

Succession of Hydrocarbon-Degrading Bacteria in the Aftermath of the *Deepwater Horizon* Oil Spill in the Gulf of Mexico

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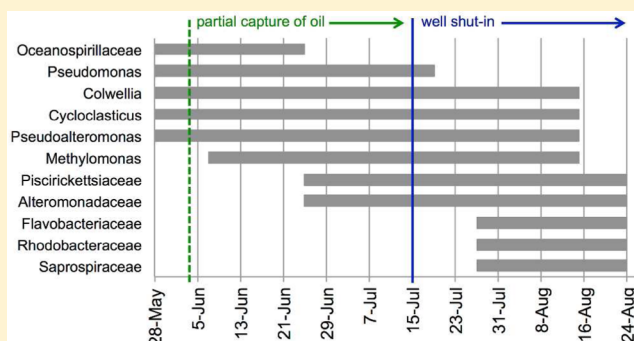
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S Supporting Information

ABSTRACT: The *Deepwater Horizon* oil spill produced large subsurface plumes of dispersed oil and gas in the Gulf of Mexico that stimulated growth of psychrophilic, hydrocarbon degrading bacteria. We tracked succession of plume bacteria before, during and after the 83-day spill to determine the microbial response and biodegradation potential throughout the incident. Dominant bacteria shifted substantially over time and were dependent on relative quantities of different hydrocarbon fractions. Unmitigated flow from the wellhead early in the spill resulted in the highest proportions of *n*-alkanes and cycloalkanes at depth and corresponded with dominance by *Oceanospirillaceae* and *Pseudomonas*. Once partial capture of oil and gas began 43 days into the spill, petroleum hydrocarbons decreased, the fraction of aromatic hydrocarbons increased, and *Colwellia*, *Cyclocloasticus*, and *Pseudoalteromonas* increased in dominance. Enrichment of *Methylobacter* coincided with positive shifts in the $\delta^{13}\text{C}$ values of methane in the plume and indicated significant methane oxidation occurred earlier than previously reported. Anomalous oxygen depressions persisted at plume depths for over six weeks after well shut-in and were likely caused by common marine heterotrophs associated with degradation of high-molecular-weight organic matter, including *Methylophaga*. Multiple hydrocarbon-degrading bacteria operated simultaneously throughout the spill, but their relative importance was controlled by changes in hydrocarbon supply.



INTRODUCTION

The blowout of the Macondo 252 (MC252) well following the explosion of the *Deepwater Horizon* drilling unit resulted in the release of approximately 4.1 million barrels of oil and 1.7×10^{11} g natural gases ($\text{C}_1\text{--C}_5$ hydrocarbons) into the Gulf of Mexico over an 83 day time period from April to July 2010.^{1–3} The complex mixture of hydrocarbons was released at a depth of 1500 m and subject to physical and chemical partitioning as it moved through the water column.^{3,4} Large subsurface hydrocarbon plumes formed at depths of 900–1300 m and largely consisted of dissolved gases ($\text{C}_1\text{--C}_5$ alkanes) and monoaromatics. These plumes reportedly contained small, neutrally buoyant oil droplets that retained some insoluble hydrocarbon fractions at depth.³ The total amount of hydrocarbons retained in deep plumes was estimated to be around 36% of the total leaked.³ On July 15, 2010 the well was shut in and flow into the Gulf of Mexico ceased. By early August monitoring cruises

failed to find elevated concentrations of hydrocarbons from the spill in the open ocean,⁵ but there were anomalous depressions in dissolved oxygen concentrations at depths equivalent to the subsurface plume that were reported around the well-head and as far as 300 km to the southwest.^{5,6}

Microbial community composition was significantly altered by subsurface hydrocarbon plumes and dominated by a few types of Gammaproteobacteria.^{7–9} Hazen et al.⁷ found samples collected between May 27–June 1 were dominated by an uncultivated *Oceanospirillales* and also enriched in 15 other families of cold-adapted Gammaproteobacteria. Two weeks later, Valentine et al.⁹ sampled the plume and found a different

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microbial community structure that was dominated by *Colwellia* and *Cycloclasticus*, and contained comparatively little *Oceanospirillales*. Microbial communities in dissolved oxygen (DO) anomalies measured in September contained high numbers of methylotrophic bacteria that were thought to be remnants of a July bloom of methane consuming bacteria,^{6,8} but this interpretation was contested.¹⁰ A modeling effort concluded that the order and timing of these shifts were a consequence of different metabolic growth rates of these bacteria combined with physical mixing patterns in the plume area.¹¹

Variations in the supply of leaked hydrocarbons may offer a different explanation for these dramatic shifts in plume microbial communities over time. Hydrocarbon inputs into the Gulf of Mexico were not constant over the duration of the spill. Flow from the wellhead was estimated at 50 000–70 000 BPD¹ and was largely unabated from April 25 until June 4. Partial capture began at the wellhead on June 4 and reduced inputs into the water column by 15 000 barrels per day (BPD). An additional 9000 BPD were captured starting June 11, further reducing flow into the water column to approximately 52–66% of inputs prior to June 4. An analysis of all available subsurface monitoring data found a significant drop in plume hydrocarbon concentrations from an average of 46.1 $\mu\text{g L}^{-1}$ before June 4 to 5.5 $\mu\text{g L}^{-1}$ after.⁴ These changes in the availability of hydrocarbon substrate could have promoted shifts in the microbial community structure in the entrainment layer.

We hypothesized that plume microbial communities were controlled by changes in hydrocarbon and nutrient availability. We examined the most continuous and complete set of samples from the deep plume region spanning six weeks before the spill to six weeks after the spill to trace the succession of the microbial community in impacted waters. Microbial community dynamics were compared to hydrocarbon chemistry, carbon isotopic compositions, and water parameter measurements to determine the influence of geochemical conditions on microbial community structure. We followed the fate of the plume microbial community in persistent oxygen anomalies after the well was shut in to understand the causes of continued oxygen demand. Finally, we isolated several different hydrocarbon-degrading bacteria that were abundant during the incident.

MATERIALS AND METHODS

Sample Collection. Prespill samples were collected during the R/V *Cape Hatteras* GulfCarbon 5 cruise in the northern Gulf of Mexico (30° 07' N, 088° 02' W to 27° 39' N, 093° 39' W; Figure 1) from March 10 to 21, 2010 (Supporting Information (SI) Figure S1, Table S1). Samples were collected using Niskin bottles and a General Oceanics rosette sampling system equipped with a SBE25 CTD data package. Approximately 1 L of water from each sample was pressure filtered (at ~60 kPa) through 0.22 μm Durapore filters (Millipore), then the filters were frozen at -20 °C in 2 mL of lysis buffer (0.75 M sucrose, 40 mM EDTA, 50 mM Tris; pH 8.3).

After the onset of the spill water samples were collected in the Gulf of Mexico during a series of monitoring cruises spanning May 27–August 26, 2010 on the R/V *Ocean Veritas* (May 25 to June 11), R/V *Brooks McCall* (May 29 to June 27) and R/V *Ferrel* (July 3 to August 29). The cruises were conducted as part of the monitoring effort to assess the effect of subsea dispersant use during the MC252 oil leak (<http://www.epa.gov/bpspill/dispersants.html#directives>). A colored dissolved organic matter (CDOM) WETstar fluorometer (WET

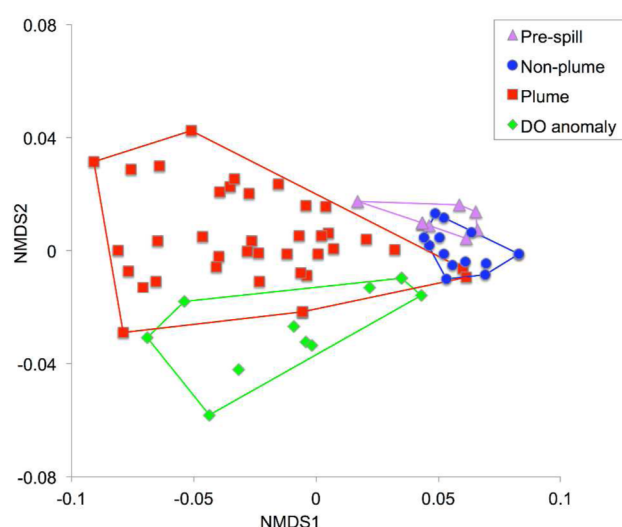


Figure 1. Differences among microbial communities in hydrocarbon plume, postspill oxygen anomalies, nonplume and prespill waters. Ordination conducted by nonmetric multidimensional scaling analysis with Bray–Curtis distance metric (2D stress = 0.08).

Laboratories, Philomath, OR) was attached to a CTD sampling rosette (Sea-Bird Electronics Inc., Bellevue, WA) and used to detect the presence of oil along depth profiles between the surface and seafloor. Fluorometer results were subsequently confirmed with laboratory hydrocarbon analysis. Dissolved oxygen concentrations at depth were continuously recorded with a Seabird Electronics electrochemical SBE-43 oxygen sensor. Niskin bottles attached to the CTD rosette were used to capture water samples. The Niskin bottles were cleaned internally with distilled water and detergents between samplings. From each sample 800–4000 mL of water were filtered through sterile filter units containing 47 mm diameter polyethysulfone membranes with 0.22 μm pore size (MO BIO Laboratories, Inc., Carlsbad, CA) and then immediately frozen and stored at -20 °C for the remainder of the cruise. Filters were shipped on dry ice to Lawrence Berkeley National Laboratory and stored at -80 °C until DNA extraction.

Sample for isotopic analyses of hydrocarbons were collected from the Niskin bottles and immediately filtered into pre-evacuated 160 mL bottles capped with 20 mm blue chlorobutyl septa (Bellco Glass, Inc., Vineland, NJ). The samples were kept refrigerated until isotopic measurements were made.

DNA Extraction and Analysis. A total of 68 samples were analyzed for microbial community characterization with PhyloChip (Second Genome, San Bruno, CA) (SI Figure S1, Table S1). DNA from prespill samples was extracted from the filters by enzymatic hydrolysis of cellular material with 40 μL of lysozyme (50 mg mL⁻¹), then 60 μL of proteinase K (20 mg mL⁻¹) and sodium dodecyl sulfate (100 μL of a 10% solution). DNA in 800 μL of the lysate was purified by phenol-chloroform extraction and elution in 50 μL of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) prior to storage at -80 °C as described in.¹² DNA extraction for the spill response samples is described in Hazen et al.⁷ Laboratory procedures of 16S rRNA gene amplification and characterization by PhyloChip methods are as in Hazen et al.⁷ except the r_{XQ3} cutoff value for subfamily detection was set to ≥ 0.10 . Microarray data are available for download at http://greengenes.lbl.gov/Download/Microarray_Data/Dubinsky_2013_EST/.

Hydrocarbons and Water Parameters. Hydrocarbon and oxygen data were compiled from NOAA-National Oceanic Data Center archive of data observations made aboard research survey vessels supporting the Subsurface Monitoring Unit in the Gulf of Mexico (www.nodc.noaa.gov/General/DeepwaterHorizon/ships.html). To determine hydrocarbon composition derived from the presence of oil on the filtered samples, 200 μL of chloroform was added to the neutral lipid extract which was then vortexed followed by a 30 s sonication. The extract was analyzed on an Agilent GC/FID and peaks were identified by GC/MS. Quantification was accomplished by comparison to a known *n*-alkane standard.

Dissolved inorganic nitrogen and total ammonia nitrogen was measured by an automated membrane diffusion/conductivity detection method using the TL-2800 ammonia analyzer (Timberline Instruments, Boulder, CO).¹³ Nitrate was calculated as the difference between dissolved inorganic N and ammonia. Total iron was measured using a reaction with phenanthroline according to SM 3500-Fe B and orthophosphate was quantified on unfiltered samples by the ascorbic acid method adapted from SM 4500-P-E.¹⁴

Carbon Isotopes. For carbon isotopic analyses of dissolved methane, 5 mL of water in the 160 mL bottles were replaced with helium and allowed to equilibrate with the dissolved gases in the sample. A portion of the gas was then introduced into a trace gas preconcentration system where water, CO_2 and higher hydrocarbons were stripped from the sample. The remaining gas was then passed through an oxidation furnace at 1050 $^\circ\text{C}$ to convert the methane to CO_2 which was then fed into a Micromass JA Series mass spectrometer for carbon isotopic analysis. The isotopic compositions are reported as per mil (‰) variations from Vienna Pee Dee Belemnite (VPDB) using the delta notation:

$$\delta^{13}\text{C} = [({}^{13}\text{C}/{}^{12}\text{C})_{\text{sample}}/({}^{13}\text{C}/{}^{12}\text{C})_{\text{VPDB}} - 1] \times 1000$$

The reproducibility of these analyses is 0.5‰ (1 s).

Microbiological Isolation. Samples were collected for bacterial isolation in September 2010 from 200 m to 1500 m depths within a kilometer of the MC252 wellhead. Enrichments were initiated under aerobic conditions at 5 $^\circ\text{C}$ and at 25 $^\circ\text{C}$ based on the ocean depth from which the samples were collected using minimal marine medium¹⁵ with 100 ppm MC252 oil as the sole carbon source. When an increase in turbidity and optical density was observed, the enrichments were transferred into fresh media. After two or three such transfers, distinct colonies were obtained by plating on marine agar plates in one or two weeks. Colonies were picked into marine broth for strain purity checks and DNA extracted using the MoBio UltraClean Microbial DNA Isolation Kit (MoBio Inc., Carlsbad, CA). PCR amplification was carried out using universal bacterial 16S rRNA gene primers 27F and 1492R with the following PCR conditions: initial denaturation at 95 $^\circ\text{C}$ (3 min), followed by 25 cycles of 95 $^\circ\text{C}$ (30 s), 53 $^\circ\text{C}$ (30 s), 72 $^\circ\text{C}$ (1 min), followed by a final extension 72 $^\circ\text{C}$ (7 min). Amplicons were sequenced in both directions at the UC Berkeley DNA Sequencing Facility, and reads were assembled with Geneious (5.67), aligned with PyNast and chimera checked with ChimeraSlayer. Sequences were classified by BLAST search in Greengenes. Isolates were grown and maintained in minimal media with MC252 oil as the sole carbon source. Confirmation of oil degradation was obtained by closed loop measurement of CO_2 production over time from

the headspace of incubations where MC252 oil was the sole carbon source using a Micro-Oxymax respirometer (Columbus Instruments, Columbus OH).

Statistics. After collection, samples were binned into the following groups: prespill, plume, nonplume and postspill DO anomaly (SI Table S1). Prespill samples were collected in March 2010 before the spill began on April 22, 2010. Plume samples were collected during or after the spill and contained detectable petroleum hydrocarbons as measured by C10–C35 *n*-alkanes, cycloalkanes (cyclohexane + methylcyclohexane), benzene, toluene, ethylbenzene, and total xylenes (BTX), or polycyclic aromatic hydrocarbon analyses. Nonplume samples were collected during or after the spill and contained no detectable hydrocarbons or DO anomalies. Postspill DO anomalies were samples with >5% deviation from background DO concentrations but containing no detectable petroleum hydrocarbons. Background DO concentrations over the plume interval were interpolated by fitting a sixth degree polynomial to DO profiles from 700 to 1300 m depth. Taxa that were commonly enriched in hydrocarbon plume or DO anomaly samples were identified by PhyloChip as having >2-fold increase in hybridization intensity over the mean background (nonplume) intensity in at least half of plume or DO anomaly samples. A 2-fold increase in \log_2 hybridization intensity is approximately a 4-fold increase in \log_2 16S rRNA gene concentration.⁷

Relationships among community structure and explanatory variables were examined with Nonmetric Multidimensional Scaling (NMDS) using the vegan package in the R computing environment (3.0.1). NMDS was performed with up to 20 random starts and 999 permutations using the Bray–Curtis distance metric. The significance of differences between groups was tested using Analysis of Similarity (ANOSIM) with Bonferroni-corrected *P* values in Primer 6. The BIO-ENV procedure was used to select environmental factors with the strongest relationships to community structure.¹⁶ The envfit function in vegan was used to display the maximal correlation of explanatory variables with the ordination configuration. The weighted averages of hybridization scores for enriched taxa were calculated with the wascores function in vegan to display abundance-weighted centroids of enriched taxa in ordination space.

RESULTS AND DISCUSSION

Succession of the Plume Microbial Community. We analyzed microbial communities in 68 water samples that were collected from 900 to 1300m deep between March 10 and August 25, 2010 (SI Figure S1, Table S1). Microbial communities in water with detected petroleum hydrocarbons were significantly different from prespill and nonplume waters collected during the spill at subsurface plume depths (Figure 1; ANOSIM *R* = 0.53 and 0.61, respectively; *p* < 0.01). Bacterial community structure in the DO anomalies detected at plume depths was also significantly different from prespill and nonplume waters (ANOSIM *R* = 0.62 and 0.74, respectively; *p* < 0.01) (Figure 1). Microbial communities in prespill and nonplume waters were more similar to each other than to either waters containing hydrocarbons or DO anomalies (Figure 1, ANOSIM *R* = 0.37; *P* = 0.01).

Community structure in the subsurface hydrocarbon plume was dynamic over time and changed in response to variations in hydrocarbon composition and quantity (Figure 2). Changes in plume microbial community structure were strongly associated

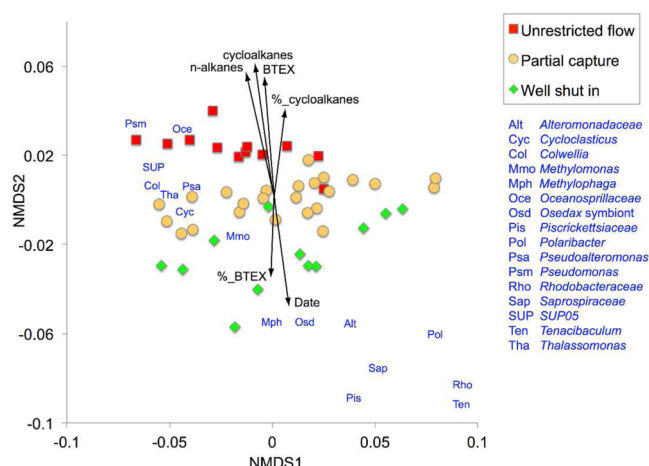


Figure 2. Changes in microbial community structure in hydrocarbon plume and DO anomalies samples over time. Periods of differing flow status at the wellhead are shown as unrestricted flow (before June 5), partial capture of hydrocarbons (June 5–July 15) and well shut-in (after July 15). NMDS 2D stress = 0.11. Vectors show maximal correlation of the ordination configuration of variables with the strongest relationships to community structure. Taxonomic codes plotted on the ordination show the abundance-weighted centroids of enriched taxa.

with sampling date and changed significantly among samples collected during periods of unmitigated flow, partial capture and well shut-in (Figure 2, ANOSIM Global $R = 0.33$, $P < 0.01$). Correspondingly, these changes in community structure were strongly associated with decreasing concentrations of *n*-alkanes, BTEX and cycloalkanes (Figures 2). Concentrations of petroleum hydrocarbons decreased sharply from the period of unmitigated flow and plume composition shifted toward soluble components, with average concentrations of BTEX, cycloalkanes, and *n*C10–*n*C35 alkanes decreased to 21%, 13%, and 5% of their averages during unmitigated flow, respectively (Figure 3).

There were no significant correlations between changes in microbial community structure and distance from wellhead or

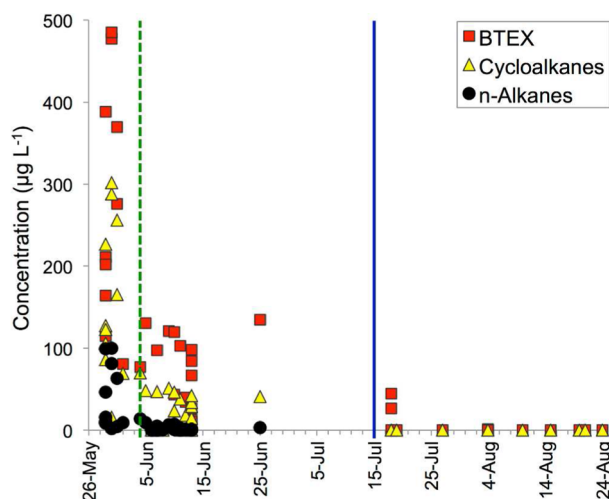


Figure 3. Concentrations of BTEX, cycloalkanes (cyclohexane + methylcyclohexane) and *n*C10–*n*C35 alkanes in the plume over time. Dashed green line and solid blue line mark the onset of partial capture and well shut-in, respectively.

indices of saturated-hydrocarbon biodegradation (*n*-C17:phytane and *n*-C18:pristine ratios). There were no significant correlations between microbial community structure and concentrations of phosphorus, iron, nitrate, ammonium or total inorganic nitrogen (SI Table S1), thus any limitation or competition for major nutrients did not substantially alter community dynamics. Only petroleum hydrocarbons and date were significantly related to changes in community structure.

Enriched taxa in the hydrocarbon plume were exclusively Gammaproteobacteria (SI Table S2), but the dominant taxa within that class shifted over time. During the period of unmitigated flow, *Oceanospirillales* and *Pseudomonas* taxa were the most enriched taxa detected in the plume relative to nonplume waters (Figure 2). The enriched *Oceanospirillales* taxon was an unclassified and uncultivated OTU within the *Oceanospirillaceae* and its enrichment was greater than any other taxa detected in the plume (Figure 4a), consistent with

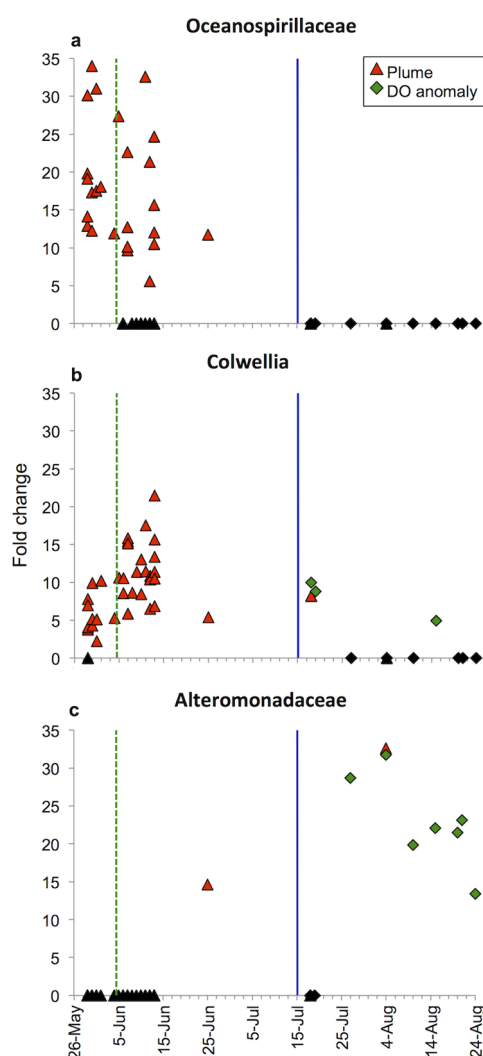


Figure 4. Temporal changes in relative abundance of taxa that were most enriched in the plume during different phases of the spill: *Oceanospirillaceae* (a) during unmitigated flow (May 29–June 4); *Colwellia* (b) during partial capture of hydrocarbons (June 5–July 15); *Alteromonadaceae* (c) after well shut-in. Values are fold change over nonplume mean of estimated 16S rRNA gene copies. Values of zero indicate nondetects. Dashed green line and solid blue line mark the onset of partial capture and well shut-in, respectively.

previous reports that it was 60–95% of sequences in plume samples collected before June 4.^{7,8,17} Samples dominated by *Oceanospirillaceae* had the highest concentrations of petroleum hydrocarbons and highest fractions of insoluble *n*-alkanes and cycloalkanes over the period of study, (Figure 2 and 3).

Shifts in hydrocarbon quantity and composition explained the changes in microbial community structure that occurred in early June (Figure 2). After partial capture began there were large increases in the relative abundance of *Colwellia*, *Pseudoalteromonas*, *Cycloclasticus* and *Thalassomonas* (Figure 4b and SI Figure S2). Methane-oxidizing *Methylomonas* was also enriched within three days after the onset of partial capture (Figure 5a). The relative abundance of *Oceanospirillaceae* and

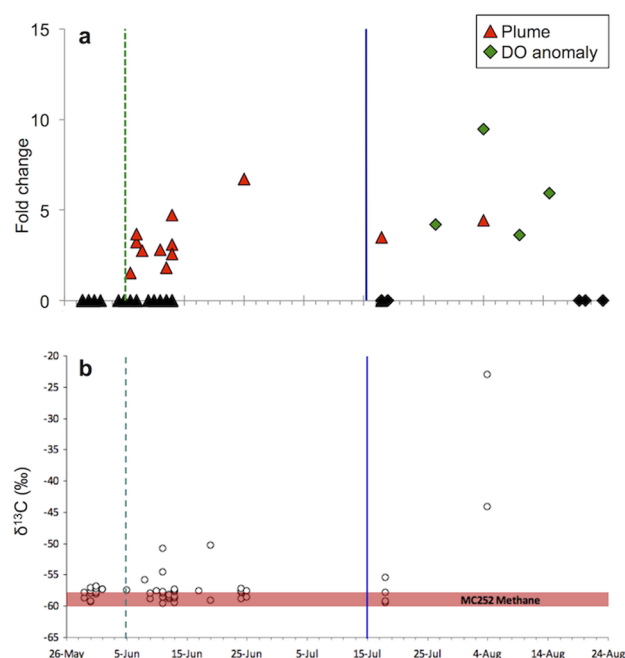


Figure 5. Temporal changes in *Methylomonas* enrichment (a) and carbon isotopic composition of methane (b). *Methylomonas* enrichment shown as fold change over nonplume mean of estimated 16S rRNA gene copies. Values of zero indicate nondetects. Dashed green line and solid blue line mark the onset of partial capture and well shut-in, respectively.

Pseudomonas concurrently decreased, and *Oceanospirillaceae* was undetectable in several samples after the onset of partial capture (Figure 4a) when longer-chain alkanes disappeared from the plume (Figure 3).

The higher availability of *n*-alkanes and cycloalkanes during unmitigated flow likely supported the growth of alkane-degrading bacteria. Metagenomic and metatranscriptomic analyses conducted on a subset of samples collected during unmitigated flow revealed alkane degradation was the dominant hydrocarbon-degrading pathway expressed when *Oceanospirillaceae* and *Pseudomonas* were dominant in the community.¹⁷ Single-cell genomics of the uncultivated *Oceanospirillaceae* showed this organism has a complete pathway for cyclohexane degradation, and it appears capable of chemotactic response to hydrocarbons, a trait that may have aided its rapid response to suspended oil droplets early in the spill.¹⁷ In addition, other *Oceanospirillaceae* that have been cultured from marine environments are capable of degrading aliphatic hydrocarbons.^{18,19} We were able to isolate *Pseudomonas* collected

within 1 km of the wellhead and confirm its growth on MC252 oil as the sole carbon source (SI Figure S3). *Pseudomonas* species are known for their ability to degrade a variety of petroleum compounds, including aliphatics and aromatics, and are frequently associated with petroleum degradation in cold marine environments.^{20–22}

Relative abundances of *Colwellia*, *Cycloclasticus*, *Pseudoalteromonas* and *Thalassomonas* increased during partial capture (Figure 4b and SI Figure S2) as petroleum hydrocarbon concentrations decreased and the more dilute plume consisted of more BTEX relative to alkanes (Figures 2 and 3). The increased predominance of soluble BTEX and natural gases,² and drop of *n*-alkanes and cycloalkanes to near nondetectable concentrations in several samples (Figure 3 and SI Table S1) likely explains why these taxa became more dominant in the community. *Cycloclasticus* and *Pseudoalteromonas* are both capable of aromatic degradation and commonly found in contaminated marine environments. Stable isotope probing experiments found *Colwellia* in the Gulf of Mexico incorporated benzene, propane and ethane.⁸ The high concentrations of BTEX and natural gases and lack of C6+ alkanes in the plume after June 4² would have provided an advantage to these organisms over alkane-degrading specialists. We also confirmed that *Colwellia* and *Pseudoalteromonas* that were isolated from plume depths were capable of growing on MC252 as the sole carbon source at 5 °C (SI Figure S3).

Valentine et al.¹¹ suggest respiratory succession and circulation patterns caused the difference between late May and mid June samples, with *Oceanospirillaceae* dominating microbial communities earlier in the spill because it was assumed to have a high growth rate consuming alkanes, primarily dissolved butane and pentane.¹¹ The model predicts that slower-growing *Colwellia* subsequently emerged in dominance as the primary consumer of more abundant propane and ethane, and then maintained high abundances in the following weeks as previously contaminated water masses recirculated toward the wellhead. Our results suggest an additional explanation for this shift in dominance was variations in the supply of hydrocarbon substrates to plume bacteria that were likely caused by interventions at the wellhead. Prior to June 3, during the period of *Oceanospirillaceae* dominance, unabated flow from the wellhead occurred at two points along the riser pipe, through a kink directly above the blowout preventer (BOP) at the wellhead and through the open end of the riser 1200 m away from the BOP. Shearing the riser on June 3 consolidated the leak to a single ejection point that was sprayed with chemical dispersant, and allowed for the installation of the Top Hat #4 containment device that reduced inputs into the water column. In addition, the surge in released hydrocarbons and oil droplets that followed the failed top kill effort on May 29 may have further stimulated *Oceanospirillaceae* growth prior to June 4.¹ Furthermore, a regional physical oceanographic model predicted an eddy in the vicinity of the wellhead in late May that may have enhanced buildup of hydrocarbons and accumulation of oil droplets at this time compared to mid-June.¹¹ Once partial capture began, the disappearance of suspended oil droplets and longer-chain alkanes from the plume appeared to lead to the demise of the *Oceanospirillaceae*, while bacteria reliant on BTEX, natural gases and other soluble hydrocarbons continued to persist.

It is important to note that the relative abundances of *Colwellia*, *Cycloclasticus*, *Pseudoalteromonas*, and *Thalassomonas* were enriched in samples collected in late May when flow was

unmitigated even though their relative concentrations were overshadowed by the vigorous *Oceanospirillaceae* bloom (Figure 6). Metagenome and metatranscriptome analyses determined

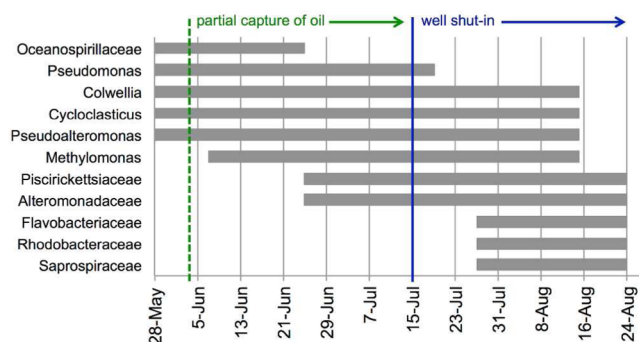


Figure 6. Periods of enrichment for plume taxa. Bars bracket the dates when relative abundance was enriched over background in any sample (>2-fold mean nonplume intensity).

that *Colwellia* were 9–11% of the community and active in samples collected in late May,¹⁷ and demonstrated that the absolute abundance and activity of *Colwellia* was high even when *Oceanospirillaceae* was dominant. Our results indicated that multiple hydrocarbon-degrading metabolisms were operating simultaneously within 5 weeks after the onset of the spill. Subsequent shifts in dominance were likely caused by disproportionate reductions in *n*-alkanes preferred by *Oceanospirillaceae* and *Pseudomonas*, and allowed already active *Colwellia* and other aromatic hydrocarbon degraders to surge in relative abundance in spite of an overall decrease in hydrocarbon concentrations (Figure 4 and SI Figure S2).

The most abundant hydrocarbon released by the spill was methane, which was inferred to cause a vigorous bloom of methanotrophic bacteria between late June and August that was responsible for most microbial respiration during that time.^{6,8,11} We attempted to analyze the $\delta^{13}\text{C}$ of dissolved methane in 77 samples collected from the water column between May 28 and August 20. Of those samples, 52 had measurable concentrations of methane, most of which were from the plume depth. The results of those analyses are plotted versus collection date on Figure 5b. Measurements of the $\delta^{13}\text{C}$ of methane in the MC252 oil range from -58 to -60‰ . The earliest samples we analyzed all had $\delta^{13}\text{C}$ values in this range. As early as June 8, however, we observed samples with $\delta^{13}\text{C}$ values as high as -56‰ in the plume. Microbial methane oxidation produces CO_2 depleted in ^{13}C , causing the residual methane to be enriched in ^{13}C . The magnitude of the fractionation between the methane and CO_2 can vary,²³ but for a reasonable fractionation factor, $\alpha_{\text{CH}_4\text{CO}_2}$ of 1.02, a shift of 2‰ in the $\delta^{13}\text{C}$ of the methane represents oxidation of 10% of the methane (SI Figure S4). Methane in samples collected between June 8 and July 18 (3 days after the shut-in) had $\delta^{13}\text{C}$ values shifted by up to 8‰, signifying oxidation of ~35% of the methane. After July 18, we were only able to find two samples with measurable concentrations of methane (of 21 attempted). Both of these samples were collected on August 4 from plume depths with significant dissolved oxygen dips and had highly enriched $\delta^{13}\text{C}$ values of -44 and -23‰ , indicating high degrees of methane oxidation (~52 and 84%, respectively). The isotope results parallel the observed changes in the relative abundance of methane-oxidizing *Methylomonas* (Figure 5). *Methylomonas* enrichment started in early- to mid-June when the first significant

enrichments in the $\delta^{13}\text{C}$ of the methane were observed and continued until mid-August when the isotope data indicated that most of the methane had been consumed.

Surprisingly, the magnitude of *Methylomonas* enrichment was less than other taxa like *Oceanospirillaceae* and *Colwellia* that bloomed during the spill, and less than *Alteromonadaceae* and *Flavobacteria* in August (Figure 4 and SI Figure S2). Methane oxidation was thought to have supported most microbial respiration that occurred in the plume and resulted in a highly dominant population of methanotrophs by the end of the spill.^{6,11} It is possible that methanotrophs peaked in early July when our sampling was sparse, but the muted enrichment in late July and August indicates that other degradation processes may have contributed more to oxygen consumption than previously suggested.¹¹

The occurrence of both *Methylococcaceae* and *Methylophaga* in September DO anomalies has been cited as evidence of the antecedent bloom in methanotrophs during the spill^{6,8} but this interpretation was met with controversy.^{10,24} Our data implicate *Methylomonas*, a member of the *Methylococcaceae*, as the primary methanotroph because it was the most enriched methane oxidizer that occurred during the period of methane oxidation (Figure 5). This finding is consistent with stable isotope probing evidence that *Methylococcaceae* are the primary methane consumers in this system.⁸ On the other hand, the importance of *Methylophaga* to methane oxidation is less certain. Neither *Methylophaga* nor other taxa in the *Piscirickettsiaceae* were enriched in mid-June when methane oxidation began and their relative abundance did not decline once methane was totally consumed by late August (SI Figure S2), suggesting they may have been growing on carbon sources other than methane in postspill DO anomalies. Transcriptomic analyses of high-molecular-weight dissolved organic matter (DOM) additions to seawater found that *Methylophaga* are likely consuming methanol and/or formaldehyde produced from degradation of DOM methyl sugars by *Alteromonas*,²⁵ consistent with the enrichment of both of these taxa throughout August (Figures 4c and S2). McCarren et al.²⁵ suggest the ubiquity of *Methylophaga* and other methylotrophs in the ocean is due to their role as terminal degraders of DOM in the aerobic food chain. In addition, *Methylophaga* are frequently enriched in oil-contaminated environments that lack methane input.^{26–29} Thus *Methylophaga* in postspill oxygen anomalies were not necessarily inactive remnants of historical methane oxidation, but were likely metabolizing other C1 compounds and actively consuming oxygen in August and September.

Our results suggest scavenging of the decaying bloom and consumption of organic residues in the plume fueled microbial respiration in DO anomalies observed throughout August and reported in September by others.⁶ Enriched taxa in DO anomalies throughout August included *Flavobacteria* (*Tenacibaculum* and *Polaribacter*), *Alteromonadaceae*, and *Rhodobacteraceae* (Figure 6, SI Table S3). The dominance of these taxa is indicative of a community optimized for the degradation of complex organic matter. *Flavobacteria* are prominent degraders of high-molecular-weight organics in marine systems,³⁰ and dominate microbial communities on marine snow and following phytoplankton blooms.^{31,32} *Tenacibaculum* and *Polaribacter* have been shown to be the most dominant bacteria in cold surface waters enriched with phytoplankton detritus.³³ Likewise, *Alteromonadaceae* and *Rhodobacteraceae* enriched in DO anomalies are also implicated in the turnover of high-

molecular-weight dissolved organic matter in seawater.²⁵ *Tenacibaculum* can also produce bacteriolytic enzymes and have been observed to lyse prey cells in social swarms,³⁴ suggesting a predatory role that may account for their abundance in the decaying bloom of hydrocarbon-degrading bacteria after the spill ceased. Interestingly, *Tenacibaculum* and *Polaribacter* were previously found to be associated with crude oil in cold marine environments.^{35,36} After the spill these *Flavobacteria* may have been feeding on recalcitrant components of MC252 oil, although the low concentrations of these components cannot account for the observed oxygen draw-down.^{6,9} *Oceanospirillales* taxa were also enriched in postspill DO anomalies (SI Figure S2, Table S3), however these taxa were distinct from the uncultivated OTU that dominated the period of unmitigated flow, and are closely related to symbionts of *Osedax* worms that degrade lipids in whale and fish bones.³⁷

The plume microbial community was dynamic and appeared responsive to interventions at the wellhead that altered hydrocarbon composition and quantity in the subsurface. Once partial capture at the wellhead commenced, the plume microbial community structure changed significantly and was associated with a sharp decline in petroleum hydrocarbons, and an increase in the relative amount of BTEX and other soluble hydrocarbons in the plume. These changes in substrate supply corresponded with large increases in the relative abundance of aromatic hydrocarbon degraders and consumers of natural gases, and concurrent declines in alkane-degrading *Oceanospirillaceae*. *Methylomonas* enrichment and methane oxidation occurred throughout June and July, but *Methylophaga* were not clearly linked to methane oxidation and were likely active in the postspill heterotrophic community that was consuming residual cell mass and organic residues in the aging plume. The deep ocean microbial community is highly sensitive to the presence of released hydrocarbons and is an effective biosensor for contamination in this environment.

■ ASSOCIATED CONTENT

■ Supporting Information

This information is available free of charge via the Internet at <http://pubs.acs.org/>.

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Notes

The authors declare no competing financial interest.

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