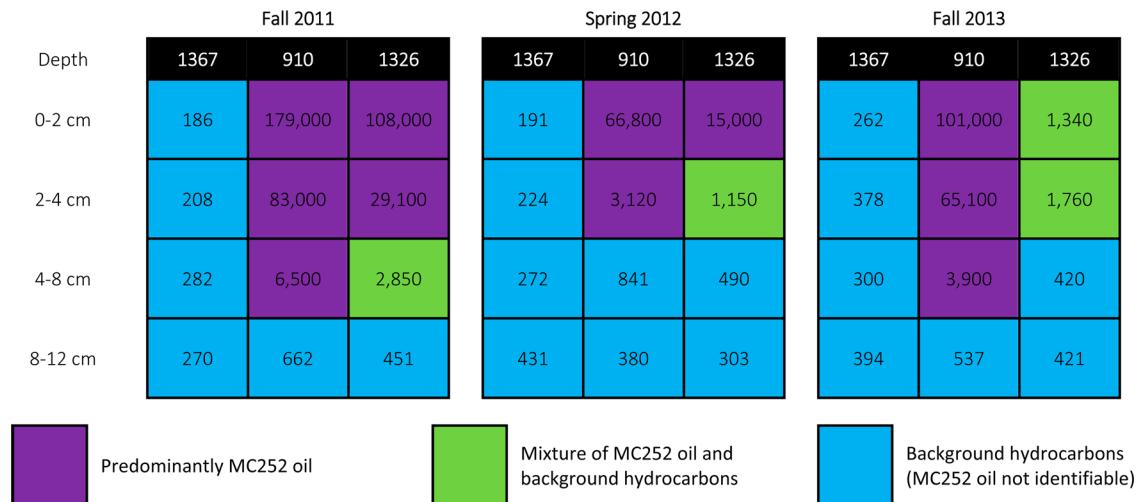


Figure 2. TPH concentrations (mg/kg) and the identification of source material corresponding to the specific cores used for microbial community analyses at sites 910, 1326, and 1367.

$$\text{TPAH depletion (\%)} = \left[1 - \left(\frac{[\text{TPAH}]_{\text{sample}}}{[\text{hopane}]_{\text{sample}}} \div \frac{[\text{TPAH}]_{\text{control oil}}}{[\text{hopane}]_{\text{control oil}}} \right) \right] \times 100 \quad (1)$$

Analogous analyses were performed to determine the losses of the three- and four-ring PAHs components of Macondo oil.

Metagenomic Analysis. The metagenomic analysis of DNA contained in sediment samples consists of four steps: (1) the extraction, concentration, and purification of DNA; (2) the shearing and preparation of DNA fragment libraries for sequencing; (3) the obtaining of the genetic sequences of shotgun library fragments; and (4) the comparison of product sequences against a database of whole genome sequences. These steps were accomplished through a combination of custom and off-the-shelf technologies as described in detail in the Supporting Information.

DNA was extracted from thawed homogenized cores, concentrated, and purified using off-the-shelf DNA extraction (MoBio, Carlsbad, CA) and inhibitor removal kits (Zymo Research, Irvine, CA); specific details are provided in the Supporting Information. Metagenome shotgun analysis was done on a total of 33 DNA extracts in support of this report; 8 of the 33 extracts were analyzed in duplicate to confirm the reproducibility of analysis. Extracts were sheared by sonication to 300 bp fragments and used to create a library for sequencing with the Illumina HiSeq 2000 (San Jose, CA), which was used to generate up to 1×10^{11} bases of genetic data as short-read fragments (1×10^8 to 1×10^9 reads of 100 bp length) per sample. Sequence data were screened against stringent quality metrics, and 250 million or 100 million (depending on the depth of coverage) high-quality sequences were compared against those in RefSeq, a curated comprehensive, integrated, nonredundant, well-annotated database of known-identity DNA sequences (NCBI, Bethesda, MD). RefSeq is accessible via BLAST, Entrez, and the NCBI FTP site. The matches of the test sequences to reference sequences with greater than 97% similarity were used to assign an organism name to test sequences, a process that has been used previously for the detection of metabolic genes³⁰ and has been named “fragment recruitment” in at least one summary of bioinformatics methods.³¹

Species identified in this way were confirmed by secondary analysis, in which the reads associated with the top taxa were aligned to the whole genome sequences in the reference database. These reads were evenly distributed across the genome, confirming the correct identification. Microbial diversity was assessed at the level of taxonomic ID by calculating the Shannon–Weaver diversity index (H), species richness, and equitability (E_H).^{32,33} Species richness was estimated by summarizing the number of unique named species represented by the list of taxonomic IDs identified for each sample. Summary statistics for the sequencing depth, sequences passing quality filters, sequences submitted for BLAST analysis, and number of sequences identified are reported in Table S2 in the Supporting Information.

RESULTS AND DISCUSSION

This study presents the results of both detailed chemical analyses on the weathering of oil in impacted salt marshes and genomic analyses of the microbial populations in these marshes, which are indicators of active hydrocarbon biodegradation and ecological recovery processes. Hydrocarbon concentrations measured at the 20 sites varied spatially and temporally (Table S1, Supporting Information). An extensive loss of hydrocarbons from the oil occurred during the rise to the surface in the water column. Approximately 60% of gasoline-range organics and 18% of TPH was lost from the Macondo oil from the wellhead to the surface. Once on the sea surface, rapid evaporative weathering and photodegradation occurred;³⁴ most of the oil reaching the shoreline was already more than 90% depleted in TPAH. DPnB was not detected above the sample-specific reporting limits in any of the samples, although the three highest concentrations were measured in the 0–2 cm horizon at the heavily oiled site 910.

For the purposes of simplifying and focusing the findings, a comprehensive discussion of results will be presented from three sites: two heavily oiled sites (910 and 1326, which were representative of the most heavily oiled sites) and one reference site (1367, which had not been impacted by entrained oil from the *Deepwater Horizon* accident). Data for other sites are presented in the Supporting Information in Tables S1 (chemical data) and S2 (microbiological data), and Figure S2 in the Supporting Information illustrates the attenuation of TPH



Figure 3. Time course of detected PAHs in the near-surface (0–2 cm) sediment layer from sites 910 (A) and 1326 (B).

concentrations in the surface sediment from all sites over the course of study.

Sites 1326 and 910 are on a shoreline segment that was still shown as heavily oiled in the March 2014 SCAT survey maps;⁷ however, the actual date for the physical survey is unknown and may have been earlier. Figure 2 illustrates the TPH concentrations over time at these three sites as well as the extent to which the hydrocarbon material measured in sediment cores was attributed to Macondo oil. The analyses demonstrate that Macondo oil penetrated below 8 cm at only one of the 20 sites (953) sampled in Barataria Bay, with a restriction to the upper few centimeters that is likely due to a heavy root mat that occurred at about 8 cm. The presence of Macondo oil in the 8–12 cm horizon at site 953 was anomalous because field notes and several shoreline surveys indicated there was no visible oil on the sediment surface.

The concentrations of TPH at site 1326 declined significantly with depth and over the time course of this study. Redox

potential measurements (Table S1 in the Supporting Information) indicated that the surface layer at site 1326 was aerobic during the fall of 2011 sampling event but anaerobic during the spring of 2012 and fall of 2013 sampling events. In contrast, the concentrations of petroleum hydrocarbons did not decline as drastically with depth or over time at site 910, which was anaerobic throughout the study. Site 910 was underwater due to tide and wind conditions on the morning of June 6, 2012, when the sample was collected. Due to the small scale spatial variability, which is reflected in the large standard deviations of the replicate analyses in Table S1 in the Supporting Information, this core was not likely collected from the precise location of maximum oiling. This circumstance may help explain why the lowest concentration of TPH and TPAH observed at this site was in the 2012 sample and not consistent with the decrease in concentrations between the 2012 and 2013 results at all other sites.

Detailed analysis of the residual oil components provided insights into the extent of biodegradation and the substrate

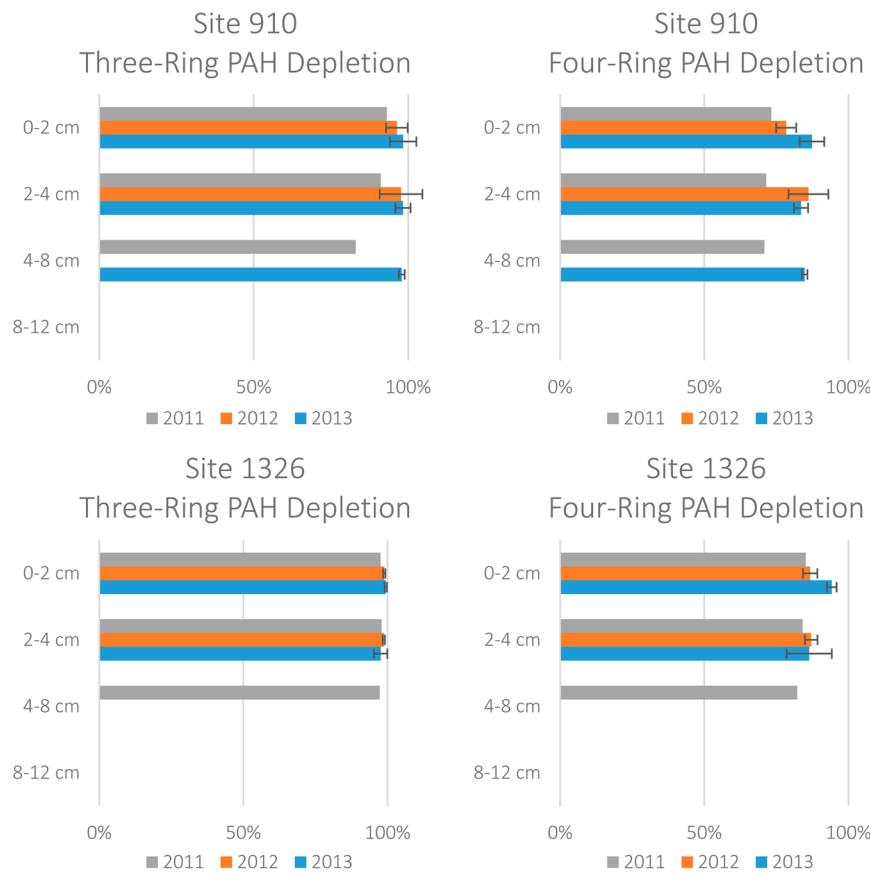


Figure 4. Data on three- and four-ring PAH depletion at sites 910 and 1326.

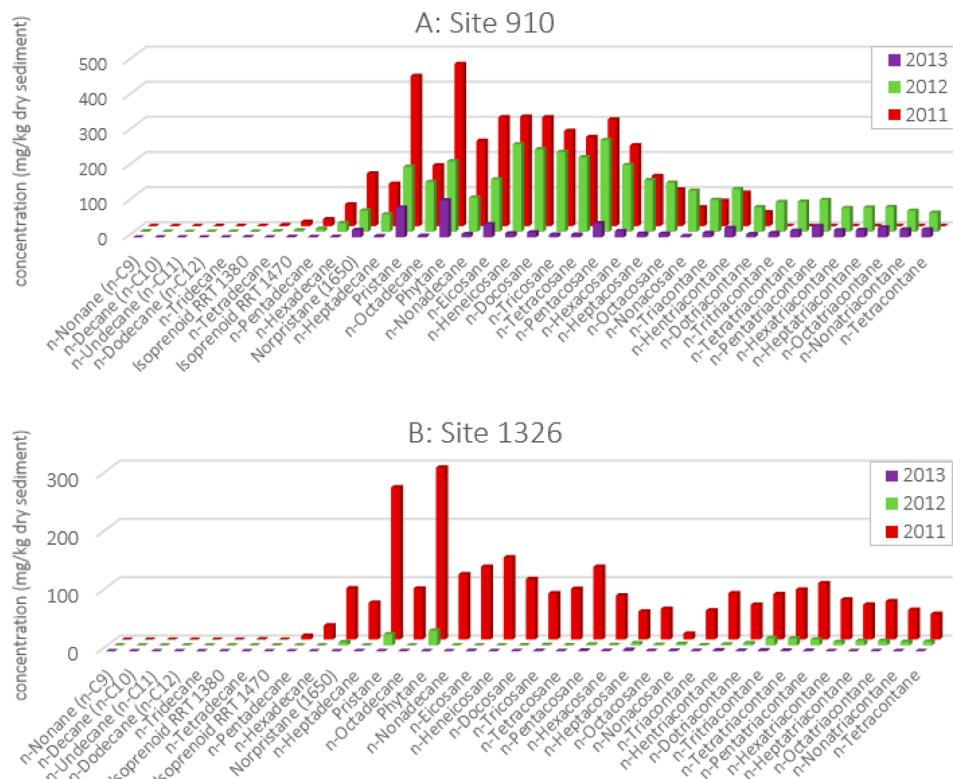


Figure 5. Time course of detected SHCs in the near-surface (0–2 cm) sediment layer from sites 910 (A) and 1326 (B).

preference exhibited by hydrocarbon-degrading microorganisms. In the fall of 2011, at the start of the study, 95.6% of the TPAH

fraction had already been lost to weathering; most of the naphthalenes and other low-molecular-weight PAH compounds

found in Macondo oil were not present at site 910 (Figure S3 in the Supporting Information and Figure 3a). The concentrations continued to decline for all categories of PAH, and by the spring of 2012, 97.0% of the TPAH components, 96.3% of the three-ring PAHs, and 78.4% of the four-ring PAHs had been lost.

The substantial loss of PAHs was especially apparent in the surface of the heavily oiled site 1326 (Figure 3b). The surface concentrations of TPAH at this site were 108 000, 29 300, and 2720 µg/kg in the fall of 2011, spring of 2012, and fall of 2013, respectively (Table S1 in the Supporting Information). Although these concentrations are higher than those reported by Turner et al.,^{35,36} our study was intentionally biased to examine the most heavily oiled sites so that biodegradation could be effectively measured. In the fall of 2011, at the start of our study we found that 98.1% of TPAH components, 97.6% of the three-ring PAHs, and 85.2% of the four-ring PAHs had already been lost relative to those in the Macondo source oil. By the spring of 2012, 98.5% of the TPAH fraction, 98.8% of the three-ring PAH fraction, and 86.7% of the four-ring PAH fraction had been lost. By the fall of 2013, almost all of the PAH components were below or near the detection limit. This site was initially aerobic, although later samples were collected during periods when even the surface layer was anaerobic. This indicates significant ongoing biodegradation of even the highly refractory four-ring PAHs under both aerobic and anaerobic conditions (Figure 4).

Evidence of naphthalene concentration increases between 2010 and 2012 as reported by Turner^{35,36} was not detected in this study (Figure 3), nor was it found in the Amoco Cadiz spill.^{37,38} An evaluation of the data set suggests that the high levels of naphthalene reported by Turner et al.^{35,36} are likely due to the inclusion of outlier samples that also have high levels of the byproducts of combustion. Whereas Turner et al. state that the concentrations of aromatic hydrocarbons from Macondo oil were not declining, our data clearly show ongoing biodegradative losses.

Saturated hydrocarbon data at site 910 were also consistent with extensive weathering over the time course of this study (Figure 5a). Low-molecular-weight alkanes were absent throughout the study. The highest concentrations measured for the mid- and high-molecular-weight alkanes were seen in the fall of 2011, and by the spring of 2012, the concentrations had declined dramatically under the anaerobic conditions found at this site. By the fall of 2013, most saturated alkane components had degraded to concentrations close to detection limits. The refractory branched alkanes norpristane, pristane, and phytane and some of the high-molecular-weight alkanes were the only resolved components remaining in the fall of 2013. Similar, but more extensive, degradation of saturated alkanes was observed at site 1326 (Figure 5b). The branched alkanes and the higher-molecular-weight alkanes were degraded to levels below the detection limit in this sample by the spring of 2012. The enhanced biodegradation of both alkanes and branched alkanes at site 1326 is consistent with the expectation of increased degradation rates with the occasional presence of aerobic conditions in the sediment. A large fraction of wetland sediment is anoxic; hydrocarbon degradation proceeds at a much faster rate under oxic conditions than it does under anoxic conditions.²⁷

The microbial populations identified in the sediments supported the conclusion that microbial degradation was ongoing at sites 1326 and 910 and that the intrusion of oxygen into the sediment at site 1326 and the consistent anaerobic conditions at site 910 influenced the prevalence of specific

hydrocarbon-degrading microbial populations. In contrast to the sites impacted by Macondo oil, the microbial population at site 1367 was highly diverse (Table 1) and lacking enriched populations of hydrocarbon degraders (Figure 6).

Table 1. Diversity and Evenness Measures for the Microbial Communities Detected in Three Sites

site ID	SCAT oiling level*	depth (cm)	date	Shannon diversity index	evenness (E_H)
910	heavy	0–2	fall 2011	3.06	0.51
			spring 2012	4.92	0.75
			fall 2013	2.60	0.44
		8–12	fall 2011	2.60	0.42
			spring 2012	5.27	0.80
			fall 2013	5.19	0.80
1326	heavy	0–2	fall 2011	4.29	0.69
			spring 2012	5.54	0.84
			fall 2013	5.34	0.81
		8–12	fall 2011	4.89	0.75
			spring 2012	5.62	0.85
			fall 2013	5.62	0.84
1367	reference (no oil)	0–2	fall 2011	5.55	0.84
			spring 2012	5.38	0.81
			fall 2013	5.85	0.87
		8–12	fall 2011	5.48	0.83
			spring 2012	5.54	0.84
			fall 2013	5.21	0.79

*As reported in the fall of 2011.⁷

The microbial community in the surface layer of site 910 was dominated by *Desulfococcus* throughout the study (Figure 6); for example, *Desulfococcus* made up 51% of the entire identified microbial community in the samples taken in the fall of 2011. Sulfate-reducing bacteria contribute largely to the organic carbon degradation in anoxic marine sediments, including coastal systems.^{39,40} Although the number of delta-proteobacteria species (which includes sulfate-reducing bacteria) in sediment samples from sites 1367, 1326, and 910 was very consistent, only varying between 46 and 59 across all three sites in two different seasons, the relative abundance of sulfate-reducing bacteria dominated in heavily oiled anaerobic surface sediments. In particular, *Desulfococcus oleovorans*, which is known to degrade alkanes and may also degrade aromatic hydrocarbons,^{41,42} constituted over half the bacterial community at sites 910 and H-01. This finding is consistent with previous studies of marsh sediments contaminated with Macondo oil, in which sulfate-reducing bacteria were indicated to be key to biodegradation activities^{23,24,26} although the prior studies did not identify *Desulfococcus* through molecular methods nor the dominance of oil-degrading sulfate-reducing bacteria at sites that were heavily impacted by Macondo oil.

Mycobacterial species, many of which degrade the higher-molecular-weight PAHs,⁴³ were also in relatively high relative abundance in the surface layer at site 910. The mycobacteria have not been previously identified as key members of the Macondo oil degrading consortium in marsh sediments^{18,23} or beach sands,^{8,14–16} possibly because of the extended time frame (36 months post-spill) of this study. The relative abundance of populations of *Desulfococcus* declined at site 910 in the spring of 2012 (along with the relative abundance of *Methanoplanus*) but with little change in the relative abundance of mycobacteria. The

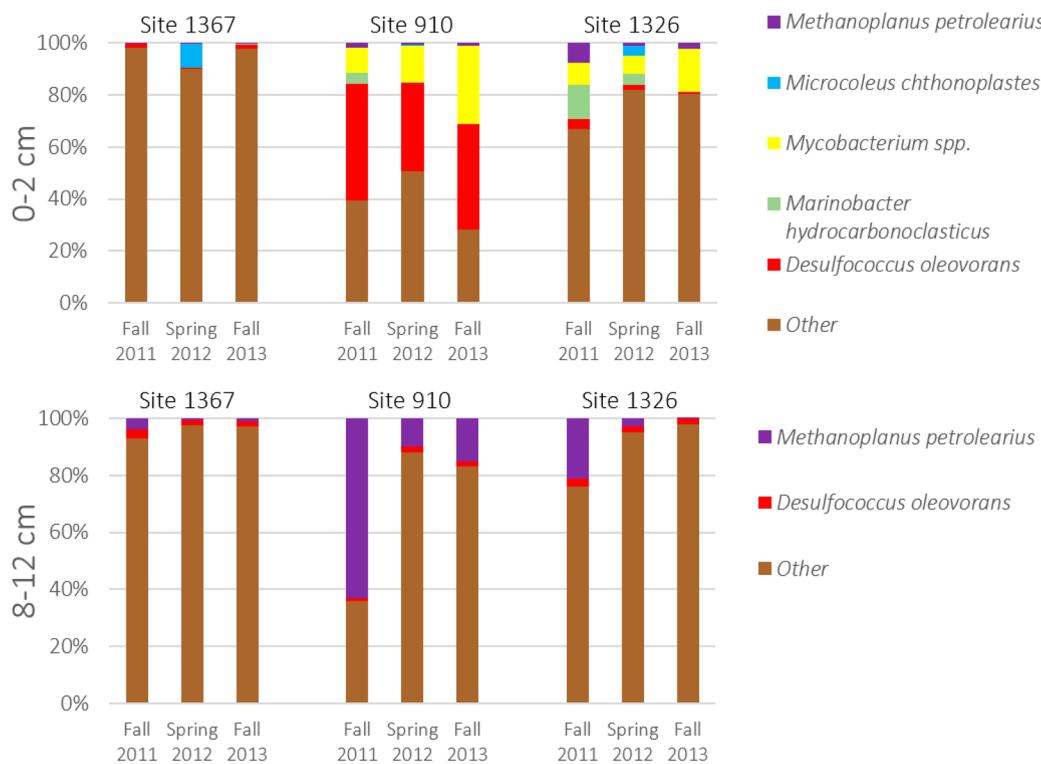


Figure 6. Relative populations of selected bacterial and archaeal species over time at selected sites during the time course of biodegradation.

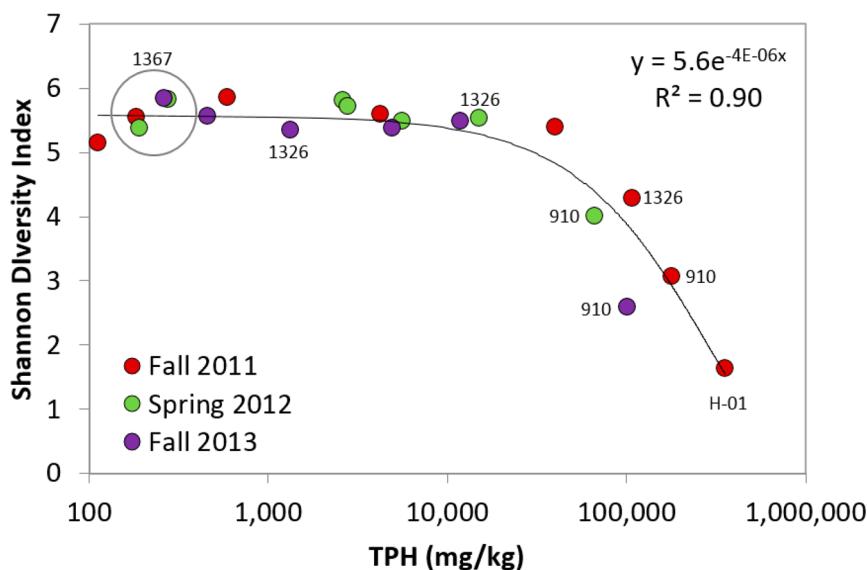


Figure 7. Relationship between the Shannon diversity index and the TPH concentration. Data from the sites discussed in the text are highlighted.

degradative metabolic capabilities of these microbial populations relate well with the observed chemical changes in the residual oil.

The surface layer at site 1326 indicated elevated proportions of several hydrocarbon-degrading bacteria but not the dominance of the anaerobic *Desulfococcus* that was evident at site 910 (Figure 6). Rather, *Mycobacterium* spp. and *Marinobacter* spp. appeared to play substantial roles in hydrocarbon degradation at site 1326. *Marinobacter* is a member of the Alteromonodales, which previously were shown to be enriched in Macondo-oil-contaminated beach sands.¹⁵ The redox condition appeared to be a driver for both the microbial population dynamics and the petroleum hydrocarbon degradation dynamics. As noted earlier, site 1326 was aerobic in the surface layer, unlike site 910, which

was anaerobic in the top 2 cm. As noted by Mills and McNeal,²⁶ the specific communities active in the degradation in this study appear to be site-specific despite the close proximity and comparable levels of impact.

At both heavily oiled sites, the 8–12 cm layer was dominated by archaea of the genus *Methanoplanus* during the fall of 2011; *Methanoplanus* persisted at lower levels at site 910 through the fall of 2013 (Table S2 in the Supporting Information). *Methanoplanus* species require acetate,⁴⁴ which is a biodegradation product of hydrocarbon metabolism. In this setting, we hypothesize that acetate is produced from hydrocarbon biodegradation in the surface layer and is introduced to deeper depths by tidal pumping to support the growth of *Methanoplanus*.

To our knowledge, this presumed specific linkage of surface biodegradation and community structure in underlying sediments has not previously been reported.

In contrast to the heavily oiled sites, the bacterial and archaeal species at reference site 1367 showed a diverse population that was not dominated by oil-degrading microorganisms (Figure 6). There was a bloom of the photosynthetic bacterium *Microcoleus* (shown in blue) in the spring of 2012 that did not appear to be related to oil contamination. The prevalence of *Desulfococcus* and any other hydrocarbon-degrading bacteria were quite low at site 1367 (Figure 6) and also at site 444 (another reference site; see data in Table S2 in the Supporting Information). The subsurface of the reference sites also shows a lack of dominance of any hydrocarbon-degrading bacterial group, although small numbers of *Desulfococcus* were detected, as was the archaean *Methanoplatus* that is sometimes associated with oil-contaminated sediments.

The diversity of the microbial communities at these sites varied with the concentration of TPH (Figure 7 and Table S2 in the Supporting Information). Diversity as measured by the Shannon index was above 5 when concentrations were less than approximately 50 000 mg/kg. Where the TPH concentrations were greater than approximately 50 000 mg/kg, the diversity was below 3. As the oil was biodegraded, the diversities of the microbial communities returned to levels that were observed at the reference sites.

Marsh sediments have not previously been characterized by deep sequence analysis of populations *in situ* over extended time periods.²⁷ Our findings were consistent with prior reports of the high spatial heterogeneity of the response to Macondo oil contamination²⁶ and the substantial impact on biodegradation by sulfate-reducing bacteria.^{23,24} We also hypothesize, for the first time, that *Mycobacterium* spp. were influential in the degradation of Macondo oil in the surface sediments of coastal marshes and that the products of microbial degradation in the surface sediment layers impacted the community composition in lower, uncontaminated layers.

A separate analysis of the metagenomic data indicated that hydrocarbon-degradation genes were substantially enriched at heavily oiled sites. As shown in Table S4 in the Supporting Information, there were dramatic increases in the relative abundance of the oil-degradation genes at oiled sites, in particular, in those associated with anaerobic pathways and even more specifically those associated with the novel anaerobic alkane degradation pathway⁴⁵ represented in *D. oleovorans*. These findings support our conclusion that the decline of hydrocarbons observed in the anaerobic marshes in this study was largely due to biodegradation.

In summary, both the microbial and chemical measurements indicate that Macondo oil has undergone significant biodegradation in the marshes impacted by the *Deepwater Horizon* accident. The persistence of Macondo oil in the top 2 cm at oiled sites varied. At site 910, which was anaerobic throughout the study, oil matching the Macondo oil profile was detected 36 months after the accident. At site 1326, which experienced both aerobic and anaerobic periods, the oil was largely gone, and the hydrocarbons that persisted resembled those at the reference sites. Among all sites, oil was not detected below 8 cm except at one site (953, Table S2 in the Supporting Information), where the concentrations of TPH and TPAH were low relative to those at the shallower depths at the impacted sites. Detailed chemical analyses indicate that substantial biodegradation of the oil occurred even at sites 910 and 1326, which were heavily oiled.

Even recalcitrant hydrocarbons such as the three- and four-ring PAHs were substantially biodegraded in marsh sediments over the course of our study. Different microbial populations were found between anaerobic site 910 and fluctuating site 1326. Consistent with these differences in biogeochemistry, biodegradation of oil progressed more rapidly at site 1326 than at site 910.

This study shows that significant biodegradation of hydrocarbons had occurred within 36 months after oiling at even the most heavily impacted sites, and in many cases the hydrocarbon profiles and the microbial community have become indistinguishable from those at the reference sites. Although Macondo oil remained detectable up to 36 months after the accident at some sites, biodegradation continues to drive the concentrations and profiles of hydrocarbons and microbial populations towards reference levels.

ASSOCIATED CONTENT

S Supporting Information

Additional description of analytical chemistry and associated quality control methods, DNA extraction, metagenome sequencing, and diversity analysis. Tables showing the summary quality and throughput statistics for the metagenomic analysis; analytical values for control oil samples; and relative abundances of reads assigned to genomes from various organisms, viruses, and measures of diversity. Graphs showing chromatograms of representative samples, TPH concentrations in surface sediment, and PAH concentrations in MC-252 source oil. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b00413.

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Notes

The authors declare the following competing financial interests: This work was supported by the Gulf Coast Restoration Organization and BP Exploration & Production Inc., who did not influence study design, data collection, or data interpretation.

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