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# Responses of Microbial Communities to Hydrocarbon Exposures

By Samantha B. Joye, Sara Kleindienst,  
Jack A. Gilbert, Kim M. Handley, Pam Weisenhorn,  
Will A. Overholt, and Joel E. Kostka

“ The impact of hydrocarbon pollution on the composition, structure, and function of microbial communities is evident in the responses of taxa able to use hydrocarbons as sources of carbon and energy. ”

**ABSTRACT.** The responses of microbial communities to hydrocarbon exposures are complex and variable, driven to a large extent by the nature of hydrocarbon infusion, local environmental conditions, and factors that regulate microbial physiology (e.g., substrate and nutrient availability). Although present at low abundance in the ocean, hydrocarbon-degrading seed populations are widely distributed, and they respond rapidly to hydrocarbon inputs at natural and anthropogenic sources. Microbiomes from environments impacted by hydrocarbon discharge may appear similar at a higher taxonomic rank (e.g., genus level) but diverge at increasing phylogenetic resolution (e.g., sub-OTU [operational taxonomic unit] levels). Such subtle changes are detectable by computational methods such as oligotyping or by genome reconstruction from metagenomic sequence data. The ability to reconstruct these genomes, and to characterize their transcriptional activities in different environmental contexts through metatranscriptomic mapping, is revolutionizing our ability to understand the diverse and adaptable microbial communities in marine ecosystems. Our knowledge of the environmental factors that regulate microbial hydrocarbon degradation and the efficiency with which marine hydrocarbon-degrading microbial communities bioremediate hydrocarbon contamination is incomplete. Moreover, detailed baseline descriptions of naturally occurring hydrocarbon-degrading microbial communities and a more robust understanding of the factors that regulate their activity are needed.

## INTRODUCTION

Oil is introduced into the marine environment through natural seepage and as a result of human activities, including pipeline and tanker leaks and spills, for example, the *Exxon Valdez* oil spill in 1989, and in some cases large accidental ocean discharges, for example, the Ixtoc blowout in the southern Gulf of Mexico in 1979 and the BP/Deepwater Horizon (DWH) discharge in 2010, which ranks as the largest marine open

water hydrocarbon discharge to date. On April 20, 2010, operators lost well control on the DWH mobile offshore drilling unit. A subsequent gas-fueled explosion resulted in the sinking of the platform two days later. Upon sinking, the riser pipe separated from the drilling platform, generating an uncontrolled oil well blowout at the seafloor.

The DWH well blowout discharged approximately five million barrels of oil and at least 250,000 metric tonnes of

natural gas to the deep waters (~1,500 m) of the Gulf of Mexico (Joye, 2015). Some seven million liters of chemical dispersants, mainly Corexit 9500 and 9527A, were applied as a response measure at the sea surface and at the discharging well-head. Of the discharged oil and gas, all of the low molecular weight alkanes (methane through propane) and half of the discharged oil were entrained in a deep-water plume at a depth of approximately 1,000 m (Joye, 2015). The microbial response to this hydrocarbon infusion, especially at low deep-ocean temperatures, was swift and remarkable (Joye et al., 2014; Kleindienst et al., 2015a).

Oil is a mixture of hydrocarbons, which are organic molecules consisting of carbon atoms bonded to each other and to hydrogen atoms. Some complex hydrocarbons contain nitrogen and sulfur residues (Seidel et al., 2016), as well as metalloids; oxygen is introduced into hydrocarbons during biodegradation and weathering (Aeppli et al., 2012). The major hydrocarbon classes include saturates (e.g., linear, branched, and cyclic alkanes), aromatics (where single and double bonds exist and help to stabilize the compound), resins, and asphaltenes. A number of aromatic hydrocarbons are toxic, making it pragmatic to

understand the potential of microbial populations to moderate the impacts of hydrocarbon pollution. Identifying the microorganisms responsible for oil biodegradation and understanding the factors that regulate bioremediation in the marine environment is critical.

The ability to degrade hydrocarbons is widespread among the bacteria, methanogenic archaea, and fungi (Leahy and Colwell, 1990; Head et al., 2003, 2006). These microorganisms degrade oil and gas, either partially or completely, and reduce negative environmental

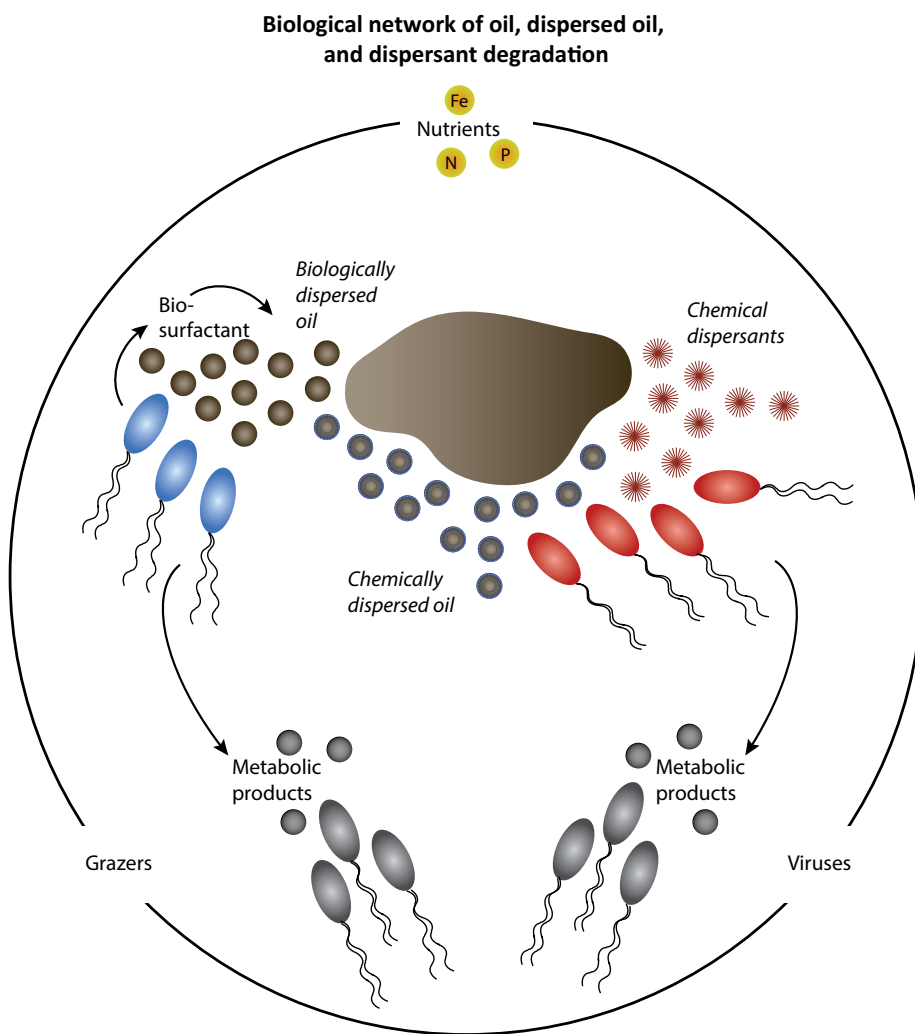
impacts (Figure 1). Microbial hydrocarbon degradation occurs under oxic, microaerophilic, and anoxic conditions (Head et al., 2003). Complete hydrocarbon oxidation is achieved through the collective action of associated, interdependent microorganisms. Though the metabolic pathways of hydrocarbon oxidation are similar at the genus level, primary pathways are linked to more taxonomically diverse secondary pathways (Heider and Rabus, 2008). Aerobic biodegradation has received more attention than anaerobic biodegradation, but

anaerobic pathways are more novel and complex (Widdel et al., 2010).

The impact of hydrocarbon pollution on the composition, structure, and function of microbial communities is evident in the responses of taxa able to use hydrocarbons as sources of carbon and energy. Many of these organisms exist as part of the “rare biosphere,” a “seed bank” of taxa (Gibbons et al., 2013) that are ecologically noncompetitive, except when exposed to hydrocarbons (Kleindienst et al., 2015a). Spatiotemporal investigations of microbial community responses to oil pollution revealed the influence that blooms of conditionally rare, opportunistic taxa have on community structure and function (Lu et al., 2012; Mason et al., 2012; Kleindienst et al., 2015a). Subsequent studies explored how community changes altered the broader ecological properties of polluted environments; for example, metagenomic analysis of oil-polluted sediments showed that their microbial communities had an elevated potential for anaerobic ammonium oxidation, or anammox (Scott et al., 2014).

Large-scale hydrocarbon inputs stimulate oxygen consumption as a consequence of accelerated aerobic microbial activity. When oxygen is depleted, anaerobic hydrocarbon metabolism is coupled to sulfate and nitrate reduction, which fundamentally shifts the nitrogen, sulfur, and carbon cycles, and promotes further changes in microbial structure and composition as a function of breakdown products and cross-feeding (Kleindienst et al., 2015b). After hydrocarbon exposure, the community may return to its original ecological functional state or be altered, with certain taxa increasing in abundance following hydrocarbon bioremediation and persisting on a time scale of years post-disturbance (Kleindienst et al., 2015a).

In this article, we describe the pathways of hydrocarbon degradation in the environment, the methods used to quantify hydrocarbon degradation rates and the microorganisms that mediate these reactions, and how microbial populations respond to hydrocarbon inputs.



**FIGURE 1.** Biological network of oil, dispersed oil, and dispersant degradation. Hydrocarbon-oxidizing microbes with the capability to produce biosurfactants to facilitate oil degradation are shown in blue. It remains a question as to whether the activity of these microorganisms is stimulated or inhibited by chemical dispersants. Different types of hydrocarbon degraders, shown in red, have the ability to degrade chemically dispersed oil as well as dispersants (e.g., *Colwellia* sp. RC25). Secondary metabolite consumers of compounds produced during oil biodegradation, for which dispersant impacts are largely unknown, are shown in gray. Parts of this network (nutrient availability, viruses, and grazers) likely influence all the above types of microorganisms. Illustration based on Head et al. (2006)

## PATHWAYS OF HYDROCARBON DEGRADATION

Petroleum is a complex mixture of ~15,000+ compounds formed from thermogenic alteration of organic matter deposited in sediments tens of millions of years ago (Marshall and Rodgers, 2004). Heterotrophic microorganisms (microbes that obtain their metabolic energy and cellular carbon from organic carbon compounds) use a plethora of metabolic pathways for consuming hydrocarbons in the largely nutrient-limited marine environment. The majority of research to date has emphasized the pathways for degradation and/or transformation of aliphatic and aromatic hydrocarbons, since these dominate crude oils and are gas chromatography (GC) amenable and thus easier to study (Fuchs et al., 2011; Abbasian et al., 2015; Ladino-Orjuela et al., 2016).

### Aerobic Degradation

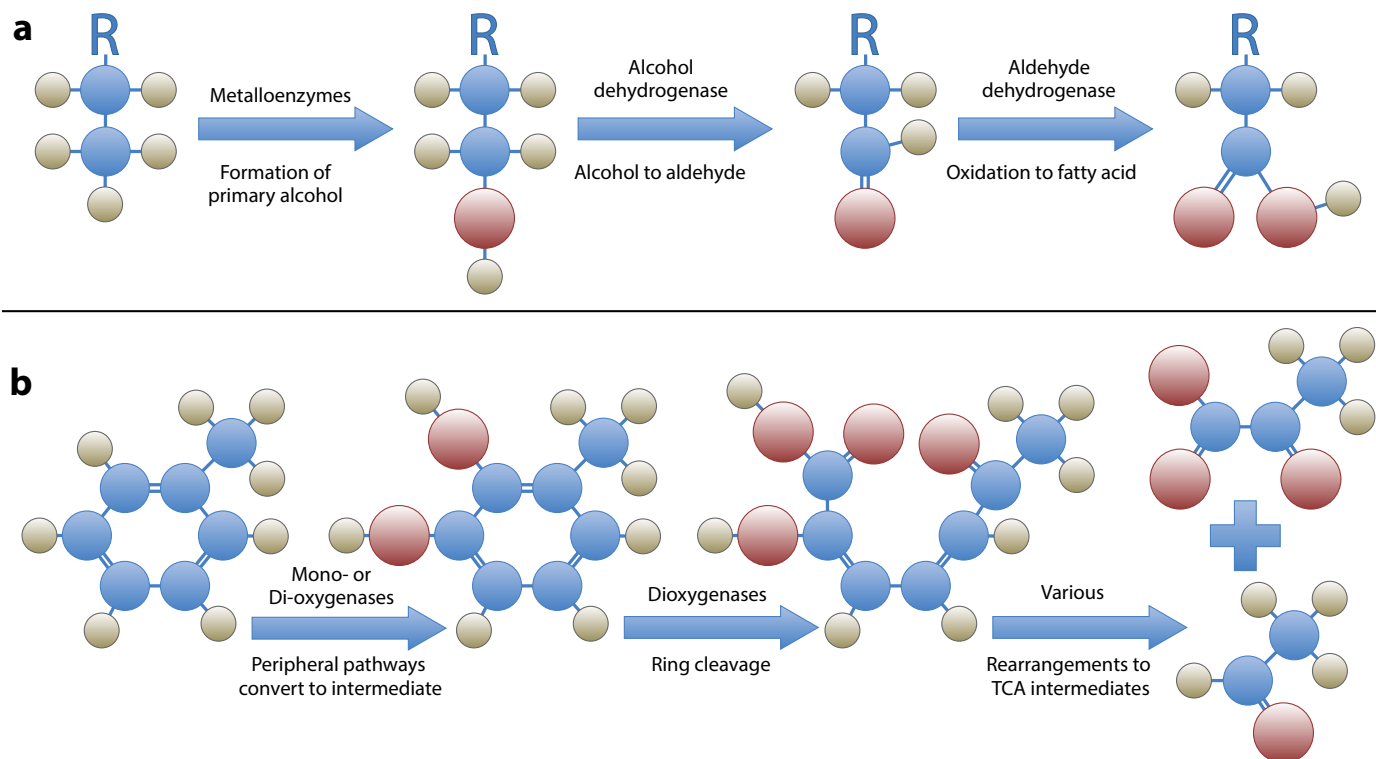
The aerobic degradation of alkanes, particularly *n*-alkanes, is well documented (Wang and Shao, 2013; Figure 2a). Alkanes are distributed ubiquitously, and a number of mechanisms activate them by breaking strong C-H bonds, an energetically demanding process. The initial step in aerobic alkane degradation transforms the terminal carbon into a primary alcohol, which is subsequently oxidized to the corresponding aldehyde via an alcohol dehydrogenase, followed by oxidation to a fatty acid by an aldehyde dehydrogenase. Fatty acids are then processed via beta-oxidation or converted to phospholipids and incorporated into the cellular membrane. Most alkane hydroxylases are metalloenzymes that incorporate metal species into the active site to activate oxygen and attack the C-H bond.

Short chain alkanes ( $C_1$ - $C_4$ , gaseous *n*-alkanes) are oxidized by two known

groups of metalloenzymes, particulate methane monooxygenase (pMMO) and soluble methane monooxygenases, and their homologs (e.g., propane monooxygenase and butane monooxygenase). Particulate methane monooxygenases use a di-copper active site and can oxidize up to  $C_5$  *n*-alkanes. Soluble methane monooxygenases lie within the large bacterial multicomponent monooxygenase family (BMM), which have a non-heme di-iron active site, and can oxidize up to  $C_8$  alkanes, including branched alkanes, cycloalkanes, and even small aromatics.

Mid-length alkane ( $C_5$ - $C_{16}$ ) hydroxylases fall into two main classes, membrane associated non-heme di-iron monooxygenases (AlkB) that share no homology to bacterial multicomponent monooxygenases, and heme-based cytochrome P450 (CYP153) enzymes. Both classes are highly diverse, often found together in hydrocarbon-degrading bacteria, and

### Key Pathways of Hydrocarbon Degradation



**FIGURE 2.** Generalized aerobic alkane and aromatic hydrocarbon degradation pathways. (a) Degradation of alkanes from primary alcohol formation on a terminal or subterminal C via metalloenzymes (e.g., pMMO, AlkB) followed by conversion of the alcohol to an aldehyde via alcohol dehydrogenase, and finally oxidation of the aldehyde to a fatty acid via aldehyde dehydrogenase. (b) Degradation of a sample aromatic hydrocarbon, toluene, to a central intermediate (e.g., 3-methyl-catechol) followed by ring cleavage and final rearrangement to TCA cycle intermediates. The first two steps in aromatic hydrocarbon degradation are performed by mono- or di-oxygenases.

have extensive overlapping substrate ranges. These two enzyme classes perform terminal alkane oxidation, resulting in a primary alcohol. The majority of AlkB alkane hydroxylases preferentially act upon C<sub>10</sub> to C<sub>18</sub> alkanes, although the best-studied AlkB from *Pseudomonas putida* GPo1 preferentially uses C<sub>5</sub> to C<sub>13</sub> *n*-alkanes (van Beilen and Funhoff, 2007; Koch et al., 2009). Many model alkane-degrading microorganisms contain multiple AlkB homologs (van Beilen and Funhoff, 2007). Genes encoding AlkB are found ubiquitously in the ocean.

Heme-containing cytochrome P450 enzymes are found in all domains of life. Bacterial alkane hydroxylase cytochrome P450s are soluble and primarily act upon *n*-alkanes between C<sub>6</sub> and C<sub>15</sub> (van Beilen and Funhoff, 2007). Long-chain alkane (C<sub>17+</sub>) oxidation enzymes are not well characterized and only a few pathways are known. The best described are two enzymes that share no apparent homology and use a flavin cofactor. AlmA, a flavin-binding monooxygenase, is thought to act upon C<sub>20</sub> to >C<sub>32+</sub> *n*-alkanes and was first identified in *Acinetobacter* sp. DMS17874. Homologues have been identified in other oil-degrading bacteria (Throne-Holst et al., 2007; Wang and Shao, 2013). LadA is a member of the SsuD bacterial luciferase subfamily that oxidizes C<sub>15</sub> to C<sub>36</sub> alkanes; this gene has been documented in a thermophilic *Geobacillus* genus (Feng et al., 2007).

### Anaerobic Degradation

Under anaerobic conditions, there are two mechanisms for alkane activation involving fumarate addition to a sub-terminal or terminal carbon (in a case of propane activation) to produce a substituted succinate compound. Enzymes in this pathway are known as alkylsuccinate synthases (ASS) or 1-methylalkyl succinate synthases (MAS), and they most likely function through the generation of a glycyl radical (Widdel and Rabus, 2001). These enzymes share homology and a similar mechanism to the

benzylsuccinate synthase involved in toluene degradation (see below). After fumarate addition, Coenzyme A (CoA) is added via a CoA transferase, followed by carbon skeletal rearrangements via a mutase, followed by decarboxylation, analogous to carbon rearrangements mediated by methylmalonyl-CoA mutase (Wilkes et al., 2002).

A diverse set of peripheral pathways transform aromatic compounds into one of a few key central intermediates (Fuchs et al., 2011; Ladino-Orjuela et al., 2016; Figure 2b). Under aerobic conditions, these are typically monooxygenases or dioxygenases that hydroxylate the aromatic compound to produce catechol, primarily protocatechuate, gentistate, or homogentistate (Fuchs et al., 2011). The aromatic ring component of these intermediates is cleaved by two oxygen-dependent strategies. Dioxygenases cleave hydroxyl-substituted aromatic rings; the  $\beta$ -keto adipate pathway is a well-known example (Ornston and Stanier, 1966). Alternately, the hydroxylated aromatic ring is further substituted with CoA followed by ring cleavage using epoxidases belonging to the bacterial multicomponent monooxygenase family, including benzoate and phenylacetate epoxidation (Fuchs et al., 2011). The resulting compounds are often incorporated into central metabolism as acetyl-CoA, succinyl-CoA, and pyruvate, and fed into the TCA cycle.

Anaerobic aromatic hydrocarbon degradation pathways are diverse and represent different mechanisms that generate a few key central intermediates, of which benzoyl-CoA is the most well known (Harwood et al., 1998; Foght, 2008; Fuchs et al., 2011). For example, toluene degradation is initiated by fumarate addition through benzylsuccinate synthase (BSS) via a glycyl radical, homologous to anaerobic alkane degradation through fumarate addition, as mentioned above. Unsubstituted aromatics may be methylated, directly carboxylated, or hydroxylated before conversion to benzoyl-CoA (Foght, 2008). Following the generation

of benzoyl-CoA, the aromatic ring is susceptible to reduction reactions outlined in Harwood et al. (1998). The first step is catalyzed by a class I benzoyl-CoA reductase (BcrABCD), which requires 2 ATP. An ATP-independent mechanism that employs a non-homologous class II benzoyl-CoA reductase (BamBCDEFGHI) is likely driven by electron bifurcation (Fuchs et al., 2011).

Little is known about the metabolic pathways involved in asphaltene and resin degradation (Lavania et al., 2012). These very high molecular weight, heteroatom-containing polar structures are resistant to biodegradation and accumulate when crude oil is biodegraded (Head et al., 2006). A few microorganisms, including *Garciaella petrolearia* TERIG02 (bacterium) and *Neosartorya fischeri* (fungus), degrade asphaltenes in heavy crude oils. *G. petrolearia* preferentially degraded asphalt under anaerobic conditions, producing CO<sub>2</sub>, H<sub>2</sub>, as well as organic acids, smaller aromatics, and *n*-alkanes (Lavania et al., 2012). The underlying mechanism and genetic pathways involved are not yet known. The fungus *N. fischeri* also grows on asphaltenes as a sole carbon source (Hernández-López et al., 2016), possibly using cytochrome P450 monooxygenases to process asphaltenes. Other fungal isolates degrade high molecular weight polycyclic aromatic hydrocarbons (PAHs) using cytochrome P450s (Syed et al., 2011). Interestingly, the resistance of asphaltenes and resins to degradation may be due to their low solubility in seawater rather than to their high molecular weight and chemical complexity (Marin-Spiotta et al., 2014).

### QUANTIFYING BIODEGRADATION RATES AND MICROBIAL POPULATIONS

Quantifying microbial oil degradation rates in environmental samples is complicated due to the composition range and differential volatility of the hydrocarbon pool. Direct and indirect approaches are used to estimate hydrocarbon oxidation

rates. Direct rate measurements involve tracking  $^{14}\text{C}$ ,  $^{13}\text{C}$ , or  $^3\text{H}$  labeled substrates into oxidized products ( $^{14}\text{C}$  or  $^{13}\text{C}\text{-CO}_2$ , or  $^3\text{H}\text{-H}_2\text{O}$ ; Richnow et al., 1998). Indirect rate assessments involve the use of proxies—such as cell counts,  $\text{CO}_2$  production rates, the rate of oxidant consumption (e.g., oxygen, nitrate, sulfate), the rate of oil depletion (approximately, the concentration change over time), or bacterial production rates following exposure to a specific hydrocarbon (e.g., hexadecane or naphthalene) or to bulk crude oil (Kleindienst et al., 2015c)—to estimate hydrocarbon oxidation rates. Proxy metrics are not specific and, as such, these data should be interpreted with caution. In particular, using changes in cell counts or bacterial production over time can be misleading because bottle effects and availability of other carbon substrates, for example, chemical dispersants, could alter these parameters in the absence of elevated hydrocarbon degradation rates (Kleindienst et al., 2015b,c).

Direct measurement of the turnover of specific hydrocarbon substrates using radiolabeled tracers provides a robust means of documenting the patterns of hydrocarbon degradation and elucidating the environmental factors that drive these patterns (Kleindienst et al., 2015b,c; Sibert et al., 2016). During the DWH response, dissolved gas (e.g., methane, ethane, and propane) oxidation rates were directly measured using stable and radio-labeled isotopic substrates (Valentine et al., 2010, and Crespo-Medina et al., 2014, respectively). However, oil degradation rates were inferred from concentration changes over time (Hazen et al., 2010, for alkanes in deep waters) or through measurements of oxygen and bulk hydrocarbon consumption (Edwards et al., 2011, in surface waters); rates were not measured directly using isotopic tracers, making it difficult to constrain the fate of hydrocarbons during the DWH incident (Joye, 2015).

Considerable knowledge of the microbial biodegradation of crude oil, with

particular focus on the bioremediation of oil spills in the environment, exists. A number of studies examined how crude-oil-associated bacteria metabolize fractions of complex hydrocarbon mixtures to optimize refining processes. Microbial processes such as biodesulfurization, biodemulsification, biodenitrogenation can enhance oil recovery, control souring, and enhance remediation. Methods used to identify these organisms range from culture-dependent approaches used to grow and isolate particular organisms to metagenomic-derived assembly of genomes of organisms associated with these processes from complex microbial populations.

Culture-dependent studies provide access to a viable organism for which the genome can be characterized, and then the specific functional potential is validated based on functional tests, for example, enzymatic activity and transformation of specific compounds. However, our ignorance of the conditions necessary for successful cultivation of many organisms, coupled to a lack of understanding of how ecological factors such as competitive exclusion and niche differentiation influence growth in vitro, mean cultivation-dependent techniques likely underestimate the range of microorganisms that can directly and indirectly access hydrocarbon mixtures for energy and biomass production.

Application of amplicon sequencing approaches is now routine, while single-cell genomic, metagenomic, and transcriptomic (“-omics”) approaches are fast becoming routine for exploring microbial system dynamics (Knight et al., 2012). An alternative approach for determining the biological contribution to hydrocarbon degradation is  $^{13}\text{C}$  DNA-based stable isotope probing (DNA-SIP). Studies during the DWH response (Gutierrez et al., 2013) identified a wide range of bacteria in the isotopically heavy DNA fraction that were potentially responsible for degrading alkane PAHs.

DNA-SIP may be susceptible to the effects of indirect heavy isotope

enrichment by organisms consuming the degradation products of primary hydrocarbon degraders (i.e., cross-feeding). However, DNA-SIP has the advantage of overcoming the uncertainty associated with interpreting the putative function of environmental genes resembling known hydrocarbon degradation genes and the substrate promiscuity of many enzymes involved in hydrocarbon degradation (e.g., van Beilen et al., 1994). A suite of -omics approaches demonstrated that hydrocarbon-infusion-induced enrichment of expressed genes associated with aliphatic hydrocarbon degradation, and plume-derived representatives of abundant *Oceanospirillales* and *Colwellia* bacteria, had the genetic capacity to degrade these hydrocarbons during the DWH incident (Mason et al., 2012, 2014).

Amplicon sequencing offers only a snapshot of the taxonomic and phylogenetic breadth of microbial community structure. Generating a detailed assessment of the functional potential of key organisms requires characterization of the metagenome, the sum of genomic information for all organisms within an ecosystem. Normally, metagenomic analyses are restricted to virus or microbial genomes, owing to their small genome sizes (Gilbert and Dupont, 2011; Knight et al., 2012). Validation of the functional role of these microorganisms, especially with relevance to specific functional genomic potentials, requires that multiple -omics technologies be applied to the same sample and/or that direct rate assays be carried out in concert with -omics studies (Kleindienst et al. 2015b).

The application of metatranscriptomics to communities of organisms reveals which genes are being transcribed into mRNA by community members under specific conditions. Metaproteomics takes this analysis one stage further to ask the question as to whether the proteins predicted to be produced from genes and mRNAs by a community of cells have actually undergone post-transcriptional modification and appropriate folding to produce a potentially active molecule; this

technique has been used to great effect to validate predictions of potential protein production by communities in complex soil systems. Finally, the outcome of microbial activity is captured by the metabolome, the metabolites and signaling molecules generated and consumed by the community. These approaches can be combined through computational modeling techniques to predict how microbial communities will change, as well as the mechanisms by which they influence the turnover of hydrocarbons in the environment (Gilbert and Henry, 2015), and they have been used to determine the impact of the DWH spill on seafloor nitrogen cycling in the Gulf of Mexico (Scott et al., 2014).

## MICROBIAL RESPONSE TO HYDROCARBON INPUTS

### Hydrocarbon Degradation in Waters, Muds, and Sands

Opportunistic microorganisms with the biochemical ability to aerobically or anaerobically degrade hydrocarbons (Head et al., 2006; Widdel et al., 2010) occur ubiquitously across marine ecosystems in the water column, in sediments, and in beach sands and marsh muds (Atlas et al., 2015). Present at relatively low abundance, these key microbial players are members of the rare biosphere (Sogin et al., 2006; Kleindienst et al., 2015a) and typically comprise <10% of microbial communities in the Gulf and elsewhere (Yang et al., 2014). Hydrocarbon-degrading seed populations can respond with incredible speed to massive perturbations (Kleindienst et al., 2015a) and even natural seepage (Ruff et al., 2015). Microbial hydrocarbon degraders fall within the *Gammaproteobacteria* (e.g., the *Oceanospirillum*, *Colwellia*, *Cycloclasticus*, *Pseudoalteromonas*, *Alkanivorax*, *Alteromonas*, and *Marinobacter*), the *Betaproteobacteria* (e.g., *Acidovorax*, *Burkholderia*), the *Alphaproteobacteria* (e.g., *Roseobacter*), numerous *Deltaproteobacteria*, as well as Actinomycetales (e.g., *Acinetobacter*), *Bacillus*, and other taxa (Figure 3).

The environment locally selects the type of microorganisms that are active, and these microbes boost their activity/abundance in response to hydrocarbon inputs. Crucial factors for enriching hydrocarbon-degrading microorganisms include the availability and concentrations of hydrocarbons and the types of bioavailable hydrocarbons (e.g., short-chain and longer-chain alkanes, PAHs). Petroleum- or natural gas-derived hydrocarbon mixtures contain similar constituents, although the relative abundance of hydrocarbons, including potentially toxic BTEX (benzene, toluene, ethylbenzene, and xylenes) and PAH compounds, varies significantly. Furthermore, abiotic processes such as weathering, absorption, and diffusion influence the concentrations and bioavailability of hydrocarbons.

The availability of electron acceptors is another factor that determines the type of hydrocarbon-degradation metabolism (e.g., aerobic or anaerobic respiration), typically favoring the most thermodynamically favorable process. However, if electron donors (i.e., hydrocarbons) are present in excess, competing respiration processes may occur contemporaneously rather than in series, dictated by the electron acceptor energy yield. Additional important selecting factors include the availability of nutrients (e.g., phosphorus, nitrogen, essential trace metals), pH, temperature, and pressure. Biotic processes further influence hydrocarbon-degrading microbial responses. Hydrocarbon degraders are part of a biological network composed of additional microbial community members, viruses, and grazers (Head et al., 2006) and thus are likely affected by interactions such as syntrophic relationships, competition, transfer of genetic material, and predation (Figure 1).

The detection and identification of key microorganisms that respond to hydrocarbon inputs is essential for understanding the environmentally relevant biogeochemical processes at natural hydrocarbon seeps and for the

(re)assessment of bioremediation and response strategies in the event of anthropogenic hydrocarbon discharges. Natural hydrocarbon seep communities harbor distinct bacterial and archaeal taxa linked to key biogeochemical functions, such as hydrocarbon degradation. Within these core groups, high diversity was observed at natural seeps (Ruff et al., 2015) and also during anthropogenic oil spills (Kleindienst et al., 2015a,b), underscoring the activity of specialized subpopulations or ecotypes. Because the environmental parameters at natural seeps are substantially different than those existing during an anthropogenic hydrocarbon release, the taxa and ecotypes endemic to natural seeps may not be active during oil spills (Kleindienst et al., 2015b) and vice versa.

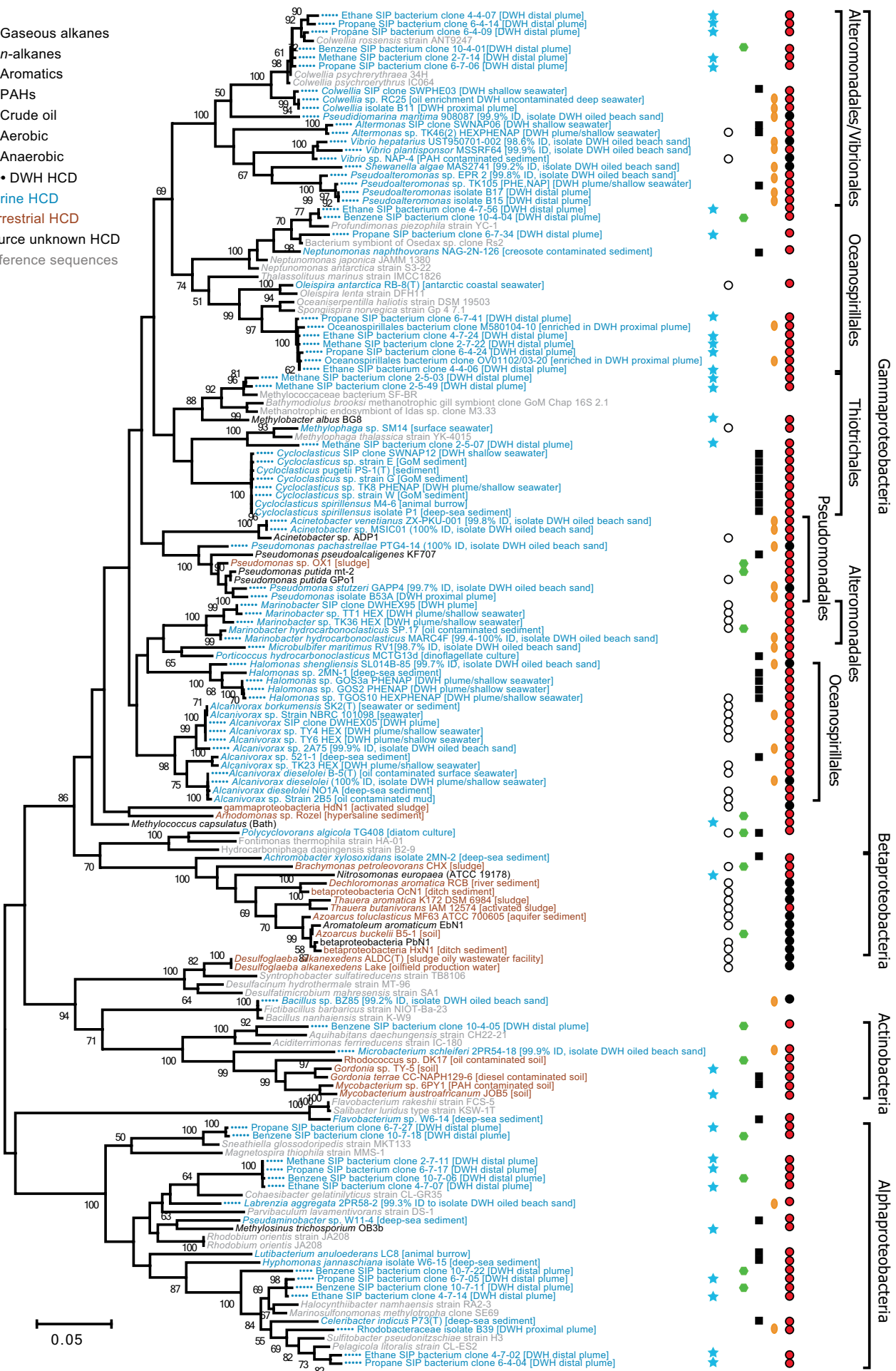
To examine the ecological roles of rare keystone taxa that provide essential ecosystem functions requires cultivation-independent 16S rRNA gene-based approaches in combination with next-generation sequencing technologies. Typically, 16S rRNA gene sequences are clustered into operational taxonomic units (OTUs), based on a sequence similarity threshold (e.g., 97%). However, rare microbial hydrocarbon degraders may not be identifiable on the OTU level and, consequently, may remain hidden in large sequencing data sets. To resolve environmentally relevant differences between sequences of closely related microbial taxa that respond to fluctuating geochemical conditions (e.g., ecotypes), bioinformatics approaches that allow sub-OTU resolution are required (Eren et al., 2013).

**FIGURE 3.** Phylogeny of 125 hydrocarbon-degrading bacteria (HCD), including isolates and bacteria enriched by the Gulf of Mexico Deepwater Horizon oil spill (DWH) or by DNA-based stable isotope probing. Maximum Likelihood tree of 16S rRNA gene sequences >1,248 bp long constructed using ClustalW alignments and 500 bootstrap replicates (MEGA v.6.06). Sequence GenBank accession numbers are given in parentheses. Where indicated in parentheses and in bold, DWH beach and water isolates are represented by proxy sequences 98.6% to 100% identical (ID).



# Phylogenetic Tree of Dominant Hydrocarbon Degrading Microorganisms

- ★ Gaseous alkanes
- *n*-alkanes
- Aromatics
- PAHs
- Crude oil
- Aerobic
- Anaerobic
- ..... DWH HCD
- Marine HCD
- Terrestrial HCD
- Source unknown HCD
- Reference sequences



Several tools and approaches are available to detect rare taxa, including oligotyping, which distinguishes subtle nucleotide variations within 16S rRNA gene amplicon reads and clusters sequences into so-called oligotypes (Eren et al., 2013). The similarity thresholds for oligotypes can be as low as 0.2%, which is

plume oxygen depletion was due to aerobic oxidation of short chain alkanes, propane, and butane (Valentine et al., 2010). Also, metabolic genes involved in hydrocarbon degradation were highly enriched in the plume (Lu et al., 2012). Stable-isotope probing laboratory studies suggested that *Colwellia* oxidized

the first few months to years after oil from the DWH spill came ashore onto beaches (Hayworth et al., 2015) and wetlands (Mahmoudi et al., 2013; Atlas et al., 2015). Whereas alkanes and low molecular weight PAHs were largely depleted in coastal sediments, high molecular weight PAHs (e.g., chrysene) persisted and could remain for many years. Oil is degraded at much reduced rates when buried; thus, submerged oil mats, tens to hundreds of meters long and up to 20 cm thick, have been reported along the inner shelf of the northern Gulf of Mexico (Dalyander et al., 2014), and tar balls, typically 0.5–5 cm in diameter and containing 5% to 10% hydrocarbons by weight continue to wash up on northeastern gulf shores. Chronic exposure to oiled sediments has severe adverse effects on juvenile benthic fish (Brown-Peterson et al., 2015), suggesting that buried oil poses a long-term ecological risk to coastal Gulf of Mexico ecosystems.

Oil contamination from the DWH spill had a profound impact on the abundance, structure, and metabolic potential of sedimentary microbial communities along beaches (Kostka et al., 2011) and marshes (Mahmoudi et al., 2013; Atlas et al., 2015) of the northern Gulf Coast. A time series study conducted at Pensacola Beach, Florida, where total petroleum hydrocarbons reached 11,000 mg kg<sup>-1</sup>, revealed a bloom of bacteria during the first four months after oil came ashore, with microbial abundance in oiled sands 10 to 10,000 times that of clean sands (Kostka et al., 2011). Geochemical evidence confirmed the role microorganisms play in the degradation of weathered oil (Ruddy et al., 2014), and the succession of indigenous microbial populations paralleled the chemical evolution of the petroleum hydrocarbons (Rodriguez-R et al., 2015).

The most extensive metagenomic time series describing microbial hydrocarbon degradation, which was collected from these Pensacola Beach sands, showed a similar progression of microbial populations linked to hydrocarbon degradation

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more than an order of magnitude lower than the dissimilarity threshold used by most OTU-clustering methods (3%). Such subtle ecotype variations distinguish key hydrocarbon degraders that respond to hydrocarbon inputs in the environment (Kleindienst et al., 2015a) and serve to reveal the taxa responding to hydrocarbon and dispersant amendments (Kleindienst et al., 2015b).

In the DWH deepwater plume, the infusion of oil and dispersants enriched for bacteria related to *Oceanospirillum*, *Cycloclasticus*, *Colwellia*, *Rhodobacterales*, *Pseudoalteromonas*, as well as to methylo-trophs (Mason et al., 2012; Reddy et al., 2012). Preferential microbial utilization of short-chain and higher-weight alkanes was inferred from compositional changes in the hydrocarbon complex (Valentine et al., 2010). Localized dissolved oxygen anomalies indicated that up to 70% of

ethane, propane, and butane (Redmond and Valentine, 2012), while single-cell genomics revealed that *Oceanospirillum* has the potential to oxidize cyclohexane (Mason et al., 2012).

A substantial, yet unconstrained, portion of DWH discharged oil reached coastal ecosystems, polluting a large (~1,800 km) swath of shoreline from East Texas to West Florida (Michel et al., 2013). Oil was transported high onto the supratidal zone of beaches by waves and tides associated with storms (Michel et al., 2013), and a portion of the oil was deposited in the intertidal and subtidal zones near the beach. Because of the dynamic nature of coastal sediments, storms often resulted in the rapid burial of oil in these environments.

Total petroleum hydrocarbons, and aliphatic and aromatic compounds were highly weathered and depleted within

observed in other coastal sediments. Oil deposition led to a decrease in taxonomic diversity. The bloom was dominated by members of the *Gamma*- and *Alphaproteobacteria*, and the abundance of genes for hydrocarbon degradation pathways closely paralleled microbial population dynamics. A clear succession pattern was observed, with early responders to oil contamination (*Alcanivorax*) likely degrading aliphatic hydrocarbons, being replaced after three months by populations capable of aromatic hydrocarbon decomposition (*Hyphomonas*, *Parvibaculum*, *Marinobacter*). After one year, a typical beach community had reestablished that showed little to no evidence of oil hydrocarbon degradation potential, but it differed significantly from the community present before the oil spill, indicating that beach microbial communities respond to crude oil perturbation according to the specialization disturbance hypothesis.

In intertidal wetlands, fine-grained sediments accumulate under relatively quiescent tidal and current conditions, producing heterogeneous, organic-rich, and anoxic conditions near the sediment surface. Hydrocarbons accumulated in marsh sediments were largely degraded within the first few years after oil came ashore (Mahmoudi et al., 2013; Atlas et al., 2015). Oxygen supply dictated the extent of hydrocarbon degradation, and anaerobic microbial populations such as sulfate-reducing members of the *Deltaproteobacteria* and methanogens increased in relative abundance in sediments where hydrocarbons were degraded (Atlas et al., 2015). Oil degradation genes associated with anaerobic pathways increased dramatically at oiled sites, and even the higher molecular weight PAHs were substantially biodegraded.

### Regulation of Microbial Processes

The fate and transport of discharged oil is determined by a complex interplay among hydrocarbon chemistry, the microbial food web, and ambient oceanographic processes, including dispersion,

dilution, dissolution, advection by ocean currents, particle flocculation and aggregation, sedimentation, and evaporation, along with biodegradation. Similar to the breakdown of terrestrially or marine-sourced organic matter, microbial communities biodegrade the majority of petroleum hydrocarbons (oil and gas) that enter the marine environment. Local temperature, oxygen levels, and nutrient availability limit the rate and extent of hydrocarbon degradation or weathering (Leahy and Colwell, 1990; Head et al., 2006); these factors are determined by physical processes that mix and ventilate water masses within the ocean. Although few data are available, pressure may also impact biodegradation rates through effects on chemical solubility and/or the physiology of hydrocarbon-degrading bacteria (Schedler et al., 2014). Relatively few studies have been conducted under high pressure and low temperature conditions that mimic deepwater conditions.

This fundamental gap in understanding microbial hydrocarbon degradation at pressure is remarkable, given the petroleum industry's trend of increasing oil and gas production in ultradeep (>1,500 m) water, which presents the implicit risk of future deep-sea oil well blowouts. Further, the impacts of chemical dispersants and their influence on biodegradation has not been studied across the full range of oceanographic conditions. More information is available on the environmental controls on hydrocarbon degradation in marine water columns than in seafloor sediments. This lack of knowledge regarding oceanographic controls on oil transport and degradation, especially in the deep sea, is a critical obstacle to effective parameterization of oil plume models, which is critical to improving model prediction.

Information from the DWH discharge indicated that oxygen is rarely completely depleted in an oil-contaminated water column, meaning that temperature and nutrients are likely the key limiting factors for hydrocarbon degradation. Laboratory studies show that

temperature strongly regulates the capacity and efficiency of petroleum hydrocarbon degradation in seawater (Bagi et al., 2013). However, kinetic constraints do not appear to be as important as previously perceived. For example, Hazen et al. (2010) observed half-lives of C<sub>13</sub> to C<sub>26</sub> alkanes to be from one to eight days at low temperatures (4°C to 6°C) in DWH deepwater plume samples. Subsequently, Brakstad et al. (2015) observed half-lives of one to two weeks for alkanes and two to four weeks for PAHs in low temperature (5°C) waters. Although these data indicate that temperature was not the overriding factor limiting degradation, in many cases, the temperature response was quantified under nutrient replete conditions. Therefore, synergies between temperature and nutrient limitation should be further explored.

Oil is an unusual carbon substrate for microbial growth. Not only is it largely insoluble, it also lacks major nutrients (N, P), a stark contrast to marine-derived planktonic organic matter. A large pulse of oil into any ecosystem could thus lead to nutrient limitation of microbial metabolism. A substantial body of research shows that nutrient availability determines the rate of microbial oil degradation in marine systems (Leahy and Colwell, 1990). These observations serve as the basis for bioremediation strategies, such as that employed in response to the *Exxon Valdez* spill. However, more than 25 years after the *Exxon Valdez* disaster, evidence remains equivocal regarding nutrient limitation of hydrocarbon degradation in studies surrounding the DWH discharge.

A study conducted using mesocosms containing Gulf of Mexico surface seawater found that nutrients appeared to limit hydrocarbon degradation and respiration rates, and microbial biomass did not increase in response to the addition of Macondo oil (Ortmann and Lu, 2015). However, under severely nutrient-limited conditions near the DWH wellhead, Edwards et al. (2011) observed enhanced respiration rates and a half-life

of 26 days for oil degradation in the surface mixed layer. Because bacterial biomass levels did not appear to differ in the surface slick relative to surrounding waters, these authors suggested that top-down processes, such as grazing or viral lysis, prevented biomass accumulation.

Data from ultra-high-resolution mass spectrometry documented that oil-derived organic matter could serve as a nutrient source (namely N) for oil degradation in deep waters collected near an active Gulf of Mexico hydrocarbon seep (Kleindienst et al., 2015b; Seidel et al., 2016). Finally, a metagenomic time series from coastal sediments exposed to oil from the DWH discharge shows that the abundance of genes associated with nutrient scavenging (nitrogen fixation, iron chelation) correlates positively with the abundance of genes for hydrocarbon catabolism (Rodriguez-R et al., 2015).

Together, these data indicate that the ocean environment dictates the efficiency and capacity of microbial communities to degrade hydrocarbons. However, we have yet to discern how environmental factors interact to regulate the final catabolic outcome of hydrocarbon bioremediation. Thus, despite an extensive knowledge base on hydrocarbon degradation, a quantitative understanding is lacking, which makes it critical to incorporate microbial biodegradation pathways and regulation(s) into numerical models of oil fate and transport. Such information is necessary to accurately construct and constrain hydrocarbon fate budgets.

Chemical dispersants emulsify oil and break up surface slicks, generating dispersant-stabilized oil micro-droplets that dissolve into surface waters, effectively increasing the volume of water polluted with discharged oil (MacDonald et al., 2015). By breaking up surface slicks, dispersant utilization can reduce the amount of thick oil stranded along shorelines and increase the oil-seawater interfacial area. During the DWH oil spill, the dispersant application was unprecedented, both because of the amount of

dispersant applied (~7 million liters) and by the location of dispersant application.

Chemical dispersants are believed to stimulate biodegradation by generating high oil-seawater interfaces that are more readily accessible to hydrocarbon-degrading microorganisms; further, the small droplet size is assumed to relieve nutrient or oxygen limitation of oil biodegradation. However, available data provide conflicting and contradictory results: some studies suggest dispersant stimulation of biodegradation while others conclude that dispersants either make no difference or inhibit biodegradation (Kleindienst et al., 2015a).

The effects of dispersants on microorganisms might be taxa-specific (Figure 4) and dependent on dispersant concentrations. For instance, certain *Colwellia* taxa responded to dispersants or oil-dispersant mixtures (Bælum et al., 2012; Kleindienst et al., 2015b), while *Marinobacter* (Kleindienst et al., 2015b) and *Acinetobacter* (Overholt et al., 2016) were suppressed by dispersants. *Alcanivorax borkumensis*, a model obligate hydrocarbon-degrading bacterium, was shown to be negatively impacted by Corexit 9500A and all anionic dispersants (Bookstaver et al., 2015). Another *Alcanivorax* strain isolated from Macondo oil contaminated beach sands demonstrated greater oil transformation efficiency on dispersed oil, albeit with a slight lag in growth (Overholt et al., 2016).

It seems clear that chemical dispersants result in a wide variation of bacterial responses through multiple mechanisms, including physically changing the oil-water interface, disruption of cell membranes causing toxicity, increasing entrained oil concentrations, and likely changing bacterial metabolic responses influencing cell growth (Kleindienst et al., 2015b). The presence of dispersants can further influence the whole food web, as indicated by reduced or blocked carbon flow to higher trophic levels (Ortmann et al., 2012). Assessing dispersant impacts across different habitats remains a crucial topic for future research.

## LOOKING FORWARD – CONCLUDING THOUGHTS

Natural oil seepage and anthropogenic oil discharges are commonplace across the world ocean. Microbes are adept and efficient at degrading hydrocarbons, even under nutrient-stressed conditions. Developing a deeper understanding of the regulation and capacity for microbial hydrocarbon remediation in a range of environments and over a reasonable suite of environmental conditions is critical. While much has been learned over the past few decades, there is still more to discover. In particular, documenting the efficiency of the microbial hydrocarbon biofilter in the presence and absence of chemical dispersants is a key area of future research.

Likewise, the DWH incident revealed a previously unrecognized rare biosphere that rapidly responds to hydrocarbon infusion (Kleindienst et al., 2015a). The use of -omics techniques has revealed a great deal about the diversity and physiology of responding microorganisms, but we do not know how effective these microbes are in situ. For example, some key microbially mediated hydrocarbon degradation processes appeared to be limited by environmental or physiological factors (e.g., methane oxidation; Crespo-Medina et al., 2014). Further, rates of complex hydrocarbon oxidation were not measured using sensitive isotopic tracer assays, making it impossible to constrain the fate of discharged oil during the DWH incident (Joye, 2015). Similarly, it is unclear whether chemical dispersants stimulated or had no effect on hydrocarbon degradation rates. These open questions and many others must be answered before the next open-ocean oil spill occurs so that a more effective response can be employed.

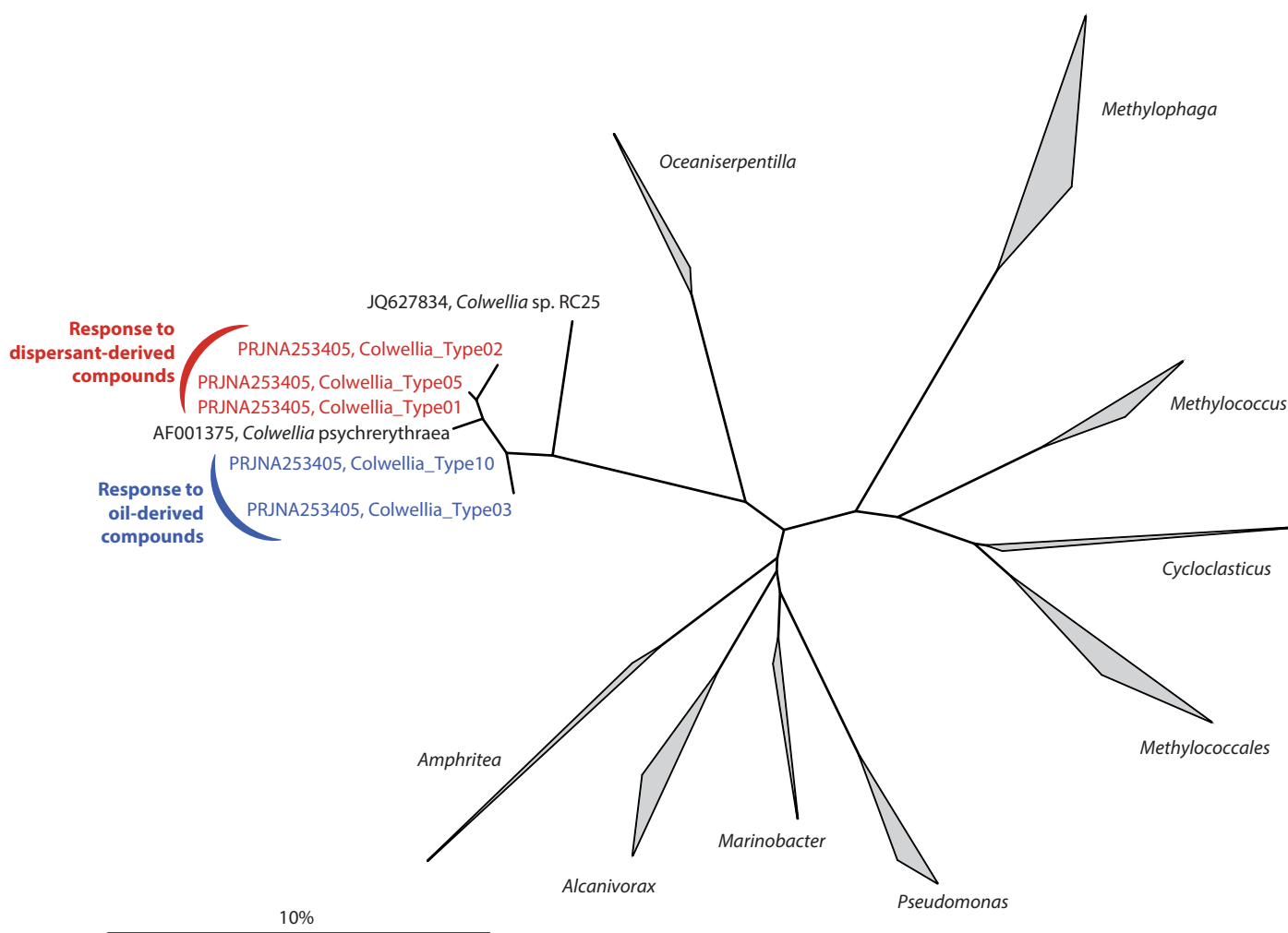
The DWH blowout was a large-scale environmental perturbation that led to rapid and remarkable microbial community shifts, raising the question as to whether, and on what time scale, these communities returned to the pre-discharge baseline. Available

evidence suggests that while the population returned to baseline at the “class” level (e.g., Kleindienst et al. 2015a), subtle changes in ecotype distributions persisted, meaning there could have been fundamental shifts in hydrocarbon metabolic dynamics in the system. The time scale of full recovery to the pre-spill baseline remains unknown.

Lessons learned from the DWH and other oil spills have advanced hydrocarbon microbiology and pointed to data that must be collected to properly describe the microbial community response in terms of microbial composition, activity, and efficiency. It is

imperative to determine hydrocarbon degradation rates directly using isotopic tracers, and full documentation of system response requires detailed spatiotemporal collections. Most importantly, environmental baselines were sorely lacking for the Gulf of Mexico ecosystem, particularly in the deepwater areas, at the time of the DWH oil well blowout (Joye, 2015). While large amounts of data have been collected in the wake of the Macondo incident, background data are lacking for much of the Gulf of Mexico system, particularly where ultra-deepwater drilling is now occurring. Such data are likewise generally unavailable for other parts of

the world ocean where oil and gas exploration and drilling are ongoing. We cannot afford to live in an “invisible present” (Magnuson, 1990). Ecological changes occur slowly or sporadically and are only apparent and quantifiable through consistent long-term observation. In what is now a classic contribution, Magnuson (1990) noted that the absence of long-term monitoring data hampers the ability of the scientific community to assess natural environmental change, manage the environment in a sustainable fashion, and document anthropogenic perturbations. ©



**FIGURE 4.** Phylogenetic tree of *Colwellia* species, highlighting environmental selection of physiologically distinct ecotypes. The figure shows subpopulations that respond to oil- (blue) and dispersant-derived (red) compounds in relation to gammaproteobacterial taxa. Responding *Colwellia* subpopulations were enriched in Gulf of Mexico deepwater microcosms, amended with oil-only, dispersants-only, or oil-dispersant mixtures (Kleindienst et al., 2015b). *Colwellia* subpopulations, representing potential ecotypes, were identified from 16S rRNA gene next-generation sequencing data using oligotyping (Eren et al., 2013). Dispersant-degrading capabilities for most marine microorganisms are largely unknown, although *Colwellia* sp. RC25 was shown to utilize hydrocarbons and dispersants as growth substrates. Globally relevant and widely distributed aerobic hydrocarbon degraders of the *Gammaproteobacteria* affiliate, for instance, with *Alcanivorax*, *Marinobacter*, and *Cycloclasticus*. The bar represents 10% sequence divergence.

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## AUTHORS

**Samantha B. Joye** (mjoye@uga.edu) is Georgia Athletic Association Professor of Arts and Sciences, Department of Marine Sciences, University of Georgia, Athens, GA, USA. **Sara Kleindienst** is Head of the Microbial Ecology Group, Center for Applied Geosciences, Eberhard-Karls-University Tuebingen, Tuebingen, Germany. **Jack A. Gilbert** is Professor, Department of Surgery, University of Chicago, Chicago, IL, USA. **Kim M. Handley** is Research Fellow, School of Biological Sciences, University of Auckland, Auckland, New Zealand. **Pamela Weisenhorn** is Postdoctoral Researcher, Argonne National Laboratory, Argonne, IL, USA. **Will A. Overholt** is PhD Candidate, School of Biology, Georgia Institute of Technology, Atlanta, GA, USA. **Joel E. Kostka** is Professor, Georgia Institute of Technology, Atlanta, GA, USA.

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