Microbial Communities Responding to Deep-Sea Hydrocarbon Spills

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Abstract

The 2010 Deepwater Horizon oil spill in the Gulf of Mexico can be considered the world's first deep-sea hydrocarbon spill. Deep-sea hydrocarbon spills occur in a different setting than surface oil spills and the organisms that respond must be adapted to this low temperature, high pressure environment. The hydrocarbon composition can also be quite different than at the sea surface, with high concentrations of dissolved hydrocarbons, including natural gas, and suspended droplets of petroleum. We discuss the bacteria that may respond to these spills and factors that affect their abundance, based on data collected during the Deepwater Horizon spill and in microcosm experiments in the following years.

1. Introduction

When the Deepwater Horizon mobile offshore drilling unit exploded on April 20, 2010 and sank two days later, it caused the world's first major deep-sea oil spill. Previous well blowouts such as Ixtoc I in the Gulf Mexico in 1979 and Platform A in the Santa Barbara Channel in 1969 also caused significant undersea spills, but the shallower depths of these spills (50-60 m) resulted in most of oil reaching the sea surface. In contrast, the Deepwater Horizon well was 1500 m below the sea surface and ~25% the hydrocarbons emitted remained dissolved or suspended in a deep-sea intrusion layer at depths between 900 and 1300 m (Ryerson et al. 2012, Gros et al. 2017). This intrusion layer, commonly referred to as the hydrocarbon plume, created an unusual set of conditions for the microbial communities that responded to this spill. Compared to a sea-surface oil spill, there was a different mix of hydrocarbon compounds, lower temperatures, higher pressure, abundant nutrients, and complete darkness. These differences in environmental conditions led to the development of microbial communities quite different from those observed in previous oil spills, dominated at different points in space and time by a novel *Oceanospirillales* (referred to as DWH *Oceanospirillales*), *Colwellia* and *Cycloclasticus* (Hazen et al. 2010, Redmond and Valentine 2012). In this chapter, we discuss the environmental features of deep-sea spills that affect microbial communities, changes in microbial community composition during deep-sea spills, and notable members of those communities.

2. Features of Deep-Sea Hydrocarbon Spills

2.1 Composition of Hydrocarbons in the Water Column

The formation of a deep-sea hydrocarbon plume was one of the most distinctive features of the Deepwater Horizon spill and had a significant effect on the microbial communities that developed, so we focus our discussion primarily on hydrocarbon degradation within this plume layer. The deep-sea plume was enriched in the most soluble hydrocarbons including: methane,

ethane, propane, butanes, pentanes (C1-C5 alkanes); cyclopentane, methylcyclopentane, cyclohexane, methylcyclohexane (C5-C7 cycloalkanes); benzene, toluene, ethylbenzene, and xylenes (BTEX); and naphthalene, methylnaphthalenes, dimethylnaphthalenes and fluorene (small PAHs), which dissolved according to their aqueous solubilities (Ryerson et al. 2012). In a surface oil spill, many of these volatile compounds would be rapidly lost to the atmosphere rather than available for consumption by microbes, likely a major factor affecting which organisms responded to the spill. In addition to dissolved hydrocarbons, the plume contained suspended petroleum micro-droplets, though the amount may have varied over time due to interventions at the wellhead. Between April 22 and June 3, oil was released at two or more points along the riser pipe. After the riser was cut, the Top Hat containment device began to capture oil, and chemical dispersants were applied consistently at the point of oil release, and the composition of the microbial community changed (Dubinsky et al. 2013). These observations suggest that hydrocarbon flux rate and the hydrocarbon composition of the plumes impacted microbial community composition, while also highlighting the potential effect of response efforts on biodegradation and the challenges in predicting microbial community response in future spills.

2.2 Dispersant

One of the most controversial aspects of the Deepwater Horizon spill was the use of chemical dispersants, particularly their unprecedented use in the deep sea. Corexit EC9500A was injected directly into the oil and gas emanating from the fallen riser and later the blowout preventer at the wellhead. The application of dispersant decreased petroleum droplet size by approximately three fold and slowed their rise through the water column, increasing dissolution of water soluble compounds into the deep-sea plume by 25% (Gros et al. 2017) and shifting the area of oil surfacing away from response vessels. This strategy appears to have been effective at reducing the risks of VOC exposure to workers at the sea surface, but dispersant's effects on biodegradation remain a point of contention (Kleindienst et al. 2016b, Prince et al. 2016a, Prince et al. 2017). Here, we focus primarily on its effect on microbial community composition. Based on closed-system microcosm experiments, Kleindienst et al. suggested that dispersant can alter community composition by selecting for organisms like *Colwellia* that are stimulated by dispersant alone and selecting against other hydrocarbon degraders like *Marinobacter,* and ultimately may inhibit degradation (Kleindienst et al. 2015). Other microcosm experiments have found no significant effects of dispersant on community composition and degradation rates (Brakstad et al. 2018) or a positive effect on degradation rate, though the stimulation of *Colwellia* by dispersant alone and inhibition of *Marinobacter* in a subset of incubations at 25°C were also noted (Techtmann et al. 2017). The growth of *Colwellia* in dispersant only treatments suggests that it may be able to metabolize the dispersant, and *Colwellia* strain RC25 was shown to degrade dioctylsulfosuccinate, a component of Corexit (Chakraborty et al. 2012). However, it is important to note that the deep plumes were an open system and the dispersant components dissolved and diluted into the ocean (Kujawinski et al. 2011). As such, the environmental relevance of microcosm experiments is somewhat limited and the primary impact of dispersants on the microbial community was likely indirect by modulating the abundance and molecular distribution of hydrocarbon substrates in the water column.

2.3 Temperature

In temperate areas, one of the most important differences between a deep-sea and a surface oil spill is water temperature. During the Deepwater Horizon oil spill, sea surface temperature in the affected area was nearly 30°C, while temperature in the hydrocarbon plume at 1100 m was only 6°C. Temperature has two major effects on biodegradation. First, it affects the physical properties of oil and can alter its bioavailability. For example, the respiration rate by *Alcanivorax borkumensis* SK2 growing on individual *n*-alkanes (C14-C20) drops significantly at the temperature that coincides with the liquid-wax phase transition for each *n*-alkane, clearly indicating that bioavailability plays a role independent of the overall microbial temperature optimum (Lyu et al. 2018). The formation of wax-like particles appears to have led to the deposition of long-chain *n*-alkanes in sediments during the Deepwater Horizon spill and slower than expected degradation relative to typically conserved biomarkers (Bagby et al. 2017).

The second effect of temperature is on microbial physiology and community composition. The effect of temperature on community composition in the Gulf of Mexico was nicely demonstrated by Liu et al (Liu et al. 2017a). In experiments with surface and bottom water inocula incubated in filtered water from each environment at 4°C and 24°C, with and without oil, they determined that temperature caused the most variation in community composition (57%). Low temperature particularly favored the development of *Cycloclasticus* and *Pseudoalteromonas*, two organisms that were abundant during the Deepwater Horizon spill (Dubinsky et al. 2013). The effects of temperature on community composition have been noted by several others, with *Colwellia*, *Cycloclasticus*, and members of the *Oceanospirillales* frequently mentioned as abundant in low-temperature communities, though they are not found exclusively at low temperature (Coulon et al. 2007, Techtmann et al. 2017, Lofthus et al. 2018).

2.4 Pressure

Hydrostatic pressure in the ocean increases by 1 atm or 0.101 MPa with each 10 m below the sea surface, resulting in a pressure of ~15 MPa at the depth of the Deepwater Horizon oil spill. While temperature is commonly manipulated in lab microcosm experiments, the equipment required to mimic deep-sea pressures is less commonly available and the effects of pressure are therefore not frequently tested. Most studies indicate that biodegradation is slower at higher pressure and that cells grow more slowly (Prince et al. 2016b, Scoma et al. 2016, Marietou et al. 2018). The only study of the effect of pressure on hydrocarbon-degrading community composition in the Gulf of Mexico showed that *Oleispira* was dominant at all pressures, while the groups common during the Deepwater Horizon spill weren't observed in any treatment (Marietou et al. 2018). Additional study is required to determine the role pressure may have played in the abundance of these organisms.

2.5 Nutrients and Oxygen

In contrast to many ocean surface environments, nitrogen and phosphorus were relatively abundant in the deep plume environment and in combination with mixing dynamics, are unlikely to have limited bacterial growth in the Deepwater Horizon spill. However, limitation of trace metals including iron and copper may have had some effect on the activity of hydrocarbon

oxidizers or the communities that developed (Bælum et al. 2012, Joung and Shiller 2013, Crespo-Medina et al. 2014). Methane oxidation may also have been affected by the depletion of light rare earth elements required for the XoxF type methanol dehydrogenase (Shiller et al. 2017), which could alter cross-feeding interactions between methanotrophs and methylotrophs (Krause et al. 2017). In microcosm experiments, nutrient amendments (nitrate, ammonium, phosphate, and trace metals) did not significantly alter the microbial community, though they did slightly increase degradation rates at later time points (Kleindienst et al. 2015). In the circumstance of Deepwater Horizon, dissolved oxygen was sufficient such that respiration in the water column was not limited by its availability. However, other circumstances may differ, such as in hydrocarbons deposited to sediment, and discharge to low-oxygen waters, such as in the Pacific Ocean's oxygen minimum zone.

3. Microbial Community Changes During Deep-Sea Hydrocarbon Spills

Given that that only one major deep-sea oil spill has occurred to date, we focus here on community changes observed during the 2010 Deepwater Horizon spill in the Gulf of Mexico, but also discuss results from microcosm experiments in the Gulf of Mexico and elsewhere.

3.1 Community Changes During the Deepwater Horizon Oil Spill

Prior to the Deepwater Horizon spill, there was very little data on microbial community composition of the water column in the deep Gulf of Mexico. However, several samples were fortuitously collected in the northern Gulf of Mexico in March 2010, including one at collected at 800 m just nine nautical miles from the spill site. These samples were later sequenced and showed a community dominated by the SAR11 clade, other *Alphaproteobacteria*, the SAR406 clade, the SAR324 clade, and a diverse group of *Gammaproteobacteria* (King et al. 2013, Yang et al. 2016). This background community persisted throughout the spill at depths above and below the main plume and returned after the spill ended (Redmond and Valentine 2012, Yang et al. 2016).

The microbial community in the first weeks after the spill began went un-sampled, as it took more than a month to obtain resources for sample collection. Several different groups of researchers obtained samples from the deep-sea plume in late May, by which point a novel group of *Oceanospirillales* (referred to as DWH *Oceanospirillales*) had become extremely abundant, dominating 16S rRNA clone libraries, pyrosequencing datasets, metagenomes, and transcriptomes (Hazen et al. 2010, Mason et al. 2012, Redmond and Valentine 2012, Yang et al. 2016). Other samples collected at the end of May showed abundant DWH *Oceanospirillales*, but also increasing relative abundance and activity of *Colwellia* and *Cycloclasticus* (Redmond and Valentine 2012, Rivers et al. 2013, Yang et al. 2016). After the riser was cut on June 3, *Colwellia* and *Cycloclasticus* replaced the DWH *Oceanospirillales* as dominant members of the plume community (Valentine et al. 2010, Dubinsky et al. 2013).

The flow of hydrocarbons into the Gulf of Mexico ended July 15, 2010, but sampling efforts were very limited between mid-June and mid-July and sparse throughout the rest of the summer. During this time, the DWH *Oceanospirillales* and then *Colwellia* abundance in plume samples returned to levels found outside of plumes, but *Cycloclasticus* persisted at moderately elevated levels, as did some of the less abundant groups of *Gammaproteobacteria* that had also

increased earlier in the spill, such as *Pseudoalteromonas* and *Neptunomonas* (Dubinsky et al. 2013). In August and September, there was also an increase in *Flavobacteria* and *Rhodobacterales* (Redmond and Valentine 2012, Dubinsky et al. 2013). Based on this timing, they were likely consuming the remnants of the primary hydrocarbon degraders, as both groups are commonly associated with the degradation of organic matter in phytoplankton blooms (Buchan et al. 2014), but they may have the ability to directly degrade hydrocarbons as well (Guibert et al. 2016, Hu et al. 2017). The abundance of methanotrophs began to increase in mid-June, followed by methylotrophs (Dubinsky et al. 2013). Both groups were still detectable in plume samples in September 2010, despite the fact that methane concentrations had decreased to below background levels for the Gulf of Mexico (Kessler et al. 2011).

3.2 Microcosm Studies

Since the 2010 spill, several studies have used microcosms to study the effects of hydrocarbons on microbial community composition. These studies vary in their ability to replicate in-situ conditions, but allow the controlled manipulation of temperature, pressure, nutrients, dispersant, and other factors. They can also be coupled with detailed measurements of hydrocarbon degradation and techniques like stable-isotope probing (SIP) to identify the organisms consuming specific hydrocarbon substrates, as well as metagenomic sequencing. It should be noted that not all of these studies were conducted with water collected from the deep sea but may still provide insight into hydrocarbon-degrading communities in low-temperature environments or organisms that play an important role in the deep sea. These microcosm studies consistently show similar changes in community composition as observed during the Deepwater Horizon oil spill: an increase in *Gammaproteobacteria*, especially *Cycloclasticus*, *Alteromonadales* (including *Colwellia*), *Oceanospirillales,* and often *Flavobacteria* and *Rhodobacterales* (Brakstad et al. 2015, Kleindienst et al. 2015, Hu et al. 2017, Liu et al. 2017a, Techtmann et al. 2017, Ribicic et al. 2018b). However, the specific organisms within the orders *Alteromonadales* and *Oceanospirillales* often vary and the DWH *Oceanospirillales* only rarely appear.

4. Organisms

There were four major groups of bacteria that responded to the Deepwater Horizon oil spill: the DWH *Oceanospirillales*, *Cycloclasticus*, *Colwellia*, and methanotrophs/methylotrophs. Relationships between these four groups are shown in a phylogenetic tree of 16S rRNA gene sequences in Figure 1. We discuss the role of each here.

4.1 DWH Oceanospirillales

The order *Oceanospirillales* contains a number of hydrocarbon degraders, including those in the genera *Alcanivorax*, *Oleispira*, *Thalassolituus*, *Oleibacter* and *Halomonas,* but the 16S rRNA gene from the DWH *Oceanospirillales* observed in May 2010 showed just 95% similarity to the closest cultured isolate, *Spongiispira norvegica* (Hazen et al. 2010). Even within uncultured sequences, there were few close matches in GenBank at the time, though they were later reported as having been abundant offshore North Carolina in 2009 (D'Ambrosio et al. 2014, Yang et al. 2016). Oligotyping analysis showed that the DWH *Oceanospirillales* operational

taxonomic units (OTUs) that responded during the spill were distinct from those observed in the Gulf of Mexico prior to the spill, suggesting that the Deepwater Horizon spill created an unusual set of conditions for this typically rare organism to rapidly respond (Kleindienst et al. 2016a). Based on its disappearance after the riser was cut in early June, the DWH *Oceanospirillale*s may have been involved in the degradation of specific hydrocarbons that were disproportionately abundant in the early stages of the spill. Attempts at cultivation have been unsuccessful, and though single-cell genomics, metagenomics and transcriptomics have provided insight, it remains unclear why this previously uncommon hydrocarbon-degrader was so abundant in this spill.

During the time that the DWH *Oceanospirillales* were dominant, metagenomes and transcriptomes suggest that alkane oxidation was the primary hydrocarbon degradation process, while genes involved in BTEX degradation were not significant (Mason et al. 2012). Single-cell-amplified genomes (SAGs) from *Oceanospirillales* cells collected at the same time contained the genes for cyclohexane oxidation (Mason et al. 2012). Cyclohexane was abundant in the deep-sea plume, but not common in sea-surface spills, providing a possible explanation for the high abundance of this organism. However, it should be noted that the 16S rRNA gene sequences from the SAGs were only 95% similar to the dominant DWH *Oceanospirillales* OTU, hinting at some of the challenges of determining which organisms are the "same" when making inferences about function. This is particularly difficult in microcosm experiments, where the differing environmental conditions increase the likelihood of observing organisms that have closely related 16S rRNA gene sequences to those observed in situ, but quite different genomes and ability to degrade hydrocarbons.

As mentioned above, the DWH *Oceanospirillales* have generally failed to dominate or sometimes appear at all in microcosm experiments. It remains unclear whether this is due to a specific set of environmental conditions (e.g. high pressure), a preference for particular hydrocarbons that may not be included in the microcosm experiments in sufficient amounts (e.g. cyclohexane), or simply low abundance in many environments. They did increase slightly and appear to take up ^{13}C-labelled ethane and propane in SIP experiments conducted in September 2010 (Redmond et al. 2010), but decreased during microcosm experiments with oil and dispersant from 2013 (Kleindienst et al. 2015). One notable exception was claimed in a recent study by Hu et al.(Hu et al. 2017), who attempted to mimic deep-sea plume conditions by dispersing oil into 10 μm diameter droplets. By day 6 of their experiments, 33.5% of metagenome sequences were assigned to a genome bin they termed *Candidatus Bermanella macondoprimitus*, with a 16S rRNA gene sequence nearly identical to those from the Deepwater Horizon oil spill (shown in Figure 1 as GOM microcosm clone). This genome bin contained just one gene for hydrocarbon degradation, an *alk*B for alkane oxidation (Hu et al. 2017). However, subsequent comparison of this genome bin to metagenomes from the oil spill showed that *Candidatus Bermanella macondoprimitus* was in fact distinct from the organism abundant during the Deepwater Horizon spill and the *alk*B gene was not found in the Deepwater Horizon plume metagenomes (Eren et al. 2015, Delmont and Eren 2017). While the DWH *Oceanospirillales* were almost certainly involved in alkane oxidation, their precise substrate preference, the relative importance of cycloalkanes vs. *n*-alkanes, and variation between individuals remains unclear.

4.2 Cycloclasticus

Unlike the DWH *Oceanospirillales* and *Colwellia*, *Cycloclasticus* was already well known as one of the most abundant marine hydrocarbon-degrading bacteria, believed to mostly consume polycyclic aromatic hydrocarbons (PAHs) (Kasai et al. 2002, Maruyama et al. 2003, McKew et al. 2007, Wang et al. 2008). *Cycloclasticus* was detected in the earliest plume samples and persisted throughout the course of the spill (Dubinsky et al. 2013). It was still readily detectable in plume remnant samples in September 2010, suggesting that it was indeed consuming the more recalcitrant PAH compounds remaining at that point (Redmond and Valentine 2012). However, its much higher abundance earlier in the summer was likely due to its ability to oxidize ethane and propane. SAGs from June 2010 show that *Cycloclasticus* was abundant when ethane and propane oxidation rates were high (Valentine et al. 2010) and contained short-chain hydrocarbon monooxygenases as well as the rest of the genes required for ethane and propane oxidation (Rubin-Blum et al. 2017). In a separate metatranscriptomic study, these hydrocarbon monooxygenases were some of the most highly expressed genes (Rivers et al. 2013), suggesting ethane and propane oxidation played a role in their rapid growth early in the spill.

It remains unclear whether short-chain alkane oxidation is common in *Cycloclasticus* and how this process affects or is affected by PAH degradation. Cultured *Cycloclasticus* were isolated as PAH degraders and lack the hydrocarbon monooxygenase genes detected in the SAGs (Lai et al. 2012, Cui et al. 2013, Messina et al. 2016). However, similar genes were observed in metagenome assembled genomes (MAGs) from the *Cycloclasticus* symbionts of mussel and sponges in the Gulf of Mexico (Rubin-Blum et al. 2017) and MAGs from *Cycloclasticus* in microcosm studies conducted in the Gulf of Mexico several years after the Deepwater Horizon spill (Hu et al. 2017). *Cycloclasticus* 16S rRNA gene sequences and similar hydrocarbon monooxygenase genes were also detected in ethane SIP experiments at hydrocarbon seep sediments in the Coal Oil Point seep field offshore Santa Barbara, CA (Redmond et al. 2010), so short-chain alkane oxidation may be common in *Cycloclasticus* living in regions where hydrocarbon seeps release both oil and natural gas. Interestingly, the mussel and clam symbionts appear to have lost the genes for PAH degradation, leaving them dependent on ethane and propane oxidation, whereas the water column SAGs and MAGs from the oil-spill and microcosm experiments retain genes for PAH oxidation (Hu et al. 2017, Rubin-Blum et al. 2017).

4.3 Colwellia

Prior to the Deepwater Horizon spill, *Colwellia* had not been widely recognized as an important hydrocarbon degrader, though it had been identified in oil-contaminated Arctic sea ice and Antarctic seawater sediments (Powell et al. 2004, Yakimov et al. 2004, Brakstad et al. 2008). *Colwellia psychrerythraea* had been studied as a model of psychrophily, with isolates unable to grow above 20°C (Methé et al. 2005), and the low temperature appears to have played a role in its dominance during the Deepwater Horizon spill (Redmond and Valentine 2012). It was abundant throughout May and June, especially after the decrease in the DWH *Oceanospirillales*. SIP suggested that *Colwellia* was involved in ethane, propane, and benzene oxidation, though it was also capable of growing on crude oil as the sole carbon source (Redmond and Valentine 2012). Other studies have shown that a *Colwellia* isolate was able to degrade *n*-alkanes and

hydrocarbon components of dispersant (Bælum et al. 2012) and that they incorporated ^{13}C-phenanthrene (Gutierrez et al. 2013), indicating that the genus has the potential for widespread hydrocarbon metabolism. It has since been observed in many microcosm studies conducted with low temperature seawater from around the world and linked to the degradation of both *n*-alkanes and aromatics (Brakstad et al. 2015, Kleindienst et al. 2015, Campeão et al. 2017, Hu et al. 2017, Bacosa et al. 2018, Lofthus et al. 2018, Ribicic et al. 2018a).

4.4 Methanotrophs and Methylotrophs

Methane was the most abundant compound released during the Deepwater Horizon spill, accounting for 15% of the total mass of hydrocarbons released (Reddy et al. 2012). It dissolved completely into the deep-sea hydrocarbon plume, where it made up approximately 60% of the mass of soluble hydrocarbons in the plume (Ryerson et al. 2012), but methanotrophs were much slower to respond than the other hydrocarbon degraders (Redmond and Valentine 2012, Dubinsky et al. 2013). In June, methane-oxidation rates were much lower that ethane- and propane-oxidation rates, and known methane oxidizers weren't detected in 16S rRNA gene clone libraries (Valentine et al. 2010). However, between June and September, methane was completely consumed, leaving a decrease in dissolved oxygen in the hydrocarbon plume and a residual community of methanotrophs and methylotrophs (Kessler et al. 2011). The methanotrophs were *Gammaproteobacteria* from the family *Methylococcaceae*, while the methylotrophs were from the *Gammaproteobacterial* genus *Methylophaga* and the *Betaproteobacterial* family *Methylophilaceae*. There was some debate over the role of the methylotrophs, as they are generally assumed to be capable of consuming methanol and other C1 compounds, but not performing the first step of methane oxidation, the oxidation of methane to methanol (Joye et al. 2011). *Methylophaga* has also been shown to consume hexadecane (Mishamandani et al. 2014) and respond rapidly to the addition of high molecular weight dissolved organic matter (McCarren et al. 2010). Its appearance in microcosm experiments with oil and no methane (Hu et al. 2017) suggests that the presence of methane certainly is not a requirement for growth. However, cross-feeding between methanotrophs and methylotrophs, presumably due to excretion of methanol, has been well documented and may be mediated by the availability of limiting nutrients (Redmond et al. 2010, Beck et al. 2013, Krause et al. 2017, Yu and Chistoserdova 2017). This makes methane oxidation an equally plausible explanation for the presence of *Methylophaga* and especially *Methylophilaceae,* and it seems likely that they acted as facultative methylotrophs incorporating carbon from both methane and petroleum.

The lack of data during the critical time period of methane loss leaves a number of unresolved questions about the response of methanotrophs during deep-sea hydrocarbon spills. Though Valentine et al. measured low methane-oxidation rates near the wellhead in mid-June (Valentine et al. 2010), Crespo-Medina et al. detected some sites with extremely high methane-oxidation rates a few weeks earlier (Crespo-Medina et al. 2014). While we suspect the elevated rates measured by Crespo-Medina are partially due to an incubation artifact resulting from an unavoidable 1-2 week delay between sample collection and tracer-rate substrate amendment (Crespo-Medina et al. 2015), they may also be explained by differences in sampling locations and spatial heterogeneity in hydrocarbon distributions and ocean circulation (Valentine et al. 2012). The microbial community data clearly shows that methanotrophs were slower to increase

than other hydrocarbon degraders and were not abundant in any samples collected in May and early June (Redmond and Valentine 2012, Dubinsky et al. 2013). The dominant "methane" monooxygenase genes measured by Crespo-Medina et al. in May and early June are related to the putative ethane and propane monooxygenases from *Cycloclasticus* (Rubin-Blum et al. 2017), though we know little about their substrate specificity and cannot exclude the possibility that they are also capable of some methane oxidation. Methanotrophs finally began to increase in mid-June (Dubinsky et al. 2013). It's unclear whether the presence of other hydrocarbons or hydrocarbon oxidizers inhibited the growth of methanotrophs, or if they were simply slower to respond to the increase in available substrate; methane oxidation has also been shown to lag ethane and propane oxidation at natural hydrocarbon seeps, suggesting this pattern may be common (Mendes et al. 2015). Despite the extended lag time, methane was completely consumed by the end of August (Kessler et al. 2011).

5. Research Needs

Another deep-sea oil spill is inevitable. In order to better predict the response of microbial communities to a future spill, several lines of research would be useful:

- **Is the Gulf of Mexico representative of the deep-sea elsewhere?** The vast majority of the research in response to the Deepwater Horizon spill has been focused on the Gulf of Mexico. Though the Gulf of Mexico is certainly a likely location for a future spill, significant deepwater and ultra-deepwater drilling also occur offshore Norway, Angola, and Brazil, and a tanker spill or large ship wreck could cause an oil spill anywhere in the world. Would a deep-sea oil spill somewhere else result in similar microbial communities and degradation rates? Studies from the Eastern Mediterranean (Liu et al. 2017b) and Amazon basin (Campeão et al. 2017) show some similarities, but more extensive study is needed, particularly in areas without natural seepage to provide a background population of hydrocarbon degraders or where low oxygen or nutrient concentrations limit microbial activity.

- **How does the presence of natural gas affect microbial communities and degradation of petroleum hydrocarbons?** The major PAH degrader in the Deepwater Horizon spill, *Cycloclasticus*, had the genetic potential to metabolize both the short-chain alkanes in natural gas and PAHs in oil (Rubin-Blum et al. 2017). Ethane- and propane-oxidation rates were high early in the summer (Valentine et al. 2010) and ethane and propane monooxygenases highly expressed (Rivers et al. 2013), suggesting that *Cycloclasticus* may have preferentially oxidized ethane and propane relative to PAHs. Did this delay the onset of PAH degradation, or did a different group of *Cycloclasticus* initiate growth on PAHs? Alternatively, did ethane and propane stimulate the growth of *Cycloclasticus* and ultimately increase PAH degradation rates?

- **What are the functions of individual members of the hydrocarbon-degrading community?** Many inferences about the function of organisms responding to the Deepwater Horizon spill were made through culture-independent techniques such as

single-cell and metagenomic sequencing, which will become even more common in future oil spills. However, the ability to make inferences about function from genome sequences is still limited in terms of specificity (e.g. substrate range of an alkane monooxygenase) and likely misses novel or poorly characterized genes involved in hydrocarbon oxidation. Culture-dependent or culture-independent efforts to better link gene sequences to enzyme function would improve such predictions.

- **How does water-column depth impact the microbial response?** Would there be a difference between a spill at 300 m and 3000 m? The difference in temperature and pressure would affect microbial communities directly and would also affect hydrocarbon solubility which could indirectly affect community composition. Nutrient and oxygen availability may vary as well. Microcosm experiments conducted at atmospheric pressure have limited utility in answering this question and additional efforts should be made to understand the effect of pressure on hydrocarbon degrading communities.

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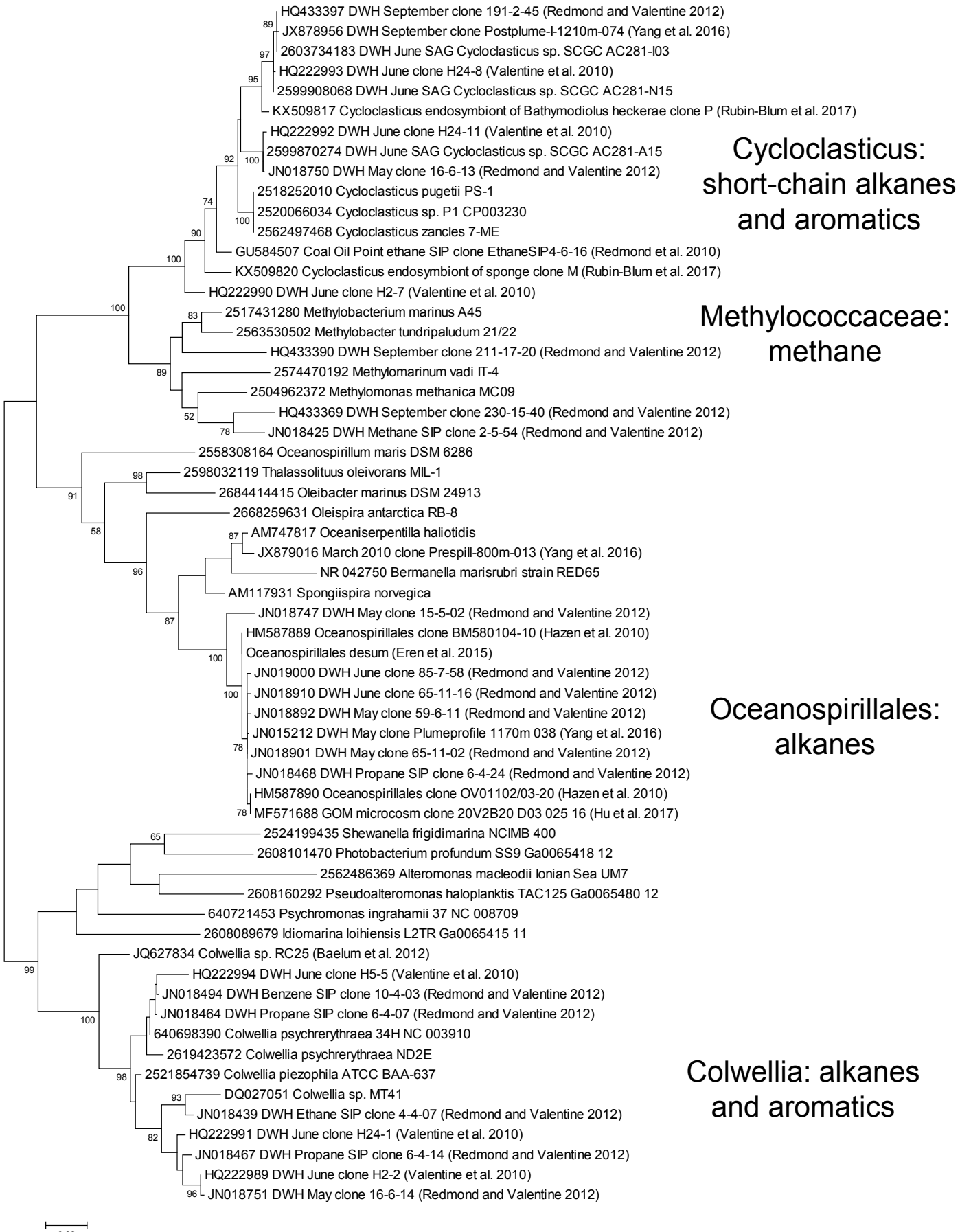

Figure 1. Maximum likelihood tree of 16S rRNA gene sequences from the major groups of Gammaproteobacteria responding to the Deepwater Horizon oil spill and their cultured relatives. Sequences are identified by GenBank accession number or IMG gene ID.