

HYDROCARBON DEGRADATION UNDER CONTRASTING REDOX
CONDITIONS IN SHALLOW COASTAL SEDIMENTS OF THE NORTHERN GULF
OF MEXICO

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Presented to
The Academic Faculty

by

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**HYDROCARBON DEGRADATION UNDER CONTRASTING
REDOX CONDITIONS IN SHALLOW COASTAL SEDIMENTS OF
THE NORTHERN GULF OF MEXICO**

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LIST OF SYMBOLS AND ABBREVIATIONS

DWH	Deepwater Horizon
GoM	Gulf of Mexico
PAHs	Polycyclic Aromatic Hydrocarbons
SOMs	Submerged Oil Mats
SRBs	Surface Residual Balls
SOAs	Sediment-Oil Agglomerates
SOMs	Sediment-Oil Mats
BTEX	benzene, ethylbenzene, toluene, or xylene
SRB	Sulfate-Reducing Bacteria
Bss	Benzylsuccinate Synthase
Nms	naphthyl-2-methyl-succinate synthase
EBDH	ethylbenzene dehydrogenase
Pps	phenylphosphate synthase
Ppc	phenylphosphate carboxylase
Blz	benzoate-CoA ligase
HMN	2,2,4,4,6,8,8-heptamethylnonane
PCoA	principal coordinate analysis
HPLC	high-performance liquid chromatography
DCM	dichloromethane
GC-MS	gas chromatography-mass spectrometry
RFLP	fragment length polymorphism
FISH	fluorescence in situ hybridization
nanoSIMS	nanoscale secondary ion mass spectrometry
AAI	Amino Acid Identity

EPFR environmentally persistent free radicals

ODC Operation Deep Clean

PICRUSt Phylogenetic Investigation of Communities by Reconstruction of Unobserved States PICRUSt

KEGG Kyoto Encyclopedia of Genes and Genomes

Alk alkane monooxygenase

SUMMARY

The overall goal of this dissertation research was to characterize sedimentary microbial populations that are active in degrading petroleum hydrocarbons that reach the seafloor during accidental oil spills. Whereas most previous work has investigated hydrocarbon-degrading microbial communities in sediments from natural hydrocarbon seep environments that are regularly exposed to high levels of oil, the focus of this study was on non-seep sediments that are not pre-exposed to high levels of petroleum. Further, oxygen is a master variable controlling the rates and metabolic pathways of hydrocarbon biodegradation in marine sediments. Therefore, this dissertation contrasts the microbial populations that mediate the degradation of petroleum hydrocarbons under anoxic conditions in shallow subtidal marine muds with their counterparts in aerobic beach sands.

Biodegradation of petroleum hydrocarbons in the marine environment is most often coupled to microbial respiration reactions. Anaerobic respiration, in particular sulfate-reduction, predominates over electron flow in fine-grained or muddy marine sediments. Thus, in chapters 2 and 3 of the dissertation, anaerobic degradation of petroleum hydrocarbons coupled to sulfate reduction was studied in fine-grained sediments collected from the continental shelf near the Mississippi River delta, in order to better predict the fate of hydrocarbons released from the Deepwater Horizon (DWH) oil spill. Specific objectives of this study were to demonstrate hydrocarbon degradation coupled with sulfate reduction and characterize the microbial populations capable of anaerobic hydrocarbon degradation in non-seep sediments of the northern Gulf of Mexico. Anaerobic enrichment cultures were initiated and successively transferred with

either hexadecane or phenanthrene as sole carbon and energy source and sulfate as a terminal electron acceptor. Microbial activity was determined by quantifying the reduction of sulfate to sulfide as well as hydrocarbon depletion, while growth was confirmed by quantitative PCR of SSU rRNA genes. Microbial communities were characterized through PCR amplification and next-generation sequencing of bacterial SSU rRNA gene amplicons as well as through clone library analysis and Sanger sequencing of abundant community members. Potential rates of sulfate reduction coupled to phenanthrene degradation were equivalent to those of hexadecane degradation over the first three culture transfers. Results showed that sulfate-reducing enrichment cultures amended with hexadecane and phenanthrene were highly enriched in *Deltaproteobacteria* (>80% sequence abundance). However, further phylogenetic analysis revealed that microbial populations were distinct according to hydrocarbon substrate, with abundant clones from hexadecane cultures showing high sequence identity to *Desulfatibacillum alkenivorans* belonging to *Desulfobacteraceae* (up to 98 % sequence similarity), while abundant clones from phenanthrene cultures were most closely related to *Desulfatiglans spp.* of the *Desulfarculaceae* (up to 95 % sequence similarity). Analysis of rRNA transcripts indicated that these same abundant microbial groups were also metabolically active in the respective cultures. Assuming complete oxidation to CO₂, observed stoichiometric ratios closely resembled the theoretical ratios of 12:1 for hexadecane and 8.25:1 for phenanthrene degradation coupled to sulfate reduction. Results provide fundamental information on anaerobic hydrocarbon degradation in marine sediments that are distant from natural hydrocarbon seeps and therefore not pre-exposed to substantial levels of hydrocarbons. Given that the ecology and biochemistry of

anaerobic PAH degradation remain largely unknown, we have identified key taxa that may be used as model organisms to elucidate the mechanisms of natural PAH attenuation in marine muds.

In contrast to marine muds, which are largely anoxic, petroleum hydrocarbon degradation occurs under mostly aerobic conditions in permeable sands found on beaches. Although the impacts of oil contamination on marine microbial communities in beach sands are well documented, few studies have addressed the microbial community dynamics associated with larger oil-sediment residues that are often trapped in coastal ecosystems. Especially in the supratidal zone of beach sand environments, low moisture content, nutrient availability, and a low surface to volume ratio of larger residues may limit bacterial hydrocarbon degradation. Thus, in chapter 4 of this dissertation, the microbially mediated degradation of petroleum hydrocarbons was investigated in sediment oil agglomerates (SOAs) in an experiment conducted *in situ* at Pensacola Beach. The main objectives of this experiment were to (i) quantify the rate of *in situ* SOA degradation in Pensacola Beach sands, (ii) interrogate the long-term succession of microbial communities that recruit onto SOAs, and (iii) to elucidate the potential coupling of SOA degradation and nitrogen fixation. Replicate homogenized SOAs, collected from the coastline immediately after the DWH disaster, were buried from 5 to 55 cm depth in Pensacola Beach sand and sampled from October 2010 to December 2013. The abundance and composition of microbial communities was determined using next generation sequencing and qPCR of SSU rRNA genes, respectively, for SOAs sampled over the three year time series. SOA degradation occurred over longer time scales in comparison to smaller, more diffuse oil particles in dry beach sands, with up to

91% of petroleum hydrocarbons degraded in three years. As has been observed for more diffuse oil contamination, taxonomic diversity of microorganisms decreased in SOAs in comparison to surrounding or pristine sands. Hydrocarbon-degrading bacteria were enriched and a succession of microbial populations was observed that paralleled the chemical evolution of petroleum hydrocarbons as determined in our companion study. Bacterial abundance was two to four orders of magnitude higher in SOAs in comparison to surrounding sands, indicating that these large oil residues are hotspots of microbial growth. Quantification of nitrogenase genes (*nifH*) in SOAs revealed a bloom in nitrogen-fixing prokaryotes or diazotrophs late in the time series. Results indicate that the high C/N ratio of SOAs causes nutrient stress and induces microbial nitrogen fixation. Thus, the coupling of nitrogen fixation to hydrocarbon degradation appears to be an important control over the fate of oil in macroscopic oil aggregates. Observations are corroborated by predictions from inferred metagenomic analysis and predictions aid in linking specific taxa to the degradation of specific hydrocarbon compounds and diazotrophy.

CHAPTER 1. INTRODUCTION

1.1 The Deepwater Horizon oil spill

In April 2010, the Deepwater Horizon (DWH) oil rig (Macondo wellhead) exploded and sank, discharging approximately 4.9 million barrels of crude oil into the Gulf of Mexico (GoM) at a depth of 1544 m over the course of 86 days (Reddy et al., 2012; Zukunft, 2010). An average of 95,500 tons of oil enters the GoM annually from natural hydrocarbon seeps (73%), oil and gas extraction activities (3%), transportation activities (4%), and oil combustion byproducts (16%) (National Research Council, 2003). Thus, the DWH disaster released the equivalent of over seven times the average annual input of oil into the GoM. Discharged oil was treated with chemical dispersants (COREXIT EC9500A and COREXIT EC9527A) at the surface ocean as well as near the wellhead. A mixture of soluble and insoluble petroleum hydrocarbons reached the sea surface where they evaporated (5%), formed oil sheens and slicks (10%), mechanically recovered (20%), or burned (6%) (Figure 1). Discharged oil also formed a lateral plume at 1000 – 1200 m water depth which was rich in water-soluble gaseous hydrocarbons along with liquid oil compounds (Camilli et al., 2010; Ryerson et al., 2012). For example, the concentration of polycyclic aromatic hydrocarbons (PAHs) reached up to 189 $\mu\text{g/L}$, and PAH levels toxic to marine organisms extended to at least 13 km from the Macondo wellhead (Diercks et al., 2010). The dissolution and biodegradation of the subsurface oil plume have been well studied (Camilli et al., 2010; Reddy et al., 2012; Valentine et al., 2010). At the ocean surface, a large amount of marine snow formed in the floating oil layer by coagulation of phytoplankton, bacteria, and lithogenic particles with oil droplets

(Passow et al., 2012; Ziervogel et al., 2012). Soluble and highly insoluble hydrocarbons were rapidly deposited to the deep sea by the sinking of marine snow particles as well as by the direct contact of deep plumes with the seafloor of the continental slope (Romero et al., 2015). Chemical analysis of sediment cores collected up to 8 km from the Macondo well demonstrated the extent of weathering of oil deposited in deep sea sediments. The dissolution and biodegradation of oil on the seafloor increased with distance from the wellhead, indicating that weathering occurred rapidly during oil transport (Stout and Payne, 2016). Deposited oil in deep sea environments may remain on the sea floor and affect the deep sea environment, since oil is resistant to chemical weathering due to its poor solubility (Figure 2). Discharged oil also rose to the surface and was transported to shorelines from East Texas to West Florida (McGenity, 2014) (Figure 3). Oil residues transported to beaches surrounding the Gulf of Mexico showed that the oil was heavily weathered and the proportion of oxygenated hydrocarbons increased tenfold (Aeppli et al., 2012). The weathered oil is heavier and more viscous and forms a water-in-oil mixture that is referred to as chocolate mousse or mousse (Figure 4). This weathered emulsion then breaks into smaller masses, forming pelagic tar balls or patties (Warnock et al., 2015). Marine tar residues vary considerably in color, shape, size, chemical composition, and aroma. The term tar ball is used to describe a discrete, roughly spherical accumulation of weathered oil less than 10 cm in diameter. Discrete tar aggregates that are larger than 10 cm in diameter are referred to as tar patties. Thick accumulations of oil-sediment residues that are partially or completely submerged by water are referred to as tar mats (Figure 4). Oil residues from the Deepwater Horizon oil spill were deposited in the intertidal and subtidal zones, and fragile tar balls with high sand content were

distributed onshore (Operational Science Advisory Team (OSAT-2), 2011). In order to make a distinction between these sedimented tar residues and typical surface-weathered tars, new terminology was defined by which the tar mats were referred to as submerged oil mats (SOMs) and the tar balls were called surface residual balls (SRBs). Recently, these macroscopic oil and sediment aggregates were renamed as sediment-oil agglomerates (SOAs) or sediment-oil mats (SOMs) (Gustitus and Clement, 2018). Tar balls collected from the shores of Louisiana and Florida showed the presence of electron paramagnetic resonance spectra characteristic of organic free radicals and transition metal ions, which might persist and result in a toxic effect in the beach environment (Kiruri et al., 2013). The chromatographic signature of petroleum biomarkers in emulsified mousse and tar balls collected from Louisiana to Alabama beaches revealed that nearly all oil residues originated from DWH oil spill (Mulabagal et al., 2013). These large oil-sediment residues were shown to be depleted in n-alkanes and low molecular weight PAHs, whereas concentrations of PAHs and alkylated PAHs increased over time (Evans et al., 2017).

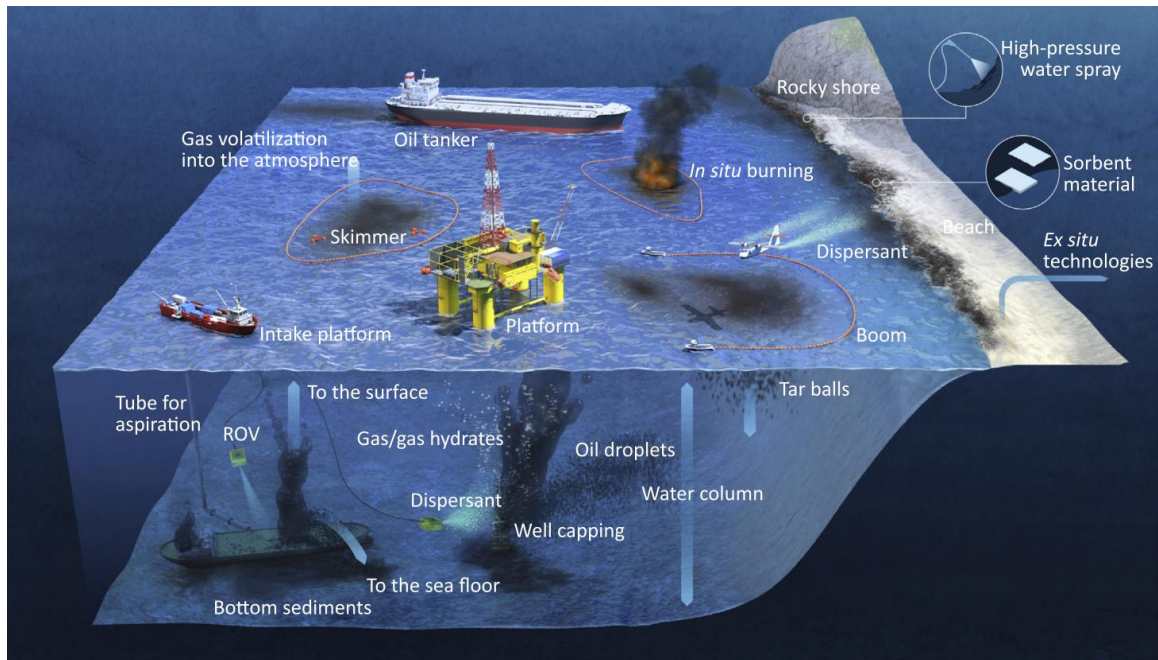


Figure 1 Technologies used in response to the Deepwater Horizon oil spill. On the sea surface, oil was chemically dispersed or physically contained by plastic booms and partially recovered with skimmers or burned *in situ*. Oil droplets from the Macondo well spread horizontally to form hydrocarbon plumes in the water column or were transported to the sea surface and eventually the coastal zone. Dispersants were used to decrease the size of oil droplets and increase the surface-to-volume ratio. Natural attenuation and dispersion phenomena in the water column and volatilization in the atmosphere additionally contributed to hydrocarbon removal (Mapelli et al., 2017).

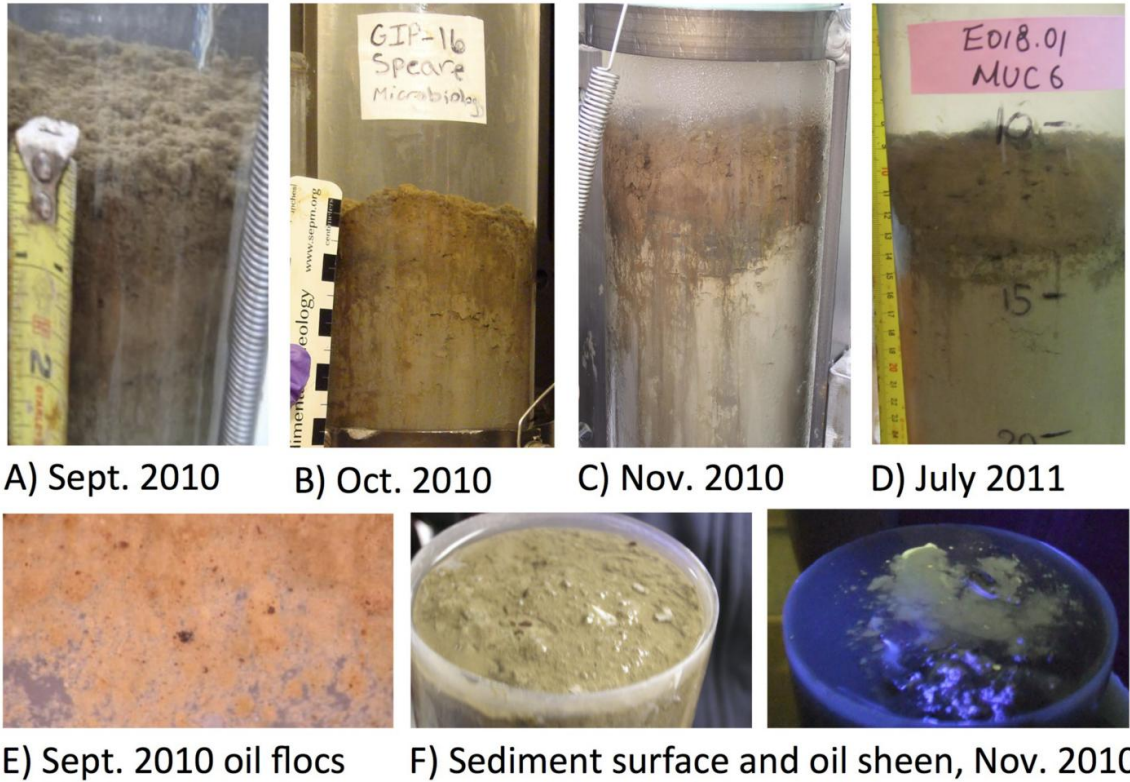


Figure 2 Oil-contaminated sediment cores, characterized by a red-brown layer at the top of the gray sediment. Photos show sediment cores collected (A) in September 2010, (B) in mid October 2010, (C) at the end of November 2011, and (D) in July 2011, (E) close-up of oil-derived marine snow flocs collected from the surface of sediment collected in September 2010, (F) surface of sediment core with and without UV illumination, indicating petroleum hydrocarbons (Yang et al., 2016).

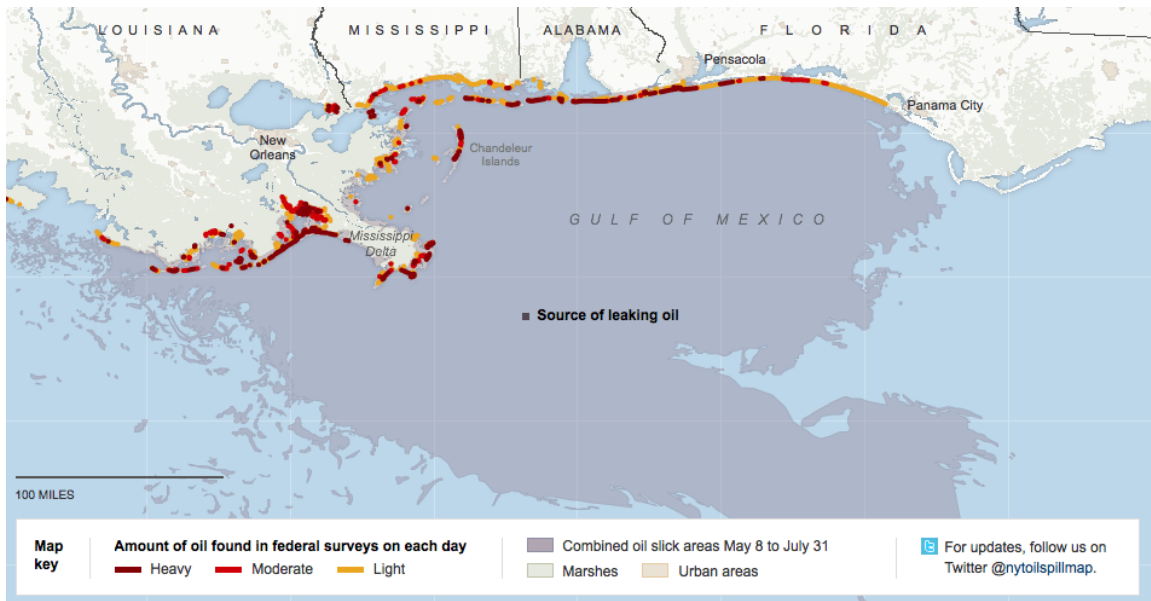


Figure 3 A map of the area impacted by the Deepwater Horizon oil spill in the northern Gulf of Mexico along with the amount of oil found in federal surveys in coastal beach environments.



Figure 4 Tar balls from the Deepwater Horizon oil spill in beach (top left), tar patty, approximately 40 cm in diameter (top right), tar mat piece recovered from Pensacola Beach in February, 2014 (bottom left), and chocolate mousse (bottom right) (Warnock et al., 2015).

1.2 Sources and chemical composition of petroleum hydrocarbons in the environment

Hydrocarbons are natural organic compounds that are widely distributed in the environment and formed either as metabolites by bacteria, archaea, plants and animals, or as abiotic transformation products of dead buried biomass (Widdel and Rabus, 2001). Petroleum hydrocarbons are highly valuable natural resources for energy generation and chemical industry. They represent the major constituents of natural oil, coal and gas, comprising 10^{12} tons of carbon stored in worldwide reservoirs that have formed over geological time scales (Rabus et al., 2016). Crude oil consists of thousands of

hydrocarbon compounds and can be divided into four classes: saturated aliphatics, aromatics, asphaltenes, and resins (Atlas, 1981). Approximately one-third of sweet light crude oil consists of alkanes including cycloalkanes and their low molecular weight derivatives (Figure 5). Aromatic hydrocarbons include monocyclic (benzene, ethylbenzene, toluene, or xylene) and polycyclic (naphthalene, phenanthrene, and anthracene) compound classes. In particular, polycyclic aromatic hydrocarbons (PAHs) were shown to have carcinogenic, mutagenic, and toxic effects on mammals and are thus considered as important contaminants in the environment (Weissenfels et al., 1992). Aromatic hydrocarbons constitute up to 50% of total hydrocarbons in crude oil (Tissot and Welte, 1984). Alkenes are also present in petroleum but in low abundance due to their relative instability. Crude oil also contains up to 14% of asphaltenes and resins (>500 molecular weight polycyclic organic molecules) (Figure 5). All together, petroleum hydrocarbons are predominantly comprised of C and H (53 - 83% and 10 - 14%, respectively), N (up to 2%), O (up to 1.5%), S (up to 6%), and various trace metals (<0.1%) (Aeppli et al., 2012).

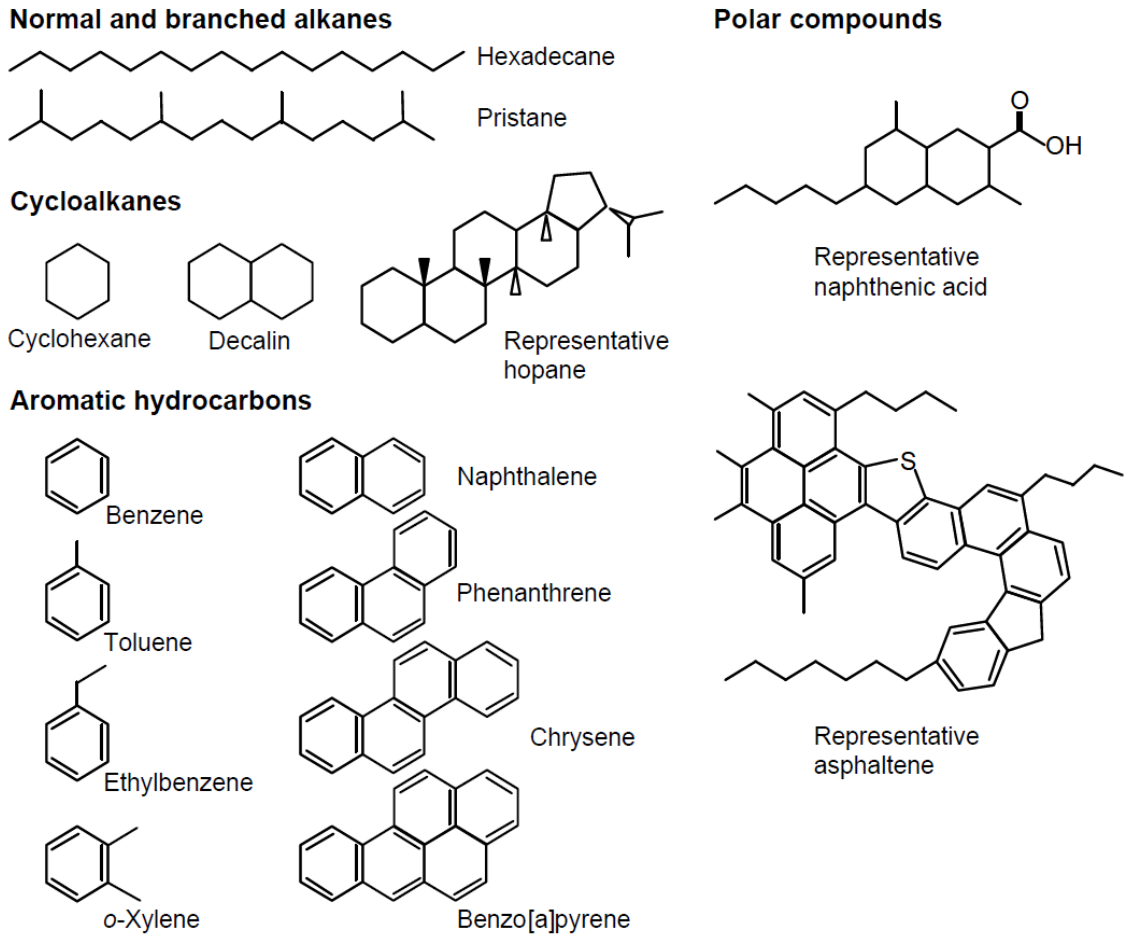


Figure 5 Examples of the main classes of compounds found in crude oil.

1.3 Bacterial hydrocarbon degradation

Bacterial hydrocarbon degradation is the primary and ultimate mechanism responsible for the removal of petroleum hydrocarbons from the environment (Atlas, 1981). Hydrocarbons must be functionalized before being metabolized in order for enzymatic reactions to activate their strong bonds. Hydrocarbons generally differ in their susceptibility to microbial degradation in the following order: linear alkanes > branched alkanes > small aromatics > cyclic alkanes > PAHs (Cooney et al., 1985). Initial

functionalization involves the cleavage of the apolar C-H bond, and high energy barriers must be overcome.

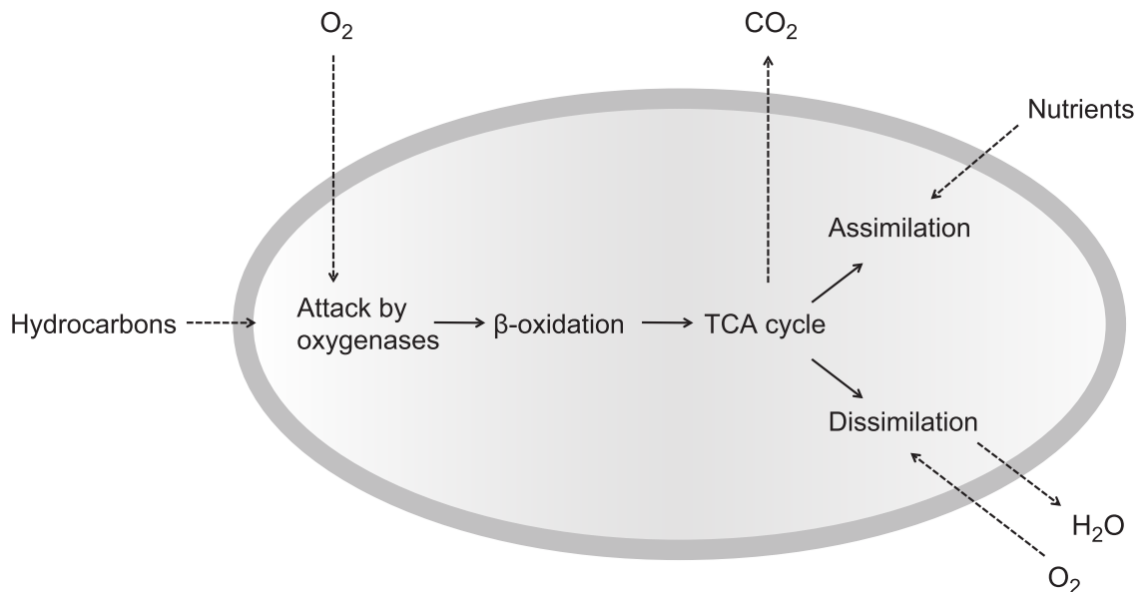


Figure 6 Metabolic pathways involved in hydrocarbon degradation by bacteria under aerobic conditions. Hydrocarbons are attacked by oxygenases that insert oxygen to activate the hydrocarbon. Hydrocarbons are then broken down by peripheral pathways. Within the tricarboxylic acid (TCA) cycle, intermediates are oxidized to gain energy, releasing carbon dioxide. Intermediates are also used to build up biomass (Kleindienst and Joye, 2017).

Hydrocarbon-degrading bacteria are ubiquitous but present at low abundance in the environment. However, these low abundance populations grow rapidly when they are exposed to an influx of hydrocarbons from natural seeps or from anthropogenic sources. Hydrocarbon degradation capability depends on many environmental factors such as electron acceptors (oxygen, nitrate, manganese, iron, sulfate, and carbon dioxide), nutrients (N, P, trace metals), temperature, and pressure. The availability of electron acceptors is one of the key factors that determines the rate and metabolic pathways of

hydrocarbon degradation. Bacteria can degrade hydrocarbons under either aerobic or anaerobic conditions. The most rapid and complete degradation of the majority of hydrocarbons is through aerobic respiration (Fritsche and Hofrichter, 2008). However, hydrocarbons are often deposited in anaerobic environments such as terrestrial soils, marine sediments or deep subsurface environment. Since anaerobic hydrocarbon degradation is a much slower process than aerobic degradation, hydrocarbon compounds can remain relatively unaltered in these environments for longer periods of time. In short, while hydrocarbons represent potential and ubiquitous substrates for microbial metabolism, they are often considered as recalcitrant under anaerobic conditions (Rabus et al., 2016).

1.3.1 Pathways and controls of aerobic hydrocarbon degradation

The essential characteristics of aerobic hydrocarbon degradation are as follows: (1) optimization of the contact between the microbial cell and the hydrocarbon compound, (2) initial intracellular attack and incorporation of oxygen catalyzed by oxygenases and peroxidases, (3) peripheral degradation that converts hydrocarbons into intermediates of the central intermediary metabolism, and (4) biosynthesis of cell biomass from the central precursor metabolites (Fritsche and Hofrichter, 2008) (Figure 6). The majority of known aerobic hydrocarbon-degrading bacteria affiliate with the *Gammaproteobacteria* (*Oceanospirillum* spp., *Colwellia* spp., *Alcanivorax* spp., *Cycloclasticus* spp., *Oleiphilus* spp., *Oleispira* spp., *Thalassolitus* spp.) or *Alphaproteobacteria* (*Roseobacter*, and *Rhodospirillales*) (Kleindienst and Joye, 2017).

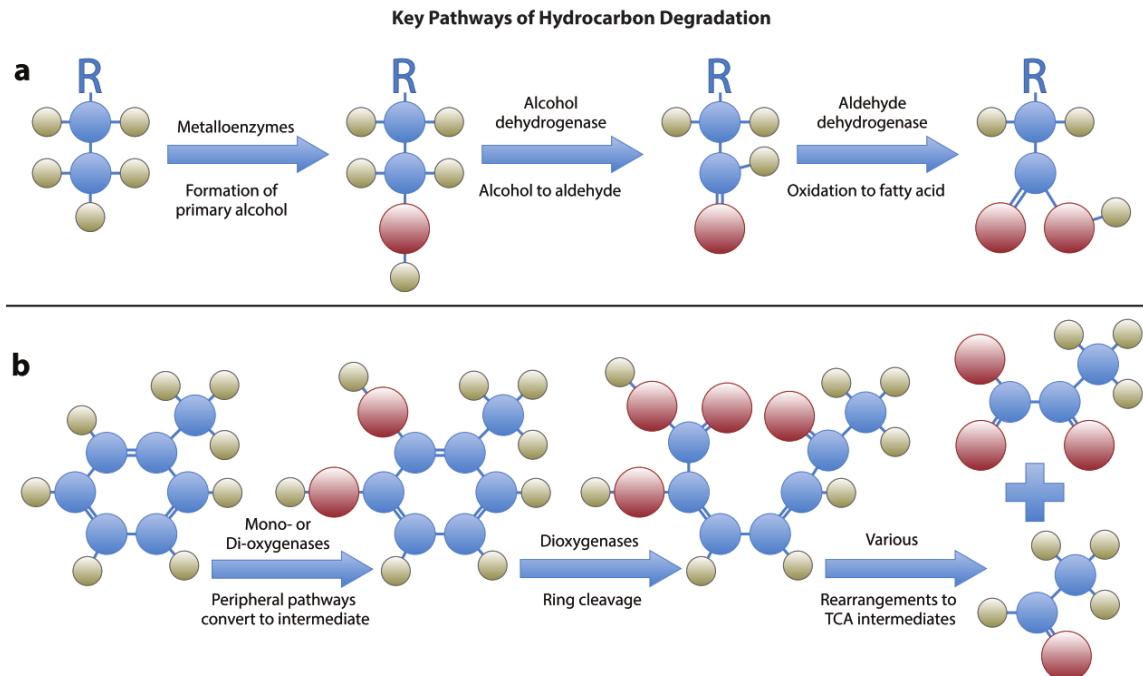


Figure 7 Enzymatic pathways of aerobic hydrocarbon degradation. (a) Degradation of alkanes from primary alcohol formation on a terminal or subterminal C via metalloenzymes (e.g., pMMO, AlkB) followed by conversion of the alcohol to an aldehyde via alcohol dehydrogenase, and finally oxidation of the aldehyde to a fatty acid via aldehyde dehydrogenase. (b) Degradation of a model 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 (Joye et al., 2016).

The aerobic degradation of saturated alkanes is carried out by breaking the strong C-H bond to form a primary alcohol. Next, the alcohol is oxidized by alcohol dehydrogenase, removing hydrogen atoms to form an aldehyde, followed by oxidation to a fatty acid catalyzed by aldehyde dehydrogenase. Fatty acids are further degraded via beta-oxidation or converted to phospholipids and incorporated into the cellular membrane (Wang and Shao, 2013) (Figure 7). Short-chain alkanes, ranging from C₁ to C₄, are degraded by particulate methane monooxygenase (pMMO), soluble methane monooxygenase (sMMO), and their homologs. Longer chain alkanes (C₂-C₁₆) are

degraded by two main classes of enzymes, alkane monooxygenase (AlkB) and cytochrome P450 (CYP153). Many groups of bacteria have been reported to degrade alkanes including *Alcanivorax* sp., *Arthrobacter* sp., *Acinetobacter* sp., *Candida* sp., *Pseudomonas* sp., *Rhodococcus* sp., *Streptomyces* sp., *Bacillus* sp., *Aspergillus japonicus*, *Arthrobacter* sp., *Acinetobacter* sp.. A subset of these taxa such as *Alcanivorax* sp. are characterized as hydrocarbonoclastic, indicating that they are highly specialized to degrade a relatively narrow range of aliphatic substrates (Schneiker et al., 2006).

Due to their toxicity and hazardous properties, microbial degradation of aromatic hydrocarbons is of particular interest (Díaz et al., 2013). In the classical aerobic catabolism, the hydroxylation and oxygenolytic cleavage of the aromatic ring is catalyzed by hydroxylating oxygenase and ring-cleavage dioxygenase. Most aerobic, aromatic degradation pathways converge to catecholic substrates, which are then metabolized by either *ortho* or *meta* cleavage by intradiol (type I) or extradiol (type II) dioxygenase. Bacterial degradation of aromatic compounds often generates non-catecholic intermediates such as gentisate, homogentisate, monohydroxylated aromatic acids, or *O*-heteroaromatic flavonols. These intermediates then undergo ring cleavage by a type III extradiol dioxygenase. Another strategy for aromatic ring cleavage is carried out by oxygenase. This is also termed a hybrid pathway since it shares some characteristics with anaerobic catabolism (Fuchs et al., 2011). All metabolites are activated to CoA thioesters through the action of an initial CoA ligase and the ring cleavage is carried out by hydrolysis. Anaerobic degradation of benzoate shares a similar initial reaction with the aerobic hybrid pathway catalyzed by a benzoate-CoA ligase. Under strictly anaerobic

conditions, the ring cleavage step is catalyzed by a CoA reductase and then resulting metabolites undergo β -oxidation-like reactions, which leads to central metabolites.

Biodegradation of hydrocarbons is influenced by many environmental factors such as temperature, the availability of oxygen and inorganic nutrients (nitrogen, phosphorus, iron, sulfur), pH, salinity, and pressure (Atlas, 1981; Leahy and Colwell, 1990). During aerobic hydrocarbon degradation, the presence of adequate inorganic nutrients is the most important limiting factor because these nutrients are scarce in crude oil. It has been widely recognized that petroleum degradation in the sea is typically limited by the availability of nutrients such as nitrogen and phosphorus. In principle, approximately 150 g of nitrogen and 30 g of phosphorus are consumed in the conversion of 1 kg of hydrocarbon to cell material (Bacosa et al., 2018). In late May 2010, nitrate concentrations notably declined inside of the oil plume from the Deepwater Horizon oil spill (mean decrease of 6 μM), whereas phosphorus concentrations did not show a significant decrease (mean decrease of 0.03 μM) (Hazen et al., 2010). Nutrient availability may also select for specific microbial populations in oil-polluted waters (Hazen et al., 2016). For example, different bacterial groups predominated in nutrient-rich bottom water collected near the wellhead in comparison to nutrient-poor surface water, showing a 10% variation in bacterial community structure (Liu et al., 2017). Oil spill events often enrich hydrocarbonoclastic bacteria in marine environments, which implies that indigenous hydrocarbonoclastic bacteria are nutritionally independent of an external nitrogen source.

Nitrogen-fixing microorganisms enzymatically transform dinitrogen gas from the atmosphere into ammonium. Nitrogen fixation is carried out by the nitrogenase enzyme and its subunits are encoded by the genes *nifH*, *nifD*, and *nifK*. The *nifH* gene has become the most widely established molecular marker for the study of phylogeny, diversity, and abundance of nitrogen-fixing microorganisms (Gaby et al., 2017). The quantification of *nifH* genes through quantitative PCR in a oil-polluted mangrove mesocosm showed increased *nifH* gene copies (Taketani et al., 2009). Another study of *nifH* genes amplified from microcosms with an added oil layer revealed sequences mostly related to those of heterocystous cyanobacteria (Musat et al., 2006). Only relatively few bacterial species have been reported to fix nitrogen during growth with oil hydrocarbons (Chen et al., 1993; Prantera et al., 2002).

1.3.2 Anaerobic hydrocarbon degradation

Under anaerobic conditions, microorganisms utilize nitrate, manganese(IV), iron(III), sulfate, or carbon dioxide as electron acceptors. In fresh water environments, nitrate-reducing bacteria affiliated with the *Betaproteobacteria* were often shown to degrade hydrocarbons. Anaerobic hydrocarbon degraders in marine environments mainly use sulfate as their terminal electron acceptor since seawater is enriched in sulfate (~28 mM) (Jørgensen and Fenchel, 1974; Rabus et al., 2013). The majority of sulfate-reducing, hydrocarbon-degraders are thought to be affiliated with the *Deltaproteobacteria*. The biochemistry involved in anaerobic hydrocarbon degradation has been studied for BTEX compounds (benzene, toluene, ethylbenzene, and the three xylene isomers), naphthalene,

methylnaphthalene, and alkanes. Degradation pathways for these hydrocarbons have been proposed by identifying key intermediates, stable isotope analysis, and genomic / proteomic data analysis. Isolates that are capable of hydrocarbon degradation under anaerobic conditions are described in Table 1.

1.3.2.1 Fumarate addition reactions

Degradation of n-alkanes under anaerobic conditions has been studied in denitrifying or sulfate-reducing pure cultures (Aeckersberg et al., 1991; Cravo-Laureau et al., 2005; Ehrenreich et al., 2000; So and Young, 1999a). The range of n-alkanes utilized extends from short-chain alkanes (propane, butane) to medium- and long-chain alkanes (C₆–C₂₀). Alkane degradation was found to be initiated by addition of fumarate to the subterminal methylene group of the respective alkanes, leading to the formation of branched (1-methylalkyl)succinates (Cravo-Laureau et al., 2005; Rabus et al., 2001, 1999). The first genome sequence of a sulfate-reducing alkane-degrader, *Desulfatibacillum alkenivorans* AK-01, revealed the presence of two separate operons for alkane-activating fumarate-adding glyceryl radical enzymes (*ass*) (Callaghan et al., 2012). A proteomic study of the pure culture HxN1 capable of n-hexane degradation under nitrate-reducing conditions identified genes coding for the subunits of expected (1-methylalkyl)succinate synthase (*masABC*) and its associated activating enzyme (*masG*) (Grundmann et al., 2008) (Figure 8).

Table 1 Characterized anaerobic hydrocarbon-degrading bacteria under denitrifying, iron-reducing, and sulfate-reducing conditions.

Organism	Phylogeny	Substrate range	Reference
Denitrifying hydrocarbon-degrading bacteria			
<i>Thauera aromatica</i>	<i>Betaproteobacteria</i>	Toluene	(Anders et al., 1995)
<i>Azoarcus tolulyticus</i>		Toluene, m-xylene	(Zhou et al., 1995)
<i>Azoarcus toluvorans</i>		Toluene	(Song et al., 1999)
<i>Azoarcus toluclasticus</i>		Toluene	(Song et al., 1999)
<i>Aromatoleum aromaticum</i> strain EbN1		Toluene, ethylbenzene	(Kühner et al., 2005; Rabus et al., 2005)
Strain HxN1		C6-8 alkanes	(Ehrenreich et al., 2000)
Strain OCN1		C8-12 alkanes	(Ehrenreich et al., 2000; Zedelius et al., 2011)
Strain HdN1	<i>Gamma</i> proteobacteria	C14-12 alkanes	(Ehrenreich et al., 2000; Zedelius et al., 2011)
Iron-reducing hydrocarbon-degrading bacteria			
<i>Geobacter toluenoxydans</i>	<i>Deltaproteobacteria</i>	Polyaromatic Hydrocarbons (PAHs)	(Lovley and Lonergan, 1990)

Table 1 continued

Georgfuchsia toluolica	<i>Betaproteobacteria</i>	Polyaromatic Hydrocarbons (PAHs)	(Weelink et al., 2009)
Sulfate-reducing hydrocarbon-degrading bacteria			
Desulfobacula toluolica	<i>Deltaproteobacteria</i>	Toluene, benzoate, phenylacetate, p-cresol	(Rabus et al., 1993)
Strain PRTOL1		Toluene	(Beller et al., 1996)
oXyS1		o-Xylene, toluene, o-ethyltoluene, o-methylbenzyl alcohol, benzoate, o-methylbenzoate, benzylsuccinate	(Harms et al., 1999)
mXyS1		m-Xylene, toluene, m-ethyltoluene, m-Isopropyltoluene, benzoate, m-methylbenzoate	(Harms et al., 1999)
EbS7		Ethylbenzene, phenylacetate	(Kniemeyer et al., 2003)
NaphS2, NaphS3, NaphS6		Naphthalene, 2-methylnaphthalene	(Galushko et al., 1999; Musat et al., 2009)
Desulfococcus oleovorans strain Hxd3		C12-20 alkanes, alkenes	(Aeckersberg et al., 1991)
Desulfabacillum alkenivorans		C8-23 alkene	(Cravo-Laureau et al., 2004b)

Table 1 continued

Desulfabacillum aliphaticivorans strain CV2803	<i>Deltaproteobacteria</i>	C13-18 alkanes	(Cravo-Laureau et al., 2004a)
Desulfoglaeba alkenexedens		C6-12 alkanes	(Davidova et al., 2006)
Strain TD3		C6-14 alkane	(Rueter et al., 1994)
Strain Pnd3		C14-17 alkane	(Aeckersberg et al., 1998)
Strain AK-01		C13-18 alkane	(So and Young, 1999b)
Desulfotomaculum strain OX39	Phylum <i>Firmicutes</i>	o-/m-Xylene, Biphenyl	(Morasch et al., 2004; Selesi and Meckenstock, 2009)

Anaerobic degradation of toluene represents a model system for the widely distributed type of initiation reaction via fumarate addition (Beller and Spormann, 1997; Spormann and Widdel, 2000). The capability for anaerobic toluene metabolism is widespread, which may be explained by the general abundance of toluene in contaminated environments as well as its relatively high solubility in water. All anaerobic toluene-degrading bacteria known to date initialize degradation of toluene via the addition of a fumarate cosubstrate to form benzylsuccinate (Beller and Edwards, 2000; Rabus and Heider, 1998; Zengler et al., 1999). Detailed toluene metabolism has been studied in denitrifying *Thauera* and *Azoarcus* species. The initial activation step of toluene is catalyzed by the oxygen-sensitive glyceryl radical enzyme benzylsuccinate synthase (BSS), which was purified from *Thauera aromatica* and *Azoarcus* strain T (Beller and Spormann, 1999; Leuthner et al., 1998). A catalytic mechanism for benzylsuccinate synthesis has been proposed to be initiated by abstracting a hydrogen atom from the methyl group of toluene, leading to the formation of a substrate-related benzyl radical intermediate (Figure 9). Benzylsuccinate is further metabolized by activation to benzylsuccinyl-CoA and further degradation via a β -oxidation reaction sequence, leading to the formation of benzoyl-CoA and succinyl-CoA (Leutwein and Heider, 2002, 2001). Benzoyl-CoA is the central intermediate of anaerobic metabolism of aromatic compounds and is further degraded via reduction of the aromatic ring by benzoyl-CoA reductase and further hydrolytic and β -oxidation-like reactions (Carmona et al., 2009; Harwood et al., 1999). All xylene isomers and cresol isomers were also shown to be activated by benzylsuccinate synthases via fumarate addition (Harms et al., 1999; Krieger et al., 1999). Anaerobic degradation of the polyaromatic hydrocarbon 2-

methylnaphthalene via fumarate addition was shown in a sulfate-reducing enrichment culture (Annweiler et al., 2000; Selesi et al., 2010). Genes encoding subunits of naphthyl-2-methyl-succinate synthase (*nmsABC*) and its associated activating enzyme (*nmsD*) were identified.

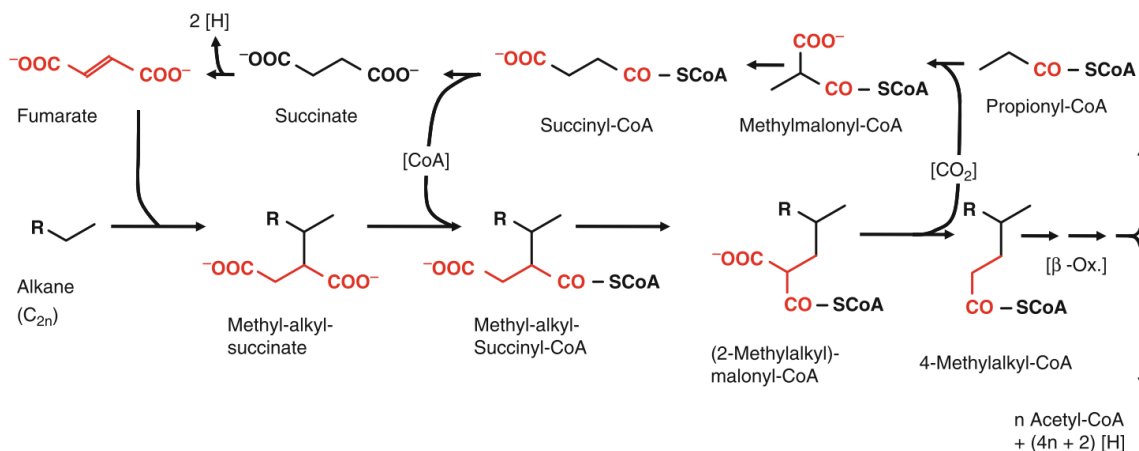


Figure 8 Anaerobic degradation of *n*-alkanes by initial addition of fumarate. The initial reaction forms a branched 1-methyl-1-alkyl-succinate adduct. Further metabolism is proposed to proceed via formation of the CoA thioester and its rearrangement to (2-methylalkyl)-malonyl-CoA, which is decarboxylated to a 4-methylalkyl-CoA derivative. The latter can be beta-oxidized to yield several acetyl-CoA units and one propionyl-CoA. It has been proposed that propionyl-CoA is used to regenerate the fumarate cosubstrate. We suggest that activation of the succinate adduct and decarboxylation of (2-methylalkyl)-malonyl-CoA may occur in coupled reactions with corresponding steps of fumarate regeneration. These suggested CoA- and CO₂-transfer reactions would allow an energy-efficient pathway for activation of the succinate adduct and fumarate regeneration. The metabolic fate of an incoming fumarate moiety over one cycle is indicated in red (Heider and Schuhle, 2013).

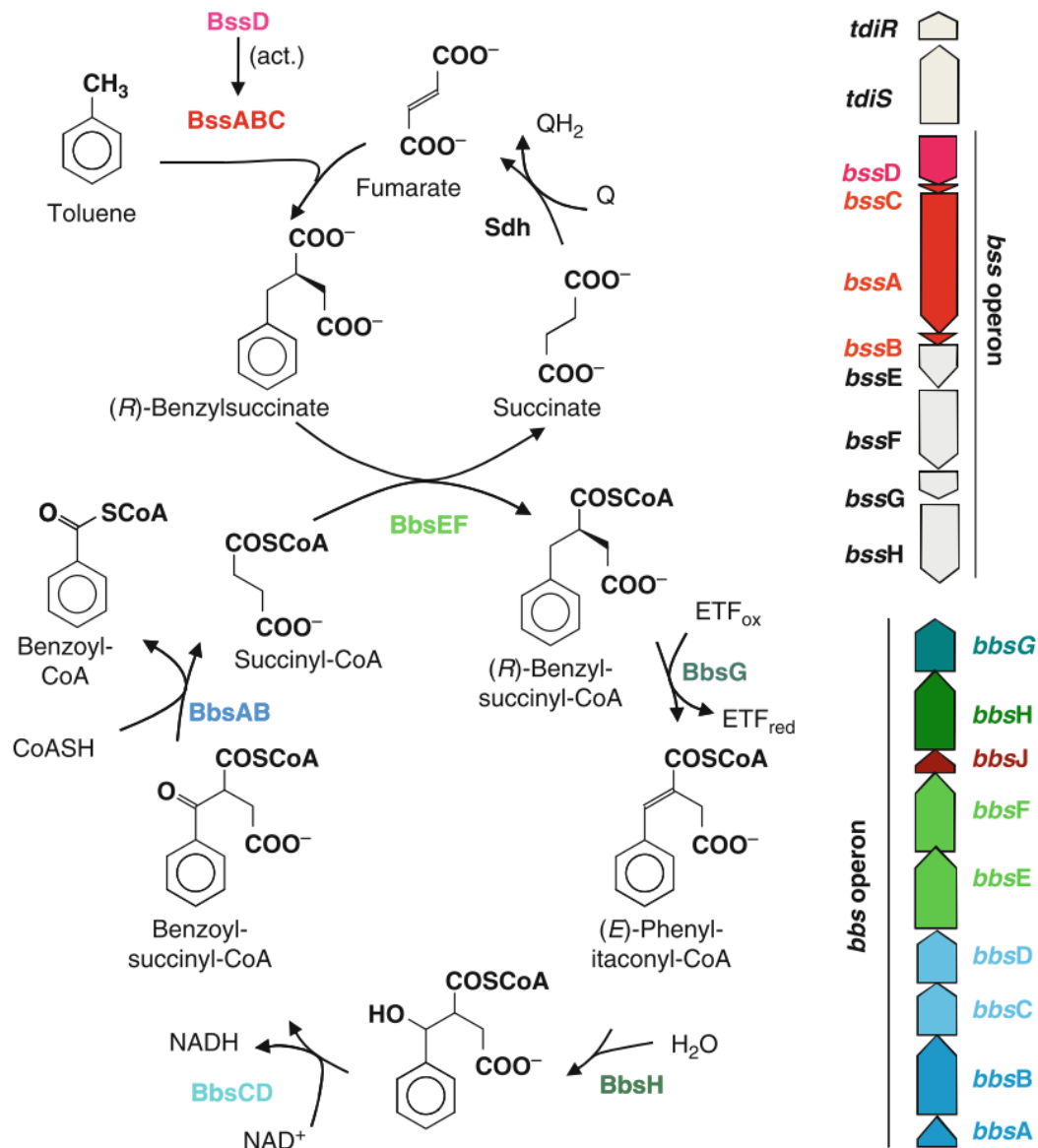


Figure 9 Anaerobic degradation of toluene by initial addition of fumarate. The initial reaction leads to the formation of (R)-benzylsuccinate, catalyzed by benzylsuccinate synthase (BSS). BSS is a glyceryl radical enzyme that needs to be activated by a specific activating enzyme (BssD). The genes coding for the subunits of BSS (*bssABC*) and activating enzyme (*bssD*) form an apparent operon together with several genes of unknown function (*bssE-H*). (R)-benzylsuccinyl-CoA is further metabolized via a beta-oxidation pathway. The genes coding for the enzymes involved are located in a second apparent operon (*bbsA-G*). The enzymes catalyzing the different steps of the pathway are indicated by the respective gene products. Both operons are regulated coordinately by the specific two-component regulation system, TdiSR (Heider and Schuhle, 2013).

1.3.2.2 Oxygen-independent hydroxylation

Ethylbenzene was shown to be initialized by oxygen-independent hydroxylation at C-1 of the side chain to yield (S)-1-phenylethanol (Ball et al., 1996; Kniemeyer and Heider, 2001a). The reaction is catalyzed by the periplasmatic molybdenum/iron-sulfur/heme-enzyme ethylbenzene dehydrogenase (EBDH). For the further metabolic pathway, (S)-1-phenylethanol needs to cross the cytoplasmic membrane and is subsequently further oxidized to acetophenone by a stereospecific NAD-dependent alcohol dehydrogenase (Kniemeyer and Heider, 2001b).

An alternative alkane metabolic pathway other than fumarate addition has been proposed. The sulfate-reducing alkane-degrading species *Desulfococcus oleovorans* and a denitrifying alkane-degrading consortium were shown to utilize alkanes by this different pathway. Based on labelling studies of cellular fatty acids with $^{13}\text{CO}_2$, it was proposed that *D. oleovorans* may initiate *n*-alkane by carboxylation at the C-3 position. However, another scenario may be an initial subterminal hydroxylation of the alkane by an EBDH-like enzyme, followed by oxidation to a ketone and subsequent carboxylation at the C-3 position (Aeckersberg et al., 1991; Callaghan et al., 2009).

1.3.2.3 Carboxylation

Enrichment cultures capable of benzene oxidation under anaerobic conditions have been reported, and benzoate was detected as a metabolite (Abu Laban et al., 2009;

Caldwell and Suflita, 2000; Coates et al., 2001; Edwards and Grbic-Galic, 1992; Kunapuli et al., 2007; Lovley et al., 1995; Musat and Widdel, 2008; Phelps et al., 2001). Benzoate or benzoyl-CoA is the central intermediate of anaerobic degradation of aromatic compounds and therefore also to be expected as an intermediate in other proposed pathways. A proteomic study of an iron-reducing enrichment culture revealed a benzene-induced protein that had sequence similarity with the α -subunit of phenylphosphate carboxylase from an anaerobic phenol-degrading bacterium of the *Thauera/Azoarcus* cluster (Abu Laban et al., 2010). Additional carboxylase-related genes and a gene encoding a putative benzoate-CoA ligase were specifically expressed (Figure 10).

Initial activation of naphthalene and phenanthrene to 2-naphthoate and phenanthrene carboxylic acid, respectively, by carboxylation was described in a previous study. Incorporation of $^{13}\text{CO}_2$ in both substrates by sulfate-reducing consortia supported carboxylation as an activation step (Zhang and Young, 1997). In the naphthalene-degrading pure culture NaphS2, 2-naphthoate was also detected as an intermediate (Galushko et al., 1999; Musat et al., 2009). A cluster of highly induced genes was identified in the sulfate-reducing enrichment culture N47, which showed similarity to genes coding for subunits of a known carboxylase involved in anaerobic phenol degradation (F. D. Bergmann et al., 2011).

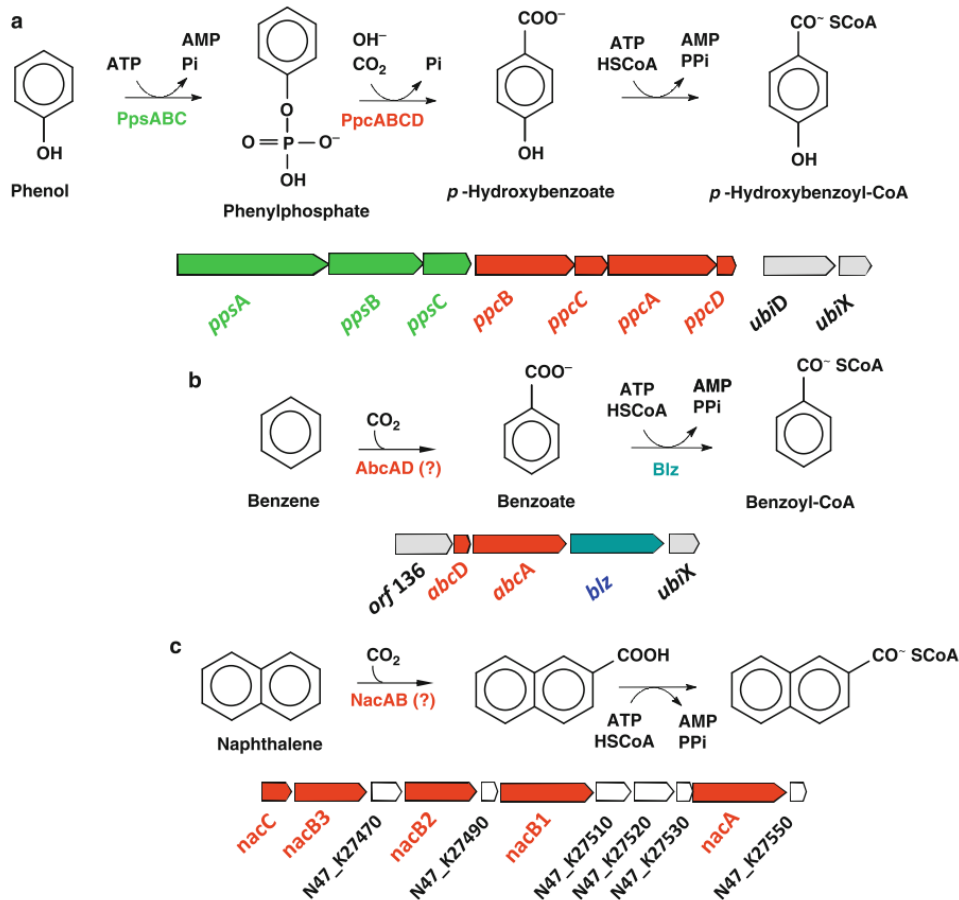


Figure 10 Anaerobic degradation of phenol, benzene, and naphthalene by initial carboxylation. (a) Anaerobic phenol degradation by initial conversion to phenylphosphate by phenylphosphate synthase (PpsABC), followed by carboxylation to 4-hydroxybenzoate by phenylphosphate carboxylase (PpcABCD). The genes coding for the subunits of the respective enzyme are shown below the reactions. Two additional genes coding for putative (de)carboxylases of unknown function similar to UbiD and UbiX, two isofunctional enzymes involved in ubiquinone biosynthesis, are also encoded in the operon. (b) Proposed pathway of benzene degradation via carboxylation to benzoate and activation to benzoyl-CoA. The genes of an induced gene cluster are shown below the reactions. They code for subunits similar to PpcA and PpcD, which are proposed to be involved in benzene carboxylation, and for a proposed benzoate-CoA ligase (Blz). Moreover, a gene coding for an ortholog of UbiX (see above) is located in the gene cluster. (c) Proposed pathway of naphthalene degradation via carboxylation. A gene cluster containing the highly induced *nacA* gene is shown below the reactions. Whereas *NacA* is similar to the *PpcA* subunit of phenylphosphate carboxylase, several potential orthologs of *PpcB* and *PpcC* are encoded in close vicinity (indicated as *nacB1-3* and *nacC*, respectively) (Heider and Schuhle, 2013).

1.4 Motivation and objectives of this study

The ultimate fate of the majority of oil deposited in coastal marine sediments and beach sand environments is degradation by indigenous microorganisms or burial. Identifying key microorganisms that degrade hydrocarbons is essential to understand environmentally relevant biogeochemical processes and for the assessment of response strategies in the event of anthropogenic hydrocarbon discharge into the oceans. Thus, this dissertation addressed the following main objectives:

1. To elucidate key microorganisms that degrade recalcitrant hydrocarbons (hexadecane and phenanthrene) in anaerobic coastal marine sediments from northern Gulf of Mexico under sulfate-reducing conditions
2. To interrogate the mechanisms of PAH activation under sulfate-reducing conditions and identify the genes involved in anaerobic PAH degradation
3. To characterize the long-term microbial community succession in SOAs in nutrient-limited, dry beach sand environments
4. To demonstrate the potential for bacterial nitrogen fixation coupled to hydrocarbon degradation and to identify key microorganisms that carry out this process

Chapter 2 investigates the anaerobic degradation of recalcitrant hydrocarbons (hexadecane and phenanthrene) under sulfate-reducing conditions. Enrichment cultures were obtained from coastal marine sediments of the northern Gulf of Mexico and supplemented with hexadecane and phenanthrene as the sole carbon and energy source.

Key microbial groups that degrade hexadecane or phenanthrene in enrichment cultures were elucidated by next generation sequencing and clone analysis of SSU rRNA genes. The stoichiometry of depleted hydrocarbons and sulfate supported oxidation of hexadecane or phenanthrene coupled with sulfate respiration. Since little is known about anaerobic phenanthrene degradation, I studied this process further by metabolite and metagenomic analysis in Chapter 3. Furthermore, long-term monitoring of aerobic degradation of SOAs in coastal beach sand environments is described in Chapter 4. Microbial community shifts were studied over a 3 year time course along with the microbial response to nitrogen limitation. Overall, conclusions from all chapters are described and future work is suggested in Chapter 5.

CHAPTER 2. ANAEROBIC DEGRADATION OF HEXADECANE AND PHENANTHRENE COUPLED TO SULFATE REDUCTION IN SEAFLOOR SEDIMENTS FROM THE NORTHERN GULF OF MEXICO

2.1 Abstract

Biodegradation of petroleum hydrocarbons in the marine environment is most often coupled to microbial respiration reactions. Anaerobic respiration, in particular sulfate-reduction, predominates over electron flow in fine-grained sediments. In order to better predict the fate of hydrocarbons released from the Deepwater Horizon oil spill, the objectives of this study were to demonstrate hydrocarbon degradation coupled with sulfate reduction and characterize the microbial populations capable of anaerobic hydrocarbon degradation in non-seep sediments of the northern Gulf of Mexico. Anaerobic enrichment cultures were initiated and successively transferred with either hexadecane or phenanthrene as sole carbon and energy source and sulfate as a terminal electron acceptor. Microbial activity was determined by quantifying the reduction of sulfate to sulfide as well as hydrocarbon depletion, while growth was confirmed by quantitative real-time PCR of SSU rRNA genes. Microbial communities were characterized through PCR amplification and next-generation sequencing of bacterial SSU rRNA gene amplicons as well as through clone library analysis and Sanger sequencing of abundant community members. Potential rates of sulfate reduction coupled to phenanthrene degradation were equivalent to those of hexadecane degradation over the first three culture transfers. Results showed that sulfate-reducing enrichment cultures

amended with hexadecane and phenanthrene were highly enriched in *Deltaproteobacteria* (>80% sequence abundance). However, further phylogenetic analysis revealed that microbial populations were distinct according to hydrocarbon substrate, with abundant clones from hexadecane cultures showing high sequence identity to *Desulfatibacillum alkenivorans* belonging to *Desulfobacteraceae* (up to 98 % sequence similarity), while abundant clones from phenanthrene cultures were most closely related to *Desulfatiglans* spp. of the *Desulfarculaceae* (up to 95 % sequence similarity). Analysis of rRNA transcripts indicated that these same abundant microbial groups were also metabolically active in the respective cultures. Assuming complete oxidation to CO₂, observed stoichiometric ratios closely resembled the theoretical ratios of 12:1 for hexadecane and 8.25:1 for phenanthrene degradation coupled to sulfate reduction. Our results provide fundamental information on anaerobic hydrocarbon degradation in marine sediments that are distant from natural hydrocarbon seeps and therefore not pre-exposed to substantial levels of hydrocarbons. Given that the ecology and biochemistry of anaerobic PAH degradation remain largely unknown, we have identified key taxa that may be used as model organisms to elucidate the mechanisms of natural PAH attenuation in marine muds.

2.2 Introduction

In April 2010, the Deepwater Horizon oil rig exploded and sank, discharging approximately 4.9 million barrels of crude oil into the Gulf of Mexico (GoM) at a depth of 1544 m over the course of 86 days (Reddy et al., 2012; Zukunft, 2010). Approximately

0.5-9.1% of the released oil was deposited to the seafloor surrounding the spill site (Chanton et al., 2014; McNutt et al., 2012; Valentine et al., 2014). Deposited oil remains on the seafloor and may undergo microbial degradation, which is a key process in terms of the ecosystem recovering from oil spill events. Microorganisms that are capable of degrading hydrocarbons are widespread in the environment and can mineralize these hydrocarbons through aerobic and anaerobic pathways. Recent studies show that aerobic hydrocarbon-degrading bacteria were enriched in the GoM water column and sediments following the Deepwater Horizon oil spill (Hazen et al., 2010; Huettel et al., 2018; Kostka et al., 2011; Mahmoudi et al., 2013; Mason et al., 2014; Rodriguez-R et al., 2015; Yang et al., 2016). Since aerobic hydrocarbon degradation tends to break down short-chain *n*-alkanes or simple polycyclic aromatic hydrocarbons (PAHs) more readily, recalcitrant oil leftover from aerobic degradation may persist in sediments for long periods of time (Aeppli et al., 2014; Gros et al., 2014; Harayama et al., 1999; Liu et al., 2012; Yergeau et al., 2015). Once hydrocarbons are buried and reach the anoxic zone, microorganisms metabolize hydrocarbons through anaerobic oxidation pathways using nitrate, iron, or sulfate as their terminal electron acceptor or by fermentation. Anaerobic hydrocarbon degradation coupled to sulfate reduction is presumably quantitatively more important in marine sediments than their degradation coupled to other electron acceptors (Widdel, 2010). Biodegradation of hydrocarbons under sulfate-reducing conditions has been demonstrated for various hydrocarbon compounds including saturated alkanes, BTEX compounds (Benzene, Toluene, Ethylbenzene, Xylene) and PAHs (Agrawal and Gieg, 2013; Heider and Schuhle, 2013; Knittel and Boetius, 2009; Meckenstock and Mouttaki, 2011; Taupp et al., 2010; Widdel et al., 2006; Widdel and Rabus, 2001).

Anaerobic degradation of long-chain *n*-alkanes as well as PAHs under sulfate-reducing conditions has been reported in pure cultures or in enrichment cultures (Aeckersberg et al., 1998, 1991; Kniemeyer et al., 2007; Rueter et al., 1994; So and Young, 1999b). Whereas aliphatic alkane degradation has been studied in sulfate-reducing enrichment cultures as well as pure cultures, PAH degradation under sulfate-reducing conditions is much less understood (Cravo-Laureau et al., 2005; Kniemeyer et al., 2007; Kropp et al., 2000). To date, only one bacterial pure culture (Deltaproteobacterial strain NaphS2) has been obtained which is capable of anaerobic PAH degradation. This strain belongs to the *Desulfobacteraceae* and was isolated from a naphthalene-degrading and sulfate-reducing enrichment culture from anoxic marine sediment (Galushko et al., 1999). Mechanisms have been proposed for anaerobic naphthalene degradation coupled to sulfate reduction in studies with strain NaphS2 and an enrichment culture, N47 (F. D. Bergmann et al., 2011; DiDonato et al., 2010; Kummel et al., 2015; Mouttaki et al., 2012; Musat et al., 2009; Safinowski and Meckenstock, 2006; Zhang and Young, 1997). The degradation pathways of all other PAHs, including phenanthrene have only been studied in enrichment cultures, as no pure cultures are available (Davidova et al., 2007; Zhang and Young, 1997).

After the Deepwater Horizon oil spill, studies demonstrated enhanced anaerobic microbial hydrocarbon degradation in various environments. Metabolic genes involved in both aerobic and anaerobic hydrocarbon degradation were enriched in deep-sea oil plumes (Lu et al., 2012). In the deep-sea sedimentary environment, metagenomic analysis and metabolite profiling revealed a high potential for anaerobic hydrocarbon metabolism (Kimes et al., 2013). Another metagenomic analysis from deep-sea sediments collected five months after the Deepwater Horizon oil spill showed an increased abundance of

genes involved in denitrification pathways (Mason et al., 2014). In salt marsh sediments, studies reported enrichment of sulfate-reducing bacteria in parallel with Macondo oil contamination (Atlas et al., 2015; Beazley et al., 2012; Boopathy et al., 2012; Natter et al., 2012). A novel oil-enrichment experiment with crude oil-amended flow-through sediment reactors provided quantification of *in situ* microbial response to oil and demonstrated elevated sulfate-reduction rate and methanogenesis at a natural hydrocarbon seep in the GoM (Orcutt et al., 2017). Anaerobic hydrocarbon degradation has been studied in hydrocarbon-rich ecosystems such as oil-contaminated sites, natural gas and oil seeps, and oil reservoirs; however, it is understudied in seafloor sediments not normally exposed to high levels of hydrocarbons (Heider and Schuhle, 2013). Thus, the objectives of this study were (i) to demonstrate hydrocarbon degradation coupled with sulfate reduction and (ii) to characterize the hydrocarbon-degrading microbial community in anoxic sediments of the northern GoM seafloor. In this study, we demonstrated microbial hexadecane and phenanthrene mineralization using sulfate as a terminal electron acceptor in GoM sediment and characterized the dominant bacterial groups catalyzing these reactions. We investigated hydrocarbon degradation activity and microbial community structure in enrichment cultures. Furthermore, we obtained SSU rRNA gene clone libraries and measured the stoichiometry of hydrocarbon degradation coupled to sulfate reduction. This study elucidates the metabolically active microbial populations that degrade petroleum hydrocarbons in sediments that are not predisposed to high levels of oil and we have identified key taxa that may be used as model organisms in conceptual models for the natural attenuation of oil contamination in marine muds of the Gulf of Mexico.

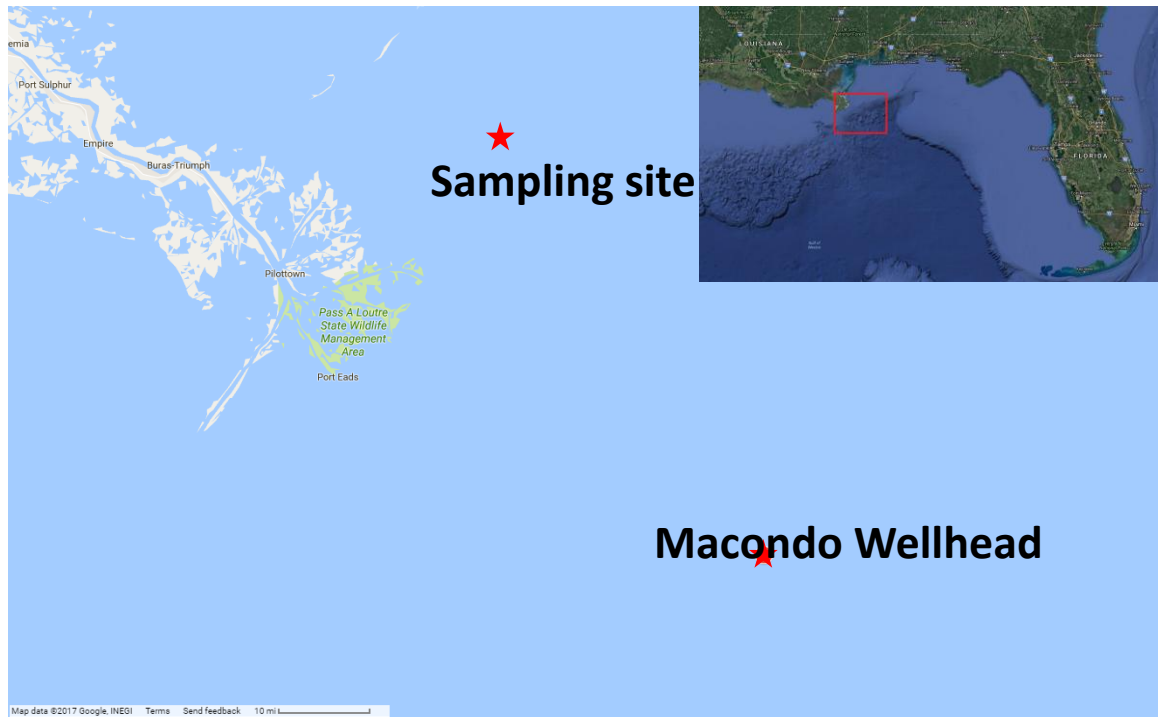


Figure 11 Location of the sampling site relative to the Macondo wellhead. Sediment samples were collected from the northern Gulf of Mexico near the Mississippi River Delta at 56 m water depth, during an expedition with the R/V Weatherbird on August 21 in 2013 (Image Landsat / Copernicus ©2018 Google; Image © 2018 TerraMetrics; Data SIO, NOAA, U.S. Navy, NGA, GEBCO from Google Earth).

2.3 Materials and methods

2.3.1 Sediment sample collection

Sediment samples were collected from the northern Gulf of Mexico (29.3989N, -88.8678W) during an expedition with the R/V Weatherbird on August 21 in 2013, 87 kilometers NW from Macondo wellhead near the Mississippi river Delta at 56 m water depth (Figure 11). To the best of our knowledge, the study site was not exposed to oil contamination from the Deepwater Horizon oil spill in 2010 (Valentine et al., 2014). A

sediment core sample was sectioned on board at 2 mm and 5 mm intervals from 0-2 cm and 2-10 cm sediment depth, respectively. Surface (0-5 cm depth) sediment core samples were collected in sterile plastic bags for cultivation and immediately stored at 4°C for 2 months until enrichment cultures were initiated. Parallel samples were frozen immediately for amplicon sequencing. At this site, oxygen is depleted within the top few millimeters of the sediment surface and the collected samples were almost entirely anoxic.

2.3.2 *Enrichment culture setup*

Sediment from the 0-5 cm depth interval was homogenized and supplemented as a 10% (w/v) inoculum with sterile anaerobic artificial seawater medium (composition per liter: 20.0 g NaCl, 3.0 g MgCl₂·6H₂O, 0.15 g CaCl₂·2H₂O, 0.3 g NH₄Cl, 0.2 g KH₂PO₄, 0.5 g KCl, 4.0 g Na₂SO₄, 1 ml trace elements A, 1 ml trace elements B, 1 ml vitamin mixture, 1 ml thiamine solution, 1 ml B₁₂ solution, and buffered with 30 mM NaHCO₃; modified from (Widdel, 2010). The medium was amended with 1 mM of sodium sulfide as a reducing agent and 0.0001% solution of resazurin as a redox indicator. Triplicate enrichment cultures were amended with either hexadecane (99%, Acros Organics, Morris Plains, NJ) or phenanthrene (98%, St. Louis, MO) as a sole carbon source in 2,2,4,4,6,8,8-heptamethylnonane (HMN) (hexadecane; 11.32 g per liter HMN, phenanthrene; 8.91 g L per liter HMN) as the inert carrier (98%, Acros Organics, Morris Plains, NJ) along with hydrocarbon-free controls (4 ml HMN per bottle). One hundred ml of enrichment culture was prepared in 165-ml serum bottles with a N₂/CO₂ (90:10 v/v) headspace and sealed with butyl rubber stoppers. Strictly anaerobic technique was used throughout all steps for all enrichment culture preparation. The bottles were incubated

nearly horizontally to maximize contact between the medium and hydrocarbon layer and to minimize contact between HMN layer and butyl rubber stopper. Enrichment cultures were incubated at 30°C in the dark without agitation. Sulfate reduction activity was monitored by measuring the accumulation of sulfide using the methylene blue method (Cline, 1969). When the sulfide concentration reached ~8 mM, enrichment cultures were successively transferred five times into fresh medium (20% v/v) to obtain sediment-free cultures.

2.3.3 *Nucleic acid extraction and analysis of SSU rRNA sequences*

Total genomic DNA was extracted from parallel frozen samples of the same 0-5 cm depth interval as that used for cultivation, and 10 ml of enrichment cultures from the first, third, and fourth generations using a MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA) with slight modifications from the manufacturer's protocol as follows. Ten ml of each enrichment culture was centrifuged at $10,000 \times g$ for 5 minutes in sterile falcon tubes and the resulting cell pellet was transferred to the provided 2 ml bead tube. Total RNA was extracted using the Direct-zol RNA miniPrep kit (Zymo Research, Irvine, CA) with slight modifications as follows: Forty ml of the fourth generation sediment-free enrichment cultures were centrifuged at $10,000 \times g$ for 5 minutes. Total RNA from cell pellets was stabilized by adding 1 ml of TRI Reagent (Zymo Research, Irvine, CA) and incubated at room temperature for 5 min. A 200 μ l aliquot of cold chloroform was added, incubated at room temperature for 3 min, and shaken vigorously for 15 seconds. Total RNA was extracted from the aqueous phase

following the manufacturer's protocol. A 10 µl aliquot of total RNA was separated by agarose gel electrophoresis to assess RNA quality (Aranda et al., 2012). Total RNA was reverse transcribed to DNA using qScript XLT cDNA SuperMix (Quanta Biosciences, Beverly, MA) according to the manufacturer's protocol. For Illumina sequencing, PCR amplification was performed using 515F and 806R primers from both DNA and cDNA as described by the Earth Microbiome Project (<http://www.earthmicrobiome.org/emp-standard-protocols/dna-extraction-protocol/>) (Caporaso et al., 2010b; Moonsamy et al., 2013). PCR products were barcoded using an Access Array Barcode Library (Fluidigm, South San Francisco, CA), purified using an E.Z.N.A Cycle Pure Kit (Omega Bio-tek, Norcross, GA), and pooled together based on DNA concentration. Purified and pooled PCR amplicons were sequenced using an Illumina MiSeq platform (Illumina, San Diego, CA). Sequence analysis was accomplished using the software QIIME ver. 1.9.1 (Caporaso et al., 2010b) and Mothur ver. 1.38.0 (Schloss et al., 2009). Sequences with a quality score below 20 were removed using Mothur ver. 1.38.0 and clustered into operational taxonomic units (OTUs) by 97% sequence identity using UCLUST (Edgar et al., 2011) implemented in QIIME ver. 1.9.1. Representative sequences were aligned against the SILVA ver. 123 database (<https://www.arb-silva.de/>) and chimeric sequences were removed using UCHIME (Edgar et al., 2011) implemented in Mothur ver. 1.38.0. Taxonomy was assigned using the RDP classification algorithm set at a 50% confidence rating with the SILVA Small Subunit rRNA Database release 123 (https://www.arb-silva.de/no_cache/download/archive/release_123/Exports/). The resultant OTU table was normalized using the CSS algorithm implemented in QIIME ver. 1.9.1 (Paulson et al., 2013). Shannon index was calculated with QIIME ver. 1.9.1. A Bray-Curtis distance

matrix was obtained from the rarefied OTU table and used to generate a principal coordinate analysis (PCoA) plot.

2.3.4 *Quantitative molecular analyses for total bacteria*

In order to quantify total bacterial SSU rRNA gene abundance, quantitative real-time PCR (qPCR) was performed using the total DNA extracted from either hexadecane- or phenanthrene-amended enrichment cultures from second, third, and fourth transfers as a template. Reactions were performed with PowerUp SYBR Green Mastermix (Applied Biosystems, Foster City, CA) using the primers 331F (5'-TCC TAC GGG AGG CAG CAG T-3') and 515R (5'-ATT ACC GCG GCT GCT GG-3') targeting bacterial SSU rRNA genes on a StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA). The following qPCR program was used: 50°C for 2 min, 95°C for 2 min; 95°C for 15 sec, 55°C for 15 sec, 72°C for 1 min (40 cycles) followed by melt curve analysis with a temperature gradient from 60°C to 95°C at an increment of 0.3°C/min. All reactions were performed in technical triplicate and analyzed using StepOne Software v. 2.3. A standard curve with an efficiency of $102.96 \pm 0.27\%$ was generated by serial dilution of pGEM-T Easy Vector plasmids (Promega, Madison, WI) containing the full-length *E. coli* SSU rRNA gene and used for absolute quantification.

2.3.5 *Cloning and Sanger sequencing*

Due to uncertainties in taxonomic affiliation and the phylogenetic placement of dominant populations from the 250bp Illumina sequences, PCR was performed in order to obtain longer (close to full length) 16S SSU rRNA gene sequences using 27F/1492R primers (Lane, 1991) with DNA extracted from third generation enrichment cultures. PCR products were purified using an E.Z.N.A Cycle Pure Kit (Omega Bio-tek, Norcross, GA) and cloned into pGEM-T Easy Vector (Promega, Madison, WI). Vectors were transformed into DH5 α *E. coli* competent cells (Invitrogen, Carlsbad, CA) and 344 positive colonies were selected by blue/white screening. PCR amplification was performed from colonies using vector-specific primers - T7P (5'-TAA TAC GAC TCA CTA TAG GG-3') and SP6 (5'-ATT TAG GTG ACA CTA TAG AA-3') - and run on a 1% (w/v) agarose gel to confirm full-length 16S SSU rRNA gene insertion. PCR products were purified using an E.Z.N.A Cycle Pure Kit (Omega Bio-tek, Norcross, GA) and digested by the HhaI restriction enzyme (New England Biolabs, Ipswich, MA). Clones were grouped according to their restriction fragment length polymorphism (RFLP) patterns and 78 representative clones were selected. Representative clones were incubated at 37°C in liquid LB media (Amresco, Solon, OH) and plasmids were extracted using a GET Plasmid Mini Prep kit following the manufacturer's manual (G-Biosciences, Louis, MO). Purified plasmids were sent to the Eurofins Genomics facility in Tucker, GA, USA for Sanger sequencing and sequencing was performed in 2 directions.

All SSU rRNA gene sequences from the clone libraries were aligned using PyNAST trained on the SILVA seed database v.128 available in Mothur and inserted into a pre-made ARB tree (SILVA v.128 Ref_NR_99) using the parsimony function implemented in ARB (Caporaso et al., 2010a; Ludwig et al., 2004; Quast et al., 2013;

Yilmaz et al., 2014). Close relatives to clone sequences were manually chosen as well as outgroups to anchor the tree structure. All illumina-derived OTU references sequences were also aligned to the SILVA seed database and inserted into the reference tree using RAxML v.8.2.10 with the environment placement algorithm (Stamatakis, 2014). The resulting JPlace file was modified using a custom script as well as the JPlace.to_iTol.rb script that can be found in the enveomics collection (Rodriguez-R and Konstantinidis, 2016). The tree was visualized using the interactive tree of life (Letunic and Bork, 2016). More details can be found at <http://waoverholt.github.io/RaXML-EPA-iToL>, including all commands and scripts used.

2.3.6 Determination of loss of hexadecane and phenanthrene coupled to sulfate reduction

Subsamples of enrichment cultures were used to measure sulfate concentrations in media and duplicates of whole cultures were sacrificed for hydrocarbon analysis. Sulfate concentrations were determined by high-performance liquid chromatography (HPLC) analysis with a Waters 1525 binary high-pressure pump coupled with a absorbance detector Waters 2487 Dual UV-vis (Beckler et al., 2014). Sulfate losses relative to carbon substrate-unamended controls were used for stoichiometric calculations. To demonstrate hexadecane and phenanthrene degradation, total hydrocarbons were extracted from enrichment cultures. In brief, three extractions of 25 ml of dichloromethane (DCM) each were performed and the resulting organic layers were collected using separatory funnels. Extracts were dried by filtering through sodium sulfate and glass wool and subsequently

evaporated using a TurboVapII (Biotage, Uppsala, Sweden) under flow of N₂ gas at 36°C. Subsamples of 1 µl from the extracts were analyzed by gas chromatography with flame ionization detection (GC-2014, Shimadzu, Kyoto, Japan) using a Rxi-5Sil MS column (30 m length, 0.32 mm internal diameter) (Restek, Bellefonte, PA). Hydrogen was used as carrier gas and the injector temperature was 315°C. The column temperature was initially set at 60°C and increased by 8°C min⁻¹ to 290°C. The total loss of hexadecane or phenanthrene was calculated by comparing with uninoculated controls.

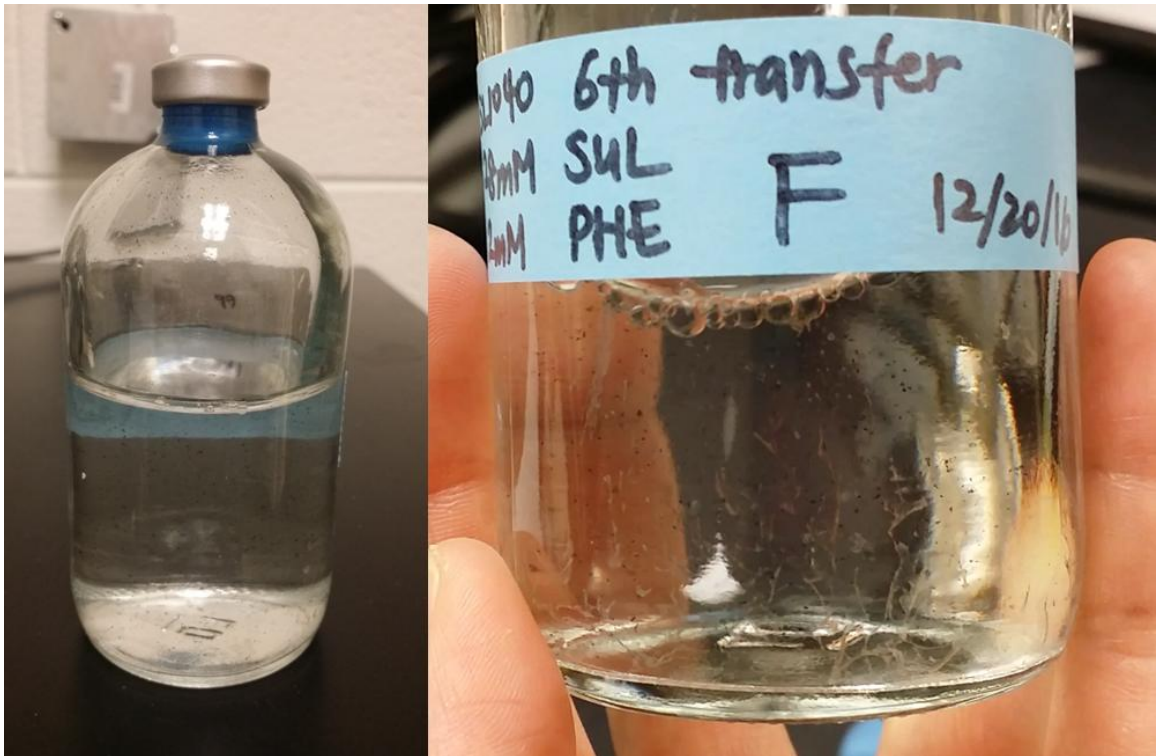
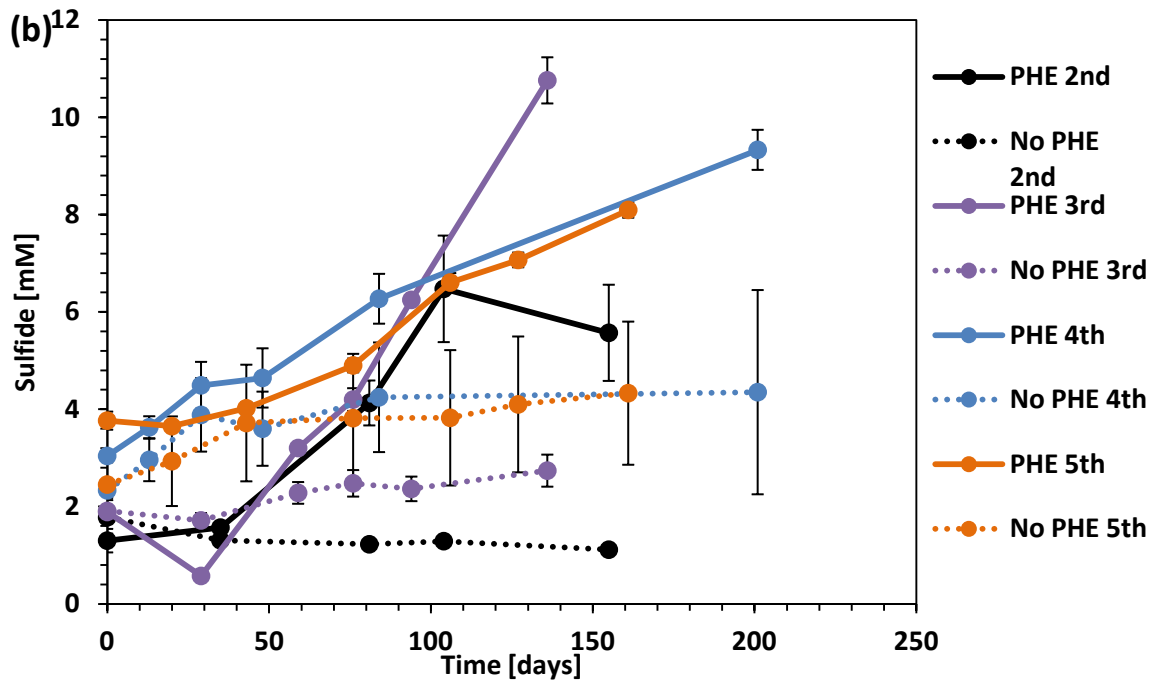
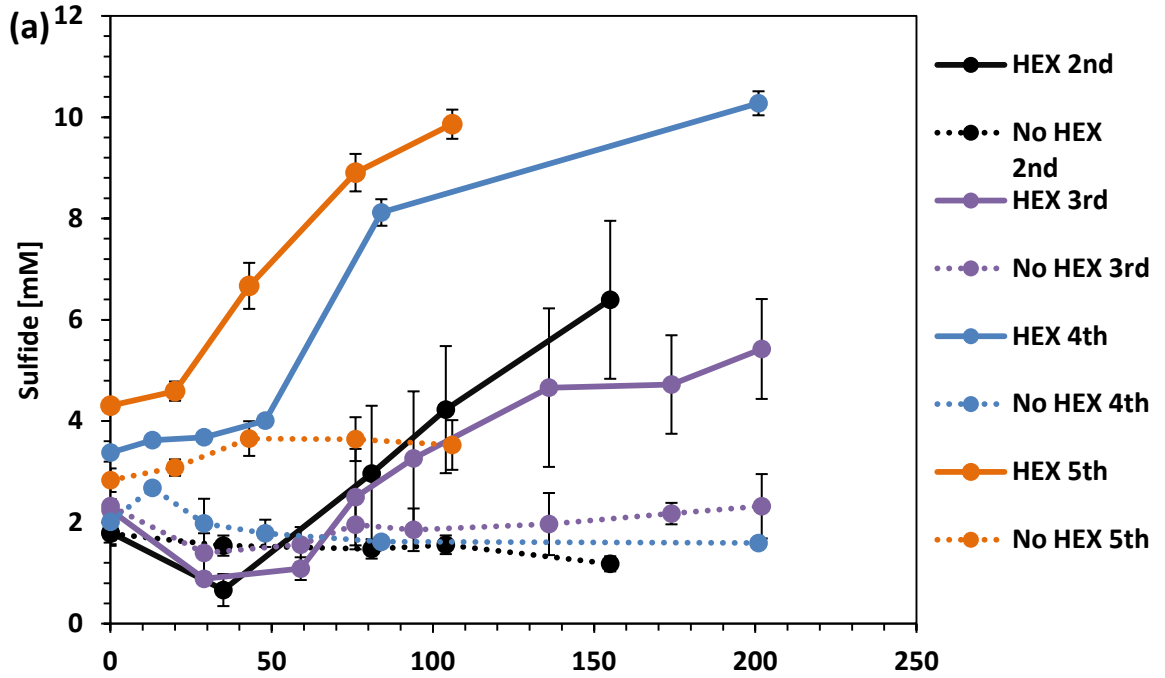


Figure 12 The sediment-free enrichment culture under sulfate-reducing conditions supplemented with either hexadecane or phenanthrene as their sole carbon and energy source.

2.4 Results

2.4.1 *Enrichment of hexadecane and phenanthrene-degrading microbial consortia*

Samples collected from the surface 0-5 cm depth interval of northern GoM sediments (Figure 11) were supplemented with an artificial seawater minimal medium and hexadecane or phenanthrene as a sole carbon source and electron donor. Sulfate reduction activity was monitored by the production of sulfide. Enrichment cultures were successively transferred to fresh media when sulfide concentrations reached 8-10 mM to obtain sediment-free cultures (Figure 12). Little to no sulfide was produced when carbon source was not added (Figure 13a and 13b). The average sulfide production rate across transfers with hexadecane was $53.21 \pm 16.44 \mu\text{mol L}^{-1}\text{day}^{-1}$ and $0.36 \pm 7.24 \mu\text{mol L}^{-1}\text{day}^{-1}$ in the controls without hexadecane. In the phenanthrene-amended enrichment cultures, an average rate of $51.22 \pm 26.69 \mu\text{mol L}^{-1}\text{day}^{-1}$ sulfide production was observed, whereas $6.20 \pm 12.04 \mu\text{mol L}^{-1}\text{day}^{-1}$ sulfide production was observed without phenanthrene addition (Figure 13c and 13d). Rates of sulfide production in phenanthrene-amended cultures equaled or exceeded those in hexadecane cultures over the first three transfers. The sulfide production rate remained stable across transfers in hexadecane-amended enrichment cultures, whereas sulfide production rate declined across transfers in phenanthrene-amended enrichment cultures. For each transfer, control cultures were created with no carbon substrate from the previously active culture. We observed that sulfide production rate increased across transfers, which suggests that bacterial growth is stimulated by transferred metabolites or phenanthrene-degradation intermediates from the previous treatment.



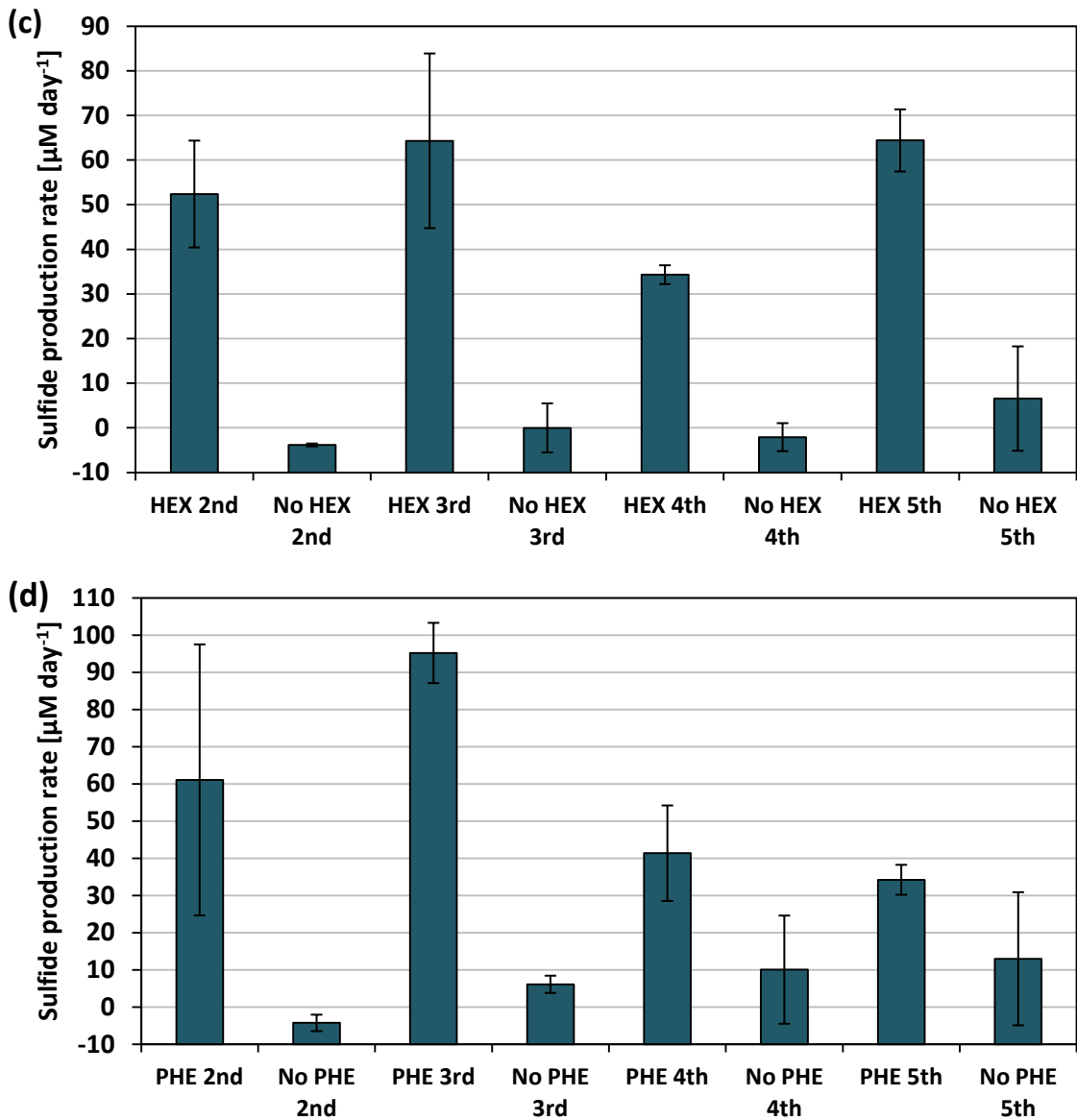


Figure 13 Sulfate respiration as determined by the accumulation of dissolved sulfide. (a) Sulfide accumulation in hexadecane-amended enrichment cultures from second, third transfers in triplicate and fourth, fifth transfers in four replicates. (b) Sulfide accumulation in phenanthrene-amended enrichment cultures from second, third transfers in triplicate and fourth, fifth transfers in six replicates. Solid lines depict sulfide increase with hydrocarbons, whereas dashed lines indicate stable sulfide concentration without hydrocarbons. (c) Sulfide production rates ($\mu\text{M day}^{-1}$) calculated from logarithmic phase of bacterial activity in sulfate-reducing enrichment cultures with or without hexadecane in triplicate for second, third transfers and in four replicates for fourth, fifth transfers and (d) with or without phenanthrene in triplicate for second, third transfers and in four replicates for fourth, fifth transfers. Error bars indicate standard deviation in biological replicate cultures.

2.4.2 *Microbial community structure in sediment and hexadecane- and phenanthrene-degrading enrichment cultures*

At the class level, *Deltaproteobacteria* constituted a relative abundance of 19.6% in the 0-5 cm depth interval from the sediment used as an inoculum, 37% in initial enrichments, and up to 86% across subsequent transfers of enrichment cultures. In contrast, members of *Nitrospira*, *Anaerolineae*, and *Gammaproteobacteria* declined in relative abundance in successive transfers (Figure 14). The family *Desulfobacteraceae* within the order *Desulfobacterales* comprised 2-3% relative abundance in inoculum sediment and was the most abundant group in hexadecane-amended cultures comprising nearly 70% of the total bacterial community in the fourth transfers with similar relative abundances in the DNA- and RNA-based libraries (Figure 15a). In the phenanthrene-degrading enrichment cultures, the family *Desulfarculaceae* within the order *Desulfarculales* comprised 0.4-1.5% relative abundance in inoculum sediment and showed the highest relative abundance throughout all transfers, especially in the RNA-based libraries where they represented 55% of the total community in the fourth transfer (Figure 15b). The dominant detected genus within this group in the phenanthrene-degrading enrichment cultures was *Desulfatiglans*, comprising 54% of the total community within the RNA-based library. The family *Desulfobacteraceae* was the second most abundant family constituting around 21% and 15% of the total community in DNA-based and RNA-based libraries, respectively. In both hexadecane- and phenanthrene-degrading enrichment cultures, *Desulfobulbaceae*, *Anaerolineaceae*, and *Nitrospiraceae* were enriched as high as 10-26% in initial enrichment cultures but their

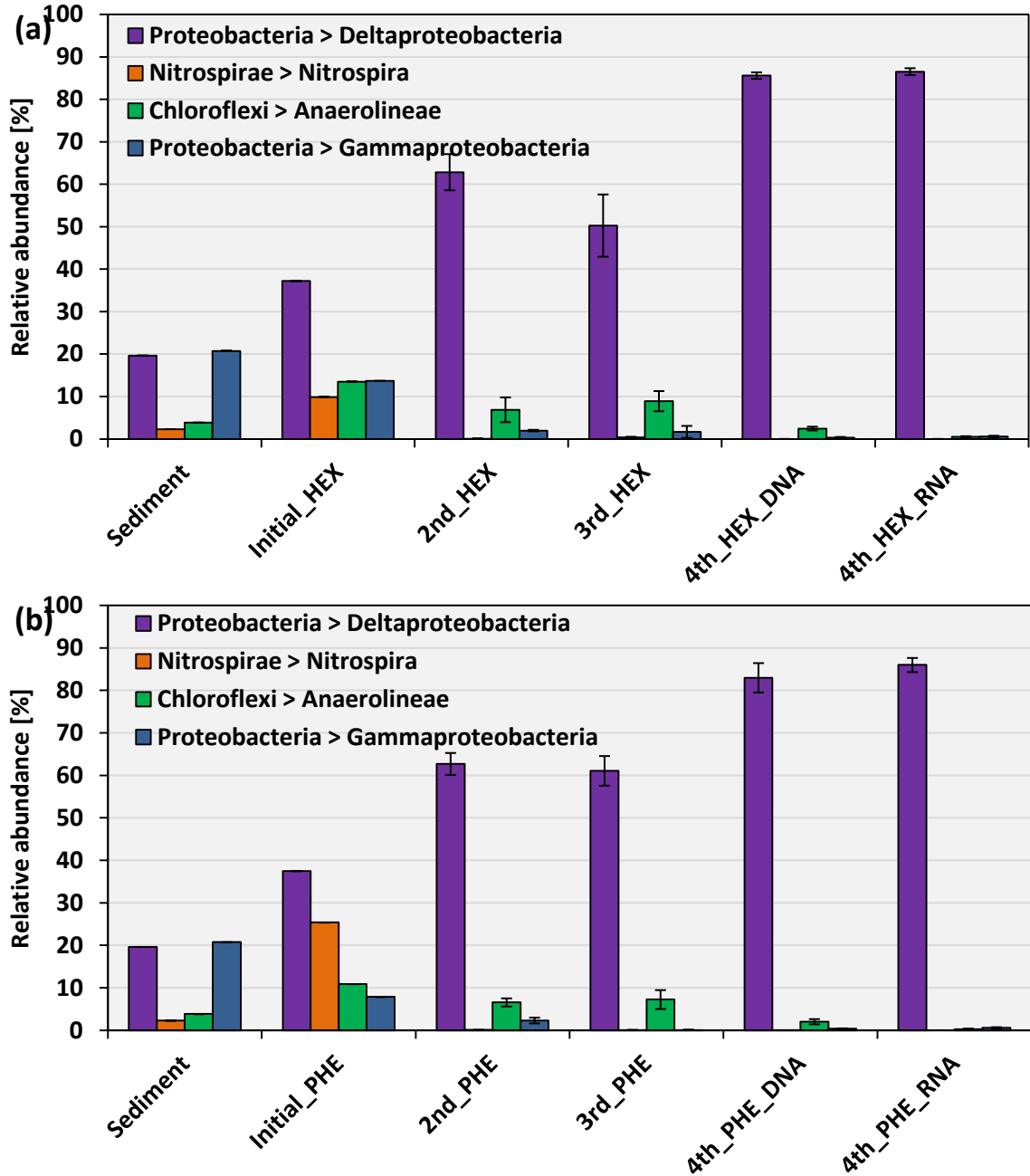


Figure 14 The relative abundance of classes *Deltaproteobacteria*, *Anaerolineae*, and *Gammaproteobacteria* in (a) hexadecane- and (b) phenanthrene- degrading enrichment cultures under sulfate-reducing conditions. Illumina next-generation sequencing was performed using genomic DNA isolated from initial, second, third, and fourth transfers and total RNA isolated from fourth transfer. Error bars indicate standard deviation among biological replicates.

abundance decreased over transfers (Figure 15). In all enrichment cultures, microbial diversity, as determined by Shannon indices, decreased across transfers (Figure 17a and 17b). Beta diversity analysis based on the Bray-Curtis distance metric showed that the initial enrichment cultures with hexadecane or phenanthrene contained more closely related microbial communities which then diverged based on carbon sources over successive transfers (Figure 17c).

2.4.3 *Quantitative molecular analysis of bacterial communities*

In hexadecane-amended enrichment cultures, bacterial abundance as determined by qPCR of SSU rRNA genes decreased from the second (5.66×10^6 copies ml⁻¹) to third transfer (8.55×10^4 copies ml⁻¹) but increased again in the fourth transfer (5.86×10^6 copies ml⁻¹). In the third transfers, no difference was observed in bacterial abundance between hexadecane-amended cultures and unamended controls. In the second transfer of phenanthrene-amended enrichment cultures, bacterial abundance was 2.46×10^6 copies ml⁻¹. In the third transfers of phenanthrene-amended cultures, we observed approximately 10 times higher bacterial abundance (2.83×10^6 copies ml⁻¹) in the phenanthrene-amended cultures in comparison to the unamended controls (2.96×10^5 copies ml⁻¹) after 98 days of incubation. After 139 days of incubation, bacterial abundance decreased to 1.04×10^6 copies ml⁻¹ in phenanthrene-amended enrichment cultures, although abundance was still 10-fold higher in comparison to unamended control cultures (9.74×10^4 copies ml⁻¹) (Figure 18).

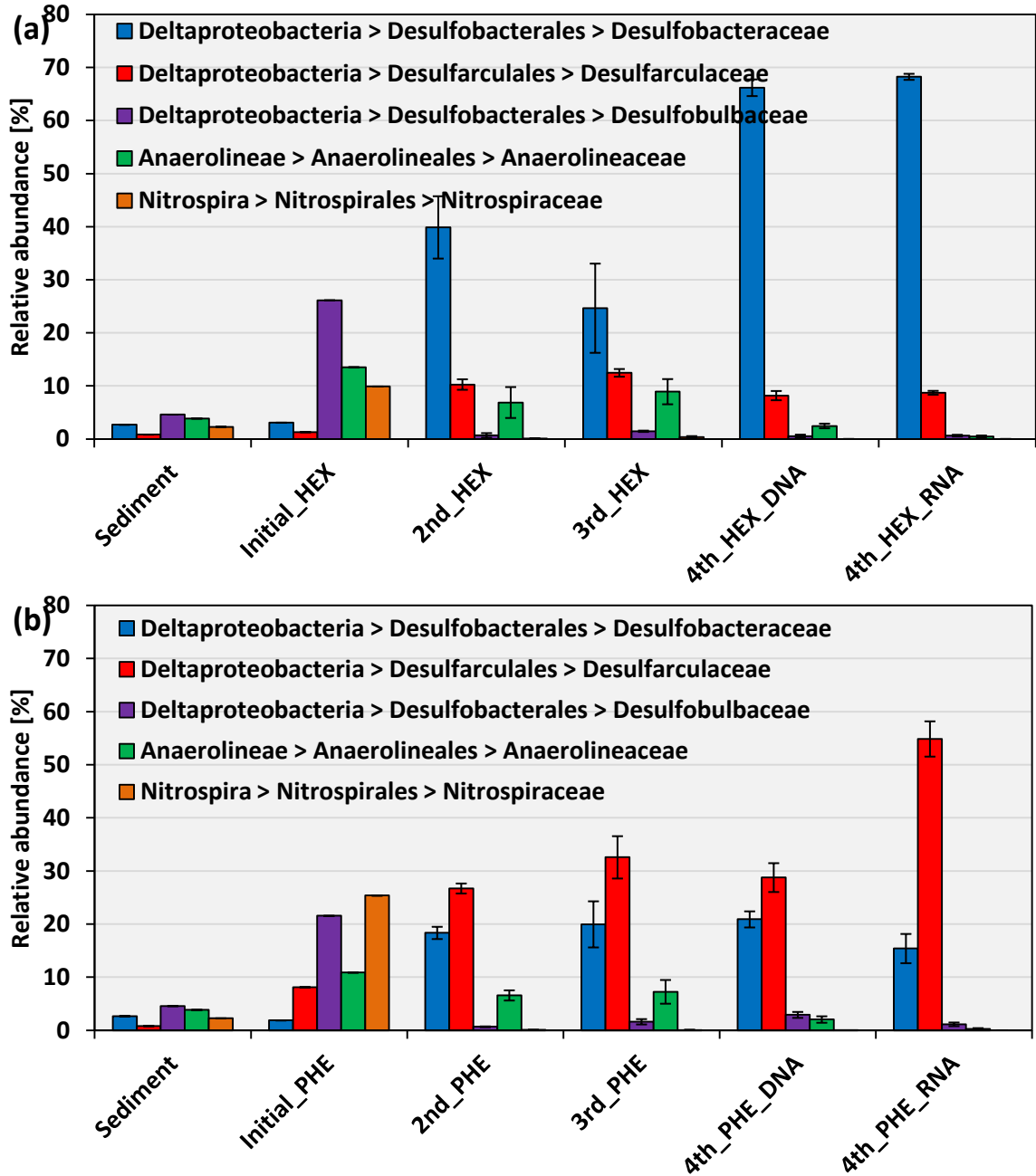


Figure 15 The relative abundance of microbial taxa at the family level in (a) hexadecane- and (b) phenanthrene-degrading enrichment culture under sulfate-reducing conditions. Illumina next-generation sequencing was performed using genomic DNA isolated from various transfer stages of enrichment cultures (initial, second, third, and fourth transfers) and using total RNA isolated from fourth transfer cultures. Error bars indicate standard deviation among biological replicates.

2.4.4 *Phylogenetic analysis of SSU rRNA gene sequences retrieved from enrichment cultures using clonal analysis and next-generation sequencing*

In order to improve phylogenetic placements and characterization of the dominant populations in the enrichment cultures, SSU rRNA gene sequences were obtained using a combination of clone library analysis and next generation sequencing. Clone libraries were screened using restriction fragment length polymorphism (RFLP) to select unique clones (data not shown). A total of 33 and 30 representative sequences (841-1525 bp) were obtained from hexadecane- and phenanthrene-degrading enrichment cultures, respectively. These sequences were inserted into the full SILVA v128 Ref_NR_99 tree using ARB parsimony. SSU rRNA gene sequences from their closest isolated relatives, previously sequenced environmental clones, sequences generated hydrocarbon-amended cultures, as well as outgroups were maintained to anchor the phylogenetic tree topology (Figure 16). The most abundant clone sequence from hexadecane-amended enrichment cultures was Clone 4_HEX (GenBank accession number MG923705), which was most closely related to *Desulfatibacillum alkenivorans* strain PF2803 and *D. aliphaticivorans* strain CV2803 with sequence similarities of 98%. These *Desulfatibacillum* strains are known to degrade alkene- and/or *n*-alkane using sulfate as a terminal electron acceptor (Cravo-Laureau et al., 2004a, 2004b). Clone 60_HEX (GenBank accession number MG923719) shared 96% sequence similarity with *Desulfobacterium cetonicum* strain

480, which was originally isolated from an oil field using butyrate as a substrate

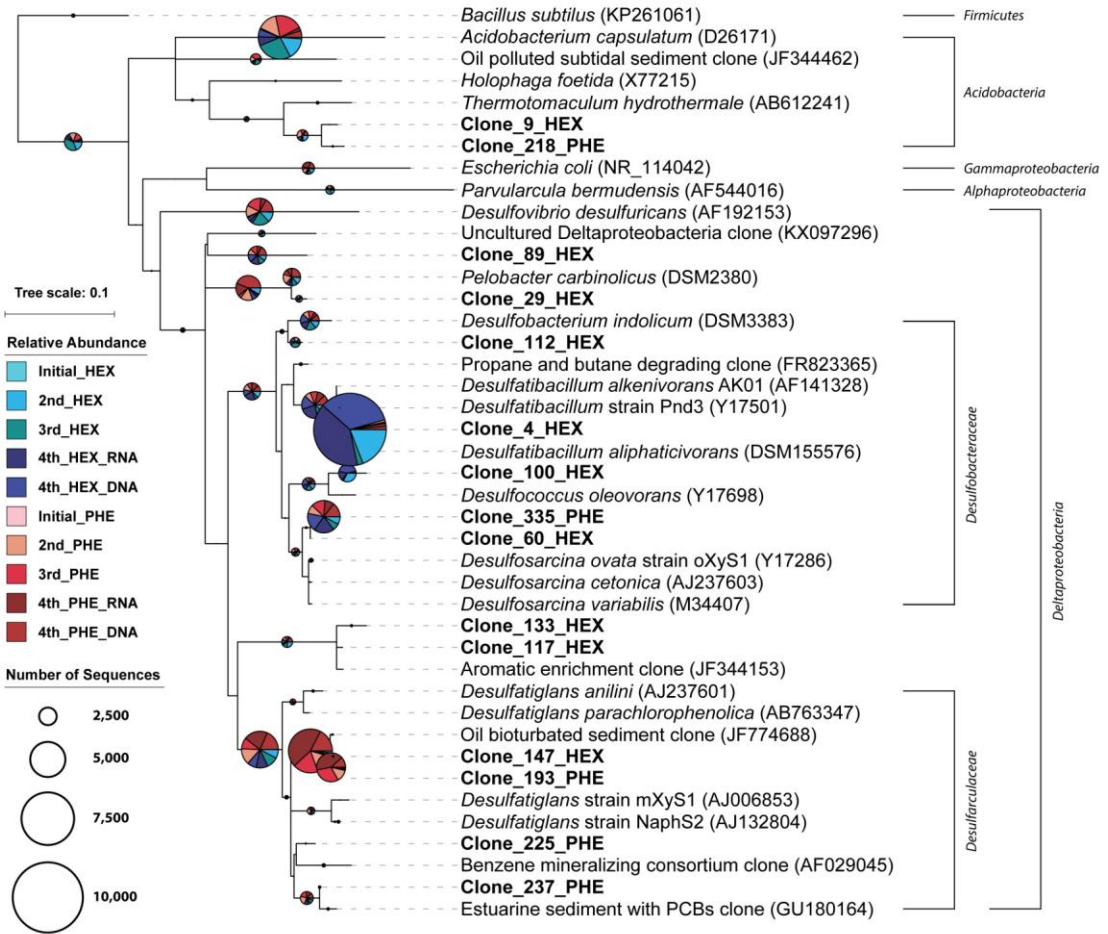
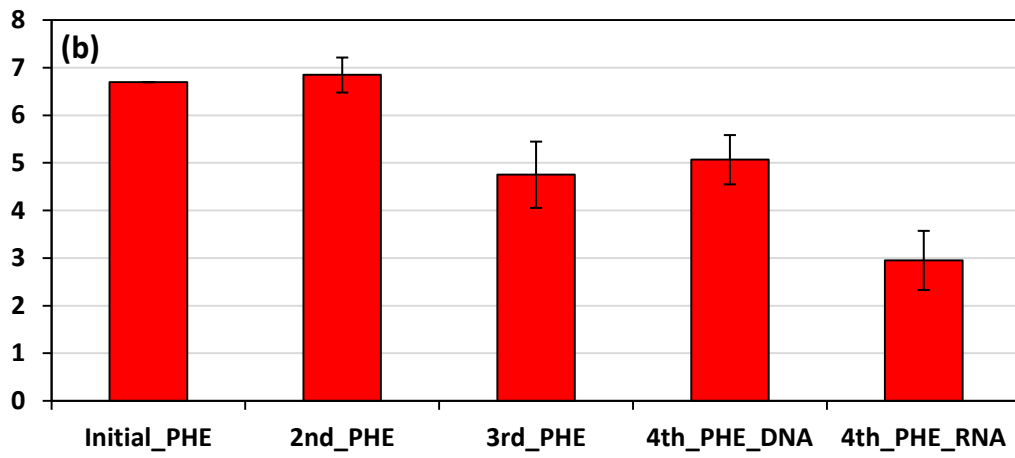
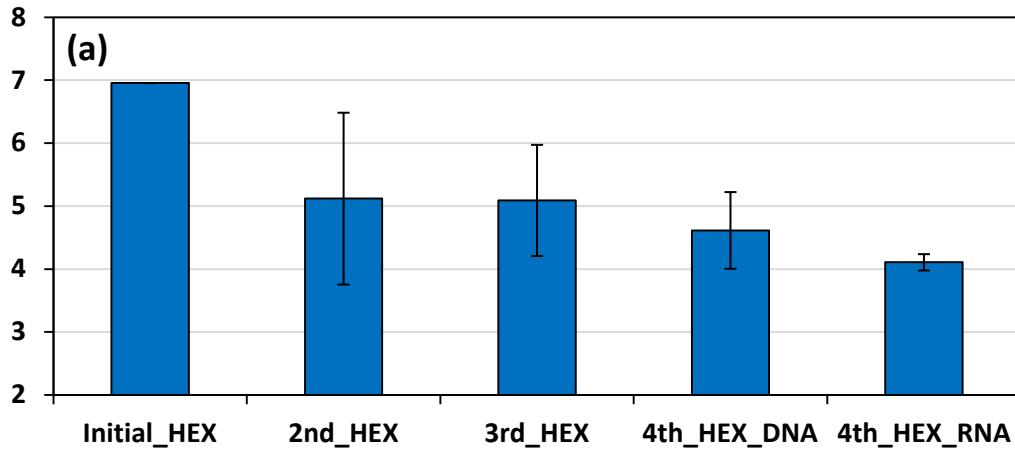


Figure 16 Phylogenetic analysis of SSU rRNA gene sequences retrieved from hexadecane- and phenanthrene-degrading enrichment cultures. SSU rRNA sequences from clone libraries, top isolated BLAST hits, similar clones were used as data set.



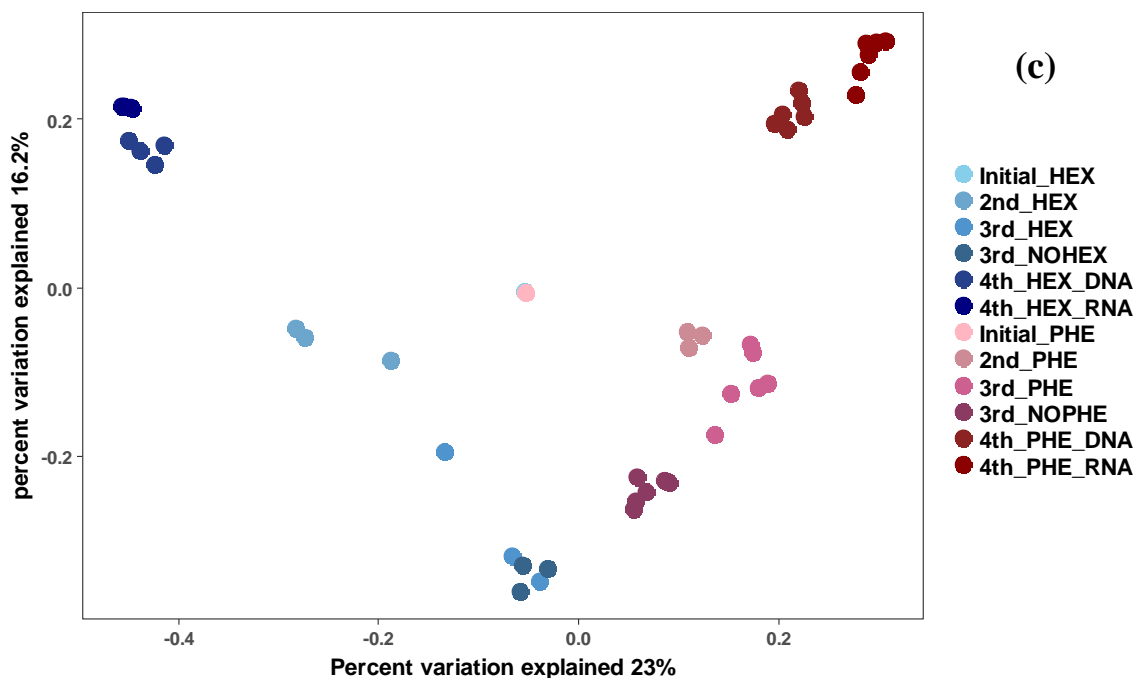


Figure 17 Alpha-diversity based on Shannon index in (a) hexadecane- and (b) phenanthrene-degrading enrichment cultures under sulfate-reducing conditions. Error bars indicate standard deviation in triplicate for second, and third transfers, four and six replicates for fourth transfer of hexadecane- and phenanthrene-amended enrichment cultures, respectively. (c) Principal component analysis plot of beta-diversity based on the Bray-Curtis distance metric.

(Janssen and Schink, 1995). From phenanthrene-degrading enrichment cultures, Clone 193_PHE (GenBank accession number MG923739) showed the second highest relative abundance among clone sequences and was related to the aniline- and chlorophenol-degrading isolates *Desulfatiglans anilini* strain Ani1 and *Desulfatiglans parachlorophenolica* strain DS (95% sequence identity). Clone 193_PHE (GenBank accession number MG923739) sequence shared 94% sequence identity with the anaerobic naphthalene-degrading pure culture strain NaphS2 (Galushko et al., 1999).

2.4.5 Degradation of hexadecane and phenanthrene coupled to sulfate reduction

Mineralization of hexadecane or phenanthrene in enrichment cultures was demonstrated by quantifying hexadecane or phenanthrene loss in the HMN layer and sulfate loss in the aqueous phase (Table 2). Observed stoichiometric ratios were close to the theoretical ratios of 12.25:1 for hexadecane and 8.25:1 for phenanthrene degradation coupled to sulfate reduction. The amount of sulfate consumed was equivalent to 133.34 %, 97.78 % in fourth and fifth transfers and 123.51% in fourth transfer of expected sulfate loss in hexadecane- and phenanthrene-degrading enrichment cultures, respectively.

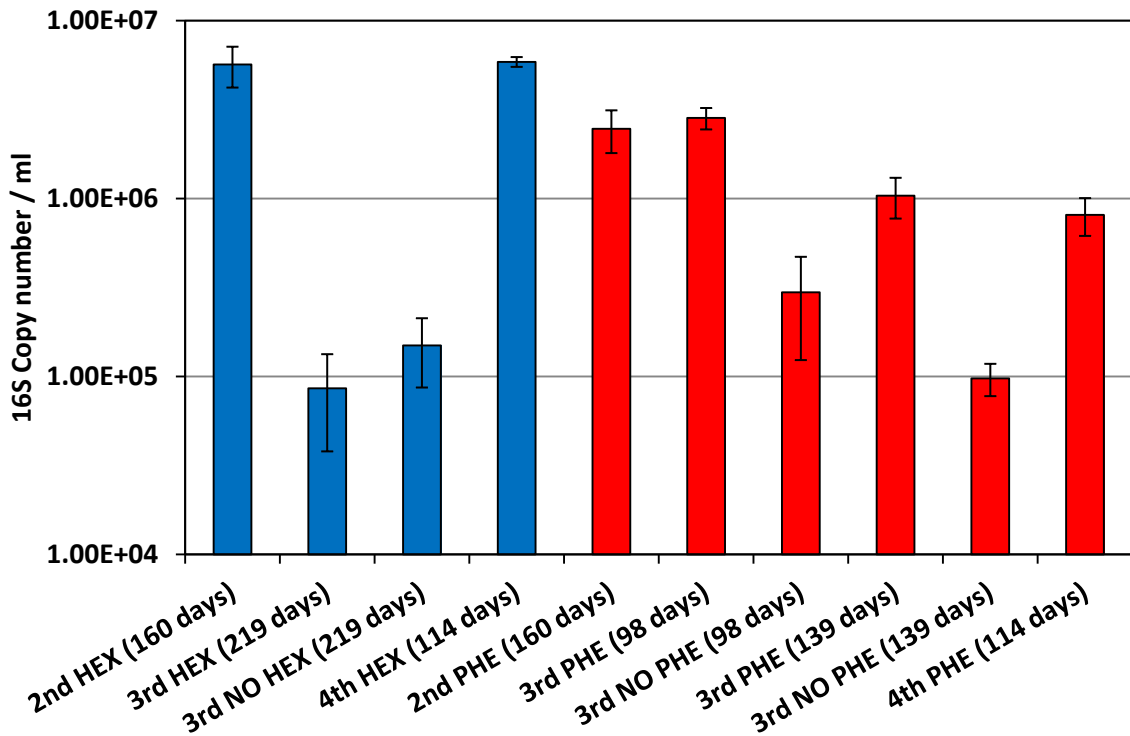


Figure 18 Bacterial abundance as determined by qPCR of SSU rRNA genes in hexadecane-amended and phenanthrene-amended enrichment cultures under sulfate-reducing conditions.

Table 2 Calculated electron balances for enrichment cultures grown on hexadecane or phenanthrene as electron donor and sulfate as electron acceptor.

Hexadecane degradation				
$C_{16}H_{34} + 12.25SO_4^{2-} + 8.5H^+ \rightarrow 16HCO_3^- + 12.25H_2S + H_2O$				
Incubation time (day)	Hexadecane loss (μ mole)	Expected sulfate loss (μ mole)	Observed sulfate loss (μ mole)	% of expected
230 (4 th transfer)	34.78 \pm 3.83	426.05	568.09 \pm 44.40	133.34
121 (5 th transfer)	29.67 \pm 3.66	484.59	473.82 \pm 55.60	97.78

Phenanthrene degradation				
$C_{14}H_{10} + 8.25 SO_4^{2-} + 9H_2O + 2.5H^+ \rightarrow 14HCO_3^- + 8.25H_2S$				
Incubation time (day)	Phenanthrene loss (μ mole)	Expected sulfate loss (μ mole)	Observed sulfate loss (μ mole)	% of expected
230 (4 th transfer)	35.83 \pm 7.2	295.62	365.13 \pm 49.64	123.51

2.5 Discussion

Anaerobic microorganisms that degrade hydrocarbons are understudied relative to their aerobic counterparts and our knowledge of anaerobic PAH degradation is still in its infancy (Meckenstock et al., 2016). A number of strains have been isolated and their metabolic pathways characterized for anaerobic alkane degradation, whereas very little

information is available on the biochemical mechanisms of anaerobic PAH degradation. In the case of both alkanes and PAHs, the ecology of anaerobes that degrade them in the environment remains unclear. Phenanthrene represents the highest molecular weight PAH compound which was shown to be degraded under anoxic conditions (Meckenstock et al., 2016), and this compound is therefore likely to persist for longer periods after oil discharge into the environment. Since PAHs are carcinogenic, mutagenic, and toxic to organisms, microbial activation and metabolism is an essential component of removing persistent PAHs from the environment (Huntley et al., 1993). Thus, we chose to study and compare the microbial populations that degrade a representative alkane (hexadecane) and PAH (phenanthrene), coupled to sulfate reduction, a dominant terminal electron accepting process, in marine sediments from the northern Gulf of Mexico (GoM). The study focused on sediments distant from natural seeps where the indigenous microbial communities are not as likely to be conditioned or primed to degrade hydrocarbons (Joye et al., 2016).

This is the first cultivation-based study that elucidates the anaerobic microbial populations which degrade alkanes or PAHs in GoM sediments that are not normally exposed to high levels of hydrocarbons. The most abundant group in the sediment inoculum was *Desulfobulbus* within the family *Desulfobulbaceae* that comprised 3.9% of total microbial community. The relative abundance of *Desulfobulbaceae* increased in initial enrichment cultures to as high as 21-26% but it decreased to 1-2% after successive transfers in both hexadecane- and phenanthrene-degrading enrichments. Cultured members of the *Desulfobulbaceae* use short-chain fatty acids or oxidize organic substrates incompletely to acetate, which suggests that their abundance increased when

carbon substrates from inoculum sediment were available but it decreased over successive transfers (Kleindienst et al., 2012). All enrichment cultures were dominated by *Deltaproteobacteria*, which rose to near 90% of all SSU rRNA gene sequences retrieved in comparison to a relative abundance of 17-21% in the sediment inoculum. However, within the *Deltaproteobacteria*, the dominant microbial populations in the phenanthrene-degrading enrichments were distinct from those of hexadecane-degrading enrichment cultures. Whereas dominant populations closely related to known alkane-degrading *Desulfatibacillum* strains in the *Desulfobacteraceae* were detected in cultures grown on hexadecane, populations more closely related to *Desulfatiglans* of the *Desulfarculaceae* predominated when phenanthrene was the sole carbon and energy source. *Desulfobacteraceae* and *Desulfarculaceae* comprised 2-3% and 0.5-1.5% of the total community in the sediment inoculums, respectively, and their relative abundance increased up to 67% and 55% in hexadecane- and phenanthrene-amended enrichment cultures. Results from RNA-based SSU rRNA amplicon libraries showed that these families of sulfate-reducers are not only abundant but represent the most active members in the enrichment cultures (Figure 15).

In the GoM region, evidence for microbial degradation of hydrocarbons was shown in Pensacola beach sands (Kostka et al., 2011; Rodriguez-R et al., 2015), surface sea water (Yang et al., 2016), deep intrusion layers of the water column (Baelum et al., 2012; Hazen et al., 2010), natural hydrocarbon seeps (Orcutt et al., 2017), and salt marsh sediments (Atlas et al., 2015; Koo et al., 2014; Mendelsohn et al., 2012) following the Deepwater Horizon oil spill. Microbial hydrocarbon degradation under oxic conditions is well characterized, whereas anaerobic hydrocarbon degradation in sediments from the

GoM is relatively understudied. Metagenomic analysis showed anaerobic hydrocarbon degradation potential in surficial sediments near Macondo wellhead and an increase in the abundance of genes involved in denitrification pathways in samples that exceeded the Environmental Protection Agency (EPA)'s benchmarks for PAHs compared with those that did not (Kimes et al., 2013; Mason et al., 2012). Cultivation-based studies under anaerobic conditions in salt marsh sediments also showed that sulfate-reducing bacteria were enriched in the presence of Macondo oil (Boopathy et al., 2012; Natter et al., 2012). Here we report the anaerobic degradation of hexadecane and phenanthrene in sulfate-reducing cultures from the Gulf of Mexico seafloor. We successfully obtained sediment-free enrichment cultures under sulfate-reducing conditions and identified key taxa that may be used as model hydrocarbon degraders for long-term monitoring of natural attenuation of oil contamination in anoxic marine sediment.

Community analysis of our cultures showed enrichment of indigenous *Deltaproteobacteria*, which includes most of the putative hydrocarbon-degrading strains studied under sulfate-reducing conditions (Heider and Schuhle, 2013; Teske, 2010). SSU rRNA gene clone sequences from hexadecane-degrading enrichment cultures of this study were affiliated with *Desulfatibacillum*, *Desulfobacterium*, *Desulfosarcina*, *Desulfococcus*, and *Desulfospira* within the family *Desulfobacteraceae*. In a previous study, members of the *Desulfosarcina* and *Desulfococcus* clades were identified as key alkane degraders at marine seeps (Kleindienst et al., 2014). Close relatives of SSU rRNA clone sequences from our cultures represent known alkane-degraders under sulfate-reducing conditions such as *Desulfatibacillum alkenivorans* AK-01, *Desulfatibacillum aliphaticivorans* CV2803. *Desulfatibacillum alkenivorans* AK-01 was isolated from the

active sulfate-reducing enrichments of petroleum-contaminated estuarine sediment collected from the Arthur Kill in New York and was capable of degradation of C₁₃-C₁₈ alkanes, C₁₅-C₁₆ alkenes, and C₁₅-C₁₆ alkanols (So and Young, 1999b).

In phenanthrene-degrading enrichment cultures of the present study, the family *Desulfarculaceae* were highly enriched based on amplicon sequence analysis especially in RNA-based libraries. The enrichment of *Desulfarculaceae* from sediments in the northern GoM is consistent with results from oil-polluted subtidal sediments investigated on the Spanish coast after the Prestige oil spill (Acosta-González et al., 2013). The majority of clone sequences from phenanthrene-degrading enrichment cultures in this study were affiliated to genus *Desulfatiglans* within the family *Desulfarculaceae* and were highly similar (>97% sequence identity) to Illumina MiSeq amplicon sequences that constituted 55% of the RNA-based total community. This indicates that *Desulfatiglans* spp. may play a key role in phenanthrene degradation under sulfate-reducing conditions. Characterized isolates which show high sequence identity to sequences retrieved from our cultures include *Desulfatiglans anilini* strain Ani1, *Desulfatiglans parachlorophenolica* strain DS, and *Desulfosarcina ovata* strain oXyS1, that are known aniline-, parachlorophenol-, and *o*-xylene-degrading pure cultures under sulfate-reducing conditions (Harms et al., 1999; Schnell et al., 1989; Suzuki et al., 2014). Little information is available on phenanthrene degradation under sulfate-reducing conditions (Coates et al., 1996; Davidova et al., 2007; Zhang and Young, 1997). Clone sequences retrieved from a phenanthrene-degrading enrichment of hydrocarbon-contaminated marine sediments in San Diego Bay (Davidova et al., 2007) were not closely related to clone sequences from this study. In our phenanthrene-degrading enrichment cultures,

Clone 193_PHE, within the *Desulfarculaceae* was the most abundant clone sequence in the 4th transfer, constituting 4.20% of total abundance. Overall, our results showed the genus of *Desulfococcus*, *Desulfatibacillum*, and *Desulfosarcina* may be the key players in *n*-alkane degradation, whereas *Desulfatiglans* spp. may be responsible for PAH degradation under sulfate-reducing conditions.

Another possibility for hexadecane and phenanthrene degradation in enrichment cultures in this study is syntrophic biodegradation (Sieber et al., 2012). We suggest that hexadecane or phenanthrene may have been mineralized by the coupled mutualistic interaction between hydrocarbon-fermenting and H₂/acetate/formate-utilizing microorganisms. Previous studies have characterized syntrophic consortia mineralizing hydrocarbons under sulfate-reducing conditions using stable isotope probing, identifying members of the *Pelotomaculum*, *Pelobacter*, and *Syntrophaceae* groups as putative hydrocarbon fermenting bacteria (Aklujkar et al., 2012; Berlendis et al., 2010; Herrmann et al., 2010; Kevorkian et al., 2018; Kleinsteuber et al., 2008). In this study, the putative hydrocarbon fermenter, *Pelobacter*, was detected in abundance in both hexadecane- and phenanthrene-degrading enrichment cultures (0.4 – 3.1 % and 1.8 – 10.4 % relative abundance, respectively), in corroboration of previous work. Members of *Syntrophaceae* were also detected in all enrichment cultures (0.1 – 1% relative abundance). These microbial groups could produce intermediates such as H₂ and/or acetate that are supplied to sulfate-reducing bacteria in a syntrophic relationship. The *Desulfobacteraceae* have been implicated as hydrogenotrophs in hexadecane-degrading enrichment cultures that consume hydrogen molecules produced from initial fermentation of hydrocarbons (Herrmann et al., 2010). In phenanthrene-degrading enrichment cultures, the family

Desulfarculaceae may play a role as a formate and/or acetate utilizing microbial group. The only isolated member of the family *Desulfarculaceae*, *Desulfarculus baarsii* strain, is was shown to oxidize formate, acetate, short-chain and long-chain fatty acids to CO₂ (Sun et al., 2010). Potential secondary fermenters or scavengers such as *Ignavibacteria* and *Anaerolineae* were also detected in all enrichment cultures but their relative abundance decreased over transfers (Kleinstuber et al., 2008). However, syntrophic degradation of hexadecane and phenanthrene in enrichment cultures from this study requires confirmation through additional experimentation using approaches such as stable isotope probing, -metagenomics, and fluorescence in situ hybridization (FISH)/nanoscale secondary ion mass spectrometry (nanoSIMS).

In this study, the consumption of hexadecane or phenanthrene in sulfate-reducing cultures agreed well with sulfate depletion expected from the stoichiometry of each reaction and corroborated previous studies. So and Young observed a loss of sulfate that corresponded to 89% in comparison to the expected stoichiometry calculated from hexadecane consumption in their enrichment cultures (So and Young, 2001). In this study, 97.78 and 133.34 % predicted sulfate loss was observed based on hexadecane consumption. Sulfate reduction rates in the So and Young study were 6 times higher (approximately 315 $\mu\text{mol L}^{-1}\text{day}^{-1}$) than those observed in this study ($53.21 \pm 16.44 \mu\text{mol L}^{-1}\text{day}^{-1}$); however, this may be explained by the fact that So and Young used a 3.4 times higher initial hexadecane concentration. In phenanthrene amended enrichment cultures, Davidova et al. showed a predicted 109% sulfate loss based on consumption of carbon substrate (Davidova et al., 2007), whereas we observed a 124% sulfate loss calculated from phenanthrene consumption. Higher sulfate depletion in our

study may be explained by sulfate reduction coupled with the oxidation of dead biomass from carried over from previous culture transfers. These results confirm that hexadecane and phenanthrene degradation are closely coupled to sulfate reduction in enrichment cultures from this study.

In summary, our understanding of the biochemical pathways of anaerobic hydrocarbon degradation is a key knowledge gap for predicting the long-term fate of recalcitrant oil compounds in fine-grained sediments that cover much of the seafloor. Anaerobic PAH biodegradation remains particularly understudied. This study describes the establishment of enriched sulfate-reducing consortia capable of the mineralization of hexadecane and phenanthrene. To our knowledge, this is the first characterization of anaerobic hydrocarbon-degrading microbial populations in non-seep marine sediments that were not pre-exposed to extensive hydrocarbon inputs in the GoM. This is significant since most of the seafloor is not exposed to high levels of petroleum hydrocarbons prior to a spill. In addition, we have identified key taxa that may be used as model organisms in conceptual models for the natural attenuation of oil contamination in anoxic marine muds.

CHAPTER 3. Metagenomic insights into phenanthrene degradation under sulfate-reducing conditions

3.1 Abstract

Biochemical pathways of anaerobic degradation of phenanthrene are reported in only a few studies. We detected a phenanthrene carboxylic acid in phenanthrene-degrading enrichment culture supplemented with sulfate as a terminal electron acceptor, providing an evidence to indicate carboxylation as an activation mechanism for phenanthrene degradation. Hydroxybenzoic acid was also detected as a key intermediate, suggesting that phenolic compounds were formed during phenanthrene degradation. Total 9 bins with good qualities were retrieved and 8 out of 9 bins were assigned to novel taxonomic groups. Results from metagenomic analysis revealed that genes involved in salicylate and gentisate, homogentisate, aromatic compound degradation pathways were detected in all good quality bins. Moreover, all bins contained genes encoding 4-hydroxybenzoate carboxylase, which agrees with detection of hydroxybenzoate from metabolite analysis. Majority of bins also possessed genes responsible for ABC transporter system. To the best of our knowledge, this study is the first demonstration of genomic analysis from phenanthrene-degrading anaerobic enrichment culture. Additional research such as stable isotope probing, metatranscriptomic, proteomic approaches are needed to elucidate pathways involved in phenanthrene-degradation under sulfate-reducing conditions.

3.2 Introduction

Hydrocarbons are formed either via geochemical or biological processes and are highly abundant in nature. High C–H bond dissociation energies of hydrocarbons are responsible for low chemical reactivities. Many hydrocarbons such as polycyclic aromatic hydrocarbons (PAHs) are considered as hazardous organic compounds due to their mutagenic and carcinogenic properties. The biological degradation of PAHs is thus of great interest as a means for the removal of these potentially harmful compounds from the environment. Under aerobic conditions, hydrocarbon metabolism is initiated by mono- or dioxygenase enzymes and these pathways are relatively well studied. However, oxygen is quickly depleted at the top few millimeters to centimeters in contaminated marine sediments or aquifers, which represent typical natural environments contaminated with hydrocarbons. In the absence of oxygen, hydrocarbons undergo biodegradation under nitrate-, iron-, sulfate-reducing, or methanogenic conditions. Anaerobic hydrocarbon-degrading bacteria use a number of enzymatic reactions for the mechanistically sophisticated process of C–H bond activation. In the last few decades, studies have demonstrated the pathways involved in anaerobic hydrocarbon degradation under different terminal accepting conditions. A substantial amount of research has been devoted to the pathways for saturated alkane degradation under anaerobic conditions. Characterization of fatty acid metabolites in alkane-degrading strain AK-01 under sulfate-reducing conditions showed that alkanes are activated by the addition of fumarate to form (1-methylalkyl)succinate and this reaction is catalyzed by the glycyl radical enzyme, alkylsuccinate synthase (Callaghan et al., 2006; So and Young, 1999a). Genome analysis of strain AK-01 provided a blueprint for anaerobic alkane metabolism including

substrate activation, CoA ligation, carbon-skeleton rearrangement and decarboxylation (Callaghan et al., 2012). *Desulfatibacillum aliphaticivorans* CV2803 was isolated from a hydrocarbon-polluted marine sediment in France. Strain CV2803 was able to oxidize C₁₃-C₁₈ alkanes and C₇-C₂₃ alkenes but growth on aromatic hydrocarbons was not observed (Cravo-Laureau et al., 2004a). Similar to findings from the previous study of strain AK-01, metabolite characterization revealed that alkane activation by strain CV2803 occurred by fumarate addition to yield methyl-branched fatty acids (Cravo-Laureau et al., 2005).

The anaerobic degradation of monoaromatic hydrocarbons (benzene, toluene, ethylbenzene, and xylene, BTEX) is relatively well studied. In contrast, the degradation of PAHs under anoxic conditions remains in its infancy, the ecology and biochemistry of microorganisms capable of these processes mostly unknown. Few studies have been conducted in enrichment cultures and only a single pure culture is available. Evidence is available for anaerobic degradation of naphthalene, 2-methylnaphthalene, and phenanthrene under sulfate-reducing conditions in both pure cultures and enrichment cultures (Coates et al., 1996; Galushko et al., 1999; Musat et al., 2009; Zhang and Young, 1997). The peripheral pathway of 2-methylnaphthalene degradation was elucidated in enrichment culture N47 that was mainly comprised of *Deltaproteobacteria*. Previous studies have shown that 2-methylnaphthalene is activated by fumarate addition to the methyl group (Meckenstock et al., 2000; Safinowski and Meckenstock, 2006). Catabolic genes that have been proposed as molecular markers for anaerobic degradation of aromatic hydrocarbons include benzylsuccinate synthase (*bsc*) which catalyzes anaerobic toluene degradation and naphthyl-methylsuccinate synthase (*nmsA*) that catalyzes 2-methylnaphthalene degradation (Beller et al., 2002; von Netzer et al., 2013). Genes for

carboxylation of benzene and naphthalene have been identified (Abu Laban et al., 2010; F. D. Bergmann et al., 2011; DiDonato et al., 2010), however, there are too few sequences known to derive specific primer pairs. To elucidate the initial activation of naphthalene, metabolite analyses were conducted and carboxylation and methylation of naphthalene were suggested (Safinowski and Meckenstock, 2006; Zhang and Young, 1997). Proteogenomic approach of the sulfate-reducing culture N47 elucidated that some of the gene products shared sequence similarity to subunits of phenylphosphate carboxylase, one of the key enzymes in anaerobic phenol degradation (F. Bergmann et al., 2011; F. D. Bergmann et al., 2011). A similar gene cluster was up-regulated in the marine *Deltaproteobacterium* NaphS2 during growth with naphthalene (DiDonato et al., 2010). Anaerobic degradation of the three-ring PAH phenanthrene was proposed to be activated via carboxylation based on the detection of phenanthrene-carboxylic acid as major metabolite in culture supernatant (Davidova et al., 2007; Zhang and Young, 1997). Zhang and Young showed mineralization of ^{14}C -phenanthrene to $^{14}\text{CO}_2$ and incorporation of ^{13}C -bicarbonate to phenanthrene (Zhang and Young, 1997). Another study also showed that ^{13}C -bicarbonate was incorporated into carboxyl group at C-2 position of phenanthrene carboxylic acid (Davidova et al., 2007). To date, enzymes involved in phenanthrene degradation under anaerobic conditions are unknown. Thus, the main objectives of this study were to elucidate the biochemical pathways that activate phenanthrene under sulfate-reducing conditions as well as to explore potential molecular markers for anaerobic phenanthrene degradation. A stable, sediment-free enrichment culture that couples phenanthrene degradation to sulfate reduction was generated previously from shallow muddy sediments in the Gulf of Mexico (see chapter 2). This

enrichment culture was employed to investigate metabolic potential through a combination of metabolomics and metagenomics.

3.3 Materials and methods

3.3.1 Cultivation of the enrichment culture

The enrichment culture was cultivated in artificial seawater medium as described previously (composition per liter: 20.0 g NaCl, 3.0 g MgCl₂·6H₂O, 0.15 g CaCl₂·2H₂O, 0.3 g NH₄Cl, 0.2 g KH₂PO₄, 0.5 g KCl, 4.0 g Na₂SO₄, 1 ml trace elements A, 1 ml trace elements B, 1 ml vitamin mixture, 1 ml thiamine solution, 1 ml B₁₂ solution, and buffered with 30 mM NaHCO₃) (Widdel, 2010). The medium was amended with 1 mM of sodium sulfide as a reducing agent and 0.0001% solution of resazurin as a redox indicator. Triplicate enrichment cultures were amended with phenanthrene (98%, St. Louis, MO) as a sole carbon source in 2,2,4,4,6,8,8-heptamethylnonane (HMN) (phenanthrene; 8.91 g L per liter HMN) as the inert carrier (98%, Acros Organics, Morris Plains, NJ) along with hydrocarbon-free controls. One hundred ml of enrichment culture was prepared in 165-ml serum bottles with a N₂/CO₂ (90:10 v/v) headspace and sealed with butyl rubber stoppers. Strictly anaerobic technique was used throughout enrichment culture preparation and transfers. Serum bottles were incubated nearly horizontally to maximize contact between the medium and the hydrocarbon layer as well as to minimize contact between the HMN layer and the butyl rubber stopper. Enrichment cultures were incubated at 30 °C in the dark without agitation. Sulfate reduction activity was monitored by measuring the accumulation of sulfide using the methylene blue method (Cline, 1969). When the

sulfide concentration reached ~8 mM, enrichment cultures were successively transferred into fresh medium (20% v/v) to obtain sediment-free cultures. A stable sediment-free enrichment was obtained after 5 successive transfers over a 4 year period.

3.3.2 *Metabolite extraction and analysis*

Duplicates of 50 ml enrichment culture were acidified to < pH 2 with 6N HCl in clean serum bottles that were prewashed with acetone. Each sample was extracted three times using ethyl acetate. Organic extracts were combined and concentrated to 50 – 100 μ L by rotary evaporation and then reacted with N,O-bis(trimethylsilyl)trifluoroacetamide (Thermo Fisher Scientific, Waltham, MA, USA) to form trimethylsilyl derivatives. Derivatized metabolites were separated by gas chromatography–mass spectrometry with a 50 m HP-1 capillary column (Agilent, Santa Clara, CA, USA) using a previously published protocol (Gieg and Suflita, 2002). The oven temperature was held at 45°C for 5 min, then increased at 4°C per min to 270°C and held for 15 min. Metabolites were identified based on matching mass spectral profiles with authentic standards as described previously (Davidova et al., 2007).

3.3.3 *Nucleic acid extraction and library preparation*

Total genomic DNA was extracted using a MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA) with slight modifications from the manufacturer's protocol as follows. Ten ml of enrichment cultures was centrifuged at 10,000 \times g for 5

minutes in sterile falcon tubes, the resulting cell pellet was transferred to the provided 2 ml bead tube, and total DNA was extracted following the manufacturer's protocol. DNA sequence libraries were prepared using the Illumina Nextera XT DNA library prep kit according to manufacturer's instructions except the protocol was terminated after isolation of cleaned double stranded libraries. Library concentrations were determined by fluorescent quantification using a Qubit HS DNA kit and Qubit 2.0 fluorometer (ThermoFisher Scientific) and samples run on a High Sensitivity DNA chip using the Bioanalyzer 2100 instrument (Agilent) to determine library insert sizes. Libraries were sequenced using a MiSeq reagent v2 kit for 500 cycles (2 x 250 bp paired end run) on an Illumina MiSeq instrument (located in the School of Biological Sciences, Georgia Institute of Technology). Additional sequencing of the same library samples was carried out on an Illumina HiSeq 2500 instrument (located in the High Throughput DNA Sequencing Core, Georgia Institute of Technology) using the HiSeq Rapid PE Cluster Kit v2 and HiSeq Rapid SBS Kit v2 (Illumina) for a rapid run of 300 cycles (2 x 150 bp paired end). Adapter trimming and demultiplexing of sequenced samples was carried out by each respective sequencing instrument.

3.3.4 Metagenomic shotgun sequencing

Total genomic DNA was sent to Georgia Institute of Technology sequencing facility to run on both Illumina HiSeq and MiSeq platforms. The DNA was fragmented and the library was prepared using TruSeq Kit (Illumina) according to the manufacturer's protocol. Triplicates of total DNA extracts from enrichment cultures were sequenced in

one flow cell lane with a 2×150 bp and 2×250 bp paired-end format for the HiSeq and MiSeq platforms, respectively. Raw Illumina sequence reads were trimmed using the SolexaQA package (Cox et al., 2010). Reads were trimmed where Phred quality scores dropped below 20. Reads shorter than 50bp were discarded and adapter trimming was performed with scythe. Nonpareil was employed for read redundancy and to estimate the coverage of the microbial community achieved by the metagenomic dataset (Rodriguez-R and Konstantinidis, 2014). Reads were merged using PEAR (Zhang et al., 2014) and assembled with IDBA-UD using concatenated merged and unmerged reads. SSU rRNA gene sequences were extracted using parallel-META (Jing et al., 2017). OTUs were picked by a closed-reference OTU picking method with 97% sequence identity using QIIME (Caporaso et al., 2010b). Representative OTUs were picked and their taxonomy was assigned using the RDP classification algorithm set at a 50% confidence rating with the SILVA Small Subunit rRNA Database (https://www.arb-silva.de/no_cache/download/archive/release_123/Exports/). The resultant OTU table was used for further phylogenetic analysis. Resulting contigs were binned using MaxBin and checkM was used to estimate completeness and contamination of each bin (Parks et al., 2015; Wu et al., 2014). Quality of bins was calculated (Quality = Completeness – $5 \times$ Contamination) and bins with a quality score above 50 were used for further gene annotation. Bins with good quality were uploaded on the Rapid Annotation using Subsystem Technology (<http://rast.nmpdr.org/>) and BlastKORALA and GhostKOALA on Kyoto Encyclopedia of Genes and Genomes (KEGG) for functional annotation. Assembled contigs were taxonomically annotated using MyTaxa (Luo et al., 2014). MiGA was used based on the NCBI prokaryotes genome database to determine the most

likely taxonomic classification and novelty rank from good quality bins (<http://enve-omics.ce.gatech.edu:3000/>). Read recruitment plots were obtained as described previously with minimum cut-off for a match of 70% of identity and 60 bp alignment length (Rodriguez-R and Konstantinidis, 2016).

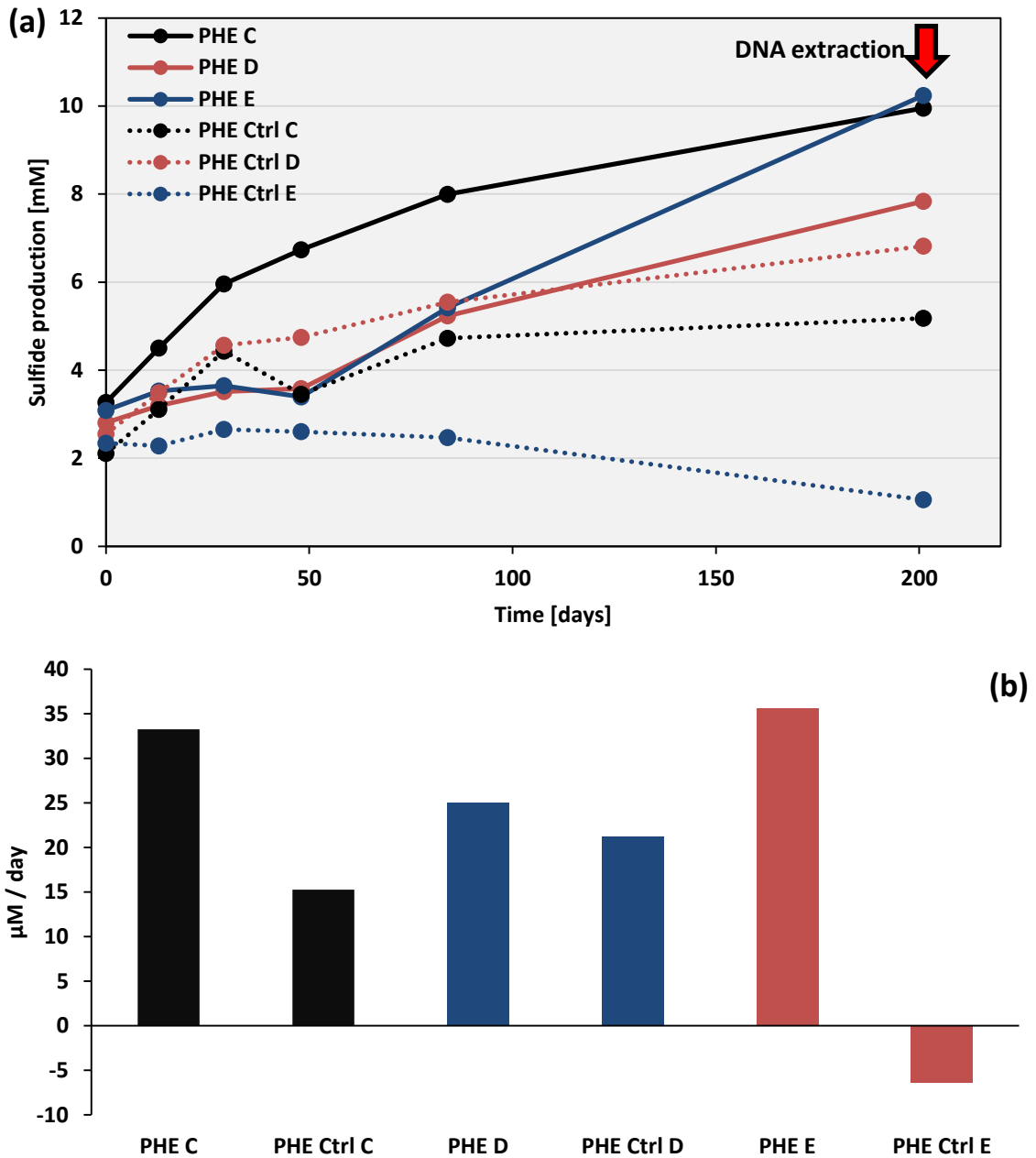


Figure 19 (a) Sulfate reduction activity as determined by the accumulation of sulfide with time and (b) sulfate reduction rates ($\mu\text{M}/\text{day}$) from phenanthrene-degrading enrichment cultures in three replicates (PHE C, D, and E) under sulfate-reducing conditions

3.4 Results and discussion

3.4.1 *Sulfate reduction activity*

Enrichment cultures were transferred into fresh medium with or without phenanthrene from a previous active culture. Whereas little to no sulfide was produced in no carbon substrate controls from previous generations, substantial sulfide was produced in current controls without carbon substrate added (see chapter 2). This may be explained by metabolites or biomass carried over from previous transfers that act as a potential carbon source. Nonetheless, phenanthrene-amended enrichment cultures showed substantially higher sulfide production rates ($31.3 \pm 5.6 \mu\text{M} / \text{day}$) in comparison to no carbon controls ($10.0 \pm 14.5 \mu\text{M} / \text{day}$), although a large variation in rate measurements was observed. Sulfide production rate in the control PHE D culture, for example, was close to that of phenanthrene-amended cultures (Figure 19).

3.4.2 *Metabolite analysis*

In order to elucidate the activation pathway of non-substituted phenanthrene under sulfate-reducing conditions, metabolites were extracted and analyzed using gas chromatography-mass spectrometry (GC-MS). Results revealed two potential intermediates of phenanthrene degradation in two cultures. Based on a matching GC retention time and mass spectral profile with an authentic standard described previously (Davidova et al., 2007), phenanthrene carboxylic acid and hydroxybenzoic acid were detected (Figure 20). The detection of phenanthrene carboxylic acid is corroborated by previous work in phenanthrene-amended enrichment cultures under sulfate-reducing conditions. Incorporation of ^{13}C -bicarbonate and the formation of phenanthrene

carboxylic acid were observed in a previous marine enrichment culture, which indicated that phenanthrene was activated by direct carboxylation (Zhang and Young, 1997). Another study also detected phenanthrene-2-carboxylic acid in a marine phenanthrene-degrading enrichment culture (Davidova et al., 2007). Although the location of the carboxyl group is not clear, consistent detection of phenanthrene carboxylic acid supports a direct carboxylation mechanism of phenanthrene activation. Another metabolite detected in this study, hydroxybenzoic acid, is produced during the degradation of phenolic compounds (Figure 21). For example, *m*-cresol is oxidized to 3-hydroxybenzoate via the addition of fumarate to the methyl group (Müller et al., 2001). Further, anaerobic degradation of 3-hydroxybenzoate has been studied *Thauera aromatica* under denitrifying conditions. In *T. aromatica*, 3-hydroxybenzoate was converted to 3-hydroxybenzoate-CoA by CoA ligase, followed by reduction to a cyclic dienoyl-CoA (Laempe et al., 2001). In a pure culture study of *Desulfobacterium aniline*, 4-hydroxybenzoate was detected as an intermediate of phenol degradation, during which phenol was phosphorylated to phenylphosphate followed by carboxylation to 4-hydroxybenzoate (Ahn et al., 2009). However, no previous reports are available for the detection of hydroxybenzoic acid as an intermediate of PAH degradation under anaerobic conditions. The results presented here provide evidence for the carboxylation of phenanthrene and suggest that hydroxybenzoate is a potential intermediate substrate during phenanthrene degradation under sulphate-reducing conditions.

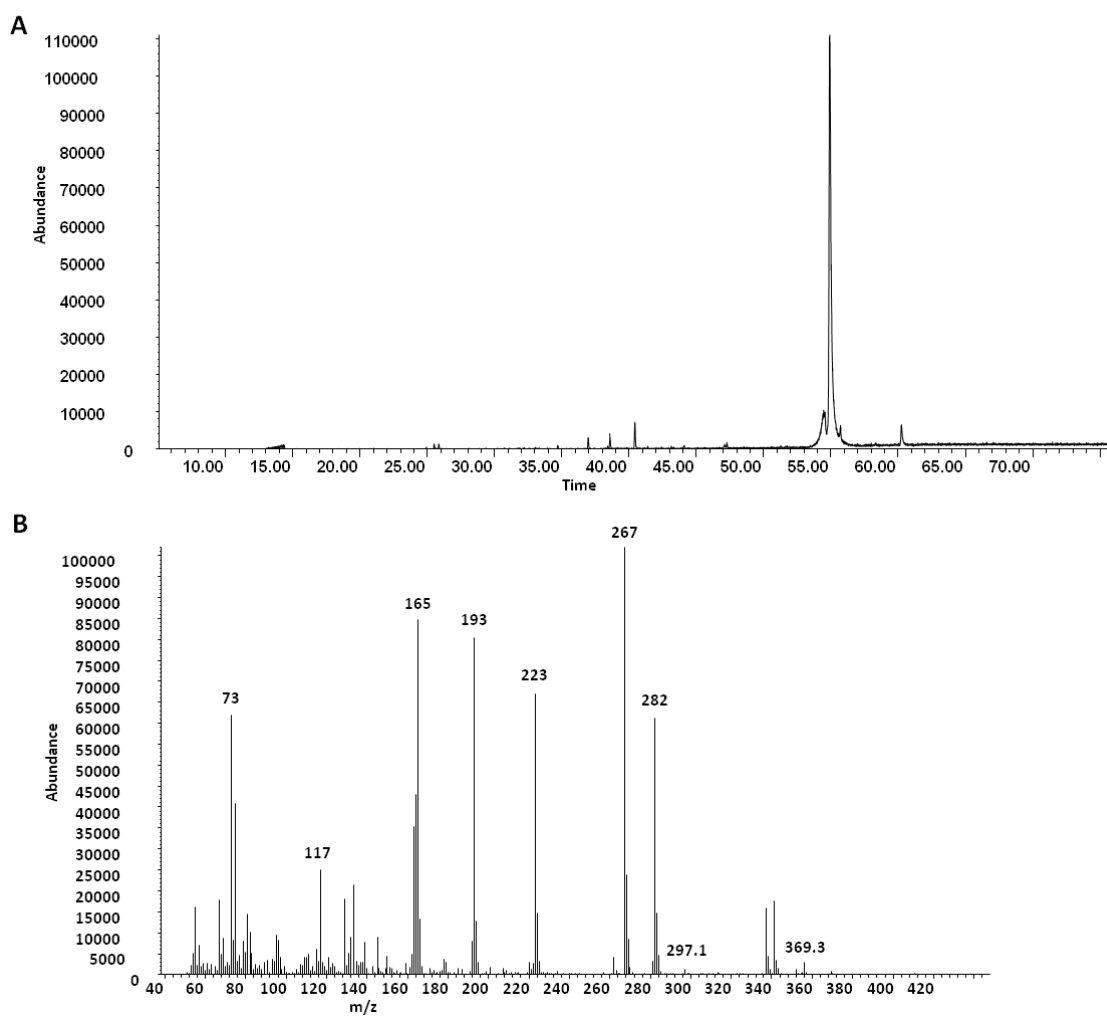


Figure 20 (a) Total ion chromatogram peak at 55 min retention time and (b) mass spectra providing evidence for the production of hydroxybenzoic acid.

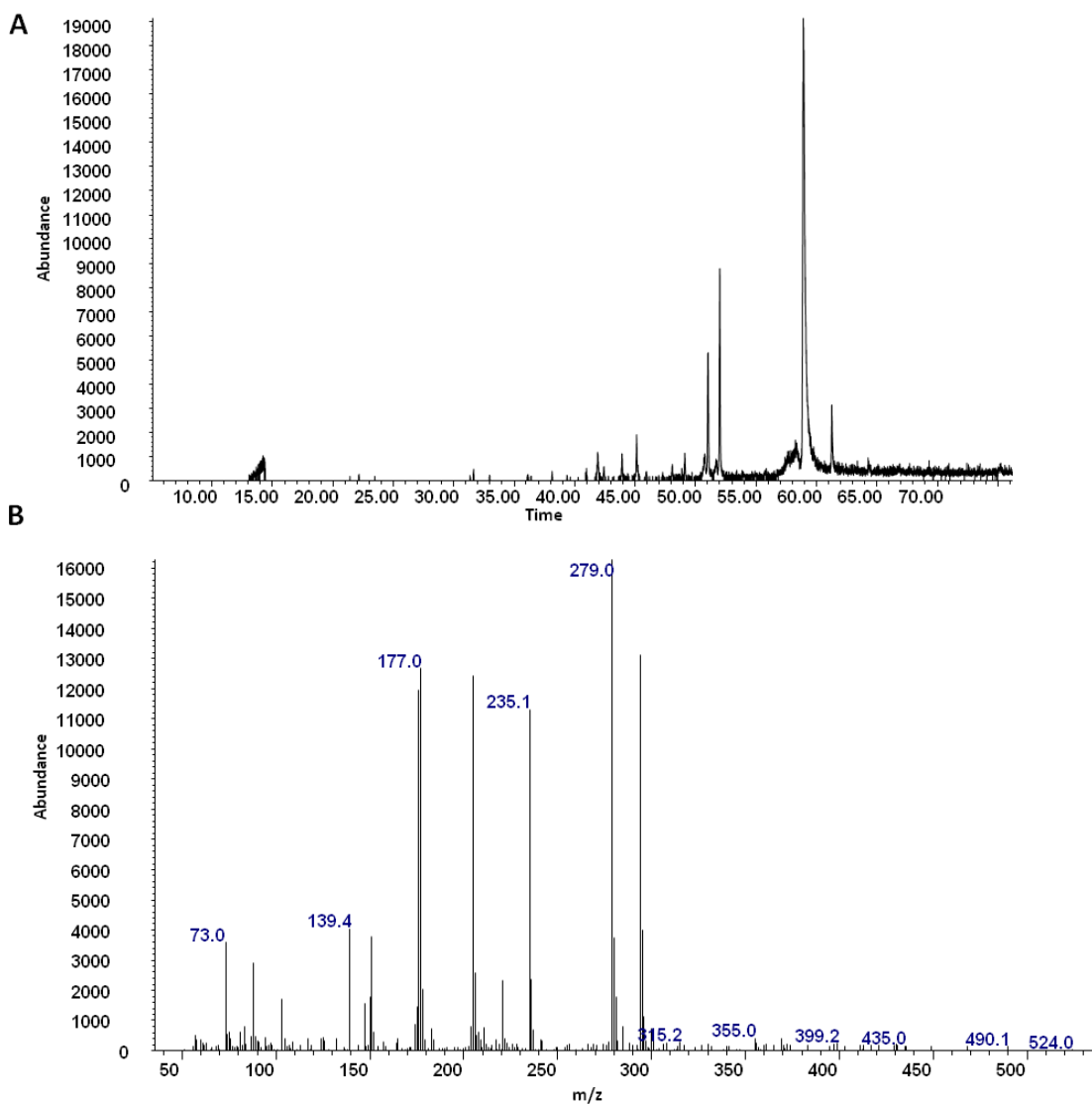


Figure 21 (a) Total ion chromatogram extracted for m/z of 279, indicating a phenanthrene carboxylic acid and (b) mass spectrum providing evidence for the production of phenanthrene carboxylic acid.

3.4.3 Microbial community composition from metagenome

In order to estimate the coverage of the microbial community in the metagenomic data set, read redundancy was employed using the statistical tool nonpareil (Rodriguez-R

and Konstantinidis, 2014). The resultant coverage of sequencing depth for the dataset was above 0.8 (Figure 22). SSU rRNA gene fragments were recovered from metagenomes and microbial community composition was assessed. At the class level, members of the *Deltaproteobacteria* predominated at up to 60, 83, 73 % relative abundance in metagenomes of the PHE-C, PHE-D, and PHE-E samples, respectively (Figure 23). Most cultivated sulfate-reducing hydrocarbon degraders are widespread within the class *Deltaproteobacteria*. The second most abundant class was *Sphingobacteria* within the *Bacteroidetes* in all samples. After the Prestige oil spill in northwestern Spain, members of *Flavobacteria* and *Sphingobacteria* within *Bacteroidetes* accounted for up to a quarter of the microbial communities in oil-contaminated beach sediments (Acosta-González et al., 2013). The most abundant taxa detected in all cultures included members of the *Desulfarculaceae* ($22 \pm 3\%$), *Desulfuromonadaceae* ($25 \pm 8\%$), and *Desulfobacteraceae* ($11 \pm 1\%$). At the genus level, *Desulfatiglans* ($20 \pm 2\%$) and *Pelobacter* ($23 \pm 7\%$) predominated. Phenol degradation initiated by phenylphosphate synthase and phenylphosphate carboxylase was reported in the sulfate-reducing bacterium *Desulfatiglans aniline* strain AK1 (Ahn et al., 2009). *Desulfatiglans aniline*-related sequences were also detected at high relative abundance in hydrocarbon and methane seeps in the Gulf of Mexico (Vigneron et al., 2017). The next most abundant genera detected in cultures were *Desulfosarcina* ($4.5 \pm 1\%$), *Thermotomaculum* ($2.4 \pm 0.7\%$), *Desulfarculus* ($2.2 \pm 1\%$), and *Desulfofutis* ($2 \pm 0.9\%$). Populations within the *Desulfosarcina/Desulfococcus* cluster were reported as key alkane-degraders at natural hydrocarbon seeps present in the Guymas basin (Kleindienst et al., 2014). Strain BuS5, which degrades short-chain alkanes under sulfate-reducing conditions, is affiliated with

the *Desulfosarcina/Desulfococcus* cluster (Kniemeyer et al., 2007). Members of *Desulfovibrio* within the family *Desulfovibrionaceae* were detected only in the PHE-D culture at a relative abundance was 26 %. *Desulfovibrio* spp. are metabolically versatile sulfate-reducers that are capable of syntrophic metabolism when sulfate is not available (Meyer et al., 2013). Some *Desulfovibrio* strains were shown to couple alkane oxidation to sulfate reduction (Davis and Yarbrough, 1966).

MyTaxa was used to identify the taxonomic affiliation of a query genome sequence or a sequence of a contig assembled from a metagenome, including short sequences (100-1000 nt) and to classify sequences representing novel taxa at phylum, genus, and species level (Figure 24).

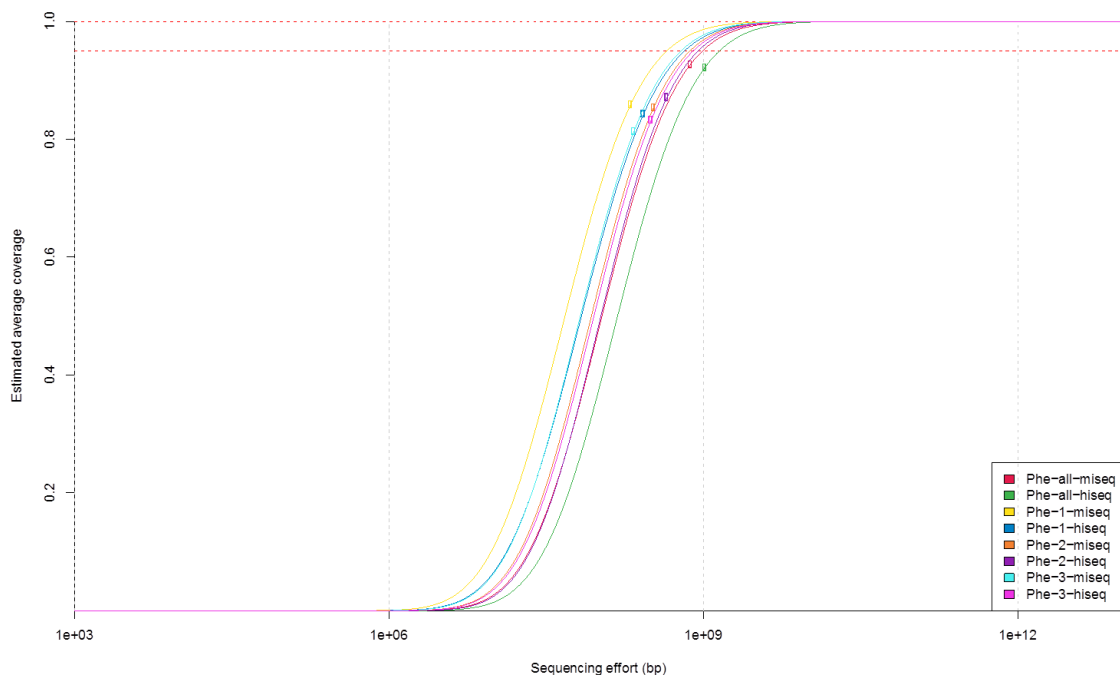


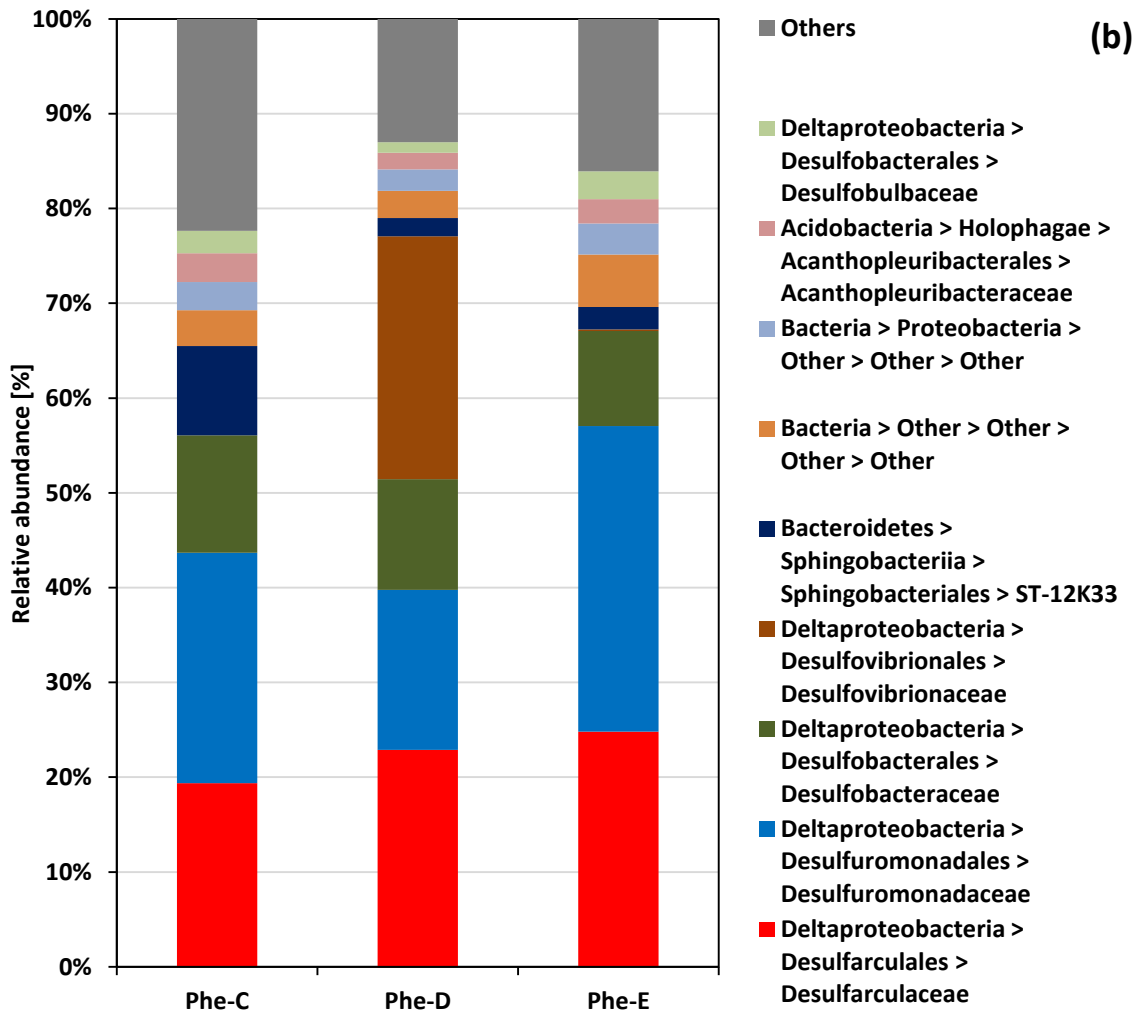
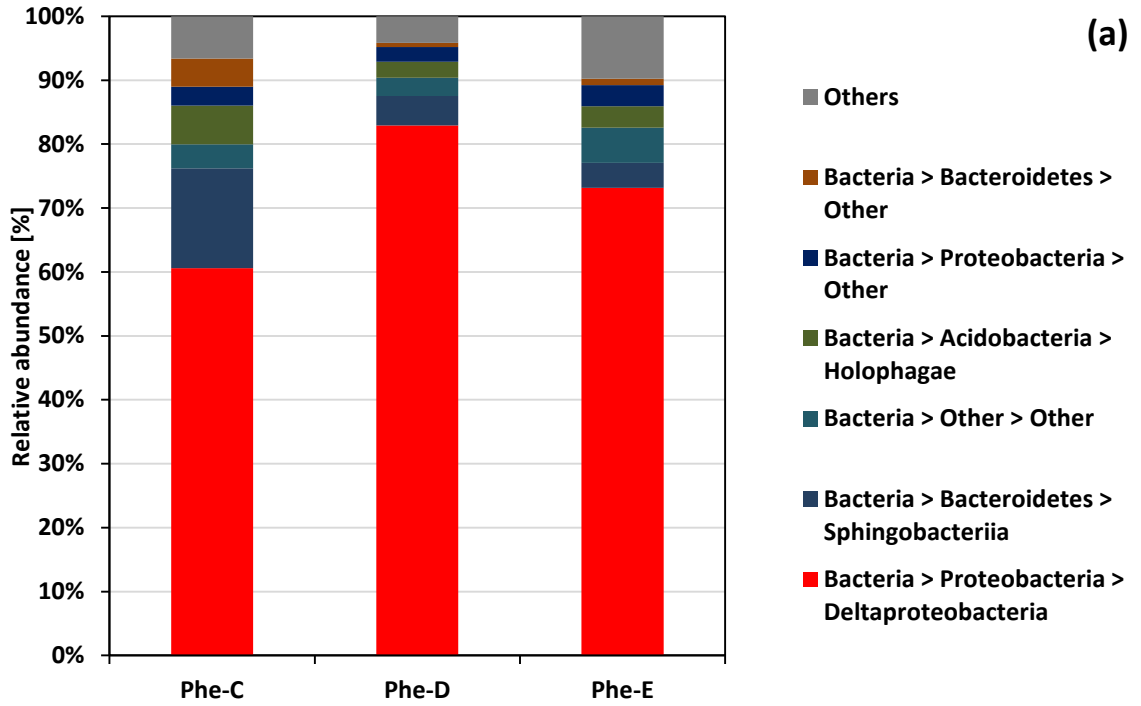
Figure 22 Nonpareil curve used to estimate dataset complexity and the amount of sequencing effort needed to achieve a desired level of coverage. Squares on curves

represent the coverage of the actual sequencing depth for each dataset in relation to the entire curve.

3.4.4 Contig binning and metabolism reconstruction

Contig binning resulted in the recovery of 31 population bins and total 9 bins were selected for further analysis with a quality score > 50 calculated based on completeness and contamination according to results from CheckM (Table 3). None of these bins contained SSU rRNA genes. However, taxonomic classification by MiGA revealed that all bins, with the exception of bin 3, were not closely related to known isolates (Table 4). The closest relative from bin 1 was *Draconibacterium orientale* strain FH5T (accession number NZ_CP007451), which shared 48.15% AAI similarity. *Draconibacterium orientale* is facultatively anaerobic bacterium isolated from marine sediments collected off the coast of Weihai, China (Li et al., 2016). Bin 2, bin 6, and bin 26 all shared 43.61, 51.24, and 48.14% of AAI with the closest relative *Desulfococcus multivorans* DSM 2059 (accession number NZ_CP015381). *Desulfococcus multivorans* DSM 2059 is reported to be able to completely oxidize a variety of organic acids (up to C₁₄) and aromatic compounds (Dörries et al., 2016). Bin 3 was affiliated with the species *Pelobacter carbinolicus* DSM 2380 (p-value 0.05) with 91.2% shared AAI. *Pelobacter carbinolicus* DSM 2380, isolated from anoxic mud, is capable of fermentation, syntrophic hydrogen/formate transfer, or electron transfer to sulphur from short-chain alcohols, hydrogen or formate (Aklujkar et al., 2012). The closest relative to Bin 4 was also *Pelobacter carbinolicus* DSM 2380 but only at 44.47% AAI similarity. Bin 5 shared 40.16% AAI with *Pelobacter acetylenicus* DSM 3246 (accession number NZ_CP015455). The closest relative to bin 30 was *Candidatus Tremblaya princeps* TPPLON1 (accession

number LN998830), which was revealed to be the primary endosymbiont of the citrus mealybug *Planococcus citri*. Bin 31 was the closest to *Desulfovibrio salexigens* DSM 2638 (accession number NC_012881) with 53.8% AAI similarity. *Desulfovibrio salexigens* was isolated from sling mud of British Guiana and is capable of nitrogen fixation.



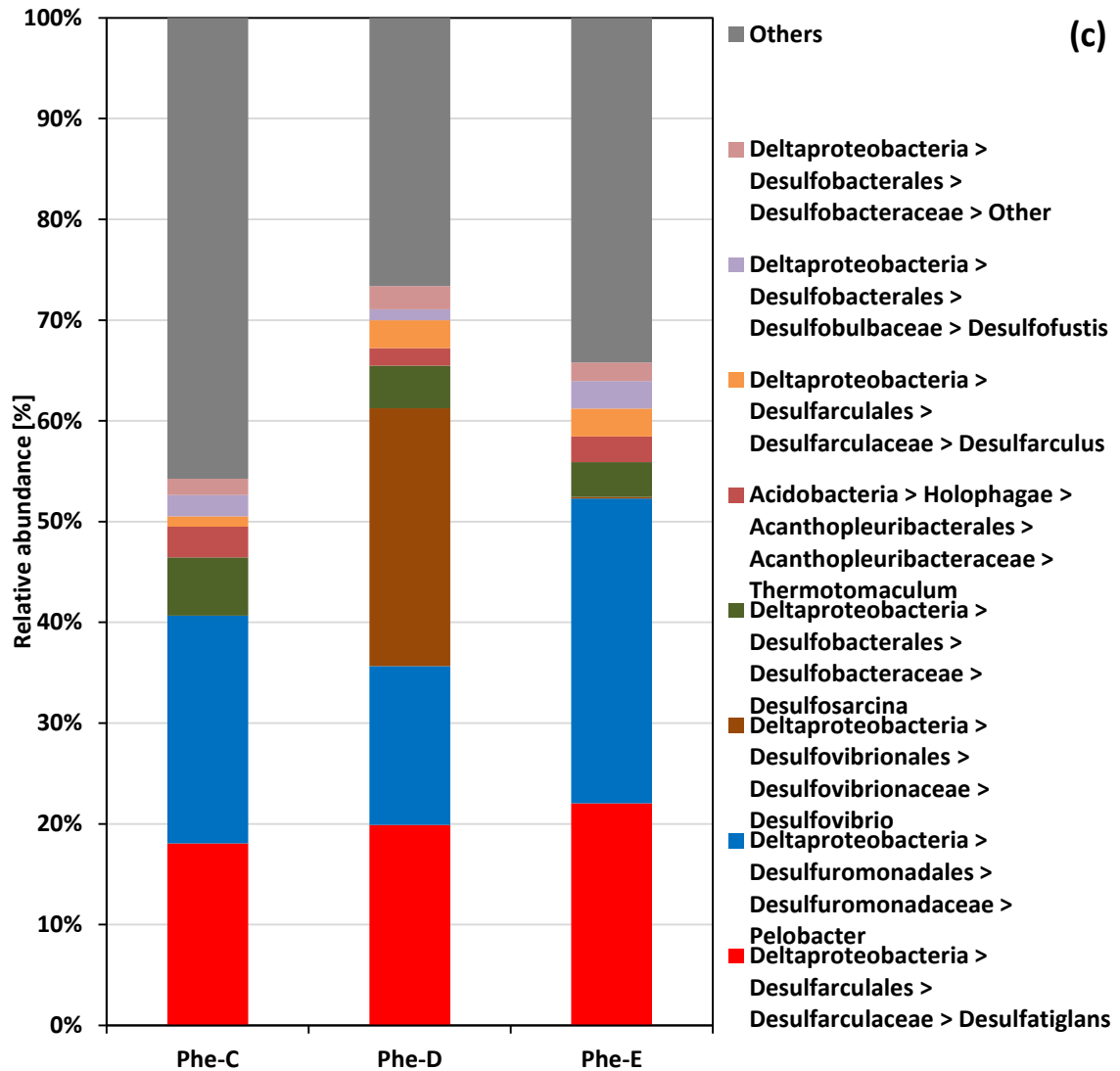


Figure 23 Microbial community structure analyzed by SSU rRNA genes retrieved from the metagenome of the phenanthrene-degrading enrichment culture incubated under sulfate-reducing conditions at various taxonomic levels including the (a) class , (b)family , and (c) genus level.

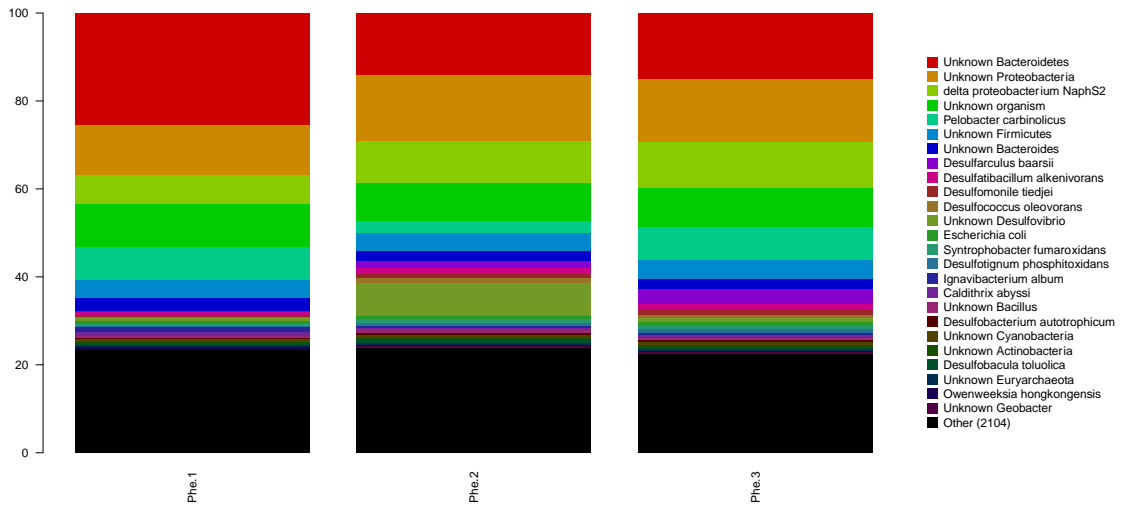
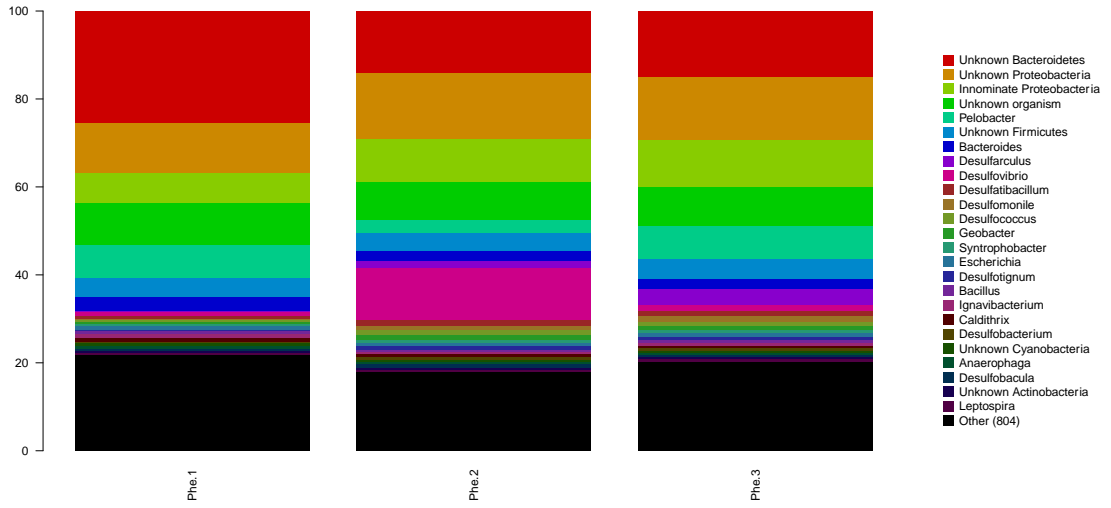
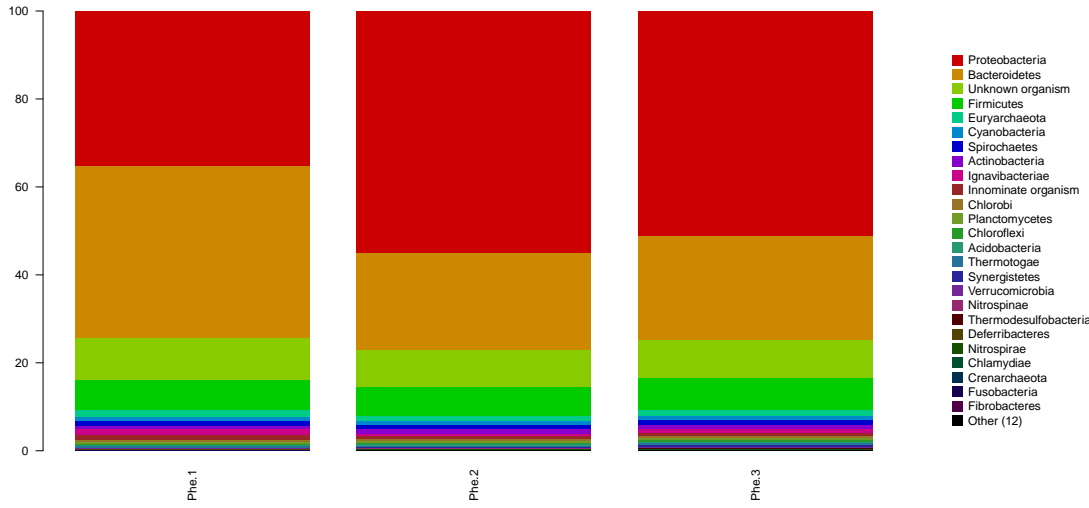


Figure 24 MyTaxa represents a new algorithm that extends the Average Amino Acid Identity (AAI) concept to identify the taxonomic affiliation of a query genome sequence or a sequence of a contig assembled from a metagenome, including short sequences (100-1000 nt) and to classify sequences representing novel taxa at phylum, genus, and species level.

Table 3 CheckM results from the most complete population bins.

Bin Id	Completeness	Contamination	Strain heterogeneity	Quality
1	97.58	1.88	0	88.18
2	98	5.18	21.43	72.1
3	91.04	1.96	50	81.24
4	97.1	4.18	5	76.2
5	96.15	1.71	0	87.6
6	84.91	3.87	25	65.56
26	91.79	8.23	0	50.64
30	92.69	6.69	62.5	59.24
31	99.41	0	0	99.41

Table 4 Taxonomic classification in MiGA using the NCBI prokaryote database. Significance at p-value below *0.5, **0.1, *0.05, ****0.01, p > 0.5 if there is no *.**

Bin Id	Taxonomic classification		
1	class Bacteroidia ****	order Bacteroidales *	family Prolixibacteraceae
2	phylum Proteobacteria ****	class Deltaproteobacteria *	order Desulfobacterales
3	genus Pelobacter ****	species Pelobacter carbinolicus ***	
4	phylum Proteobacteria ****	class Deltaproteobacteria **	order Desulfuromonadales
5	phylum Proteobacteria ***	class Deltaproteobacteria *	order Desulfuromonadales
6	class Deltaproteobacteria ****	order Desulfobacterales *	family Desulfobacteraceae *
26	class Deltaproteobacteria ****	order Desulfobacterales *	family Desulfobacteraceae
30	phylum Proteobacteria **	class Betaproteobacteria *	
31	class Deltaproteobacteria ****	order Desulfovibrionales *	family Desulfovibrionaceae *

Protein annotation of high quality bins revealed that all bins contained genes involved in salicylate (*sala* – salicylate hydroxylase) and gentisate catabolism as well as homogentisate pathway of aromatic compound degradation. Salicylate (2-

hydroxybenzoate) is formed during the degradation of phenolic compounds. For example, *Desulfobacterium cetonicum* was previously shown to oxidize *m*-cresol to 3-hydroxybenzoate by fumarate addition to the methyl group (Müller et al., 2001), while *Desulfobacterium aniline* strain AK1 was shown to degrade phenol via phosphorylation to phenylphosphate, followed by carboxylation to 4-hydroxybenzoate (Ahn et al., 2009). Also, genes affiliated with 1-(hydroxymethyl)naphthalene NAD⁺ oxidoreductase, which has been linked to PAH degradation, were also present in the majority of bins. All bins contained anaerobic 4-hydroxybenzoate carboxylase (*bsdBCD*, UbiX, PAD1), which suggests the potential for hydroxybenzoate oxidation, in agreement with our metabolite analysis. *bsdBCD* is well characterized in *Bacillus subtilis* and is reported to catalyze (de)carboxylation under both aerobic and anaerobic conditions (Lupa et al., 2005). Previous studies showed that UbiD/UbiX-like carboxylase (3-octaprenyl-4-hydroxybenzoate carboxylase) was specifically expressed during benzene degradation in an iron-reducing enrichment culture (Abu Laban et al., 2010). The genome of *Desulfatiglans aniline* strain Anil1 identified two gene clusters containing genes encoding UbiD-like proteins and a UbiX-like protein. In bin 3, a protein sequence of the fumarylacetoacetate hydrolase family that is affiliated with *Pelobacter carbinolicus* was detected with 95% amino acid sequence similarity. Another protein sequence of 4-hydroxyphenylacetate 3-hydroxylase affiliated with candidate division Zixibacteria was detected in bin 30 with an amino acid sequence similarity of 79%. Bin 31 contained a protein sequence of 3-dehydroquinate dehydratase affiliated with *Pseudodesulfovibrio profundus* with an amino acid sequence similarity of 79%. A complete set of dissimilatory sulfate reduction and oxidation genes such as dissimilatory sulfite reductase

alpha and beta subunits (*dsrAB*), adenylosulfate reductase subunit AB (*aprAB*), and sulfate adenylyltransferase were detected in bins 2, 4, 26, 30, 31. Bin 1 contained genes involved in denitrification (*NarGHIRK*, *NorBCDEFQ*, *NosDFLRXYZ*), suggesting that this enrichment culture has a capability of respiring nitrate. Genes involved in aryl-alcohol dehydrogenase (*adhCP*), alcohol dehydrogenase (*frmA*, *viaY*, *ADH5*), and acetaldehyde dehydrogenase (*adhE*) were detected in the majority of bins, indicating that the enrichment culture may be able to degrade aromatic intermediates such as toluene, xylene, methylnaphthalene, cyclohexanol, and phenol. Bin 3, bin 6, and bin 26 contained genes encoding nitrogen fixation (*nif*, *vnf*, *anf*). With the exception of bin 3, all bins contained genes encoding methanogenesis including *mcrABCDEG*, *fdhABCDEFG*, *mtrABCDEFGHX*. A significant number of membrane transporters were induced under anaerobic growth with aromatic compounds in previous studies (Carmona et al., 2009). All bins except bin 30 possessed genes for ABC transporter systems for alkylphosphonate (*phnCDE*), tungstate (*TupABC*, *VupABC*, *WtpABC*, *ModABC*), branched-chain amino acid (*livFGHJK*), oligopeptide (*oppABCDF*), and dipeptide (*dppABCDF*). These types of ABC transporters were also present and expressed when enrichment culture N47 was growing with naphthalene, 2-methylnaphthalene and 2-naphthoic acid (F. Bergmann et al., 2011). However, genes encoding ABC transporter systems for sulfate were not detected. All bins had genes involved in fermentation including pathways of acetyl-CoA fermentation to butyrate and butanol biosynthesis. *Deltaproteobacterium* enrichment culture N47 has shown expression of enzymes related to butanol production during growth with naphthalene, 2-methylnaphthalene and 2-naphthoic acid (F. Bergmann et al., 2011).

3.5 Conclusions and future directions

Polycyclic aromatic hydrocarbons (PAHs) originating from a range of natural and anthropogenic sources, are widespread in the environment. Little is known about the microorganisms that degrade PAHs under anaerobic conditions in comparison to their aerobic counterparts. Pathways involved in naphthalene and 2-methylnaphthalene degradation were described, however, very little is known about the degradation of phenanthrene. Previous studies have proposed carboxylation as an activation mechanism for phenanthrene under anaerobic conditions, however, the genes or enzymes involved in anaerobic phenanthrene degradation are unknown to date. Here we detect potential metabolic intermediates and construct genomes from metagenomic data for microbial populations that may be involved in phenanthrene degradation under sulfate-reducing conditions. Detection of phenanthrene carboxylic acid suggests that phenanthrene is degraded by carboxylation in our enrichment culture. Another metabolite detected in enrichment culture, hydroxybenzoic acid, is suggested as a key intermediate, although this requires confirmation. The metagenomes were screened for genes that code for enzymes in putative phenanthrene activation pathway. However, this search was not successful. Nonetheless, the metagenomes yielded 9 high quality bins from contigs, and the protein annotation of these bins was investigated. Although the assembled population bins were comprised of organisms from diverse phyla, they shared many metabolic pathways. The majority of binned populations contained genes for salicylate ester, quinate, and gentisate degradation. A smaller subset of populations contained genes encoding homogentisate and aromatic amin degradation, sulfite reduction, and

thioredoxin-disulfide reductase. All bins contained genes encoding 4-hydroxybenzoate carboxylase (*bsdBCD*, UbiX, PAD1), implying the degradation of hydroxybenzoate, which was detected from metabolite analysis. The results also showed that our phenanthrene-enrichment cultures possess genes involved in denitrification and methanogenesis.

Although the detection of phenanthrene carboxylic acid points to one potential mechanism of phenanthrene activation under sulfate-reducing conditions, further experiments are needed to confirm this observation and link it to the metabolism of cultured organisms. One possible approach would be to perform tracer experiments by adding deuterated phenanthrene or ^{13}C -bicarbonate to enrichments. Moreover, metatranscriptomics or proteomics could be applied with the proper control cultures to identify transcripts or proteins that are upregulated in the presence of phenanthrene or other PAH substrates but not with downstream metabolites such as fatty acids. Since the genes involved in anaerobic phenanthrene degradation are unknown, a hypothesis-based, thorough experimental design will be required. Of course, isolation of a pure culture would also greatly aid efforts.

CHAPTER 4. Succession of microbial populations linked to sediment oil agglomerate (SOA) degradation in Pensacola Beach sands impacted by the Deepwater Horizon oil spill

4.1 Abstract

The Deepwater Horizon (DWH) oil spill contaminated large areas of coastline from Louisiana to Florida and oil was buried up to 55cm depth in sandy beaches. Although the biodegradation of more diffuse oil sources has been studied extensively in beach sand, less information is available on the microbial communities associated with the degradation of larger oil residues such as sediment oil agglomerates (SOAs), which were often stranded on shorelines after the DWH disaster. Thus, the main objectives of this study were to (i) determine the rate of SOA degradation *in situ* in dry beach sand, (ii) investigate the succession of microbial populations that recruit onto simulated SOAs, and (iii) elucidate the linkage between nitrogen fixation and hydrocarbon degradation. Replicate homogenized SOAs, collected from the coastline immediately after the DWH disaster, were buried from 5 to 55cm depth in Pensacola Beach sand and sampled from October 2010 to December 2013. The abundance and composition of microbial communities was determined using next generation sequencing and qPCR of SSU rRNA genes, respectively, for SOAs sampled in a time series. SOA degradation occurred over longer time scales in comparison to smaller, more diffuse oil particles in dry beach sands, with up to 91% of petroleum hydrocarbons degraded in three years. As has been observed for more diffuse oil contamination, taxonomic diversity of microorganisms decreased in SOAs in comparison to surrounding or pristine sands. Hydrocarbon-degrading bacteria

were enriched and a succession of microbial populations was observed that paralleled the chemical evolution of petroleum hydrocarbons as determined in a companion study. Bacterial abundance was two to four orders of magnitude higher in SOAs in comparison to surrounding sands, indicating that these large oil residues are hotspots of microbial growth. Quantification of nitrogenase genes (*nifH*) in SOAs revealed a bloom in nitrogen-fixing prokaryotes or diazotrophs late in the time series. Results indicate that the high C/N ratio of SOAs causes nutrient stress and induces microbial nitrogen fixation. Thus, the coupling of nitrogen fixation to hydrocarbon degradation appears to be an important control over the fate of oil in macroscopic oil aggregates. Our observations are corroborated by predictions from inferred metagenomic analysis and predictions aid in linking specific taxa to the degradation of specific hydrocarbon compounds and diazotrophy.

4.2 Introduction

In April 2010, the Deepwater Horizon oil rig exploded and sank, discharging approximately 4.9 million barrels of crude oil into the Gulf of Mexico (GoM) at a depth of 1544 m over the course of 86 days (Reddy et al., 2012; Zukunft, 2010). Released MC252 BP oil that reached the ocean surface was transported to coastal environments, impacting approximately 850 km of beaches from east Texas to west Florida (Barron et al., 2015; Hayworth et al., 2011; Michel et al., 2013; Wang and Roberts, 2013). When weathered oil reaches the shoreline, it is generally in the form of a highly viscous, neutrally buoyant emulsion (also known as mousse), and a large portion then mixes with solids to form oil-

sediment residues that have been termed tar balls, sand mats or patties, and surface residual balls (Aeppli et al., 2012; Fingas and Fieldhouse, 2009; Lemelle et al., 2014; Urbano et al., 2013). In order to avoid confusion, we will henceforth refer to these macroscopic oil-sediment residues as sediment oil agglomerates (SOAs), according to a recent review of nomenclature (Gustitus and Clement, 2018). SOAs are oval-shaped residues, ranging from a few millimeters to centimeters in diameter, predominantly composed of sand (75-96% by mass) with a moisture content of less than 0.5 % (Elango et al., 2014; Hayworth et al., 2015; Operational Science Advisory Team (OSAT-2), 2011; Yin et al., 2015).

After the DWH oil spill, oil-sediment residues were trapped and buried up to meters in depth in beaches from Louisiana to Florida (Elango et al., 2014; Hayworth et al., 2015; Huettel et al., 2018). SOAs in highly contaminated beaches of Louisiana were found to contain elevated concentrations of recalcitrant and toxic PAHs including C1- and C2-phenanthrenes, C2- and C3- dibenzothiophenes, along with other high molecular weight oil components (Elango et al., 2014). After monitoring SOAs for four years in Alabama's beaches, Yin et al. (2015) showed that high molecular weight PAHs-such as chrysene and alkylated chrysenes persisted with time. Studies have also demonstrated the toxicity of SOAs due to persistence of PAHs, oxygenated hydrocarbons, environmentally persistent free radicals (EPFRs), and human pathogens such as *Vibrio vulnificus* (John et al., 2016; Kiruri et al., 2013; Lemelle et al., 2014; Tao et al., 2011; White et al., 2016). Although BP conducted Operation Deep Clean (ODC) to mechanically remove larger SOAs from the beach surface, SOAs remained buried to 50 cm depth (Huettel et al., 2018; Wang and Roberts, 2013). Moreover, once SOAs are buried in the sediments, degradation

of SOAs cannot occur by photooxidation, a critical weathering process for hydrocarbons in surficial environments (Prince et al., 2003; Radović et al., 2014). Therefore, even after cleanup efforts, SOAs containing toxic PAHs persist in the beach system for years and represent a potential long-term risk to ecosystem and human health (Yin et al., 2015).

The biodegradation of larger, macroscopic oil-sediment residues such as SOAs is likely to be distinct from that of smaller oil droplets or particles in coastal zones (Bacosa et al., 2016; Elango et al., 2014; Hayworth et al., 2015). The small surface area to volume ratio of SOAs may limit the access of hydrocarbons for biodegradation (Salleh et al., 2003). Depending on the porosity of the aggregate, biodegradation is also likely to be limited by the delivery of substrates, oxygen and nutrients, to sites where microorganisms mediate enzymatic breakdown of hydrocarbons. For example, Elango et al. (2014) observed that the C/N molar ratio, often used as a diagnostic variable for hydrocarbon biodegradability, ranged from 111 to 474 in SOAs, which is well above optimal C/N ratios (approximately 60) for aerobic hydrocarbon degradation (Dibble and Bartha, 1979). These findings suggest that bioavailable nutrients are often a limiting factor for microbial SOA degradation. Pure culture studies have shown that some hydrocarbon-degrading bacteria have the potential to fix nitrogen (Chen et al., 1993; Coty, 1967; Prantera et al., 2002; Toccalino et al., 1993). However, a direct linkage between SOA degradation and nitrogen fixation is lacking.

Although the impacts of oil contamination on marine microbial communities are well documented, few studies have addressed the microbial community dynamics associated with larger oil-sediment residues that are often trapped in coastal ecosystems. Especially in the supratidal zone of beach sand environments, low moisture content,

nutrient availability, and a low surface to volume ratio of larger residues may limit bacterial hydrocarbon degradation (Medina-Bellver et al., 2005). Thus, the main objectives of this study were to (i) quantify the rate of *in situ* SOA degradation in Pensacola Beach sands, (ii) elucidate long-term succession of microbial communities that recruit onto SOAs, and (iii) to explore the potential coupling of SOA degradation and nitrogen fixation. This study employed SOAs that were collected on shorelines after the DWH oil spill. SOA were homogenized and replicates were buried in Pensacola Beach sand and monitored for weight loss, succession of microbial communities, and nitrogen fixation potential for over 3 years.

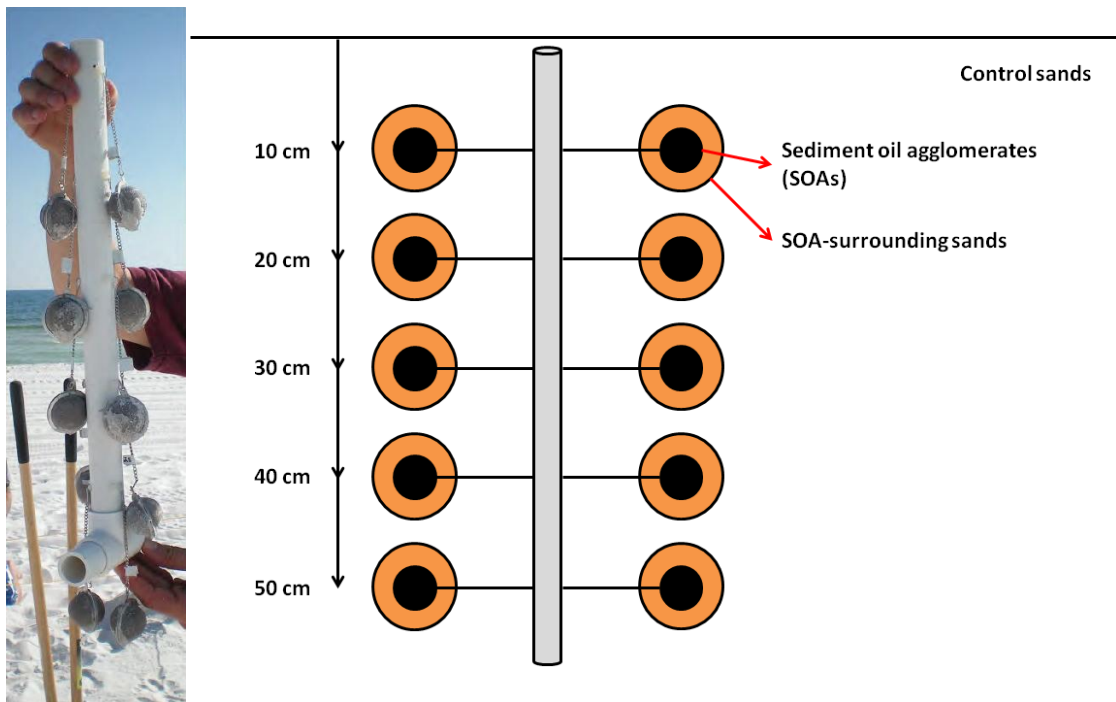


Figure 25 Experimental design employed to investigate the *in situ* biodegradation of sediment oil agglomerates (SOAs), showing replicate SOAs attached to PVC pipe at Pensacola Beach.

4.3 Materials and methods

4.3.1 Sample collection and experimental design

Sediment oil agglomerates (SOAs) were collected at Pensacola Beach, FL, USA (30119.57N, 087110.47W) on 30 June, 2010. SOAs were homogenized, and then filled into 3.8 cm diameter-stainless steel meshballs. Ten filled meshballs were attached to PVC pipe (1.3 cm diameter) and buried from 10 cm to 50 cm at 10 cm depth intervals with biological duplicates sufficient for collection of ten time points (Figure 25 and Supplemental Figure 1). Initial SOA masses were determined and all meshballs were buried in the supratidal zone on 22 October, 2010. SOAs from 10cm and 50cm depth intervals were sampled at 41, 89, 131, 181, 235, 279, 327, 445, 735, and 1152 days after burial. The mass of each SOA was determined before freezing at -20°C in clean glass jars for further microbial community analysis. Sandy sediments from the region surrounding each deployed SOA from 10 cm and 50 cm depth intervals were also collected at 41, 89, 131, 181, 235, 279, 327, 445, 735, and 1152 days after burial to identify possible impacts to indigenous microbial communities. Control sand samples without oil contamination were collected from the supratidal zone of a nearby pristine of the same beach after 41, 89, 279, 445, 735, 1152 days of incubation.

4.3.2 Nucleic acid extraction and microbial community analysis

Total genomic DNA was extracted from SOAs using a MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA) with slight modifications from the

manufacturer's protocol. Briefly, 0.25 g of thawed SOA sample was provided into a 2 ml bead tube and disrupted for 1 min using a Talboys High Throughput Homogenizer (Troemner, Thorofare, NJ). Overall microbial communities and nitrogen-fixing prokaryotes were characterized by targeting SSU rRNA and nitrogenase (*nifH*) genes, respectively. PCR amplification of SSU rRNA genes was performed using 515F and 806R primers as described by the Earth Microbiome Project (<http://www.earthmicrobiome.org/emp-standard-protocols/dna-extraction-protocol/>) for Illumina sequencing. For *nifH*, the primer set IGK3 (5'-GCI WTH TAY GGI AAR GGI GGI ATH GGI AA-3') and DVV (5'-ATI GCR AAI CCI CCR CAI ACI ACR TC-3') was used for PCR amplification as described previously (Gaby et al., 2017). PCR products were barcoded using an Access Array Barcode Library (Fluidigm, South San Francisco, CA), purified using the E.Z.N.A Cycle Pure Kit (Omega Bio-tek, Norcross, GA), and pooled based on DNA concentration. Purified and pooled PCR amplicons were sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA). Sequence analysis was accomplished using the software QIIME ver. 1.9.1 (Caporaso et al., 2010b) and Mothur ver. 1.38.0 (Schloss et al., 2009). Sequences with quality score below 20 were removed using Mothur ver. 1.38.0 and clustered into operational taxonomic units (OTUs) by 97% and 92% sequence identity for SSU rRNA and *nifH* genes, respectively using UCLUST (Edgar et al., 2011) implemented in QIIME ver. 1.9.1. Representative sequences were aligned against the SILVA ver. 123 database (<https://www.arb-silva.de/>) and chimeric sequences were removed using UCHIME (Edgar et al., 2011) implemented in Mothur ver. 1.38.0. Taxonomy assignment for SSU rRNA genes was performed using the RDP classification algorithm set at a 50% confidence rating with the SILVA Small

Subunit rRNA Database release 123 (https://www.arb-silva.de/no_cache/download/archive/release_123/Exports/). The resultant OTU table was normalized using the CSS algorithm implemented in QIIME ver. 1.9.1 (Paulson et al., 2013). Shannon indices were calculated with QIIME ver. 1.9.1. A Bray-Curtis distance matrix was obtained from the rarefied OTU table and used to generate a principal coordinate analysis (PCoA) plot. Based on microbial community composition in SOAs as determined by SSU rRNA gene amplicon sequencing, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was employed in order to predict metagenome functional content of differentially abundant OTUs (Langille et al., 2013) and predict Kyoto Encyclopedia of Genes and Genomes (KEGG) Ortholog functional profiles. The OTU table was normalized using the software QIIME ver. 1.9.1 (Caporaso et al., 2010b) and each OTU was divided by SSU rRNA gene abundance. The resultant OTU table was used to create the final metagenome functional predictions.

4.3.3 *Quantitative molecular analyses*

Quantitative PCR was performed with PowerUp SYBR Green Mastermix (Applied Biosystems, Foster City, CA) and 331F (5'-TCC TAC GGG AGG CAG CAG T-3')/515R (5'-ATT ACC GCG GCT GCT GG-3') primers targeting bacterial SSU rRNA genes or Nh21F (5'-GCI WTY TAY GGN AAR GG-3')/NifH3 (5'-ATR TTR TTN GCN GCR TA-3') primers targeting the *nifH* marker gene for nitrogen-fixing *Bacteria* and *Archaea* following the manufacturer's protocol on a StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA). All reactions were performed

in triplicate and analyzed using StepOne Software v. 2.3. Serially diluted pGEM-T Easy Vector plasmids (Promega, Madison, WI) containing either a full-length *E. coli* SSU rRNA gene or *nifH* gene were used to generate standard calibration curves for quantification of gene abundances.

4.4 Results and discussion

4.4.1 Mass loss of sediment-oil agglomerates (SOAs)

To estimate the rate of hydrocarbon degradation, the mass of homogenized sediment-oil agglomerates (SOAs) was measured before burial and immediately after sampling. Whereas no significant mass loss was detected for 327 days, a reduction of 15.5% was observed 1152 days after placement of the SOAs into the beach sediment (Figure 26). Based on a summary report for fate and effects of remnant oil in the beach environment, the mass percent of oil in SOAs is estimated at 3 to 17 % (Operational Science Advisory Team (OSAT-2), 2011). Thus, in this study, it is estimated that up to 91% of oil by mass in SOAs was reduced. However, mass loss alone is an imperfect method to estimate hydrocarbon degradation over time. Further analysis of total petroleum hydrocarbon measurement and chemical characterization are needed to investigate which oil components in SOAs were degraded over time in Pensacola Beach sand environment. Thus, in a companion study, the chemistry of petroleum hydrocarbons in SOAs was characterized using chromatography-mass spectrometry (GC-MS) in parallel with microbial community analysis.

Alkanes ranging from C₁₅ to C₄₀ and PAHs from C₁₀ to C₂₄ were observed in the parts per million (ppm) range. Analysis of total alkanes revealed that up to 80 % were depleted for over the three year time course. Hydrocarbon components were degraded at different rates based on molecular weight. Relatively short-chain alkanes (C₁₅) and low molecular PAHs such as naphthalene (C₁₀H₈) were rapidly depleted during the first ~100 days. The majority of mid-chain alkanes from C₁₆ to C₃₀ and heavier PAHs including fluorene (C₁₃H₁₀), phenanthrene (C₁₄H₁₀), dibenzothiophene (C₁₂H₈S) were degraded over ~300 days. However, long-chain alkanes (C₃₀ – C₄₀) and high-molecular weight PAHs such as pyrene (C₁₆H₁₀), chrysene (C₁₈H₁₂), benzo(c)phenanthrene (C₁₈H₁₂), benzo(a)pyrene (C₂₀H₁₂), 7,12-dymethylbenz(a)anthracene (C₂₀H₁₆), benzo(g,h,i)perylene (C₂₂H₁₂) persisted over time.

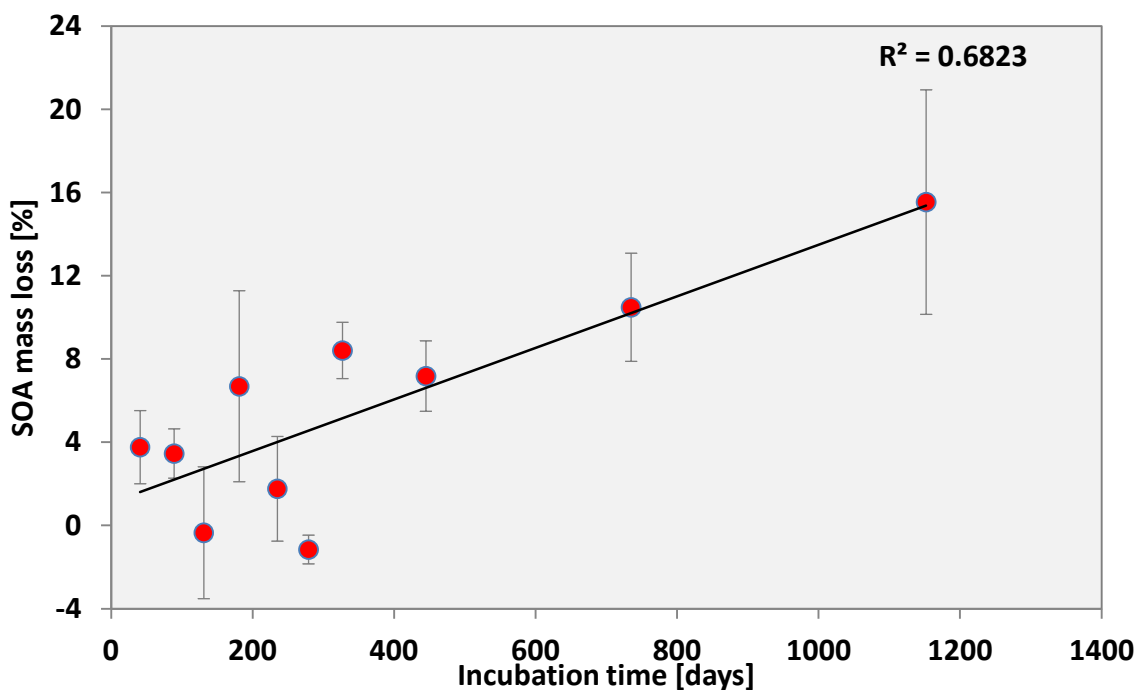
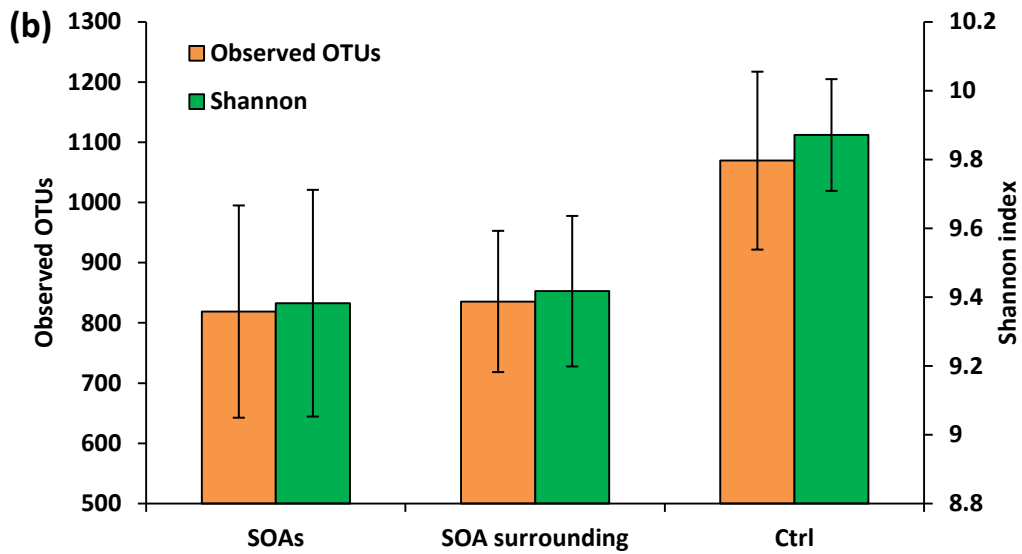
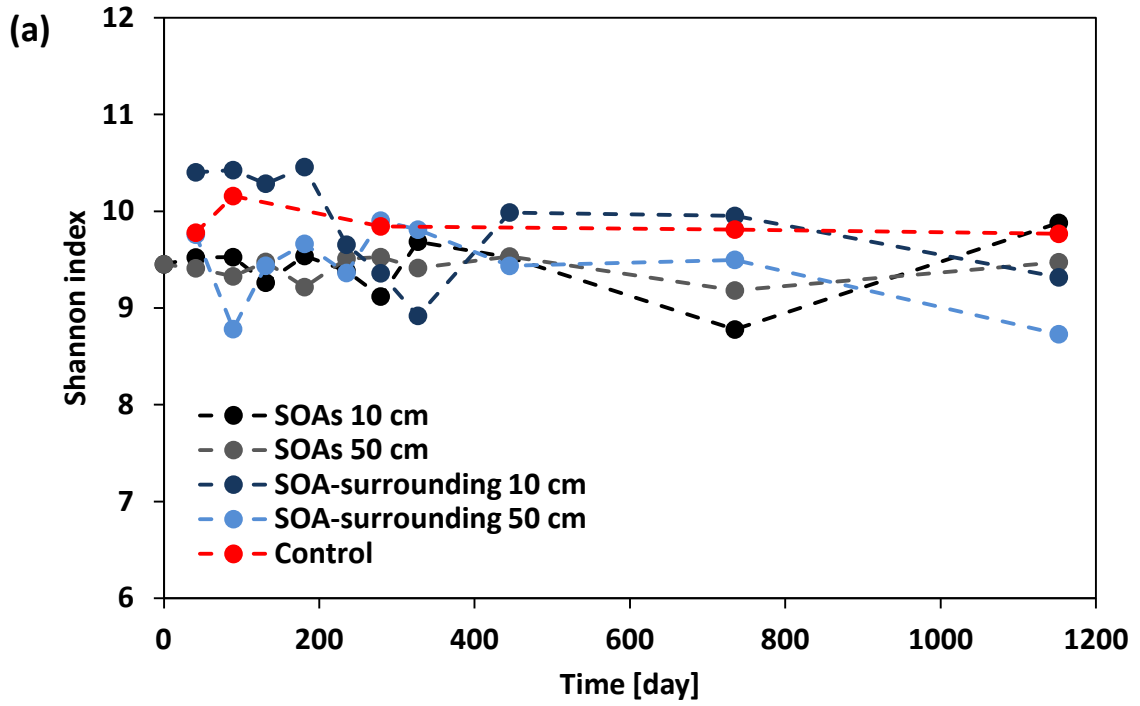


Figure 26 Mass loss of sediment oil agglomerates (SOAs) quantified over the 3 year time course in Pensacola Beach sands.



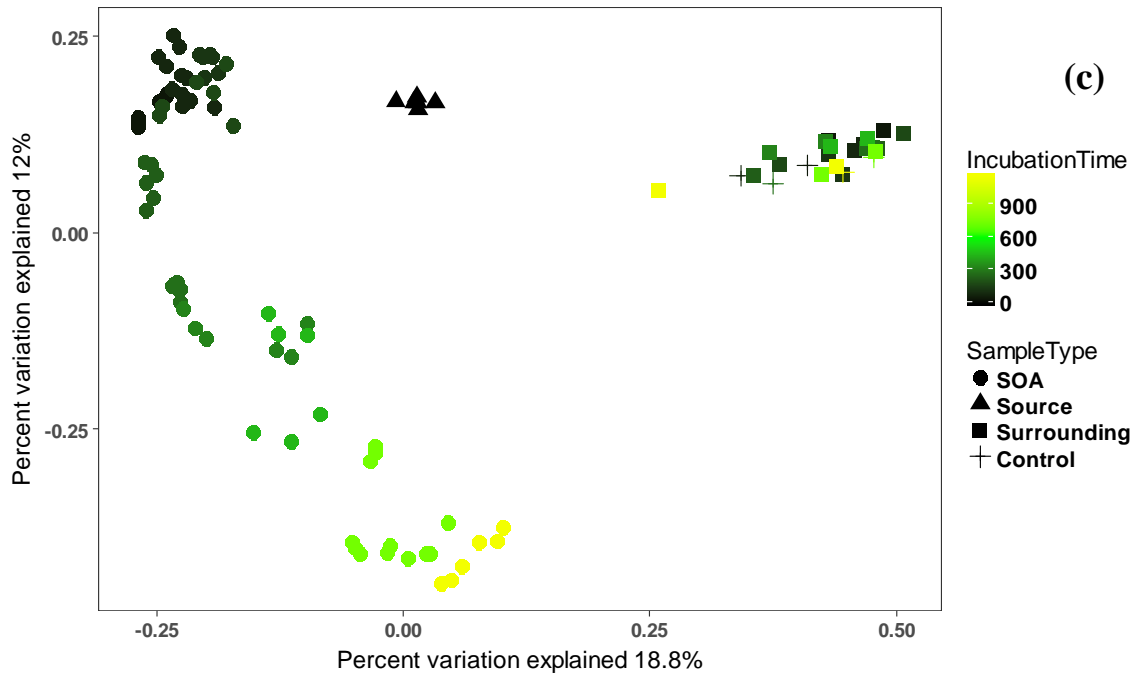


Figure 27 Diversity of microbial communities in sediment oil agglomerates (SOAs), SOA-surrounding sands, and control sands over the 3 year time course. Alpha-diversity is calculated based on (a) Shannon indices and (b) the number of observed OTUs. (c) Beta diversity of microbial communities visualized by a principal coordinate analysis (PCoA) plot of Bray-Curtis distance matrices.

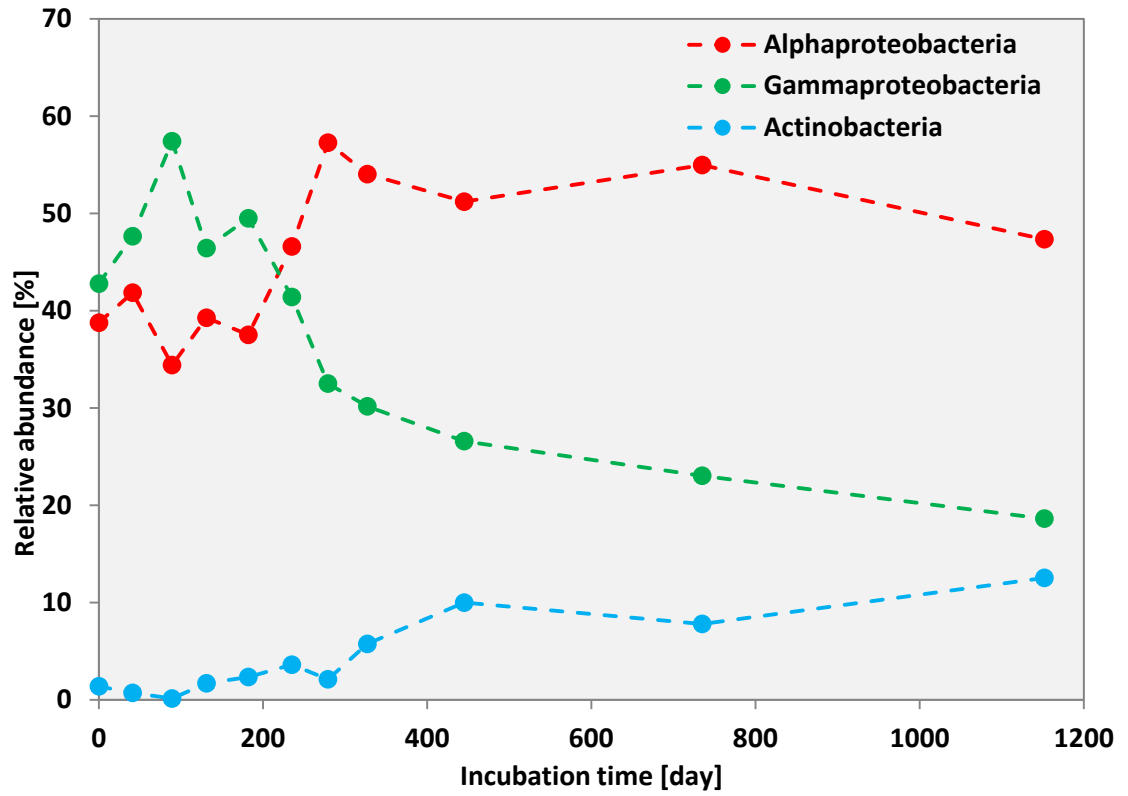


Figure 28 Relative abundance of taxa detected at the phylum to class level in sediment oil agglomerates (SOAs) over the 3 year time course. Abundance is determined based on the total SSU rRNA gene sequences retrieved.

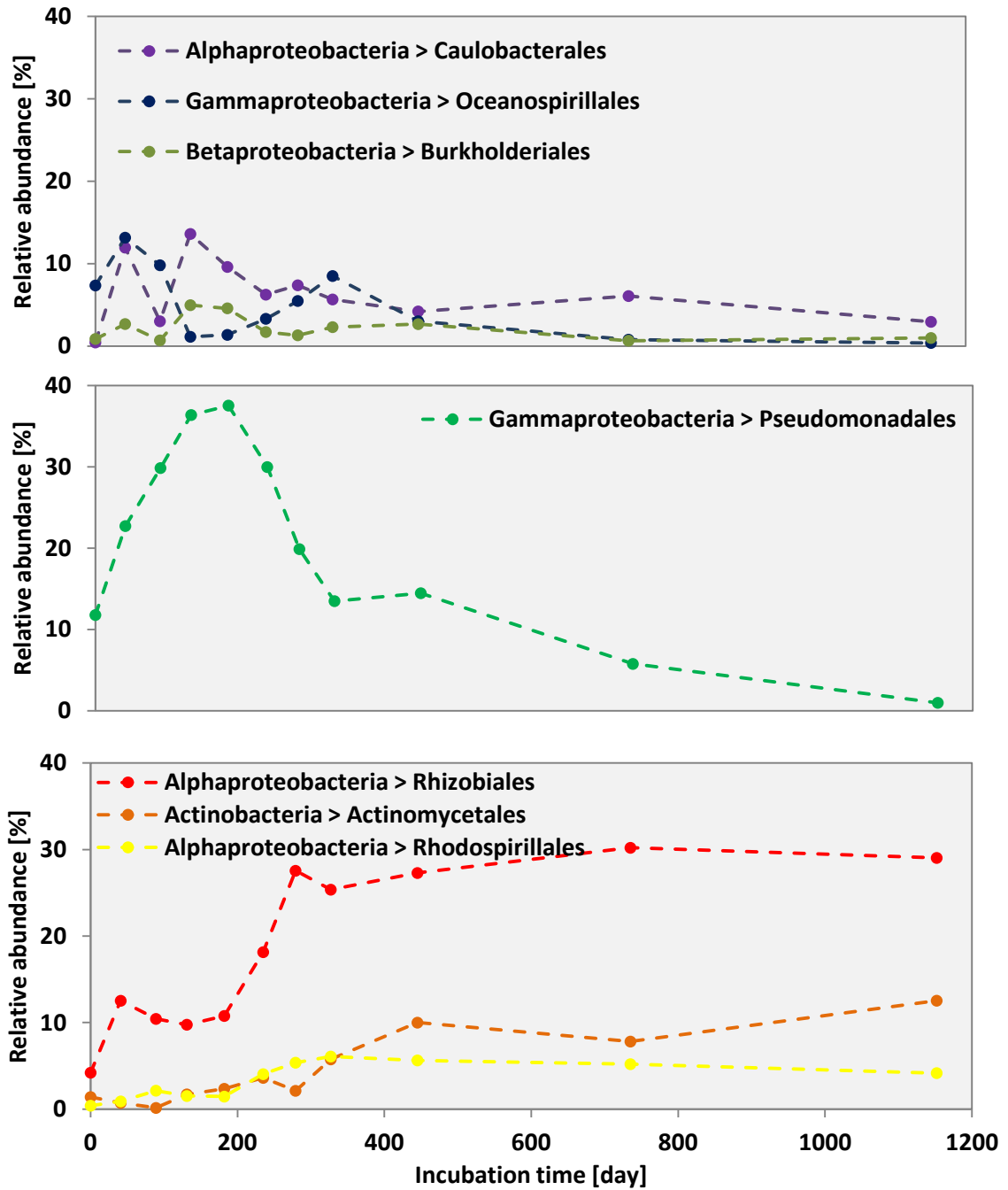


Figure 29 Relative abundance of taxa detected at the order level in sediment oil agglomerates (SOAs) over the 3 year time course. Abundance is determined based on the total SSU rRNA gene sequences retrieved.

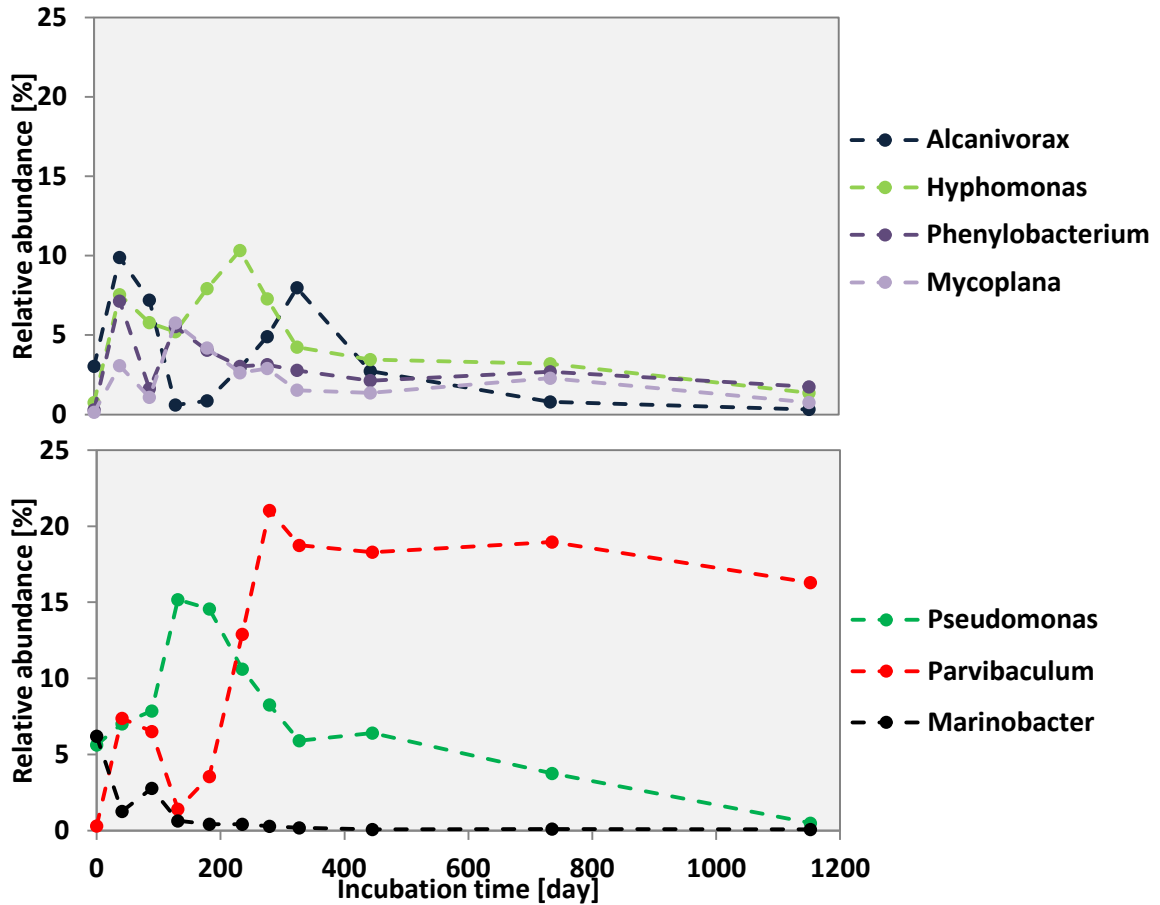


Figure 30 Relative abundance of taxa detected at the genus level in sediment oil agglomerates (SOAs) over the 3 year time course. Abundance is determined based on the total SSU rRNA gene sequences retrieved.

4.4.2 Succession of microbial community

Microbial (alpha) diversity as determined by Shannon indices showed substantial variation (Figure 27a) in SOAs and SOA-surrounding sands from both sediment depths, while diversity in control sands was more consistent with time. No significant trends in diversity over the time course were found for SOAs and SOA-surrounding sands. Whereas a previous study of more diffuse oil particles in the supratidal zone of Pensacola Beach showed a > 50% reduction of Shannon indices over a 6 month time series during

which the majority of hydrocarbons were degraded, we detected a 27 % reduction of Shannon indices and some evidence of recovery after 1152 days in the present time series. The mean number of observed OTUs declined by 23 % in SOAs and SOA-surrounding sands (827 ± 147 observed OTUs) in comparison to control sands (1069 ± 148 observed OTUs) (Figure 27b). Microbial community composition clearly changed with time but not with sediment depth in SOA samples, as revealed by beta diversity analysis (Figure 27c). Microbial communities in SOAs were clearly distinct from those present in SOA-surrounding sands or in control sands. SOA-surrounding sands and control sands showed little or no trends with time or sediment depth.

The impact of oil contamination on microbial community composition was further examined in buried SOAs by investigating the dynamics of specific taxa over the 3 year time series. Microbial community structure did not show significant differences between the 10cm and 50cm sediment intervals. Therefore, the mean of relative abundances from the two different depths were pooled for further analysis (Figure 28 to 30). A clear succession of microbial populations was observed in SOAs with time, whereas no noticeable pattern was detected in SOA-surrounding sands and control sands (Supplemental Figure 2). At the phylum and class level, microbial communities in SOAs were dominated by the *Gammaproteobacteria*, *Alphaproteobacteria*, and *Actinobacteria* (Figure 28). While the *Gammaproteobacteria* showed a maximum abundance at approximately 100 days, the *Alphaproteobacteria* peaked later at ~ 300 days, followed by a bloom of *Actinobacteria* after 400 days. Both *Gammaproteobacteria* and *Alphaproteobacteria* were enriched in Pensacola Beach sands after the Deepwater Horizon oil spill and were identified as key players in oil degradation in previous studies

that monitored microbial community shifts after weathered oil contamination over a relatively shorter time period (Kostka et al., 2011; Rodriguez-R et al., 2015). In corroboration of previous studies of diffuse oil in Gulf beach sands (Kostka et al., 2011), hydrocarbon-degrading bacteria within the *Gammaproteobacteria* responded first to oil in SOAs followed by members of the *Alphaproteobacteria*. Kostka et al. observed an initial increase in the relative abundance of the known oil degrader *Alcanivorax* within the *Gammaproteobacteria* class, followed by an increase in the relative abundance of *Alphaproteobacteria* at later stages when recalcitrant oil hydrocarbons predominated. A similar pattern was observed in this study. Members of the *Gammaproteobacteria* were enriched in parallel with the rapid depletion of relatively short-chain alkanes (C₁₅) and naphthalene. At a later stage of incubation between ~100 to ~300 days, degradation of longer chain alkanes (C₁₆ – C₃₀) and heavier PAHs (including fluorine, phenanthrene, and dibenzothiophene) was observed along with an increase in the relative abundance of *Alphaproteobacteria*. In corroboration of the results presented here, the bacterial communities in oiled rocks and supratidal sands impacted by the Prestige oil spill revealed that members of *Alphaproteobacteria* and *Actinobacteria* replaced the *Gammaproteobacteria* when the biodegradation process reached advanced stages after one year (Alonso-Gutierrez et al., 2009). Members of *Alphaproteobacteria* and *Actinobacteria* were also highly enriched, constituting 84% and 16% of sequences retrieved, respectively, in a pyrene-degrading microbial consortium (Gallego et al., 2014).

At the genus to order level, three distinct maxima in microbial populations were observed with time. These maxima in relative abundance are interpreted as microbial populations that respond to petroleum hydrocarbons available during the early, mid, and

late stages of the time series. Early responders included the *Caulobacterales* within the class *Alphaproteobacteria* and *Oceanospirillales* within the class *Gammaproteobacteria* that increased in relative abundance during the first 100 days, from 0.5 % to 12 % and from 7 % to 13 %, respectively (Figure 29). At the genus level, the relative abundance of known alkane degraders such as *Alcanivorax*, *Hyphomonas*, *Phenylobacterium*, and *Mycoplana* increased from 0.13 – 3 % to 3 – 10 % (Figure 30). *Alcanivorax* is known to degrade relatively short-chain alkanes and not capable of degrading aromatic hydrocarbons (Liu and Shao, 2005; Schneiker et al., 2006). In a previous study from Pensacola Beach sands, *Alcanivorax* spp. were the most abundant OTU in the oil-contaminated samples (Kostka et al., 2011). A high relative abundance of *Alcanivorax* (up to 20%) was also observed in a gravel beach after the Xingang oil spill in China (Zhang et al., 2017). The genus *Hyphomonas*, known to be able to utilize aromatic hydrocarbons, was enriched in oiled sand and microcosms with crude oil (Coulon et al., 2007; Kappell et al., 2014; Rodriguez-R et al., 2015). A DNA-based and stable isotope probing (SIP) study with [U-¹³C]anthraquinone from PAH-contaminated soil showed that the genus *Phenylobacterium* is responsible for anthraquinone degradation (Rodgers-Vieira et al., 2015). The genus *Mycoplana* is also known to degrade aromatic hydrocarbons (Brinda Lakshmi et al., 2013, 2012). Our observations are corroborated by petroleum hydrocarbon analysis which revealed that short-chain alkanes (C₁₅) and relatively low-molecular weight PAHs such as naphthalene were rapidly depleted over the first ~100 days when populations of early responders were enriched.

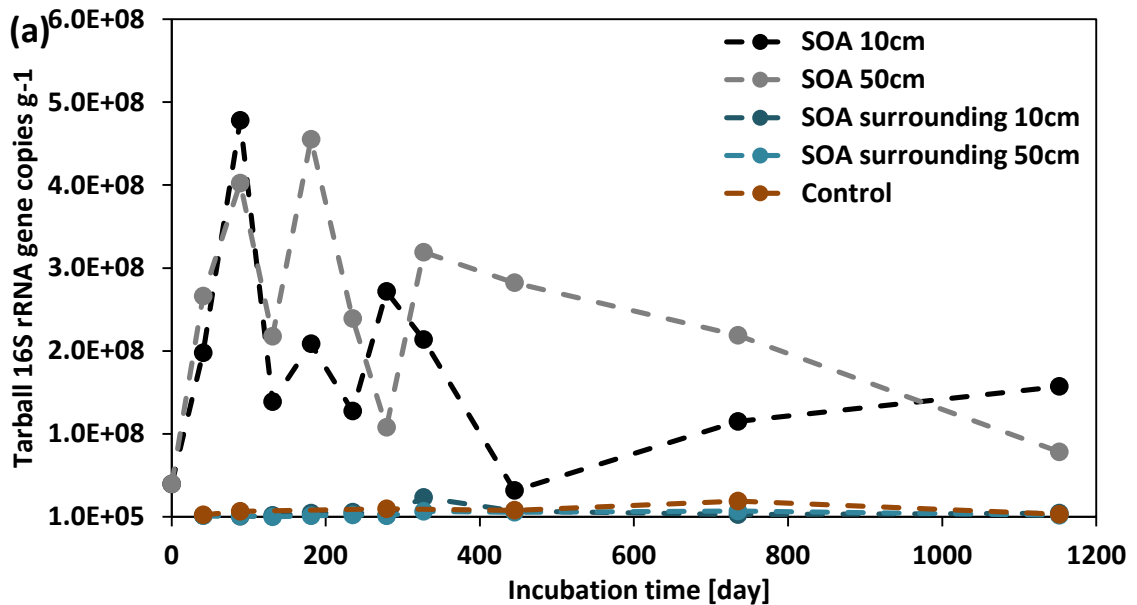
Distinct microbial groups were enriched during 131 – 181 days of the time series. The genus *Pseudomonas* within the order *Pseudomonadales* was enriched up to 15.2%

after 131 days. *Pseudomonas* has been reported to produce biosurfactant during PAH degradation and was also enriched in previous studies of oil-contaminated sands (Deziel et al., 1996; Kostka et al., 2011; Prabhu and Phale, 2003; Rodriguez-R et al., 2015). The relative abundance of *Rhizobiales*, *Actinomycetales*, and *Rhodospirillales* increased in the later stages of the time series after 279 days. *Parvibaculum* within the order *Rhizobiales* is known to degrade both aliphatic and aromatic hydrocarbons, and this genus was highly enriched late in our time series in agreement with previous work in oiled sands (Lai et al., 2011; Rodriguez-R et al., 2015; Rosario-Passapera et al., 2012). Relative abundance of *Mycobacterium*, a known PAH degraders, were nearly zero when SOA incubation started but it kept increasing until the last time point up to 9% relative abundance. *Mycobacterium* was also detected from major Denaturing Gradient Gel Electrophoresis band from SOAs collected from supratidal zone of coastal headland beach in Louisiana. *Mycobacterium* is the only genus isolated capable of degrading 4-ring PAHs such as the substituted chrysene observed in SOAs (Khan et al., 2002; Urbano et al., 2013). *Mycobacterium* is also known to be adapted to low moisture content and periods of desiccation, which resembles dry characteristic of SOAs (Harland et al., 2008). Another known PAH-degrader *Stenotrophomonas* was a major microbial group in intertidal zone of headland beach but its relative abundance in this study was very low at 0-0.22% throughout all SOA incubation time period (Urbano et al., 2013). Members of the genus *Marinobacter*, which is known to degrade both alkane and PAHs, were enriched in many oil-impacted environments (Atlas et al., 2015; Kostka et al., 2011; Lamendella et al., 2014; Wang et al., 2016; Zhang et al., 2017) but their abundances in SOAs decreased from 6 % to detection limit. The detection of genus *Marinobacter* is known to be favored

by sunlight (Bacosa et al., 2015), indicating that members of *Marinobacter* could not be enriched in buried SOAs due to absence of light. Petroleum hydrocarbon analysis showed that mid-chain alkanes ($C_{16} - C_{30}$), phenanthrene, and dibenzothiophene were degraded from ~100 days to ~300 days after the SOA burial. This implies that second responders *Pseudomonas* and some of early responders such as *Hyphomonas* and *Micoplana* were capable of degrading these hydrocarbon compounds. From 300 days of SOA incubation, degradation rate of long-chain alkanes ($C_{30} - C_{40}$) decreased, which coupled to rapid increase of relative abundance of *Parvibaculum*. A few isolates of the genus *Parvibaculum* such as *P. lavamentivorans* and *P. hydrocarboniclasticum* are known to be capable of utilizing *n*-alkanes or linear alkylbenzenesulfonates (Rosario-Passapera et al., 2012; Schleheck et al., 2004), which implies degradation of long-chain alkanes by *Parvibaculum* at a slower rate at a later stage of incubation.

Following the Deepwater Horizon oil spill, discharged oil reached along 2104 km of shoreline in the northern Gulf of Mexico (Nixon et al., 2016). Changes in hydrocarbon composition in diffuse oil extracted from oiled beach sand showed a substantial weathering in that higher-molecular-weight aliphatic ($> C_{16}$) and aromatic ($> C_{35}$) fractions (Kostka et al., 2011; Rodriguez-R et al., 2015). Chemical analysis of oil residues in sand patties and rock scrapings revealed that the remnant oil was highly weathered with decreased abundances of *n*-alkanes and PAHs and increased abundances of oxygenated hydrocarbons (OxHC) due to photooxidation and biodegradation (Aeppli et al., 2014; Gros et al., 2014; Hall et al., 2013; Lewan et al., 2014; Radović et al., 2014; White et al., 2016). Long-term weathering processes in oil residues in different state of weathering showed that prominent *n*-alkane peaks were observed in relatively less

weathered oil residues (White et al., 2016). Another study on coupling of biodegradation index and different classes of saturates in weathered oil highlighted that *n*-alkanes are the most easily biodegradable, followed by methyalkanes, cyclic and acyclic isoprenoids (Gros et al., 2014). Based on previous studies on chemistry of oil residues, microbial community structure shifts from this study may indicate dynamics of hydrocarbon composition change which altered microbial groups that are capable of feeding on available carbon source in SOAs over time. The bloom of early responders including known alkane-degraders followed by known PAH-degraders implies that microbes perform alkane degradation first followed by PAH degradation in SOAs. Moreover, this study indicated that relatively longer time scale of microbial community change accompanies by SOA degradation in Pensacola Beach sand for over 3 years, while the microbial community shifts in diffuse oil contamination showed significant recovery after 5 months to 1 year (Huettel et al., 2018; Kostka et al., 2011; Rodriguez-R et al., 2015).



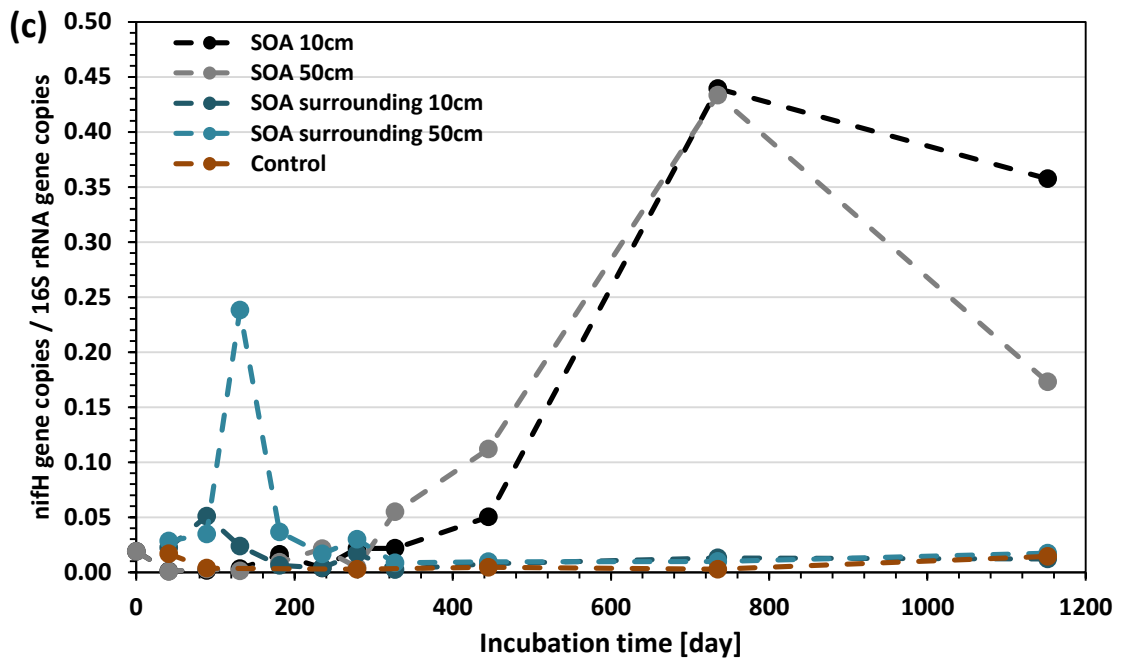
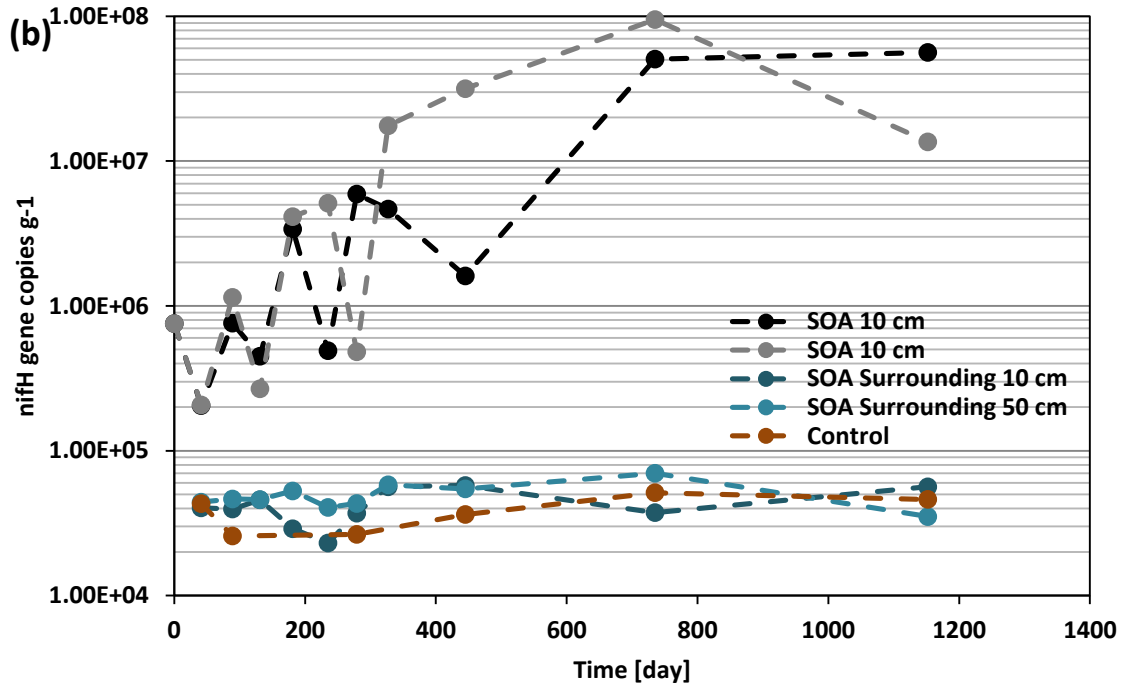


Figure 31 (a) SSU rRNA gene, (b) *nifH* gene abundance per gram sediment and (c) *nifH* gene abundance normalized to the abundance of SSU rRNA genes in 10cm and 50cm sediment depth intervals from SOAs and SOA-surrounding sands as well as control sands.

4.4.3 *The abundance of overall bacteria and diazotrophic communities*

Previous studies have shown that nitrogen fixation was enhanced during microbial hydrocarbon degradation due to nutrient limitation (Coty, 1967; Rodriguez-R et al., 2015; Tocalino et al., 1993). Characterization of microbial communities by SSU rRNA gene sequencing revealed a number of abundant groups that contain known diazotrophs including the *Rhizobiales*, *Rhodobacterales*, and *Rhodospirillales*, we hypothesized that microbial nitrogen fixation would be enhanced with time. In order to estimate nitrogen fixation potential during *in situ* SOA incubation in Pensacola Beach sand, the abundance of genes encoding nitrogenase enzyme (*nifH*), the best studied molecular marker for nitrogen fixation (Gaby and Buckley, 2012), was quantified. Results showed that both SSU rRNA gene and *nifH* gene copy numbers from SOA sands had distinct patterns from those from SOA-surrounding sands and control sands (Figure 31). The abundance of SSU rRNA genes on average was three orders of magnitude higher in SOAs in comparison to the surrounding sands or control sands. This indicates that a bloom of overall bacteria occurred and SOAs are hotspots of microbial growth at least during the early stages after being trapped in the beach. In the SOAs, overall bacterial abundance increased from 3.4×10^7 to 4.4×10^8 copies g^{-1} during the first 89 days, whereas overall bacterial abundance in SOA-surrounding sands and control sands remained at 2.0×10^5 to 2.4×10^7 copies g^{-1} . The abundance of *nifH* genes was elevated to a much larger extent, increasing in SOAs from one to three orders of magnitude higher abundance in comparison to surrounding and control sands (7.6×10^5 to 8.4×10^7 copies g^{-1} in SOAs; 2.3×10^4 to 7×10^4 in surrounding; 2.6×10^4 to 5.1×10^4 in control). The ratio of *nifH* to SSU rRNA gene abundance (*nifH* / SSU rRNA gene abundance) remained close to the detection limit for

the first 300 days and then showed a large increase to 0.44 towards the latter stages of the time course, suggesting that diazotrophs bloomed in abundance as nitrogen became limiting in the SOAs. Similar to the abundance of overall bacteria, diazotroph abundance remained low and close to the detection limit in the surrounding sands and control sands. Our results from SOAs are corroborated by previous studies of more diffuse oil in sands at Pensacola Beach which showed elevated nitrogenase abundance in oil-contaminated sand layers (Rodriguez-R et al., 2015). Results imply high C/N ratio triggered microbial nitrogen fixation to produce bio-available nitrogen nutrients from atmospheric nitrogen.

Sequencing of *nifH* amplicons was conducted to further elucidate the microbial groups responsible for nitrogen fixation (diazotrophs) in the oiled dry beach environment. Diazotroph microbial diversity as determined by Shannon indices decreased over time (Figure 32). At the phylum to class level, results showed that the *Alphaproteobacteria* and *Actinobacteria* dominated the diazotroph communities in SOAs with relative abundances of 64 – 71 % and 15 – 21 %, respectively across the time series (Figure 33). At the order level, members of the *Rhodospirillales* of the *Alphaproteobacteria* were the most abundant throughout time series (36 – 44% relative abundance). The late bloom of diazotroph abundance along with the high relative abundance of *Rhodospirillales nifH* in the time series agrees well with the relative abundance of the *Rhodospirillales* tracked by SSU rRNA gene amplicon sequencing, which peaked after 400 days. Members of the *Rhodospirillales* were found in oil slick produced from MC-252 oil contamination after the Deepwater Horizon oil spill (Chakraborty et al., 2012). In addition, a recent study reconstructed the genome of naphthalene-degrading bacteria affiliated with *Rhodospirillales* (99% similar to *Thalassospira profundimaris* WP0211) from the

Deepwater Horizon oil spill, showing that the *Rhodospirillales* were likely responsible for PAH degradation at the sea surface (Dombrowski et al., 2016). In a metagenomics study of soil microbial communities in a crude oil field, alkane reductase-dependent monooxygenase and methane monooxygenase genes affiliated with *Rhodospirillales* were detected (Abbasian et al., 2016). Therefore, multiple lines of evidence indicate that the *Rhodospirillales* are mediating PAH degradation and nitrogen fixation under nitrogen-limited conditions such as beach sand environment. The second most abundant diazotroph group at the order level was the *Chlorobiales*, which is mainly comprised of obligate anaerobic photoautotrophic bacteria, constituted up to 15 – 20 % relative abundance. *Chlorobiales* (0.1% of the microbial strains identified) and genes involved in nitrogenase and its associated proteins were also detected in metagenomic analysis of crude oil field soil (Abbasian et al., 2016).

At the genus level, *Methylobacterium* within the order *Rhizobiales* was the most abundant diazotroph group, which constituted from 25 to 33% of *nifH* gene relative abundance (Figure 33). *Methylobacterium* increased rapidly from 25 % relative abundance after 445 days of incubation to 31 % abundance at the end of the time course. *Methylobacterium* was also abundant in oil mousse collected from the sea surface and salt marshes during the Deepwater Horizon oil spill (Liu and Liu, 2013). The second most abundant genus was *Frankia* within the order *Actinobacteria*, which are filamentous bacteria that live in symbiosis with plants that are capable of fixing atmospheric nitrogen into ammonia (Benson and Silvester, 1993). *Frankia* has been shown to fix nitrogen under free-living as well as symbiotic conditions (Sellstedt and Richau, 2013). However, within our SSU rRNA gene-based analysis, the relative abundance of *Methylobacterium*

and *Frankia* comprised only 0-0.04%. This may imply that *Methylobacterium* and *Frankia* are capable of fixing nitrogen even at low relative abundance or other bacterial groups contain *Methylobacterium* or *Frankia*-associated nitrogenase genes. Members of the *Rhodobacterales* were abundant (19 %) at the beginning of the time series based on SSU rRNA gene amplicon sequencing data, and their relative abundance decreased to 3% at the end of the time course. However, *nifH* gene relative abundance affiliated with *Rhodobacterales* was 0-0.4% throughout all time period. At OTU level, denovo 1677 showed the most significant increase from 1% to 3.2% of their relative abundance (Figure 34). Interestingly, this OTU shared 100 % sequence identity with the clone PollT75-7 from mangrove sediment microcosms that were amended with oil to simulated a spill (Taketani et al., 2010). OTU denovo 1677 shared 82 % sequence similarity with *nifH* gene sequence of *Methylobacterium* sp. xct7.

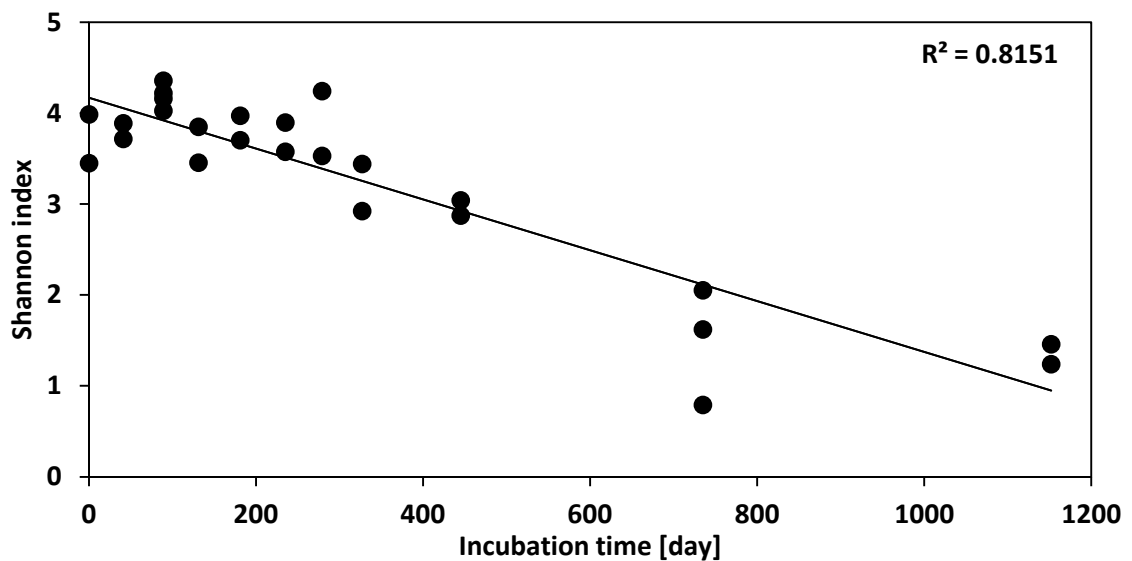


Figure 32 Alpha-diversity of nitrogenase gene sequences over the three year time course of SOA samples incubated in Pensacola Beach sand.

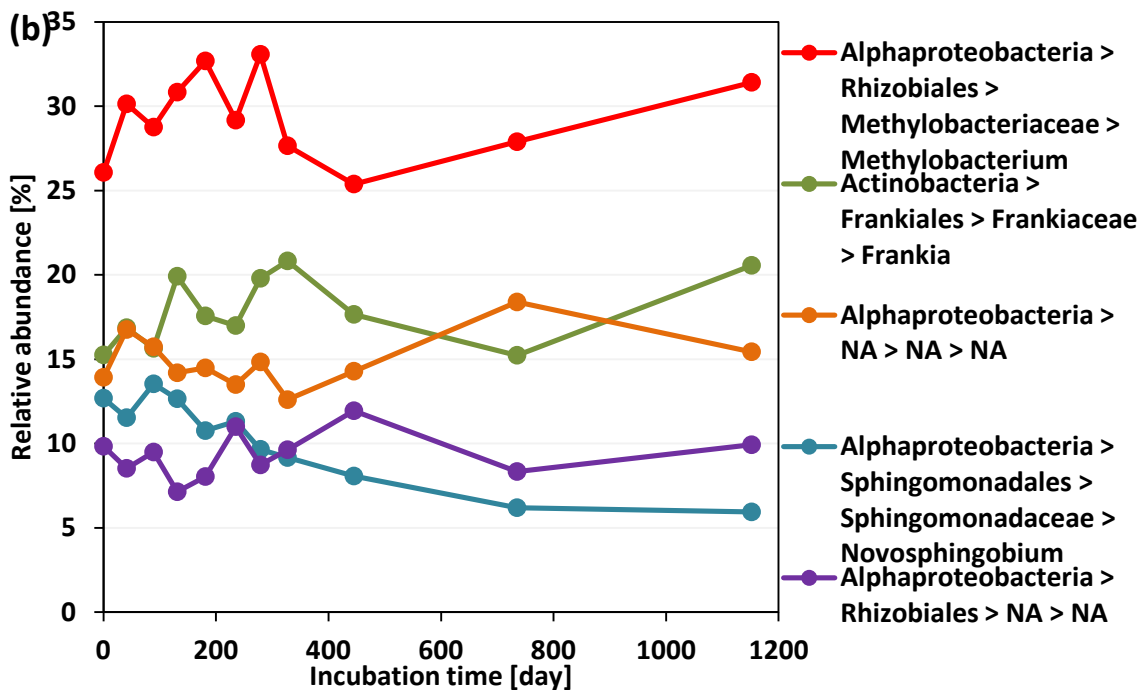
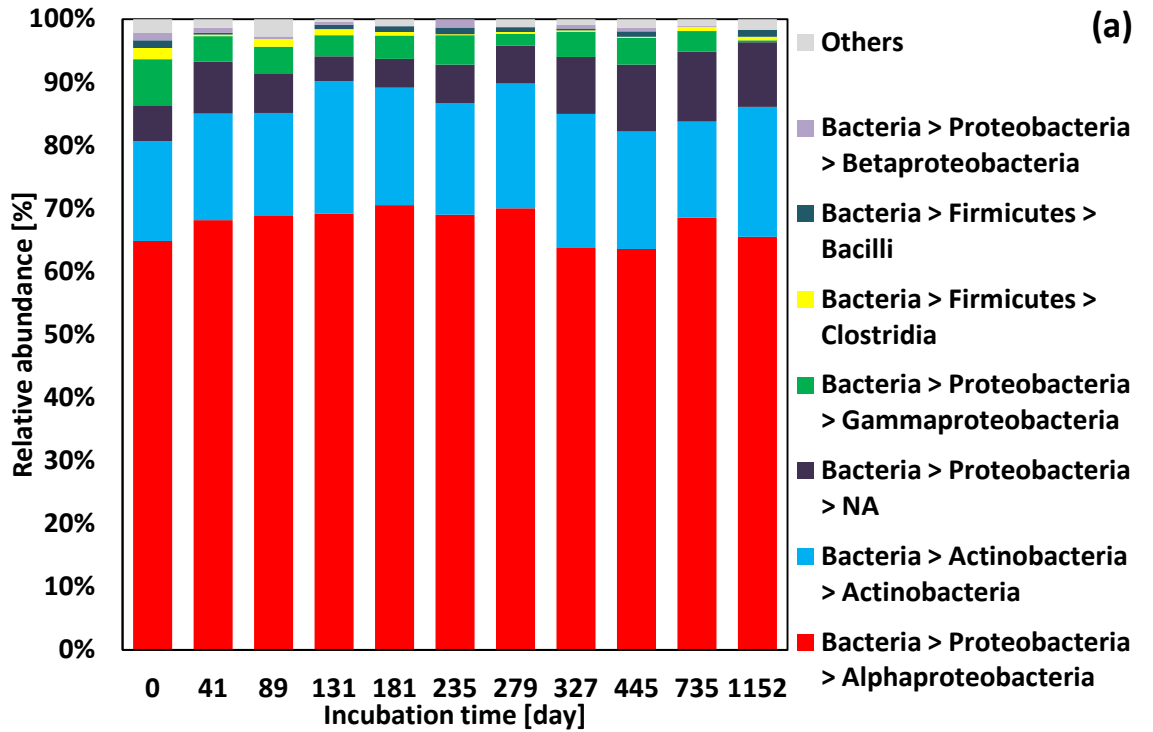


Figure 33 (a) Microbial community structure at the class level based on *nifH* gene sequences retrieved from SOAs over the three year time course. (b) Relative abundance of the genera *Methylobacterium*, *Frankia*, and *Novosphingobium* based on *nifH* gene sequence analysis over the three year time course.

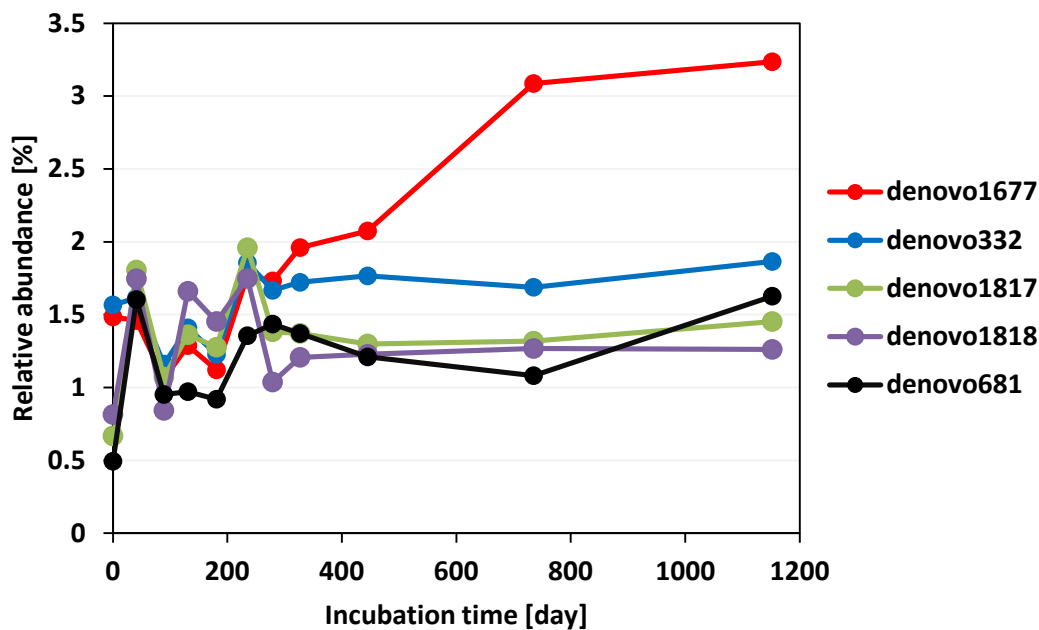


Figure 34 Relative abundance of the top five OTUs of *nifH* gene amplicons over the three year time course.

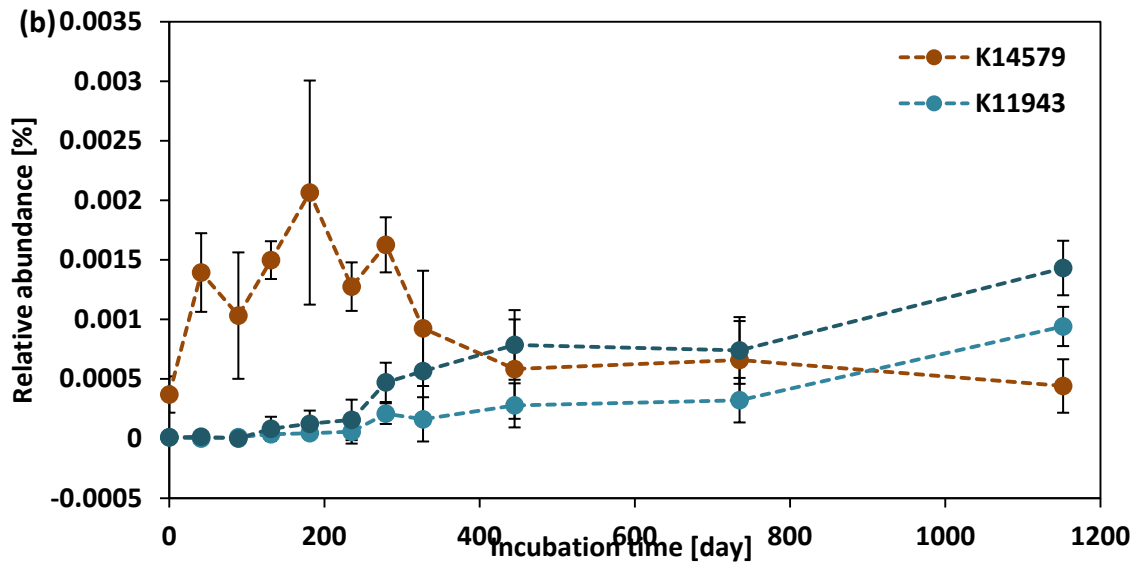
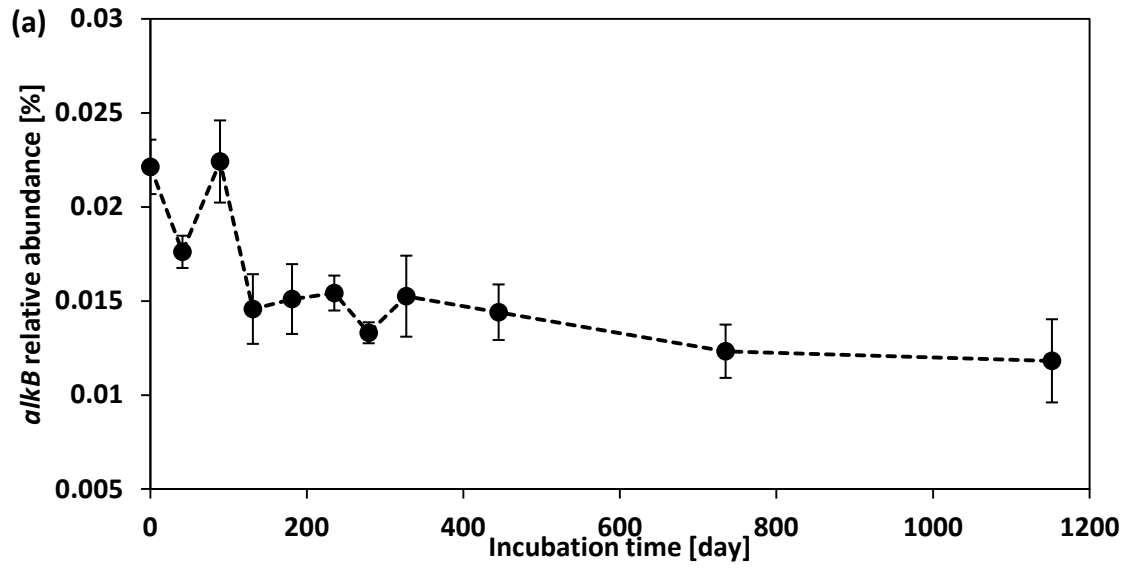
4.4.4 Inferred metagenomic analysis

Based on microbial community composition in SOAs as determined by SSU rRNA gene amplicon sequencing, inferred metagenomic analysis was performed to assess the metabolic potential of the communities across the time series. Given the abundance of hydrocarbon-degraders and diazotrophs in the time course, our analysis focused on functional genes for hydrocarbon degradation and nitrogen fixation (Figure 35). The predicted relative abundance of alkane-1-monooxygenase (*alkB*) genes peaked in the first approximately 100 days post burial and then decreased rapidly to 200 days, which suggests that relatively simple hydrocarbon substrates such as alkanes were utilized by bacteria at this early stage of the time series. Microbial groups that were

predicted to contribute to alkane degradation include members of the *Rhodobacteraceae*, *Pseudomonadaceae*, *Alcanivoraceae*, and *Alteromonadaceae*. The predicted relative abundance of naphthalene 1,2-dioxygenase genes (*nahAc*, *ndoB*, *nbzAc*, *dntAc*) and other PAH dioxygenase genes (*nidA*, *nidB*) reached a maximum later in the time series, at approximately 200 and 300 days post initiation, respectively. *Pseudomonas* was predicted to contribute to the degradation of more recalcitrant, aromatic hydrocarbons later in the time series. Lastly, predicted relative abundance of nitrogenase genes increased rapidly after 400 days and peaked at approximately 750 days, indicating enhanced bacterial nitrogen fixation at a later stage of SOA incubation. Microbial groups that are predicted to contribute to nitrogenase gene abundance include *Rhizobiales*, *Rhodobacterales*, and *Rhodospirillales* (Figure 36).

Predictions from PICRUSt were corroborated by the chemical evolution of petroleum hydrocarbons, as determined in our companion study, along with temporal trends in the abundance of overall bacteria and diazotrophs. The predicted abundance of hydrocarbon degradation genes peaked during the first 400 days in parallel with overall bacterial abundance as well as the degradation of alkanes and aromatic compounds. Predictions of alkane monooxygenase (*alkB*) abundance showed good agreement with the consumption of short chain (C₁₅) alkanes, with both showing maximum changes during the first 100 days, suggesting that early responders (*Alcanivorax*, *Hyphomonas*, *Phenyllobacterium*, *Mycoplana*) are utilizing these compounds. Between 100 and 400 days, the consumption of longer chain alkanes (C₁₈-C₂₂) was not concurrent with the predicted *alkB* abundance, indicating that other enzyme pathways are responsible for the degradation of these compounds. Finally, the predicted abundance of nitrogenase shows

good agreement with the observed nitrogenase abundance, with both showing the largest increases between 400 and 750 days. Thus, multiple independent lines of evidence support the coupling of hydrocarbon degradation to diazotrophy in SOAs.



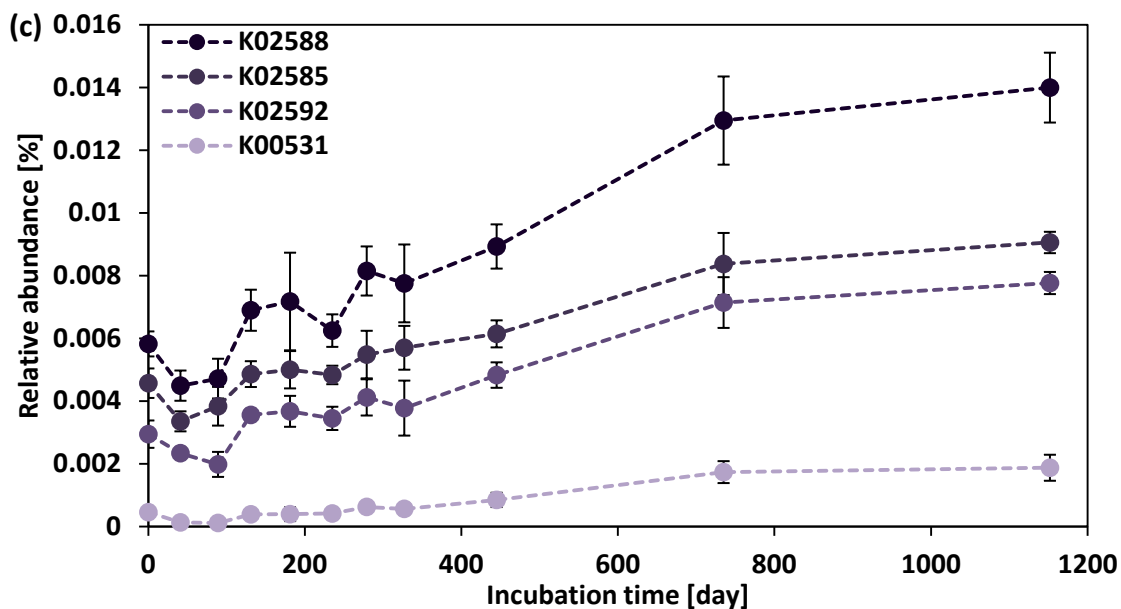
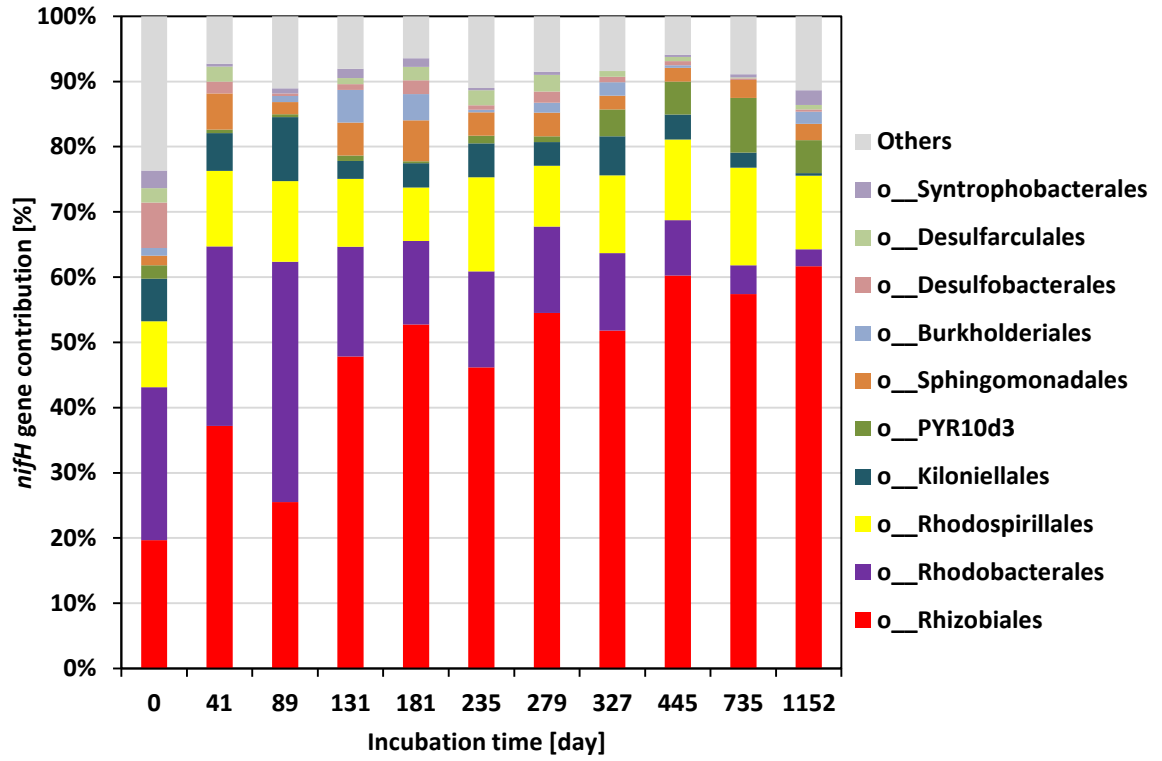


Figure 35 Predicted relative abundance of functional genes over the three year time course as determined by PICRUST. Relative abundance of genes encoding (a) alkane-1-monooxygenase (AlkB) – K00496, (b) naphthalene 1,2-dioxygenase subunit alpha – K14579, PAH dioxygenase large subunit – K11943, PAH dioxygenase small subunit – K11944, (c) nitrogenase iron protein NifH – K02588, nitrogen fixation protein NifB – K02585, nitrogenase molybdenum-iron protein NifN – K02592, and nitrogenase delta subunit – K00531.



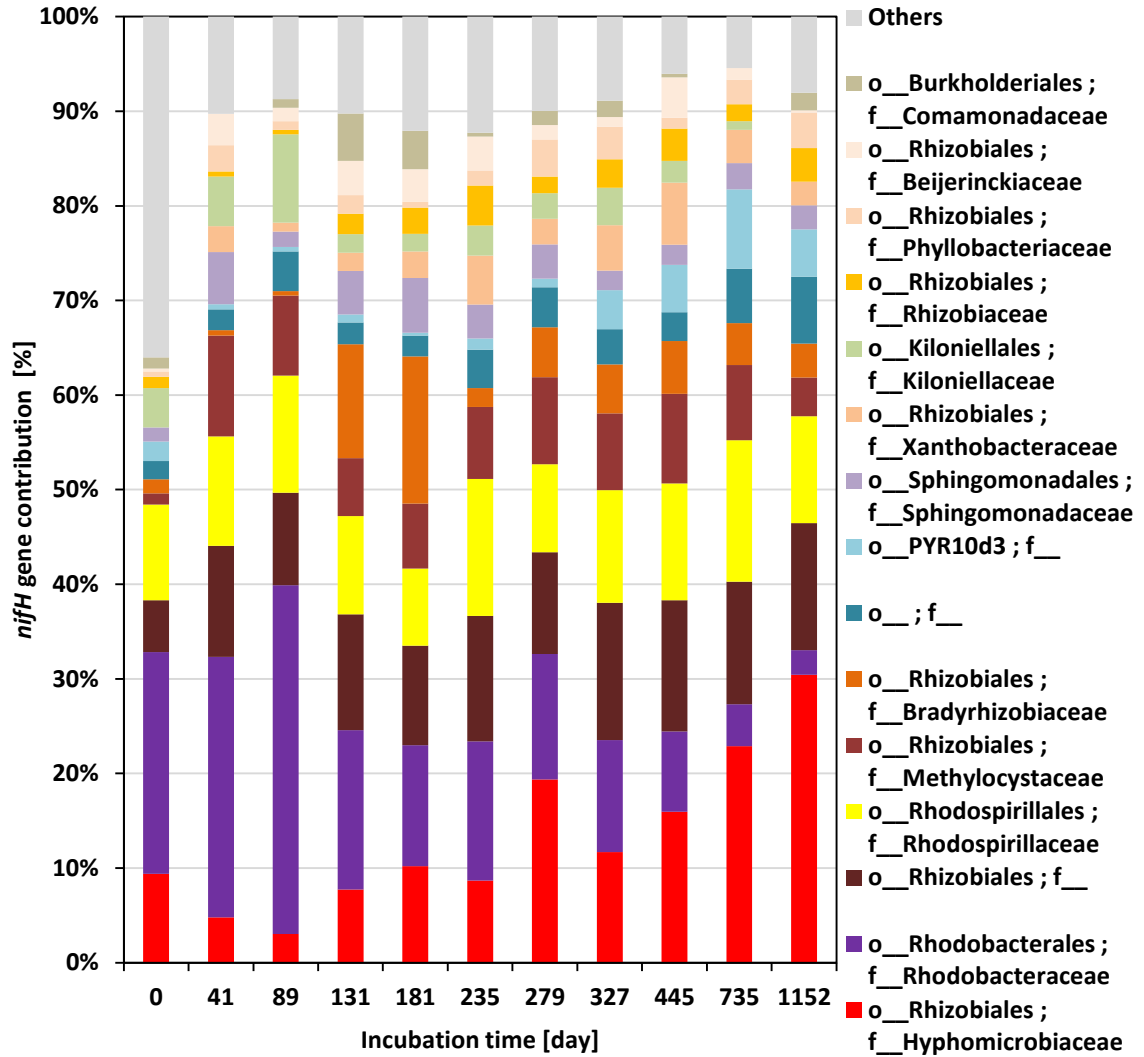


Figure 36 Predicted community composition of diazotrophs determined by inferred metagenomic analysis (PICRUST) of *nifH* genes.

4.5 Conclusions

After the Deepwater Horizon oil spill event, weathered oil came ashore along the coastline from east Texas to west Florida and was deposited into sandy sediment. Macroscopic sediment-oil agglomerates (SOAs) were formed when oil and sediment interacted, and biodegradation is understudied in these larger oil-sediment aggregates in

comparison to more diffuse oil contamination in nearshore environments. Thus, the main objectives of this study were to investigate the rate of SOA degradation *in situ* in Pensacola Beach sands, to interrogate the microbial communities that recruit onto these larger aggregates, as well as to elucidate linkage between hydrocarbon degradation and nitrogen fixation. Up to 91% of petroleum hydrocarbons were degraded in SOAs over a three year time series. As has been observed for more diffuse oil contamination in beach sands, the taxonomic diversity of microorganisms is lowered in SOAs in comparison to surrounding or pristine sands. Hydrocarbon-degrading bacteria are enriched and a succession of microbial populations is observed that parallels with the chemical evolution of petroleum hydrocarbons as determined in our companion study. Temporal trends in oil chemistry and microbial succession suggest that SOA degradation occurs over longer time scales in comparison to smaller, more diffuse oil particles in dry beach sands. We provide the first evidence of a bacterial bloom in SOAs, indicating that these large aggregates are hotspots of microbial growth. In addition, we provide the first quantification of diazotrophs in large aggregates, revealing that nitrogen-fixing taxa predominate in microbial communities during the late stages of the time course. Results indicate that the high C/N ratio of SOAs causes nutrient stress and induces microbial nitrogen fixation. Thus, the coupling of nitrogen fixation to hydrocarbon degradation appears to be an important control over the fate of oil in macroscopic oil aggregates. Our observations are corroborated by predictions from inferred metagenomic analysis and predictions aid in linking specific taxa to the degradation of specific hydrocarbon compounds and diazotrophy.

CHAPTER 5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

After the Deepwater Horizon (DWH) oil spill in 2010, discharged oil rose to the surface ocean and was transported to coastal environments of northern Gulf of Mexico. Surfaced oil was buried in coastal marine sediments from fine-grained muds of the continental shelf to permeable beach sands. Biodegradation is the primary means by which petroleum hydrocarbons are removed from the marine environment, and oxygen is a master variable controlling the rates and metabolic pathways of hydrocarbon biodegradation. Thus, characterizing the structure and function of microbial communities that degrade hydrocarbons under aerobic vs. anaerobic conditions is crucial to understand the fate of oil after accidental oil spill events. This study contrasts the microbial populations that mediate the degradation of petroleum hydrocarbons under anoxic conditions in shallow subtidal marine muds with their counterparts in aerobic beach sands.

Anaerobic respiration, in particular sulfate-reduction, predominates over electron flow in coastal marine muds. Thus, anaerobic degradation of petroleum hydrocarbons coupled to sulfate reduction was studied in fine-grained sediments collected from the continental shelf near the Mississippi River delta, in order to better predict the fate of hydrocarbons released from the DWH oil spill. Sediment-free enrichment cultures were established with either hexadecane or phenanthrene as sole carbon and energy source and sulfate as a terminal electron acceptor. All sulfate-reducing enrichment cultures amended with either hexadecane or phenanthrene were highly enriched in *Deltaproteobacteria* (>80% sequence abundance). However, further phylogenetic analysis revealed that

microbial populations were distinct according to hydrocarbon substrate, with hexadecane cultures enriched in *Desulfatibacillum alkenivorans* belonging to *Desulfobacteraceae* (up to 98 % sequence similarity), while phenanthrene cultures were enriched in *Desulfatiglans spp.* of the *Desulfarculaceae* (up to 95 % sequence similarity). Analysis of rRNA transcripts indicated that these same abundant microbial groups were also metabolically active in the respective cultures. Assuming complete oxidation to CO₂, observed stoichiometric ratios closely resembled the theoretical ratios of 12.25:1 for hexadecane and 8.25:1 for phenanthrene degradation coupled to sulfate reduction. Results provide fundamental information on anaerobic hydrocarbon degradation in marine sediments that are distant from natural hydrocarbon seeps and therefore not pre-exposed to substantial levels of hydrocarbons. Given that the ecology of anaerobic PAH degradation remains largely unknown, this dissertation research has identified key taxa that may be used as model organisms to elucidate the mechanisms of natural PAH attenuation in marine muds.

Since the biochemical pathways of anaerobic phenanthrene degradation are virtually unknown, sediment-free enrichment cultures were employed to explore the metabolic potential of phenanthrene-degrading consortia under sulfate-reducing conditions using a combination of metagenomic and metabolomic approaches. Results indicate that phenanthrene is activated by carboxylation under sulfate-reducing conditions. Genes encoding 4-hydroxybenzoate carboxylase were detected in all high quality bins and corroborated by the detection of hydroxybenzoate from metabolite analysis. Hydroxybenzoate is suggested to be a key intermediate of polycyclic aromatic hydrocarbon degradation under sulfate-reducing conditions.

In contrast to marine muds, which are largely anoxic, petroleum hydrocarbon degradation occurs under mostly aerobic conditions in permeable sands found on beaches. Although the impacts of oil contamination on marine microbial communities in beach sands are well documented, few studies have addressed the microbial community dynamics associated with macroscopic oil-sediment residues that are often trapped in coastal ecosystems. Especially in the supratidal zone of beach sand environments, low moisture content, nutrient availability, and a low surface to volume ratio of larger residues may limit bacterial hydrocarbon degradation. Thus, the microbially mediated degradation of petroleum hydrocarbons was investigated in sediment oil agglomerates (SOAs) in an experiment conducted *in situ* at Pensacola Beach. SOA degradation occurred over longer time scales in comparison to smaller, more diffuse oil particles in dry beach sands, with up to 91% of petroleum hydrocarbons degraded in three years. As has been observed for more diffuse oil contamination, taxonomic diversity of microorganisms decreased in SOAs in comparison to surrounding or pristine sands. Hydrocarbon-degrading bacteria were enriched and a succession of microbial populations was observed that paralleled the chemical evolution of petroleum hydrocarbons as determined in our companion study. Bacterial abundance was one to three orders of magnitude higher in SOAs in comparison to surrounding sands, indicating that these large oil residues are hotspots of microbial growth. Quantification of nitrogenase genes (*nifH*) in SOAs revealed a bloom in nitrogen-fixing prokaryotes or diazotrophs late in the time series. Results indicate that the high C/N ratio of SOAs causes nutrient stress and induces microbial nitrogen fixation. Thus, the coupling of nitrogen fixation to hydrocarbon degradation appears to be an important control over the fate of oil in

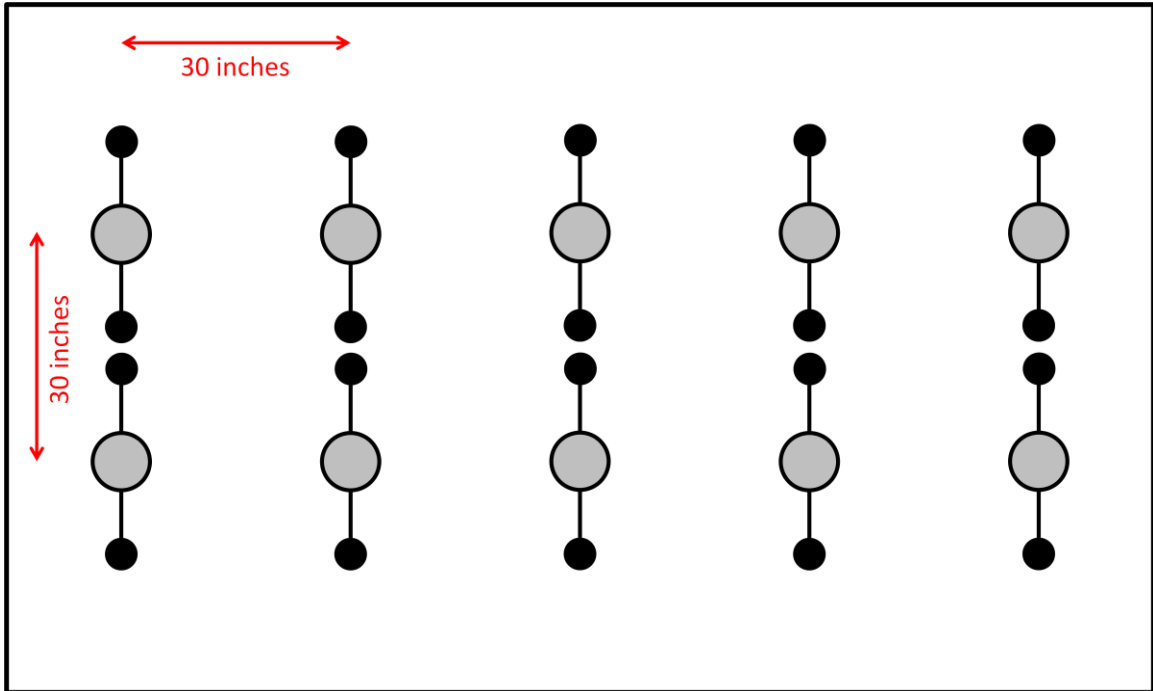
macroscopic oil aggregates. Observations are corroborated by predictions from inferred metagenomic analysis and predictions aid in linking specific taxa to the degradation of specific hydrocarbon compounds and diazotrophy.

Overall, this dissertation provides fundamental science to improve predictions of the fate of petroleum hydrocarbons that are deposited in marine sediments after an oil spill, contrasting processes and microorganisms that mediate hydrocarbon degradation in oxic vs. anoxic environments. From shallow anoxic marine sediments of the continental shelf in the northern Gulf of Mexico, sulfate-reducing bacteria that degrade the 3 ring polycyclic aromatic hydrocarbon, phenanthrene, were identified for the first time. The biochemical mechanisms of phenanthrene degradation under sulfate-reducing conditions were further elucidated in enrichment cultures using a combination of metagenomics and metabolomics. In contrasting aerobic environments from the supratidal zone of Pensacola Beach, the *in situ* degradation of petroleum hydrocarbons was quantified for macroscopic oil-sediment residues in an unprecedented time series. Large oil-sediment residues were shown to be hotspots of microbial growth and nitrogen fixation

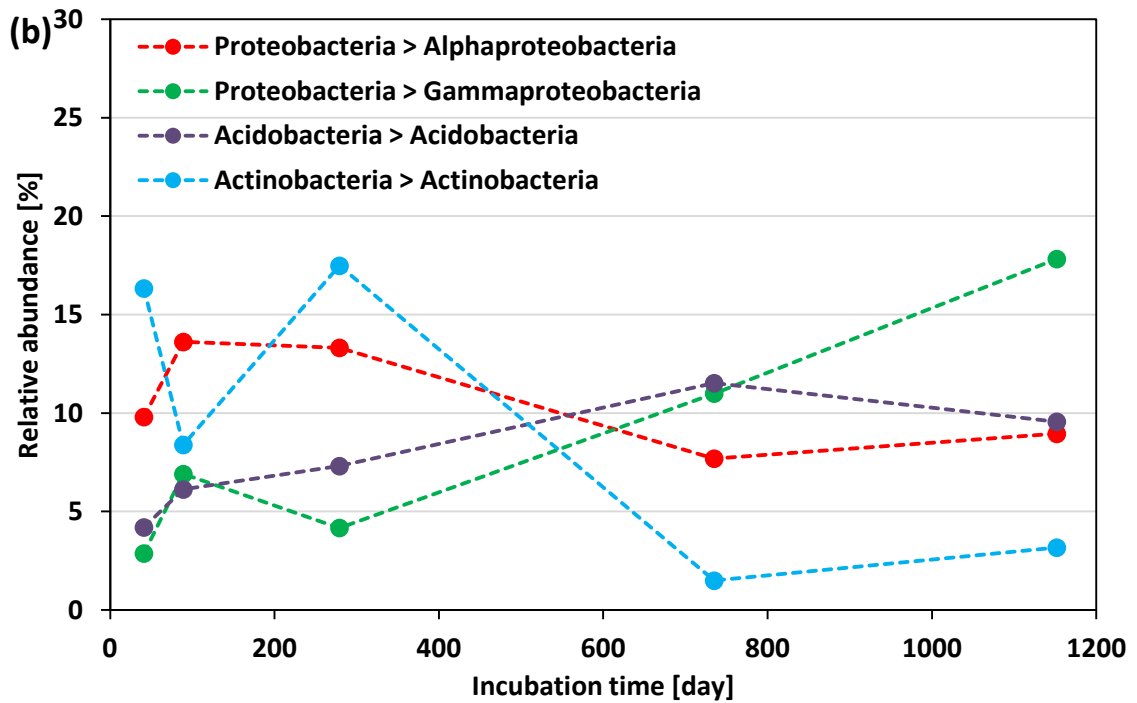
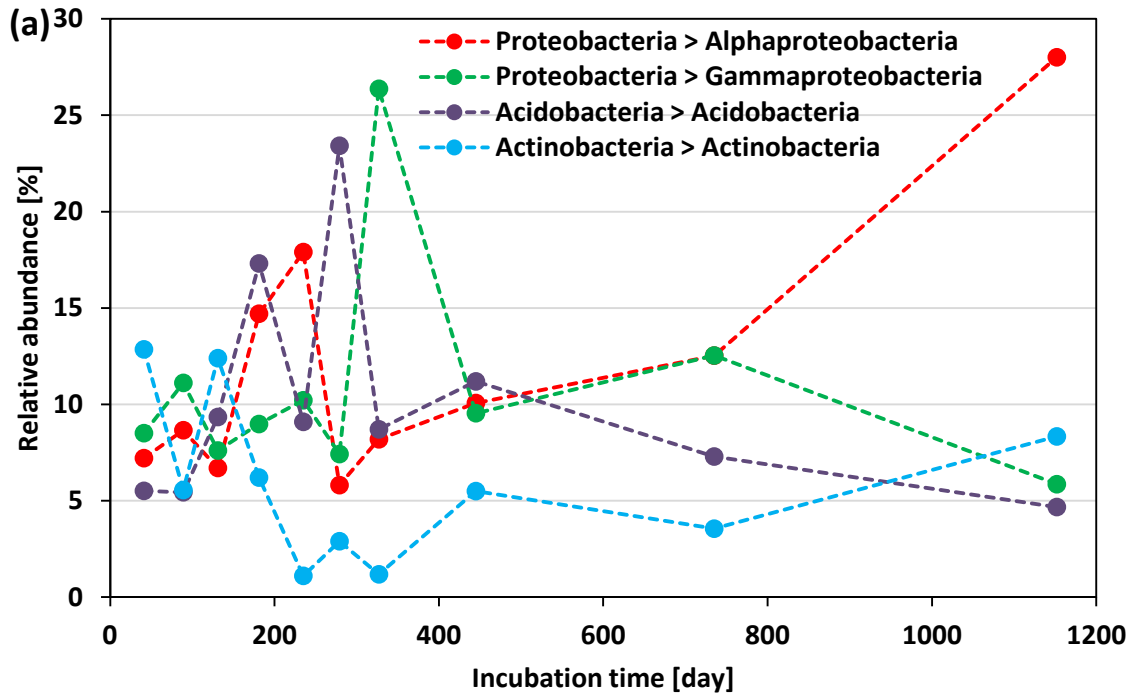
Further investigations of the biochemistry of microbial hydrocarbon degradation under anaerobic conditions are warranted. Stable isotope probing with ^{13}C -bicarbonate or ^{13}C -phenanthrene could be employed along with metabolite analysis to further elucidate the activation of phenanthrene under sulfate-reducing conditions. In addition, metatranscriptome analysis of phenanthrene-amended vs. phenanthrene carboxylic acid-amended enrichment cultures may help to elucidate the mechanism of phenanthrene activation. Isolation of pure cultures that carry out sulfate respiration with phenanthrene

as the sole carbon source would also greatly help to understand the biochemistry of PAH degradation under anaerobic conditions.

APPENDIX. SUPPLEMENTARY DATA FOR CHAPTER 4



Supplemental Figure 1 SOA *in situ* incubation in Pensacola Beach. Ten SOA-filled meshballs were attached with their chain to a PVC pipe that kept them at a defined location and defined sediment depth from 5 cm to 55 cm. Duplicate SOA-filled meshballs were attached at each sediment depth at 10 cm depth intervals. SOA arrays were buried at Pensacola Beach on October 22, 2010 in the high dry beach that could only be reached by seawater during big storm events.



Supplemental Figure 2 Relative abundance change of classes of *Alphaproteobacteria*, *Gammaproteobacteria*, *Acidobacteria*, and *Actinobacteria* from (a) SOA-surrounding sands and (b) control sands.

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