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THE ROLE OF GAMMAPROTEOBACTERIA IN AEROBIC ALKANE DEGRADATION IN OILFIELD PRODUCTION WATER FROM THE BARNETT SHALE

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THE ROLE OF GAMMAPROTEOBACTERIA IN AEROBIC ALKANE DEGRADATION IN OILFIELD PRODUCTION WATER FROM THE BARNETT SHALE

A THESIS APPROVED FOR THE
DEPARTMENT OF MICROBIOLOGY AND PLANT BIOLOGY

BY

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I would like to dedicate this work to my family. To my loving parents, Tim and Donna, who have sacrificed so much for me and continue to provide for and support my wildest aspirations. To my sister, Mackenzie, who inspires me to set an example worthy of following. And to my future husband, Clifford Dillon DeGarmo, who motivates me to work harder, dream bigger, and pursue the best version of myself.

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Abstract

Despite nearly a century's worth of research focused on investigating the activities of microorganisms in petroleum production systems, a large gap in the literature exists on the role of heterotrophic microorganisms in petroleum-impacted systems. This study aimed to review the current literature examining the major topics in petroleum microbiology while also contributing new findings towards understanding how heterotrophic bacteria, especially members of the Gammaproteobacteria, function in instigating hydrocarbon degradation and downstream biocorrosion in petroleum production water. Several isolates were obtained from the petroleum production water, examined for hydrocarbon degradation ability, and identified via 16S rRNA gene sequencing. One isolate, *Halomonas* (Gammaproteobacteria) A11A completely oxidized C_5 *n*-pentane and C_{10} *n*-decane and partially oxidized C_{16} *n*-hexadecane and toluene as estimated through oxygen respirometry. Whole genome sequencing of A11A revealed it possesses an alkane monooxygenase (*alkB*) and also contains pathways for complete oxidation of *n*-alkanes and incomplete oxidation of aromatic hydrocarbons (BTEX). Fifteen additional heterotrophic isolates were identified via 16S rRNA ribosomal gene sequencing as *Arcobacter* (7 isolates, Epsilonproteobacteria), *Thalassospira* (5 isolates, Gammaproteobacteria), *Marinobacterium* (2 isolates, Gammaproteobacteria) and *Salinicola* (1 isolate, Gammaproteobacteria). One strain each of *Arcobacter* and *Marinobacterium* were tested for growth on hydrocarbons. The *Marinobacterium* strain showed moderate growth, but the *Arcobacter* strain was unable to grow on any of the hydrocarbon substrates. However, *Arcobacter* species contribute to biocorrosion by the oxidation of sulfide. Other studies have found that *Thalassospira*

and *Salinicola* strains can degrade polyaromatic hydrocarbons. Collectively, these findings suggest that aerobic heterotrophs, such as Gammaproteobacteria, could contribute to biocorrosion by providing substrates, fully and partially degraded hydrocarbons, which foster the activities of sulfidogenic and fermentative microorganisms. Here, we propose that the underappreciated, seemingly innocuous, and often ignored general heterotrophs be considered as problematic and be monitored more closely since they can indirectly threaten the integrity of production water systems.

Preface

Decades of research have investigated the functions of microorganisms in the petroleum production process. Microorganisms possess the ability to both induce harm to the oil and gas industry and the environment and mediate petroleum pollution. Efforts to understand the mechanisms employed by these microorganisms are necessary to anticipate and prevent biocorrosion/MIC. Hydrocarbon-degrading and aerobic heterotrophic microorganisms remain largely uncharacterized regarding their role in biocorrosion. This thesis aims to shed light on the topics of aerobic hydrocarbon degradation and the microbial ecology of production water systems. This work is the product of collaboration among many academic peers and industry partners and would not have been possible without their guidance and support.

The first chapter is a review of literature focused on selected topics in petroleum microbiology. These topics include biocorrosion and microbially-influenced corrosion (MIC), petroleum biodegradation and hydrocarbon degradation, hydrocarbonoclastic microorganisms, and gene systems involved in aerobic and anaerobic hydrocarbon degradation. Reviewing these topics sets the stage for the main chapter of this thesis, chapter 2, and provides a foundation for the rationale used to support the hypotheses presented throughout this work. Without the efforts and contributions made by decades of researchers before, the findings, implications, and methods themselves would not be possible. I am grateful to contribute a small piece of work to the collection of scientific knowledge from which I have gleaned my own learning.

Chapter 2 and supporting materials in Appendix I and II have been made possible by collaborations with the University of Oklahoma Biocorrosion Center (OUBC). The industrial sponsors collaborating with the OUBC provided the samples and funding for the study. The overall goal of the study was to elucidate the causes, mechanisms, and solutions to corroded production water tanks in the Barnett Shale. Members of the OUBC investigated the differences in chemistry and microbiology among problematic and non-problematic production water tanks to expose key variances correlated with corrosion. The team aimed to answer i) if MIC was occurring, ii) if hydrocarbons were subject to biodegradation, and iii) what mechanisms could be linking the two processes.

A series of chemical and metabolic analyses, led by Drs. Duncan, Nanny, Suflita, McInerney, Lenhart, Davidova, Aktas and members of their laboratories, were made to quantify sulfate reduction rates, metabolic activities, iron reduction rates, and microbial hydrocarbon degradation. My role was to compare aerobic microbial hydrocarbon degradation rates to determine the preferred carbon substrates: *n*-alkanes, *n*-fatty acids, BTEX compounds, or oxidized BTEX compounds. The team's initial findings suggested that sulfate reduction and dissolved iron were linked to microbial sulfate reduction activity and targeted metabolomics found evidence for aerobic hydrocarbon degradation (Dr. Deniz Aktas and Brian Harriman). Molecular analysis revealed that a significant portion of the microbial community in the problematic tanks consisted of Deltaproteobacteria, Clostridia, and Gammaproteobacteria (Dr. Kathleen Duncan and Dr. Sylvie Le Borgne). From here, I continued to investigate the role of Gammaproteobacteria and other heterotrophs in the production water, as described in

Appendix II; we hypothesized that these aerobic heterotrophs were initiating the degradation of condensate hydrocarbons that ultimately feed the sulfate-reducing and fermentative bacteria.

The remainder of my study consisted of enriching and isolating aerobic microorganisms from the production water. I isolated several heterotrophic microorganisms from the production water, including *Arcobacter* and *Marinobacterium* strains (discussed in Appendix 2) from an enrichment initiated by Dr. Sylvie le Borgne. Through amendment with *n*-alkanes, I also obtained several *Halomonas* (Gammaproteobacteria) isolates that are capable of hydrocarbon degradation; I hypothesized that Gammaproteobacteria in the production water degrade *n*-alkanes using monooxygenase enzymes such as *alkB* or cytochrome p450. Investigating this hypothesis involved genome sequencing of isolate A11A and oxygen respirometry experiments to obtain hydrocarbon oxidation rates. Annotation and analysis of the isolate genome was made possible by the MGMIC pipeline designed by Dr. Boris Wawrik, and Vince Sandifer assisted me in the bioinformatics analysis. Oxygen respirometry methods were mastered with guidance from Brian Harriman. Throughout the study, I was responsible for cultivation, DNA extraction, sequencing preparation and sequence analysis, oxidation rates calculations, and analysis. We found evidence that Gammaproteobacteria, such as the *Halomonas* A11A isolate, use *alkB* monooxygenase enzymes to degrade a wide range of *n*-alkanes and toluene hydrocarbons; thus, reinforcing the hypothesis that aerobic heterotrophs degrade the condensate hydrocarbons in the production water.

Together, the findings of this work suggests that microbially-driven aerobic hydrocarbon degradation occurring in production water tanks contributes to downstream biocorrosion and MIC by providing nutrients for known biocorrosive sulfate-reducers and fermentative species. I anticipate publishing Chapter 2 upon completion of parallel degradation studies to quantify substrate loss initiated by *Halomonas* A11A.

Chapter 1:

A review of selected topics in petroleum microbiology: biocorrosion, aerobic and anaerobic hydrocarbon degradation genetic systems, and aerobic hydrocarbonoclastic microorganisms

Abstract:

Hydrocarbons are ubiquitous compounds released by both natural seeps and anthropogenic processes. Accidental release of petroleum hydrocarbons is a problem that concerns the petroleum industry, human health, and the environment. Biogenic sulfide production from sulfate-reducing microorganisms and direct iron oxidation by lithotrophic microorganisms are some of the many mechanisms that contribute to biocorrosion of production machinery; corrosion events often lead to accidental release of hydrocarbons and toxic gases. Conversely, microorganisms also play a major role in reducing pollution by degrading petroleum hydrocarbons and other pollutants through natural biodegradation processes. Microorganisms possess the power to both induce and mitigate harm to the environment and consequently, the oil and gas industry. Recognizing the natural abilities employed by microorganisms will allow researchers and industry leaders to better protect human health and the environment while optimizing the recovery of fossil fuels by preventing product losses through accidental releases and subsequent environmental contamination.

In contrast to the vast amount of literature investigating the impact and activities of sulfate-reducing organisms and biocorrosion, the role of hydrocarbonoclastic organisms in partially aerobic production water systems where biocorrosion occurs is largely unknown. Rather than focus on the direct players and

pathways involved in biocorrosion of carbon steel in the oil and gas industry, the aim of this thesis is to examine the role of aerobic hydrocarbonoclastic Gammaproteobacteria and how they can initiate a cascade of hydrocarbon degradation and therefore influence downstream biocorrosion.

1.0 Microbially influenced corrosion (MIC)

Corrosion leads to the accidental release of hydrocarbons and hazardous compounds, damage to machinery and manufacturing materials, and contamination of environments. Corrosion events impact every facet of the oil and gas industry including engineering, economics, safety, environmental science, and human health (Beech et al., 2004; Duncan et al., 2009; NACE Standard TM0212, 2010; Vigneron et al., 2016). The origins of these corrosion problems are both biotic and abiotic. Abiotic events include chemical oxidation of metals when metal, water, and oxygen intermingle; metals become oxidized through the loss of electrons from the metal surfaces and weakening the pipelines and storage tanks (Hamilton et al., 2003; Suflita et al., 2008). Biotic corrosion also functions as an electrochemical process but implicates microbial physiology. The two most widely accepted mechanisms of MIC are chemical (CMIC) and electrical (EMIC). CMIC is described as the reduction of iron to produce iron sulfide instigated by sulfate-reducing microorganisms (Dihn et al., 2004; Venzlaff et al., 2013). EMIC occurs through direct iron oxidation; lithotrophic microorganisms withdraw electrons directly from iron in metal surfaces (Enning et al., 2012; 2014). While MIC is not directly linked to a single microbial species,

sulfidogenic, iron-oxidizing, and fermentative microorganisms are characteristic of petroleum systems (Dihn et al., 2004; Suflita et al., 2008; Vigneron et al., 2016).

1.1 Sulfidogenic bacteria

Microbially influenced corrosion (MIC) begins with formation of biofilms on metal surfaces (Beech et al., 1999; 2004; Vigneron et al., 2016). The rate of corrosion depends greatly on the metabolites produced by the microbial community, availability of electron acceptors and donors, and abiotic factors such as temperature, pH, and salinity. Biofilms on carbon steel often consists of sulfate-reducing bacteria (SRB), sulfide-oxidizing bacteria (SOB), methanogens, fermentative bacteria, and iron-oxidzing/reducing bacteria (Videla, 2002; Baker et al., 2003; Beech et al., 2003; 2004; Kjellerup et al., 2003; Zhang et al., 2003; Suflita et al., 2008). Sulfate-reducing bacteria are consistently associated with biocorrosion of carbon steel in the petroleum industry due to their production of corrosive sulfuric acid and sour hydrogen sulfide gases from sulfate and thiosulfate; whereas some SRBs are able to use steel directly as an electron donor without needing a hydrogen intermediate (Venlaff et al., 2013; Enning et al., 2014). The anaerobic conditions maintained in oil production equipment are ideal for SRBs; the abundance of carbon substrates (hydrocarbons, organosulfur compounds, organic acids, etc.), steel infrastructure of the equipment itself, and produced and injection waters provide SRBs with the necessary electron donors and acceptors to induce MIC. While SRBs are obligate anaerobes, they can be active in oxygen-rich environments when protected by a biofilm (Beech et al, 2002). Generally, SRBs rely on other microorganisms to degrade complex substrates and use the byproducts as substrates (Muyzer and Stams, 2008). Some SRBs can utilize

hydrocarbons as a carbon source, but most cannot; the majority of SRBs oxidize a range of organic acids and alcohols, such as lactate, acetate and fatty acids, completely to carbon dioxide or in the absence of sulfate may grow fermentatively (Hansen, 1993; Rueter et al., 1994; Daumas et al., 1988; Liu et al., 1997; Dihn et al., 2004; Parshina et al., 2005; Aüllo et al., 2013; Vigernon et al., 2016). Oil reservoirs and production waters play host to a wide range of substrates suitable for SRBs. As primary production slows secondary recovery methods are implemented to increase production; water flooding with seawater introduces high concentrations of sulfate to the reservoir and provides SRBs with their preferred electron acceptor and may catalyze sulfate-reduction.

The majority of sulfate-reducing bacteria are characterized as Deltaproteobacteria. Some of the most common Deltaproteobacteria that contribute to sulfate reduction or are associated with reservoirs include members of the *Desulfovibrionaceae* family and are characterized as mesophilic. Specifically, *Desulfovibrio* species, all of which can utilize lactate, and *Desulfomicrobium* species, are known to grow autotrophically and are thermophilic, thus are often associated with high-temperature petroleum reservoirs (Rozanova et al., 1988; Magot et al., 1992). Others, characterized as part of the *Desulfobacteriaceae* family include *Desulfacinum*, which are thermophiles and can use a wide range of substrates oxidizing them fully to CO_2, and *Desulfobacter* species, which have a more limited range of substrate preferences (Rees et al., 1995; Lien & Beeder, 1997). In contrast, *Desulfobacterium* species have been shown to oxidize benzoic acid, *p*-cresol and *m*-cresol, and toluene, compounds commonly found in association with production fluids (Galushko &

Rozanova, 1991; Muller et al., 1999, 2001; Harms et al., 1999). A different group of SRBs characterized to be endospore forming and Gram-positive include *Desulfotomaculum* species; many are described as thermophilic or mesophilic with some halophilic and capable of lactate and ethanol oxidation (Nazina & Rozanova, 1978; Tardy-Jacquenod et al., 1998). A thermophilic SRB, *Thermodesulfobacterium*, isolated from Yellow Stone National Park was the first thermophilic organism isolated from produced water from an oil well (Rozanova & Khudyakova, 1974). Further, archaeal sulfate reducers have been isolated and characterized; *Archaeoglobus fulgidus* was isolated from a hydrothermal system and characterized as a hyperthermophile capable of hydrocarbon degradation (Stetter et al., 1987). The diverse physiological preferences of sulfate-reducing microorganisms allow them to inhabit a variety of niches; their optimal growth conditions are supported by natural reservoir and production water conditions allowing them to play a major role in the cycling of carbon and sulfur in the subsurface.

1.2 Other contributors to biocorrosion

Depending on the availability of sulfate in production systems sulfate reducers may compete or grow syntrophically with methanogens. In sulfate-limited environments, sulfate reducers may be forced to compete with methanogens for acetate and hydrogen substrates (Stams et al., 2003). Hydrogen-utilizing SRBs rapidly out-compete the methanogens and acetogens due to higher affinity of hydrogen for the sulfate reducers (Stams et al., 2003). Conversely, SRBs may grow syntrophically with homoacetogens when they require acetate as a substrate; again the SRBs will out-compete acetoclastic methanogens due to higher affinity of and lower threshold for

acetate (Schonheit et al., 1982; Brysch et al., 1987; Stephanie et al., 1994; Weijma et al., 2002). Syntrophic relationships between microbial communities also contribute to biocorrosion, hydrocarbon degradation, and methane production. Methane oxidation coupled to sulfate reduction is one example of syntrophic archaeal communities working to reverse methanogenesis while SRBs oxidize some of the intermediates produced (Hoehler et al., 1994; Boetius et al., 2000; Orphan et al., 2001; Nauhaus et al., 2002; Vigneron et al., 2016).

Sulfate as a terminal electron acceptor is chemically unfavorable; the $E°'$ of sulfate to sulfite redox couple is too low at -516 mV for reduction of ferredoxin at -398 mV or NADH at -314 mV (Muyzer and Stams, 2008). This unfavorable reaction requires the formation of adenosine-phosphosulphate (APS) and pyrophosphate via ATP sulphurylase. Ferredoxin or NADH now can reduce APS since the $E°'$ of APS to sulfite plus AMP redox couple is -60 mV. Ultimately, activation of sulfate expends 2 ATP molecules, eventually producing sulfide. Upon availability of a more thermodynamically and redox favorable terminal electron acceptor, many sulfate reducers can shift their ability to use different electron acceptors (Muyzer and Stams, 2008). Thus, high numbers of SRBs in an environment do not indicate sulfate reduction as the dominant terminal electron accepting process. Thiosulfate, sulfite, sulfur, nitrate, and nitrite can serve as electron acceptors for some SRBs (Keith and Herbert, 1983; Dalsgaard and Bak, 1994; Moura et al., 1997; Lopez-Cortes et al., 2006).

Oilfield machinery and production equipment are designed to operate in a primarily anaerobic environment to prevent the direct oxidation of steel. Anoxic

environments foster the growth and activity of SRBs, as discussed above. If the steel structure of pipelines and production tanks are exposed to oxygen, hydroxide ions are formed (Mand et al., 2017). This shift in oxygen availability also grants access to a more thermodynamically favorable terminal electron accepting process, oxidation; the presence of oxygen ultimately dictates the dominant microbial physiology active in the production machinery and waters. Aerobic and facultative anaerobes hydrocarbon-degrading microorganisms compose a significant portion of the microbial community in production systems (Stevenson et al., 2011). Many aerobic or facultatively anaerobic hydrocarbon-degraders are halotolerant or halophilic, ideal for salty production waters (Gauthier et al., 1992; Alva et al., 2003; Ananina et al., 2007; de Lourdes Moreno et al., 2011; Lu et al., 2015). Members of the Alpha- Epsilon- and Gammaproteobacteria classes demonstrate hydrocarbon degradation in seawater and production water systems (Prabargaran et al., 2007; Luo et al., 2009). Aerobes, such as *Marinobacter* sp., *Roseobacter* sp., *Arcobacter* sp., *Pseudomonas* sp., among many others, have been identified in association with oily seawater, suggesting their involvement in the aerobic degradation of hydrocarbons or cycling of electron acceptors like sulfate (Prabargaran et al., 2007; Stevenson et al., 2011). Together, aerobic hydrocarbon degraders may affect MIC by supplying partially oxidized hydrocarbons and byproducts (lactate, acetate, fatty acids, etc.) for fermentative and sulfidogenic microorganisms to use as carbon sources.

2.0 Biocorrosion mitigation strategies

The impact of biocorrosion is obvious when considering the effect on production machinery and potential economic risks. Methods to mitigate the effects of corrosion are implemented at many points in the production process. Specifically, methods may include microbial monitoring, biocide treatments, and coatings on metal surfaces. Microbial monitoring predicts when and where corrosion or souring may occur. Strategies often include microbial counts through ATP measurements, staining procedures, and the use of microscopes for direct counting (Dexter et al., 1991; Kjellerup et al., 2003; Ollivier et al., 2005). Unfortunately, these methods can be biased and give inaccurate estimates on active microbial numbers (Ollivier et al., 2005). Another treatment strategy is the use of biocides; production machinery is routinely flushed with biocides to kill microorganisms (Xue et al., 2015). Biocide treatments have differing effects due to the broad specificity of the chemicals and varying operating conditions of the production machinery. Injection method and frequency of treatment can be adjusted to the type of organism that is being targeted. For example, pulsing biocides at long intervals and low concentrations have been noted to lead to increased resistance of biocides compared to short intervals of high concentrations of biocides (Xue et al., 2015). Unfortunately, biocide treatments are often expensive and can contribute to additional corrosion problems. Moreover, microorganisms often develop resistance to the biocides or are unaffected by them due to the protective properties provided by the formation of a biofilm. Further, coating metals with corrosion-resistant materials has been used to mitigate corrosion effects. This method is expensive but works well as long as the coating is applied evenly and

does not leave areas unprotected. Epoxy-based and coal tar coatings have been the most successful at protecting against microbial corrosion, while coatings made from PVC often perform poorly (Videla, 1996).

3.0 Hydrocarbon degradation & petroleum biodegradation

3.1 Overview of petroleum biodegradation

Biodegradation of petroleum products is a concern for the oil and gas industry. In the event of an oil spill in terrestrial or marine systems, petroleum products are introduced to environments they would not otherwise be present. The ultimate fate of these products is of great concern to human and animal health and environmental safety. While some petroleum constituents are nontoxic and volatilize with air, others may be recalcitrant or solubilize in water and serve as dangers to human and environmental health (Keith et al., 1979; Alfreider et al., 2007). Petroleum fluids consist of a number of constituents that may be subject to microbial attack and serve as growth substrates. The majority of petroleum fluids consist of alkanes and paraffins, cycloalkanes and naphthenes, and aromatic hydrocarbons (Boylan et al., 1971; Morlett-Chávez et al., 2010; Doherty et al., 2016). The biodegradation of petroleum products is influenced by several factors, both biotic and abiotic. Abiotic factors include the temperature of the reservoir, salinity and oil residence, while the biotic factors are due to microbial metabolism (Ollivier et al., 2005). Whether biotic or abiotic, biodegradation of petroleum products alters the physical composition of the oil. In general, oils that have been subject to biodegradation are less desirable due to increased viscosity, higher levels of sulfur, acid, and resins, and they are more difficult

to refine (Ollivier et al., 2005). Monitoring the quality and level of biodegradation of petroleum products is necessary as a measure of quality control for the oil and gas industry. Different classes of hydrocarbons have different rates and susceptibilities to biodegradation (Davidova et al., 2005). The shorter-chain aliphatic hydrocarbons (C_5-C_{15}), *n*-alkanes, are the most readily biodegraded; higher molecular weight alkanes have decreasing solubility which can affect bioavailability to the cell. Aromatic hydrocarbons, and branched-chain and cyclic alkanes are more recalcitrant to biodegradation and depend greatly on position and number of methyl groups and alkyl substitution (Masterson et al., 2001; George et al., 2002). Polycyclic aromatic hydrocarbons (PAHs) biodegraded based on the number of rings; PAHs may consist of two or three rings, such as naphthalene and anthracene respectively, or be constructed of as many as 222 carbon atoms (Feng et al., 2009). Biodegradation of these hydrocarbons by heterotrophic organisms yield organic acids and alcohols that are used by other organisms, including sulfate-reducing bacteria, and may contribute to downstream reservoir souring or biocorrosion.

Quantifying the level of biodegradation in petroleum samples allows diagnosis of what types of organisms are responsible for the degradation and potential downstream effects. Specifically, the detection of signature intermediates and metabolites of biodegradation can be indicative of degradation occurring under aerobic versus anaerobic mechanisms. Many methods exist to allow examination of petroleum biodegradation, including isotopic fractionation, metabolomic analysis and identification, and genomic analysis to identify key genes involved in different degradation pathways (Fiehn et al., 2001; Bombach et al., 2010). Contamination of

environments by crude petroleum constituents is a problem that plagues all aspects of the petroleum industry.

Biodegradation of these compounds plays a major role in the quality of hydrocarbons and in efforts to clean up contaminated environments. One of the most successful methods employed to clean up polluted environments is through bioremediation; this strategy harnesses the metabolic activities of the endogenous microbial community to optimize the degradation of contaminants (Shim et al., 2010; Deng et al., 2017).

3.2 Aliphatic hydrocarbon degradation genes and pathways

Alkanes compose a major fraction of crude oil but are also produced from other natural sources such as plants, animals, and other bacteria and microorganisms (Ladygina et al., 2006). Some eukaryotic organisms may use the hydrophobic properties of alkanes and other hydrocarbons to guard against desiccation or predators, while prokaryotic organisms and some fungi use hydrocarbons as a food source (van Beilen et al., 2003; Wentzel et al., 2007). Long chains of saturated carbon atoms contain high amounts of energy, making them ideal substrates for organisms possessing the appropriate metabolic machinery to access it. Access to this energy requires organisms to possess mechanisms capable of activating and selectively oxidizing substrates without denaturing or compromising or its own enzymes to reactive oxygen (van Beilen et al., 2005).

Alkanes are most completely and rapidly degraded in the presence of oxygen. Aerobic degradation of alkanes begins via oxidation of a terminal methyl group yielding a primary alcohol. Further oxidation of the alcohol produces an aldehyde,

which is further oxidized to a fatty acid. Fatty acids are oxidized by a wide variety of microorganisms; they are directly transformed to acetyl-CoA through β-oxidation. (Ashraf et al., 1994; Rehm and Reiff, 1981; van Hamme et al., 2003; Watkinson and Morgan, 1990; Wentzel et al., 2007). Metabolic machinery used in the hydroxylation of alkanes differs depending on alkane chain length. Short C_2-C_4, gaseous alkanes are targeted by methane monooxygenases (Ashraf et al.,1994; van Beilen and Funhoff, 2007). Specifically, evidence in *Pseudomonas butanovora* shows sequential oxidation of terminal groups on alkanes via butane monooxygenase, consisting of a non-heme iron monooxygenase (Arp et al., 1999; Sluis et al., 2002). Conversely, some species oxidize alkanes at sub-terminal positions, such as *Gordonia sp.*, which utilizes propane via a propane monooxygenase but cannot oxidize other gaseous alkanes (Kotani et al., 2003). Mid-length alkanes, C_5-C_{10}, are subject to attack commonly by soluble cytochrome p450 or integral membrane non-heme iron proteins, such as AlkB (Rojo et al., 2009; Groves et al., 2011). Longer chain length alkanes seem to be hydroxylated via a different group of enzyme pathways. Some studies have characterized AlmA as a long-chain alkane monooxygenase found in *Acinetobacter*, and has led to the identification of *almA* in *Alcanivorax*, *Marinobacter*, and *Parvibaculum* (Throne-Holst et al., 2007; Wentzel et al., 2007). Similarly, LadA, described as a thermophilic soluble long-chain alkane monooxygenase from *Geobacillus* has been described (Feng et al., 2007). These systems are currently understood to be a group of alkane hydroxylases, such as flavin-binding or -dependent monooxygenases, which are different from those used for shorter and mid-length alkanes.

3.2.1 Aerobic alkane oxidation systems

One of the most well characterized systems for alkane oxidation is the *alkB* family of alkane hydroxylases. The *alk* system was first described in *Pseudomonas putida* GPo1 for its ability to oxidize C_5-C_{12} *n*-alkanes to alkanols, aldehydes, acids and ultimately β-oxidation pathways (Baptist et al. 1963; van Beilen et al., 1994; Throne-Holst et al., 2007). The system consists of three major components. First is the alkane hydroxylase, *alkB*, described as an integral membrane non-heme diiron monooxygenase that functions to hydroxylate alkanes at a terminal methyl group (Kok et al., 1989; van Beilen et al., 1992). The diiron cluster permits activation of an alkane via a substrate radical intermediate (Austin et al., 2000; Bertrand et al., 2005; Shanklin et al., 1997). The second component is the rubedoxin, *alkG*, which functions to bind iron and consists of two domains, AlkG1 and AlkG2 (Kok et al., 1989). The third component is *alkT* and is a rubedoxin reductase (Smits et al., 2002). Today, more than 60 *alkB* homologs have been characterized in both Gram-positive and Gram-negative organisms (Marin et al., 2001, 2003; Smits et al.,1999, 2002, 2003; van Beilen et al., 2002b, 2004; Rojo et al., 2009; Wang et al., 2010; Pérez-de-Mora et al., 2011; Nei et al., 2014).

Cytochrome p450 hydroxylases are ubiquitous in all forms of life, functioning as protein catalysts oxygenating a wide array of compounds. Their ubiquitous nature has lead to a wide range of substrates they are able to catalyze, including: fatty acids and steroids, as well as some materials of anthropogenic origin, including, anesthetics, solvents, pesticides, and drugs (Bernhardt et al., 2006). Specifically, bacteria capable of C_5-C_{10} alkane degradation often employ soluble cytochrome p450 monooxygenases.

The CYP153 class of cytochrome p450s is responsible for alkane degradation in microorganisms (Scheps et al., 2011). This system requires ferredoxin and ferredoxin reductase proteins as an electron-delivery system and is the first to exhibit specific hydroxylation of alkanes at the terminal position. Studies suggest the CYP153 system favors linear alkanes while omitting others due to the shape of a substrate-binding pocket and steric hindrance properties (Funhoff et al. 2006).

3.2.2 Anaerobic alkane degradation

While aerobic degradation of hydrocarbons is observed and characterized extensively, anaerobic degradation of hydrocarbons has taken longer to understand and characterize. Many environments are oxygen limited, preventing the biodegradation of hydrocarbons and other pollutants from degrading as quickly or at all. For many years the degradation of hydrocarbons under anoxic conditions was believed to be impossible or occur at such slow rates the degradation was negligible. Further, if oxygen-dependent remediation methods have been used, the rapid loss of oxygen may leave sites anoxic, overwhelmed with biomass, or with recalcitrant compounds that remain a threat to health and the environment. For these reasons, the anaerobic degradation of hydrocarbons has been more fully examined in recent years (Heider et al., 1998; Aitken et al., 2004; Chakraborty et al., 2004; Haritash et al., 2009; Rabus et al., 2016).

Electron acceptors such as sulfate, nitrate, iron, manganese, and carbon dioxide can be coupled to the oxidation of hydrocarbons. The main pathway of anaerobic alkane oxidation is the fumarate addition pathway; some have considered the carboxylation pathway but it is not verified. Instead, the new hypothesis is that

anaerobic hydroxylation is followed by the formation of a ketone, which is then carboxylated and oxidized (Aitken et al., 2013; Callaghan et al., 2013). Fumarate addition involves the addition of the alkane at the C_2 position to the double bond of fumarate, yielding an alkylsuccinate (Beller et al., 1992; Evans et al., 1992). At this point, further degradation occurs via carbon rearrangement of the alkylsuccinate, decarboxylation, and β-oxidation (Wilkes et al., 2002). Evidence of this pathway has been documented and characterized in sulfate-reducing conditions (Kropp et al., 2000; Cravo-Laureau et al., 2005; Davidova et al., 2005; Callaghan et al., 2006; Kniemeyer et al., 2007). Nitrate-reducing conditions and denitrifying bacteria have also given evidence of fumarate addition coupled to alkane oxidation (Rabus et al., 2001).

3.2.3 Anaerobic alkane degradation gene systems

Activation of alkanes can be catalyzed through glycyl radical enzymes. One of which is the alkylsuccinate synthase (ASS) enzyme, demonstrated in *Desulfatibacillum alkenivorans* AK-01; (Callaghan et al., 2008; Herath et al., 2016) the other is the methylalkylsuccinate synthase (MAS) enzyme, characterized in the model strain '*Aromatoleum'* HxN1 (Grundmann et al., 2008). These mechanisms are also exhibited in the activation of aromatic hydrocarbons where benzylsuccinate synthase (BSS) acts to catalyze the oxidation of the aromatic rings; BSS was initially recognized to oxidize toluene in *Thaura aromatica*, a denitrifying bacterium (Altenschmidt et al., 1991; Leuthner et al., 1998; Heider et al., 2016). The initial characterization of toluene activation via BSS is initiated through removal of hydrogen from the methyl group of toluene. This step yields a benzyl radical intermediate which adds across the double bond of fumarate forming benzylsuccinate. Several similarities between the *bss*, *ass*,

and *mas* genes have been found; *D. alkenivorans* AK-01 and '*Aromatoleum'* HxN1 harbor genes for putative glycyl radical activating enzyme identified in the *S*-adenosylmethionine (SAM) superfamily which is similar to the BSS activase (Leuthner et al., 1998; Krieger et al., 2001; Verfurth et al., 2004). Additional studies have shown the *ass* and *mas* gene systems play a role in the degradation of short chain alkanes and even solid paraffins under methanogenic conditions (Kniemeyer et al., 2007; Callaghan et al., 2010; Savage et al., 2010; Davidova et al., 2011; Wawrik et al., 2016).

With some exceptions, anaerobic hydrocarbon degradation occurs much more slowly than aerobic degradation (Gieg et al., 2009). Anaerobic degradation will exhaust the available electron acceptors one at a time according to availability of the most thermodynamically favorable electron acceptor and redox potential of the environment unless kinetics override. Identifying the dominant terminal electron acceptor in an environmental can be identified by monitoring hydrogen levels and can estimate the rate of *in-situ* degradation (Lovley et al., 1988; Harris et al., 2007).

3.3 Aerobic and anaerobic aromatic hydrocarbon degradation and gene systems

A significant portion of petroleum products consist of highly soluble BTEX (*n*-benzene, *n*-toluene, *n*-ethylene, *n*-xylene) and other aromatic hydrocarbons that are volatile aromatic compounds containing a benzene ring (Deng et al., 2017). BTEX compounds can account for up to 59% (w/w) of gasoline products, 80% of total VOC in refineries, and are present in a wide variety of environments due to anthropogenic and natural releases (Adams et al., 2001; Barona et al., 2007; Gadd et al., 2009; Doherty et al., 2016). High solubility values for BTEX compounds (benzene, 1791 mg/L, toluene, 535 mg/L, ethylbenzene, 161 mg/L, xylenes, 175-146 mg/L, at 25° C)

make them useful as solvents for chemical companies but dangerous for human exposure (Dean et al., 1990; Howard et al., 1991). The U.S. Environmental Protection Agency classifies these compounds as priority pollutants due to their high solubility and risks to human health such as their toxic, mutagenic, and carcinogenic properties (Keith et al., 1979; Alfreider et al., 2007). Considering these risks, extensive research has been focused on how to safely degrade these compounds to less toxic or non-toxic forms.

A wide variety of microorganisms are characterized to degrade BTEX compounds under aerobic conditions; including: *Pseudomonas*, *Acinetobacter*, *Bacillus*, *Serratia*, *Sphingomonas* and others (Huang et al., 2014; Shadi et al., 2015; Avanzi et al., 2015; Zhou et al., 2016). To date, the most successful studies for BTEX degradation have been performed with enriched microbial consortia obtained from oil-impacted environments rather than single isolates or exogenous degraders (Prenafeta-Boldu et al., 2004; Mukherjee et al., 2012; Jin et al., 2013). Several factors can impact microbial degradation of BTEX compounds, such as the concentration and chemical interaction of the pollutants, temperature, pH, inorganic nutrient availability, how adapted the endogenous microbial community is to the compounds, and other kinetic restraints (Singh et al., 2010l El-Naas et al., 2014).

Aerobic oxidation of these aromatic compounds is initiated by activating the aromatic ring, which is catalyzed by dioxygenase and monooxygenase enzymes to produce catechol intermediates (El-Naas et al., 2014; Karigar et al., 2011; Li et al., 2014). Ring substituents targeted by monooxygenases (TOL pathway) are transformed to pyrocatechols or phenyl glyoxals while dioxygenases (TOD pathway) oxidize rings

to produce 2-hydroxy-substituted compounds (Tsao et al., 1998; Jindrova et al., 2002; Zhang et al., 2013). Further cleavage produces catechol compounds via intra- (catechol 1,2-dioxygenase) or extradiol (catechol2,3-dioxygenase) oxygenases cleave between (ortho) or proximal (meta) to the hydroxyl groups (Smith et al., 1991; Jindrova et al., 2002; Andreoni et al., 2007). The intermediates are all further degraded to form pyruvate and further metabolized through Krebs cycle (Cozzarelli et al., 2003). These degradation pathways are only possible in oxygen-rich environments and are often stunted when oxygen becomes depleted.

The most widely accepted and characterized pathway for anaerobic BTEX and aromatic hydrocarbon degradation is the addition to fumarate (Boll et al., 2010; Meckenstock et al., 2011; Callaghan et al., 2013). This pathway is catalyzed by benzylsuccinate synthase (BSS), a glycyl radical enzyme, and its homologs (Beller et al., 2002; Boll et al., Meckenstock et al., 2011). Metabolite analyses in a few studies have suggested alternative mechanisms for benzene degradation, including: methylation, hydroxylation, or carboxylation, but they are not widely accepted mechanisms and remain controversial (Meckenstock et al., 2011).

4.0 Aerobic hydrocarbonoclastic microorganisms

A number of aerobic bacteria have been characterized as hydrocarbonoclastic due to their ability to degrade hydrocarbons (Yakimov et al., 2007; Rojo et al., 2009). Some of the most common aerobic hydrocarbon-degrading microorganisms detected in oil production facilities include: *Marinobacterium, Pseudomonas*, *Marinobacter*, *Alcanivorax*, *Acinetobacter*, *Rhodococcus*, and others (Yakimov et al., 2007; Vila et

al., 2010; Stevenson et al., 2011; Long et al., 2017). *Alcanivorax borkumensis* is a marine bacterium that can degrade and assimilate linear and branched alkanes but is unable to use aromatic hydrocarbons, sugars, amino acids, fatty acids and other simple carbon sources (Schneiker et al., 2006; Yakimov et al., 1998). Other *Alcanivorax* sp. are able to degrade pristine and phystane, allowing them to survive and proliferate in heavily oil-contaminated environments and even be used as bioremediation agents in contaminated soils (Hara et al., 2003; Harayama et al., 2004; Kasai et al., 2002; McKew et al., 2007a, b; Yakimov et al., 2007). The genes responsible for alkane degradation in *A. borkumensis* strain SK2 are described as part of the *alkB* family of alkane hydroxylases and bear codon similarity to those found in *Pseudomonas putida;* to date, studies have quantified *Alcanivorax spp.* to contain three p450 systems and two *alkB* systems (Kok et al., 1989; Golyshin et al., 2003; Hara et al., 2004; van Beilen et al., 2001; 2003; 2004).

As described herein, *Pseudomonas putida* GP01 was the first organism characterized to contain the three-component *alkB* alkane hydroxylase system mapped on the OCT plasmid (Peterson et al., 1966; van Beilen et al, 2001). This gene system and those similar have since been detected in isolates from many oil-contaminated environments; some claim it may represent the genetic foundation found in similar species capable of hydrocarbon degradation (Sotsky et al., 1994; Smits et al., 1999). Further, some strains of the opportunistic pathogen *Pseudomonas aeruginosa* are able to grow on crude oil as their sole carbon source (Belhaj et al., 2002). Belhaj and others described a set of *P. aeruginosa* strains isolated from polluted effluents from an oil refinery in Morocco to degrade hexane, heptane, octane, and decane using *alkB* and

alkB-related genes, further suggesting that the alkane-degrading genes are on a mobile OCT plasmid and may be shared via horizontal gene transfer (van Beilen et al., 2001).

Another well-characterized bacterium known for degrading hydrocarbons is the Gammaproteobacterium *Marinobacter hydrocarbonoclasticus* (Gauthier et al., 1992). *M. hydrocarbonoclasticus* is a halotolerant, Gram-negative denitrifying marine bacterium isolated from hydrocarbon-contaminated sediments. Growth on alkanes C_{16}-C_{20} revealed no evidence of cytochrome p450 activity for *M. hydrocarbonoclasticus*; however, *M. aquaeolei* VT8 contains homologs of both CYP153A and two copies of *alkB* (Wang et al., 2010). Many microorganisms are able to oxidize multiple types of hydrocarbons and alkanes and therefore contain multiple copies and gene pathways for aerobic hydrocarbon degradation; *Alcanivorax spp.* and *Bacillus spp.* were noted to contain both p450 and *alkB* systems, suggesting these organisms can use a wider range of substrates and ultimately confer higher adaptability (Smits et al., 2003; van Beilen et al., 2005; van Beilen and Funhoff, 2007; Rojo et al., 2009; Wang et al., 2010; Groves et al., 2011). Regulation of these pathways is tightly regulated and activated only in the presence of the appropriate alkane or hydrocarbon.

Another Gammaproteobacterium, related to *Marinobacter*, *Halomonas* sp. exhibits some hydrocarbon degradation potential but is not widely noted as a hydrocarbonoclastic microorganism (Pepi et al., 2005; Mnif et al., 2009; Wang et al., 2010). Few studies have characterized the alkane degradation pathway that is used and have relied on GC-MS analysis to measure and identify metabolites. Specifically, a *Halomonas* strain C2SS100 produced hexadecanol, hexadecanaldehyde and hexadecanoic acid as intermediates through monoterminal oxidation of hexadecane

(Minf et al., 2009). Only recently, (Wang et al, 2010) the CYP153 gene pathway was identified in a strain of *Halomonas ventosae* but did not detect an *alkB* homolog or grow the organism on diesel oil. *Halomonas* spp. could be useful as bioremediation agents in marine systems due to their halotolerance; further studies need to elucidate the pathway these organisms use to oxidize alkanes and other hydrocarbons. *Halomonas* sp. and other Gammaproteobacteria are ideal targets for additional investigation for hydrocarbon degradation potential due to their high abundance in production waters (Stevenson et al., 2009; Vila et al., 2010).

Concluding Remarks

The petroleum industry is affected greatly by the activity of microorganisms. Microorganisms appear to be the key to both preventing damage during production and degrading pollutants once they are released in the environment. While the attention is often directed to sulfate reducing microorganisms other microorganisms may be responsible for initiating the biocorrosion process. Hydrocarbon and petroleum biodegradation contributes to both biocorrosion and the quality of fuel products. Identifying organisms capable of hydrocarbon degradation will allow researchers to predict when and where biocorrosion may occur. In oxygen-exposed and microaerophilic oil production environments aerobic hydrocarbonoclastic microorganisms indirectly contribute to MIC by initiating hydrocarbon degradation and "feeding" sulfidogenic and fermentative microorganisms that directly contribute to corrosion. In this thesis, we hypothesize aerobic Gammaproteobacteria in production water oxidize *n*-alkanes using monooxygenase enzymes (*alkB* or cytochrome p450). If

supported, this hypothesis may serve as a model for investigating a wider range of hydrocarbon-degrading microorganisms, different hydrocarbon substrates, and as a tool to monitor for MIC.

Chapter 2:

The role of Gammaproteobacteria in aerobic alkane degradation in oilfield production water from the Barnett Shale

Abstract

Microorganisms from production water from the Barnett Shale were enriched with various hydrocarbons to select for hydrocarbon-oxidizing microorganisms. Gammaproteobacteria increased in relative abundance under *n*-alkane and *n*-fatty acid amendment. Nine isolates were obtained and all were identified as *Halomonas* using 16S rRNA gene sequencing. *Halomonas* isolate A11A was capable of aerobic hydrocarbon degradation as evidenced by growth studies and oxygen respirometry to quantify hydrocarbon oxidation rate. Respirometry studies showed *Halomonas* A11A could completely oxidize (>99 % of theoretical) C_5 *n*-pentane and C_{10} *n*-decane, partially oxidize C_{16} *n*-hexadecane (<76 %) and toluene (37 %). The culture also showed the ability to completely oxidize C_6-C_9 *n*-alkanes. Genomic analysis of A11A suggests that *n*-alkane oxidation is initiated by the *alkB* alkane hydroxylase system and toluene is activated via the *tod* toluene dioxygenase system. *Halomonas* A11A and other hydrocarbonoclastic Gammaproteobacteria in the production water have the potential to play a major role in hydrocarbon degradation and biodegradation as well as an indirect role in the biocorrosion cycle by contributing partially oxidized hydrocarbon metabolites as carbon sources for sulfidogenic and fermentative microorganisms. We propose therefore that aerobic hydrocarbonoclastic Gammaproteobacteria, such as *Halomonas*, should be considered as threats in production water systems.

1.0 Introduction

Anthropogenic petroleum releases and natural crude oil seeps are major sources of environmental hydrocarbon contamination. Petroleum products and contaminants are the most widespread pollutants in the environment, contributing to significant groundwater, aquifer, soil and ocean contamination (Albaiges, 1989; Magot et al., 2000; Duncan et al., 2009; Vila et al., 2010; Stevenson et al., 2011). Further, petroleum spills pose a threat to human health, various ecosystems, and impact on the economy by adding cost for remediation, fines for environmental contamination, loss of product, and healthcare costs for impacted individuals. Studies conducted by the National Association of Corrosion Engineers (NACE) and the U.S Federal Highway System estimated corrosion costs in the oil and gas industry reach $1.372 billion dollars annually (NACE Standard 1C184, 2008) and suggest that up to 40% of the industry's corrosion is triggered by microbially-influenced corrosion (MIC) (Sooknah et al., 2007: NACE International). Corrosion often results in the loss of the structural integrity and spilling of pipeline or tank contents (Beech et al., 1999; 2004; Videla, 1996; Ollivier & Magot, 2005; Suflita et al., 2014). Complex microbial communities are the instigators of these biocorrosion events and yet remain poorly understood (Magot et al., 2000; Hamilton, 2003; Vigneron et al., 2016). Moreover, methods to bioremediate contaminated environments through degrading petroleum products to less toxic or non-toxic forms through the metabolic pathways of endogenous microorganisms is one of the most cost effective and sustainable strategies for cleaning contaminated sites; however, the variability in environmental conditions and effect on microbial populations can limit the effectiveness of bioremediation methods

(Vila et al., 2010). Thus, identifying microorganisms and characterizing the pathways and enzymes they employ to degrade the petroleum hydrocarbons or participate in the biocorrosion cycle is of increasing importance (Rahman et al., 2003).

Microorganisms can be the source of many problems in the oil and gas industry including corrosion of the pipelines and storage tanks and biodegradation of the hydrocarbon products (Suflita et al., 2014). The structural integrity of pipelines and storage tanks can be compromised by microbially-influenced corrosion (MIC) or biocorrosion resulting in the release of petroleum products and severe environmental contamination (Vigneron et al., 2016). Sulfate-reducing, sulfide-producing, iron-reducing, and acid-producing microorganisms are thought to be the main players in MIC due to their production of corrosive acids and gases (Magot et al., 2000; Vigneron et al., 2016). To date, researchers consider biofilm formation on metal surfaces as one of the initial stages of biocorrosion (Beech et al., 1999; Vigneron et al., 2016). Moreover, the biofilm can function as a protective barrier from biocide treatments and oxygen for obligate anaerobes in dynamic environments such as pipelines and tanks (Beech et al., 2002). Some iron-oxidizing strains, sulfate-reducing bacteria (SRB), methanogens, and lithotrophic bacteria can withdraw electrons directly from iron and steel surfaces in a process known as electrical MIC (EMIC) (Dihn et al., 2004; Enning et al., 2012; 2014; Vigneron et al., 2016). Additionally, SRBs and other sulfidogenic bacteria generally oxidize a range of organic acids, like lactate, acetate, some hydrocarbons, and other fatty acids coupled to the reduction of sulfate and thiosulfate in a process called chemical MIC (CMIC) (Hansen, 1993; Rueter et al., 1994; Dihn et al., 2004; Enning et al., 2014). These, and other partially oxidized metabolites that feed sulfidogenic bacteria are the

by-products of general heterotrophs and hydrocarbonoclastic (hydrocarbon-degrading) bacteria (Muyzer and Stams, 2008). While a great volume of research is focused on understanding the direct mechanisms for biocorrosion and MIC, the microbial participants and conditions that foster these events remain largely unknown, poorly characterized and unappreciated.

Biodegradation and biodeterioration of the petroleum products is also a problem for the oil and gas industry because it can reduce the quality of petroleum products (Head et al., 2003). Biodegraded petroleum products are less desirable because of the marked compositional changes (both chemical and physical), selective degradation of specific isomers, and acidification of some compounds (Head et al., 2003). Oil biodegradation occurs at the base of the oil column, deep in the reservoir, where electron donors and water are plentiful, but also occurs during petroleum production. Hydrocarbon-degrading microorganisms are present in many environments; they have been described and identified in a wide range of environments and conditions including: oxic, sulfate-reducing, iron-reducing, denitrifying and methanogenic conditions (Hutchins et al., 1991; Coates et al., 1996; Zengler et al., 1999; Rapp et al., 2003; Jones et al., 2008; Gray et al., 2010; Wawrick et al., 2016).

Depending on the origin of the petroleum products, *n*-alkanes may constitute 20-50% of crude product and are detected in trace quantities in most environments (van Beilen et al., 2003). In aerobic alkane degradation, aerobic microorganisms use O_2 as an electron acceptor. Oxygen is reduced to H_2O and the alkane is oxidized to an alcohol via terminal, biterminal, or subterminal oxidation. Ultimately, the alkane is converted to a

fatty acid and processed in β-oxidation reactions (Boulton et al., 1984; Britton, 1984; Watkinson et al., 1990). Aerobic microorganisms degrade hydrocarbons using mono- and dioxygenase enzymes. For example, aliphatic hydrocarbons such as *n*-alkanes are oxidized by alkane hydroxylases such as the *alkB* system or cytochrome p450 pathways (Wang et al., 2010). Additional proteins such as AlmA and LadA are involved in long chain alkane degradation.

With some exceptions, petroleum hydrocarbons and other compounds are not as readily degraded under anoxic conditions (Gieg et al., 2009). Anaerobic degradation requires a non-oxygen electron acceptor, such as sulfate or nitrate, to couple the reduction of the carbon substrate and allow for degradation; these reactions will proceed according to most thermodynamically favorable and available electron acceptor based on the redox potential of the environment (Widdel & Rabus, 2001). Under anaerobic conditions microorganisms activate hydrocarbons via anaerobic hydroxylation or through the addition to fumarate via alkylsuccinate synthase or benzylsuccinate synthase enzymes (Watkinson et al., 1990; Aeckersberg et al., 1991; 1998; van Beilen et al., 1994; Callaghan et al., 2013; Heider et al., 2016).

Aromatic hydrocarbons such as benzene, toluene, ethylene, and xylene (BTEX) that may constitute up to 59% of gasoline products are also targets of microbial degradation under both aerobic and anaerobic conditions (Dagley et al., 1984; Gibson et al., 1984; Coates et al., 1997; Rapp et al., 2003; Deng et al., 2017). In aerobic environments, BTEX compounds are activated through mono- and diooxygenase enzymes and form catechol compounds (El-Naas et al., 2014). In anoxic environments, most of these aromatic compounds are targeted by benzylsuccinase synthase enzymes,

which catalyze the additional to fumarate (Beller et al., 1997; Leuthner et al., 1998; Chakraborty et al., 2004). Benzene degradation is more of a mystery; some evidence suggests benzene is activated via carboxylation, hydroxylation, methylation, or reduction of the ring (Coates et al., 2002). Ultimately, these aromatic rings are cleaved and pushed through to complete degradation and various end products. Degradation of BTEX compounds have been characterized predominantly under sulfate- and nitrate-reducing conditions (Ball et al., 1996; Rabus et al., 1995), but studies have also witnessed degradation to occur under Fe(III)-reducing and methanogenic conditions (Coates et al., 1999; Caldwell et al., 2000; Phelps et al., 2001).

Recovering crude products from the subsurface and refining them thereafter consume and produce significant volumes of water. The initial stages of production begin with drilling a well to draw fluids, including water and oil, from the reservoir to the surface (Ollivier et al., 2005). Throughout production, wells produce water from the reservoir, but as wells near the end of their production life, they produce up to 95% water and very little oil; the large volumes of water are stored in steel surface facility production water tanks until disposal. The production water tanks also serve as another facility to allow condensate and oil to separate from the water. Characteristically, production water direct from the well does not contain any dissolved oxygen, is highly saline (>300 g/L), has a high temperature (40-90° C), and contains significant amounts of condensate hydrocarbons and a variety of organic compounds and acids (Ollivier et al., 2005). Production waters typically harbor a wide variety of microorganisms capable of hydrocarbon degradation and corrosion (Lyles et al., 2013; Liang et al., 2017). By the

time produced water reaches the storage tanks it has been transformed according to the chemical and environmental parameters. The storage tanks shift in their volume of production water, vapor space, microbial population, oxygen concentration, and temperature; this dynamic nature contributes to creating an environment conducive to several types of corrosion and the accidental release of their petroleum-based contents (Heidersbach, 2010; Davis et al., 2012). Depending on the combination of environmental parameters, bicorrosion can occur in different areas of storage tanks, including the water-condensate interface, in sludge deposits on the bottom of the tank, cracks or fissures in the steel, and the exterior of the tank. Corrosion of the production machinery is studied extensively, but a complete understanding of the microbial interactions and community structure that contribute to the corrosion is lacking.

In this study, we examined a production water tank drawing from the Barnett Shale in north central Texas. Shale gas produced from the Barnett Shale is completely thermogenic and supplies over a third of the shale gas in the United States (Pollastro, 2007). High temperatures and narrow pore size (<0.005 μm) of the shale formation does not support the growth of microorganisms within the formation (Bowker, 2007). However, cooled (82 °C) natural gas wells appear to harbor diverse microbial populations (Bowker, 2007). Several sites throughout the Barnett Shale have reported problems with biogenic sulfide production and biocorrosion despite the formation being abiotic (Fichter et al., 2009). One study characterized microbial populations collected from gas-water separators and storage tanks from two newly drilled wells (Davis et al., 2012). Here, they monitored two sites over six months using 16S rRNA pyrosequencing to gauge community shifts and attempted to track the origin of the microorganisms.

Overall, they noted that the communities consist of halophilic and halotolerant Firmicutes and Proteobacteria, specifically Beta-, Gamma-, Epsilon-, and Deltaproteobacteria; several were classified as sulfate-reducing, thiosulfate-reducing, and sulfur-reducing species. Other microorganisms of interest identified throughout the study include chemolithoautotrophs (*Arcobacter*), aerobic heterotrophs belonging to orders *Alteromonadales* and *Oceanospirillales* (*Pseudomonas*, *Marinobacter*, and *Halomonas*) and anaerobic fermenters (*Halanaerobium*). They also established that the bacterial communities reflect the unique geochemical conditions of the reservoir rather than from those found in drilling muds or fracturing fluids used to create the wells. Considering the diverse microbial community, it is clear the production water is a very dynamic system and plays host to aerobic, microaerophilic, facultative anaerobic, and obligate anaerobic microorganisms. Specifically, water flow from the well to the separators and finally to the production water tanks is not homogeneous and may result in stagnation and areas of anaerobic water in the pipes and at the bottom of the water storage tanks (van der Krann et al., 2010; Davis et al., 2012). Thus, a more complete understanding of community interactions and interplay between the aerobic and anaerobic populations is necessary before biocorrosion and hydrocarbon biodegradation prevention strategies can be applied and relied upon.

The purpose of this study was to enrich for and identify aerobic microorganisms in petroleum production water, examine their hydrocarbon degradation potential, and detect the genes coding for the enzymes involved in hydrocarbon degradation pathways. We hypothesized that aerobic microorganisms, specifically aerobic

Gammaproteobacteria, initiate the degradation of *n*-alkanes via monooxygenases such as *alkB* or cytochrome p450 gene systems. We addressed our hypothesis through analysis of the microbial community composition in the production water before and after amendment with various hydrocarbons. Relative abundances of microbial community members were assessed through 16S rRNA gene libraries. The dominant culturable *n*-alkane-degrading microorganisms were isolated after amendment of production water with C_5-C_{10} *n*-alkanes. Nine isolates were obtained and identified as *Halomonas* sp. using 16S rRNA gene sequencing. Isolate A11A was analyzed for possession of *n*-alkane monooxygenases through genome sequencing, and evaluated for hydrocarbon degradation ability and oxidation rate estimated by oxygen respirometry. Together, these methods provide valuable information regarding microbial degradation of petroleum hydrocarbons, suggest gene targets for hydrocarbon degradation screening, and provide targets to monitor groundwater, aquifer and subsurface contamination.

2.0 Materials and Methods

2.1 Production water sampling and analysis

Production water was obtained in June 2015 from a production water tank containing oil and gas from the Barnett Shale in the Fort Worth Basin (Montgomery et al., 2005). The production water tank sampled was chosen based on repeated detection of high numbers of sulfate-reducing bacteria by single serial dilution into media designed to facilitate the growth of sulfate-reducing bacteria (personal communication: L. Woods, ConocoPhilips, Decatur, TX, USA, NACE Standard TM0212, 2012).

Production water was obtained by lowering sterile 1 L bottles through the thief hatch; bottles were anaerobically sealed once samples were collected.

2.1.1 Production water chemistry

On-site detection of temperature, pH, oxygen, sulfide, and thiosulfate were measured; pH was measured using Hydron pH papers range 2-10 (Microessentials Laboratory, Brooklyn, NY, USA), oxygen was measured using an FireSting O_2 probe (OXROB10, PyroScience, Aachen, Germany). In-field measurements of sulfide (K-9510D) and thiosulfate (K-9708) were collected using Chemetrics (Chemetrics Inc. Midland, VA, USA) kits according to the manufacturer's instructions (Chemetrics Inc. Midland, VA, USA). Upon returning to the laboratory, sulfate (SO_4^{2-}) and salinity, estimated by chloride (Cl^-) anion, measurements were calculated by ion chromatography (Dionex model IC-1000, Sunnyvale, CA) according to Lyles et al., 2013. Briefly, sulfate and chloride in the production water was measured by ion chromatography fitted with an IonPac AS4A column, ASRS 300 4 mm self-regenerating suppressor, and a conductivity detector. A 1:100 dilution of sodium carbonate/sodium bicarbonate buffer served as the eluent from an AS4A ready-to-dilute concentrate (Dionex, Sunnyvale, CA). The electrochemical suppressor was set at 27 mA, and the system was operated isocratically at 1.5 mL/min and. Chromatograph peaks were analyzed with the Chromeleon software package (Thermo-Scientific). Cells were aseptically pipetted from the collected production water and counts estimated by direct methods (NACE, 2012). Briefly, counts were determined by epifluorescense microscopy under blue excitation using a micrometer grid on an

Olympus BX-61 microscope. Cells obtained from the production water were fixed using a 1/10 volume of 37% formaldehyde and a dilution of the fixed samples was stained with 5 μL of 5 ng/mL 4,6-diamidino-2-phenylindole (DAPI) in the dark for 45 min at room temperature. Following staining, cells were filtered onto 0.22 μm black stained 25 mm diameter polycarbonate membrane filters (Sterlitech, Kent, WA, USA). An average of 30 fields were counted to estimate cell counts on each slide. Production water samples were analyzed for putative polar metabolites and other compounds present in the gas condensate and production water. Extractions were performed and analyzed according to Aktas et al., 2010 and 2012. Briefly, extractions from the production water were concentrated, derivatized, and analyzed via Gas Chromatography-Mass Spectroscopy (GC-MS). Metabolite identities were made by comparing GC retention times and fragmentation by MS profiles of commercially available standards.

2.1.2 Molecular analysis of the microbial community

To preserve cells for 16S sequencing for clone libraries and metagenomic analysis, 5 mL of DNAzol (DN127, Molecular Research Center, Cincinnati, OH, USA) were added to 1 L of the production water within 6 hours of sampling. The following day, the remaining gas condensate was removed from the water sample and 250 mL of the production water was filtered with a 0.2 μm cellulose nitrate analytical test filter funnel (145-2045, Fisher Scientific, Fair Lawn, NJ, USA). The filter was preserved by freezing at -80° C until DNA was extracted. DNA was extracted from the filtered production water samples using PowerMax Soil DNA Isolation Kit (12888,

Mo Bio Laboratories, Inc., Qiagen, Carlsbad, CA, USA) according to manufacturer's instructions with minor modifications: 1.2 mL of the provided C1 solution was added to a 50 mL centrifuge tube containing the preserved filter. The tube was vortexed and incubated in 50° C water bath for 30 minutes and frozen at -80° C for 10 minutes; this was repeated three times. The PowerBead tube, 1 tube of Lysing Matrix E Beads (Biomedicals, Santa Ana, CA, USA), and 15 mL of bead solution were added to the tube and vortexed for 10 minutes and frozen at -80° C overnight. The remaining steps followed the manufacturer's instructions. Following extraction, 4 mL of the DNA was concentrated by adding 0.2 mL of 5 M NaCl and inverting 5 times to mix , followed by adding 10.4 mL of 100% cold ethanol and inverting 5 times. After mixing, the tube was centrifuged at 2500 x g for 30 minutes at ambient temperature. Following centrifugation, all liquid was decanted without dislodging the pellet and allowed to dry. Concentrated DNA was resuspended in 500 µL nuclease-free H_2O (Promega, Madison, WI) and quantified via fluorometry with the Qubit® dsDNA HS Assay (Life Technologies, ThermoFisher Scientific, Carlsbad, CA) according to the manufacturer's instructions. Sample DNA was diluted 1:10 and 1:100 and stored at -20° C.

Extracted DNA was amplified with 519-M13 forward primer, 5'-<u>GTA AAA CGA CGG CCA GCA</u> GCM GCCG CGG TAA-3', with the M13 unique barcoded sequence (underlined). The 785 reverse primer, 5'-TAC NVG GGT ATC TAA TCC-3', "S-D-Bact07850b-A-18" was from Klindworth et al., 2013. The PCR product spans the V4 region of the 16S rRNA gene from position 519 to 802. The PCR reactions consisted of 12.5 µL of 2x Phusion High-Fidelity PCR Master Mix (New

England Biolabs, Ipswich, MA, USA), 0.5 µL of each 20 pmol/µL forward and reverse primer, 7.0 µL of PCR grade water, and 5 µL of DNA. Thermal incubation cycling was conducted on Techne Prime Thermal Cycler (Techne, Cambridge, United Kingdom). Cycling conditions consisted of 1 cycle of initial denaturation at 98° C for 30 seconds, 30 cycles of denaturation at 98° C for 10 seconds, DNA annealing at 52° C for 20 seconds, and extension at 72° C for 10 seconds. Final extension was held at 72° C for 5 minutes for one cycle. Amplified products were visualized via UV-illumination in 1% agarose (Thermo Fisher Scientific, Fair Lawn, NJ, USA) with 0.5 TBE (Tris/Borate/EDTA) Buffer stained with 0.1% SybrSafe (Invitrogen, Carlsbad, California, USA) and assessed against the GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific, Fair Lawn, NJ, USA). Product bands were cut from the gel and added to 50 µl of sterile nuclease-free H_2O and frozen at -20° C for at least 2 hours. A second thermal cycling reaction consisting of 12 cycles of the same conditions was used to add a unique barcode on the forward primer to each DNA sample (Wawrik et al. 2012). The 50 µL reaction consisted of Phusion 2x Master Mix, 5 µL of the 785 reverse primer, 15 µL PCR grade water, 4 µL of the product band DNA, and 0.5 µL of unique M13 barcode. Products were visualized via UV-illumination after gel electrophoresis in 1% agarose with 0.5 TBE Buffer stained with 0.1% SybrSafe and assessed against the GeneRuler 1 kb DNA ladder. Barcoded PCR products were purified using Ampure® XP paramagnetic beads (Beckman Coulter, Indianapolis, IN, USA), and quantified using the Qubit® dsDNA HS assay. All barcoded products were pooled in equal concentrations and submitted to the Oklahoma Medical Research

Foundation (OMRF, Oklahoma City, OK) for 300 bp paired end sequencing via the Illumina Miseq platform.

The 16S library sequences from the original production water, *n*-alkane, and fatty acid amendments (described below) were analyzed using QIIME (Quantitative Insights Into Microbial Ecology); the pair-end reads were joined and demultiplexed in the QIIME software package (Caporaso et al., 2010). Operational Taxonomic Units (OTUs) were set at 3 percent dissimilarity and chimeras were removed using the USEARCH algorithm (Edgar, 2010). Taxonomy was assigned against the small subunit rRNA sequences from the RDP Naïve Baysian classifier (Wang et al., 2007b) and SILVA database Release 111 (www.arb-silva.de) (Caporaso et al., 2010; Pimenov et al., 2012).

2.2 Aerobic enrichment of microorganisms in petroleum production water

Production water was used as an inoculum and amended with hydrocarbons to determine the degradation potential of the endogenous aerobic microbial community (enrichment design shown in Figure 1). Production water was inoculated into triplicate incubations of Widdel's medium prepared per liter with: 10 mL of Widdel's 10x salts (g/L) (NaCl, 60.0; $MgCl_2$, 3.0; $CaCl_2$, 0.5; Na_2SO_4, 3.0; KH_2PO_4, 0.2; KCl, 0.5) and supplemented with 10 ml 100x RST trace metals (g/L) (nitrilotriacetic acid adjusted to pH 6.0 with KOH, 2.0; and $MnSO_4 \bullet H_2O$. 1.0; $Fe(NH_4)_2(SO_4)_2 \bullet H_2O$, 0.8; $CoCl_2 \bullet 8H_2O$, 0.2; $ZnSO_4 \bullet 7H_2O$, 0.2; $CuCl_2 \bullet 2H_2O$, 0.02; $NiCl_2 \bullet 6H_2O$, 0.02; $Na_2MoO_4 \bullet 2H_2O$, 0.02; Na_2SeO_4, 0.02; Na_2WO_4, 0.02), and 3.5 g of Na_2SO_4 (7757-82-6 anhydrous, reagent grade, Carolina Biological Supply Company, Burlington, NC,

USA) per liter. The medium was adjusted to 7.2 pH (Widdel & Bak, 1992). Hydrocarbon and heterotrophic substrates (Table 1) were prepared anoxically in sterile 160 ml serum bottles sealed with black butyl rubber stoppers (20 mm × 16 mm, Geo-Microbial Technologies, Inc., Ochelata, OK) and secured with aluminum crimp seals. For every 9 mL of media, the following was added: 0.1 mL of 10 mg/mL yeast extract, 0.3 mL 10% $NaHCO_3$, and 1.0 mL 100x RST vitamins (mg/L): pyridoxine•HCl, (10); thiamine•HCl, 5.0; riboflavin, 5.0; calcium pantothenate, 5.0, thioctic acid, 5.0; *p*-Aminobenzoic acid, 5.0; nicotinic acid, 5.0; vitamin B_{12}, 5.0, biotin, 2.0; folic acid, 2.0 (Tanner, 1989). Hydrocarbon substrates and concentrations for amendment were designed based on organic extractions from the production water, described in Aktas et al., 2010 and Dalvi et al., 2012.

2.2.1 Initial enrichment of aerobic microorganisms from production water

Bottles received a 10% (v/v) inoculum of production water into Widdel's medium containing one of the substrates (Table 1) for a total volume of 30 mL. Incubations were kept in 160 ml serum bottles sealed with black butyl rubber stoppers (20 mm × 16 mm, Geo-Microbial Technologies, Inc., Ochelata, OK, USA) and secured aluminum crimps. Unamended controls were inoculated into Widdel's medium without any added substrates and heat-killed sterile controls were made by inoculation into Widdel's medium and then autoclaved for 30 minutes. Active enrichments, showing growth based on visual turbidity, were transferred (10%; (v/v) on three successive occasions into fresh media every 30-40 days to confirm that growth resulted from hydrocarbon amendment rather than carry-over of other

substrates from the production water. Enrichments were incubated at 25° C without shaking. Upon completion of three successful transfers, 2 mL of culture was retained for further molecular analysis by centrifuging the sample at 10,000 x g for 3 minutes to pellet the cells. The supernatant was discarded, and 100 µl DNAzol (DN127, Molecular Research Center, Cincinnati, OH, USA) was added to the pellet and stored at -20° C.

The DNA was extracted from the pellet via the Maxwell ®16 Tissue LEV (low elution volume) Total RNA purification kit (AS1220, Promega, Madison, WI). To preserve the sample DNA, the DNA removal steps of the Total RNA Purification Kit protocol were not used. Instead, samples were loaded into the prepared reagent cartridges along with equal parts RNA lysis buffer and RNA dilution buffer, each 400 µL. The instrument was loaded with the elution tubes containing 100 µL nuclease-free water (Promega, Madison, WI, USA), provided plungers, and prepared cartridges. Extraction was performed with the DNA extraction program; as modified by Oldham et al., 2012. The DNA was quantified fluorometrically with the Qubit® dsDNA HS Assay. Extracted DNA from each enrichment sample was diluted 1:10 and 1:100 in nuclease-free H_2O and stored at -20° C for subsequent molecular analysis. Extracted DNA from the *n*-alkane and *n*-fatty acid-amended enrichments was amplified and analyzed to form 16S libraries representative of each enrichment condition and compared to the original production water community using the same methods outlined above.

2.2.2 Progressive aerobic enrichment of microorganisms from production water

Additional progressive enrichments were prepared by serial dilution (1:10) to select for the dominant *n*-fatty acid- and *n*-alkane-degrading microorganisms in the aerobic enrichments (Figure 1). The active fatty acid and *n*-alkane incubations were serially diluted (1:10) into fresh media reaching 10^{-4} dilution, amended with the same *n*-fatty acid (C_5-C_{10}) or *n*-alkane (C_5-C_{10}) substrates above, and incubated in sterile 20 mm x 150 mm borosilicate glass tubes with sterile removable caps. All dilutions were performed in triplicate with heat-killed and substrate unamended negative controls. Enrichments were prepared, monitored, and tranferred according to the methods outlined above. Samples were collected and preserved for subsequent molecular analysis according to the methods outlined above.

2.3 Isolation and characterization of Halomonas A11A

Several *n*-alkane and fatty acid degrading bacteria were isolated from the active *n*-alkane- and fatty acid-amended progressive enrichments (Figure 1). Two isolates, A11A and A11B, were obtained from the 10^{-4} dilution of the first round of *n*-alkane-amended progressive enrichments. Three isolates, B29A, B29B, and B29X, were obtained from the 10^{-2} dilution of the second round of *n*-alkane-amended enrichments. Two isolates, C53A and C53B, were obtained from the 10^{-4} dilution of the third round of *n*-alkane-amended enrichments. Two isolates, C44A and C44B, were obtained from the 10^{-4} dilution of the third round of *n*-fatty acid-amended enrichments. Isolates were obtained by streaking multiple rounds of isolated colonies on Widdel's medium agar containing 15 g/L agar (Bacto agar, Difco, Detroit, MI,

USA). Prior to inoculation, plates were supplemented with 80 uL 0.1 M *n*-alkanes or 200 uL 0.1 M fatty acids (e.g. "substrates") by spotting the agar with the substrates then spreading the substrates over the agar surface with a sterile loop. Plates were incubated for 7 to 10 days at 25° C. Pure cultures were maintained on Widdel's medium agar and in Widdel's medium broth with added *n*-alkanes or fatty acids. A 1.5 mL sample of each pure culture was transferred to a 2 mL microcentrifuge tube and centrifuged at 6000 x g for 5 minutes to pellet the cells. The supernatant was removed without dislodging the pellet, discarded and 50 µL DNAzol was added. Following homogenization with DNAzol, 100 µL nuclease-free H_2O was added and cultures were stored at -20° C to be used for DNA extraction and subsequent 16S rRNA gene amplification.

Isolates obtained from *n*-alkane and *n*-fatty acid-amended enrichment were analyzed via their 16S rRNA gene sequence. Amplification of the bacterial 16S rRNA gene was performed via PCR using protocols outlined by Weisburg et al., 1991. Briefly, 1:100 diluted isolate DNA was amplified using 1.25 uL of primer fD1 (5pmol/uL, 5' AGAGTTTGATCCTGGCTCAG 3') and primer rP2 (5pmol/uL, 5' ACGGCTACCTTGTTACGACTT 3') to yield an approximately 1500 bp product. PCR amplification was performed in the Techne Prime Thermal Cycler as follows: an initial denaturation step at 94° C for 4 minutes; 30 cycles with denaturation at 94° C for 1 minute; annealing occurred at 48° C for 1 minute; extension ran at 72° C for 1 minute; and final extension occurred for 1 cycle at 72° C for 10 minutes. Products were visualized via UV-illumination after gel electrophoresis in 1% agarose with 0.5 TBE Buffer stained with 0.1% SybrSafe and assessed against the GeneRuler 1 kb DNA

ladder. Desired PCR products were purified for DNA sequencing using Exo-SAP IT (USB Co., Cleveland, OH, USA) by transferring 5 μL of the PCR product to a sterile PCR tube and adding 1.5 μL ExoSAP-IT reagent and cycled on the Techne Prime Thermal Cycler according to manufacturer's instructions. Cleaned and amplified samples and sequencing primers were submitted for sequencing by OMRF. The sequencing primers P1 and D907R (Santegoeds et al., 1998) cover a region of approximately 550 bp and were used for all isolates except A11A. Isolate A11A PCR product was sequenced with 4 primers: fd1, rp2, P1, and D907R to cover approximately 1400 bp. Quality trimming of sequence chromatographs and consensus sequence formation from the forward and reverse reads were performed via Sequencher (Gene Codes Corp. Ann Arbor, MI, USA) Trimmed and assembled sequences were compared to existing sequences in GenBank via BLASTN and RDP (http://rdp.cme.msu.edu/, Ribosomal Database Project) (Altschul et al., 1990; Larsen et al., 1993; Cole et al., 2009; Edgar, 2010).

2.3.1 Genomic and phylogenetic analysis Halomonas A11A

The 1432 bp 16S rRNA gene sequence of *n*-alkane-degrading isolate A11A was analyzed using an online tool, http://www.phylogeny.fr/alacarte.cgi using "a la carte" mode with the following parameters. The 16S rRNA gene was aligned using Clustal W v. 2.1 (Dereeper et al., 2008; 2010; Thompson et al., 1994) with available gene sequences (≥ 1411 bp) of selected species of the genera *Halomonas*, *Pseudomonas*, *Marinobacter*, and *Alcanivorax* as representatives of other Gammaproteobacteria with an emphasis on hydrocarbon-degrading species. An

Alphaproteobacterium, *Roseovarius pacificus* strain 81-2 was used as the outgroup. Sequences were also aligned using MUSCLE v. 3.8.31 using default settings (Edgar., 2004) but no differences in branching order or clade membership were observed (data not shown). After alignment, positions with gaps were removed. The phylogenetic trees were reconstructed via the neighbor-joining method implemented in Neighbor from the PHYLIP package v. 3.66 (Felsenstein J., 1989). Bootstrap values (≥ 50 %) representing percentage of 1000 replicate trees in which associated taxa clustered as shown in both neighbor-joining and maximum-likelihood methods (Guindon et al., 2003) are displayed on nodes (Figure 4). Distances were calculated using FastDist (Elias et al., 2007). TreeDyn v. 198.3 was used to render graphical representations of phylogenetic trees.

DNA was extracted from isolate A11A from the *n*-alkane–amended enrichment for whole genome sequencing for identification of genes associated with hydrocarbon degradation. The culture was inoculated in marine broth and incubated at 27° C with 50 rpm shaking for 1 week. Once turbid, 1 mL was collected and DNA was extracted via Maxwell ®16 Tissue LEV Total RNA purification kit according to the instructions outlined above and quantified via fluorometric Qubit® dsDNA BR Assay according to methods outlined above.

Following DNA extraction, the bacterial genome was sequenced by OMRF via the Illumina MiSeq platform using 300 bp paired end sequences. The metagenome was analyzed using an in house pipeline (MGMIC, Dr. Boris Wawrik, OU Biocorrosion Center). In brief, the sequences were assessed for quality via FastQC v. 0.11.2. Adapter sequences were trimmed via TrimGalore and Cutadapt (Martin, 2011). Reads

were trimmed further to a quality score of 30 and poly-AA tails and artifacts cut using HomerTools. Unpaired reads and sequences < 100 bp were removed via Trimmomatic (Bolger et al., 2014). Sequences were assembled using Meta-Ray (Boisvert et al., 2010; 2012) with Kmer setting 31, all contigs <500 bp were discarded. Prodigal (Hyatt et al., 2012) predicted all contigs for the open reading frames (ORFs). Assembled DNA sequences were binned into genome scaffolds based on tetranucleotide frequency data using MaxBin (Wu et al., 2014). Assembled genomes were screened for relevant functional genes using KEGG KOBAS (KEGG Orthology Based Annotation System) v. 3.0 to obtain gene, enzyme, or pathway frequencies (Wu et al., 2006; Xie et al., 2011). Briefly, the genome sequence is BLASTed against the sequences in the KEGG KOBAS database using the default cutoffs: *E*-value $<10^{-5}$ and rank ≤5 (Xei et al., 2011). The sequences are assigned KEGG Orthology (KO) terms of the first BLAST hit with either a known KO assignment, an *E*-value $<10^{-5}$, and has less than 5 other hits with a lower *E*-value that do not have KO assignments (Mao et al., 2005; Xei et al., 2011).

Annotated genes with KO identifiers were submitted to KEGG Mapper (http://www.genome.jp/kegg/mapper.html) to illustrate select gene pathways in the *Halomonas* A11A genome against the KEGG reference pathways (Kanehisa et al., 2011). Pathways were reconstructed for fatty acid, long-chain fatty acid, alkane, benzene, toluene, ethylbenzene, and xylene metabolisms to map and identify genes of interest and demonstrate which pathways may be complete or incomplete.

2.3.2 Physiological analysis of Halomonas A11A

Isolate A11A was screened for hydrocarbon degradation ability by visual evaluation of growth in liquid medium containing various hydrocarbons. Single bottles of aerobic incubations received a 10% (v/v) 0.5 OD_{610} inoculum of isolate A11A, grown on the same hydrocarbon they were tested in, into Widdel's medium containing 20 μM of one of the hydrocarbon amendments: C_5-C_{10} alkane mix, C_5 (*n*-pentane), C_6 (*n*-hexane), C_7 (*n*-heptane), C_8 (*n*-octane), C_9 (*n*-nonane), C_{10} (*n*-decane), C_{16} (*n*-hexadecane), BTEX (benzene, toluene, ethylbenzene, and xylene), or Toluene as the only carbon source. No yeast extract or tryptone was used in the growth medium from this point forward. Marine broth was used as the positive control for visual growth, and a substrate unamended negative control, prepared with Widdel's medium without substrates and a bottle containing inoculum plus substrate, sterilized by autoclaving for 30 minutes, served as an abiotic control (heat-killed control). Actively growing cultures, showing growth based on visual turbidity, were transferred (10%; v/v) three successive times into fresh media every 7-10 days. The bottles were incubated at 25° C with 50 rpm shaking.

Isolate A11A was grown in Widdel's medium with 20 μM of the hydrocarbon being tested as the only carbon source in preparation for respirometry. Prior to inoculation for oxygen respirometry, cells were quantified via optical density and corresponding CFU (colony forming units) per mL. Inoculum was obtained from the third successive transfer of the isolate grown in 20 μM of the hydrocarbon being tested and inoculated in fresh Widdel's medium at 1:1, 1:2, 1:5 and 1:10 dilutions amended with 100 nM of the selected *n*-alkane. The optical density (OD) was measured

spectrophotometrically at 610 nm against uninoculated media as a blank. Following OD measurements, colony forming units were quantified by plating 50 uL of 1:10, 1:100, 1:1000, 1:10000, and 1:100000 dilutions on marine agar in duplicate and incubated for 7 days at 25° C. After visible colonies formed, colonies were counted and used to estimate CFU/mL (# of colonies)(dilution factor)/(volume plated in mL) used in oxygen respirometry experiments.

2.3.3 Oxygen respirometry and hydrocarbon degradation by Halomonas A11A

Isolate A11A was assessed for aerobic hydrocarbon degradation via oxygen respirometry. Bottles were incubated at room temperature on the 10-channel Micro-Oxymax Respirometer system (Columbus Instruments, Columbus, OH, USA) housing an electrochemical oxygen sensor and assembled according to manufacturer's instructions. Oxygen respiration was measured by purging the headspace every 4 hours for up to 96 hours.

Experimental yields of hydrocarbon oxidation rates were inferred from graphical representation of the rate of oxygen uptake (μM/min) and total oxygen consumption (μM) generated from oxygen respirometry and compared to theoretical stoichiometric mass balances for complete hydrocarbon oxidation (Table 2). The highest peak value in oxidation rate (μM/min) for the substrate unamended 2,2,4,4,6,8,8-heptamethylnonane (HMN) negative control was subtracted from the highest peak value of each hydrocarbon-amended peak to establish the active oxidation rate of each hydrocarbon. This difference was divided by the theoretical μM of O_2 required to completely oxidize the hydrocarbon. The value was converted to a

percentage and represents the experimental yield of μM of O_2 consumed to oxidize each respective hydrocarbon.

The optimal inoculum size for respirometry was determined by comparing the peak in oxidation rate (uM/min) for inoculation concentration at 5% and 10% (v/v). The cells were grown in Widdel's medium for 10 days with 0.334 mM concentration of the C_5-C_{10} *n*-alkane mix solution. Fresh bottles were inoculated with 5% and 10% inoculum measured spectrophotometrically to equal 0.5 OD_{610} and amended to a final concentration of 0.334 mM C_5-C_{10} *n*-alkane mix solution. Duplicate replicates of each inoculum size culture with corresponding unamended controls and a sterile heat-killed control bottle were incubated on the respirometer for 60 hours.

Following optimization, the isolate was screened for hydrocarbon degradation ability with a suite of *n*-alkanes and other hydrocarbons. A 10% (v/v) inoculum of *Halomonas* A11A grown on the 20 μM C_5 *n*-pentane, at 0.5 OD_{610}, was inoculated in 30 mL of fresh Widdel's medium and screened for active aerobic hydrocarbon degradation. To examine the substrate range of the isolate and oxidation rate of each hydrocarbon each bottle received one of the following hydrocarbon substrates in concentrations that yield similar total-carbon concentrations: 40 μM C_5 (*n*-pentane), 36 μM C_6 (*n*-hexane), 32 μM C_7 (*n*-heptane), 28 μM C_8 (*n*-octane), 24 μM C_9 (*n*-nonane), 20 μM C_{10} (*n*-decane), 2.5 μM C_{16} (*n*-hexadecane), and 32 μM $C_7H_5CH_3$ (toluene) solution in acid-washed 250 mL Schott bottles. To avoid toxic effects, solubility limitations, and volatilization the hydrocarbon stocks were prepared in 2,2,4,4,6,8,8-heptamethylnonane (HMN) and added as diluted solutions to amend culture stocks and respirometry bottles for the remaining tests (Rabus et al., 1993; 1995). Substrate

concentrations were selected to be higher than effector thresholds for monooxygenase and dioxygenase enzymes (Attaway et al., 2002; Rojo et al., 2009). Substrate unamended controls were prepared with HMN and Widdel's medium and heat-killed sterile controls were made by inoculating cells into Widdel's medium and autoclaved for 30 minutes.

Oxygen respirometry tests were used to obtain average experimental yields of oxygen consumption during hydrocarbon oxidation for cells grown on one substrate then transferred to a different substrate for testing. Cells grown with 20 μM C_5 (*n*-pentane) were used to inoculate bottles prepared with C_5 (*n*-pentane), C_{10} (*n*-decane), C_{16} (*n*-hexadecane), or toluene (anhydrous, 99.8%, Sigma-Aldrich St. Louis, MO, USA): all at a final concentration of 20 μM. The test was repeated using cells grown on C_{10} (*n*-decane) and amended with C_5 (*n*-pentane). All substrates were prepared in HMN and tested in triplicate replicates as previously described. Triplicate sterile heat-killed and unamended controls prepared in HMN were used as previously described.

3.0 Results

3.1 Production water chemistry

Production water was tested in the field and in the laboratory to determine the background water chemistry parameters (Table 3). The in-field analysis of the water revealed a neutral pH (6.9) and an ambient temperature of 30 °C. Additional in-field chemical analysis of the water estimated oxygen at 0 ppm, sulfide was undetectable, and thiosulfate was 6.5 mg/L. Laboratory testing measured sulfate at 150 uM and

salinity estimated by Cl^- anions to be 2.7%. Direct cell count methods estimated the production water to contain approximately ≤ 1.68e +6 cells/ mL.

Production water samples were analyzed for putative intermediates of hydrocarbon biodegradation. This analysis detected several *n*-alkanes, ranging from C_5 to C_{12}, as well as iso-alkanes ranging from C_7-C_{17} (Table 4). The water also contained a range of fatty acids between C_4-C_6 and alkanoic acids, including: C_5-C_{10}, C_{16}, C_{18} and unsaturated C_{18} (Table 4). Aromatic hydrocarbons were also detected, including: alkylated benzene, toluene, alkylated toluene, xylene, alkylated xylene, phenol, alkylated phenol, *p*-cresol, *o*-cresol, benzoic acid, and benzenediol (Table 4). Furthermore extractions detected naphthalene, alkylated naphthalene, alkylated cyclohexane, and cyclohexanol compounds. Notably, there was no evidence of alkyl- or benzyl-succinate compounds in the production water (Table 4). The extractions also detected several organic acids such as: α-hydroxy acids (glycolic, lactic, hydroxypropanoic, and hydroxybutanoic acids), dioic acids (succinic, pentanedioic, and hexanedioic acids), and other acids (2-butoxy acetic acid) (Table 4).

3.2 Molecular analysis of the microbial community

Barnett Shale production water was examined to determine the endogenous microbial community. Summaries of the 16S rRNA gene sequence libraries depicting the dominant classes and genera of bacteria in the production water are represented in Table 5. Proteobacteria was the most abundant phylum in the production water; of the Proteobacteria, Deltaproteobacteria were the most abundant and represent 41% relative abundance of the microbial community in the production water (Table 5). Clostridia

compose 10.2 %, Thermotogae 11.4%, and Gammaproteobacteria 10.5% of the relative abundance of the microbial community (Table 5). The most abundant genera in the production water were *Desulfobacter* (29.1%, Deltaproteobacteria), *Geotoga* (Thermotogae, 9.0%), *Marinobacterium* (Gammaproteobacteria, 7.4%), and *Desulfotignum* (Deltaproteobacteria, 4.6%) represented by relative abundance (Table 5).

3.3 Aerobic enrichment of microorganisms from production water

3.3.1 Initial aerobic enrichment of microorganisms in production water

Production water was amended with various hydrocarbon and heterotrophic substrates to determine if the endogenous microbial community can degrade them aerobically; the enrichment design is illustrated in Figure 1. Overall growth score is shown after three transfers, one transfer occurred every 30-40 days (Table 6). Growth was monitored via turbidity. The turbidity scores indicate the endogenous microbial community from the production water tank can grow with *n*-alkanes, fatty acids, and the positive control substrate (yeast extract plus tryptone). However, the microbial community did not grow in the presence of BTEX or oxidized BTEX compounds.

3.2.2 Additional aerobic enrichment of microorganisms in production water

Initial and progressive enrichments aimed to select for the dominant *n*-alkane and *n*-fatty acid-degrading bacteria in the production water. Summaries of the 16S rRNA sequence libraries for the initial and progressive amendments with *n*-alkanes and *n*-fatty acids are illustrated in Figures 2 and 3, respectively. The libraries depict a marked shift in the dominant classes of bacteria with continued *n*-alkane and *n*-fatty

acid amendment; Gammaproteobacteria increase and dominate while Thermotogae, Clostridia, and Deltaproteobacteria decrease or disappear. Synergistia and Alphaproteobacteria remain at low abundance in the progressive *n*-alkane-amended enrichments; Mollicutes are enriched in the *n*-fatty acid but not in the *n*-alkane-amended enrichment.

Summaries of the 16S rRNA sequence libraries for the initial and progressive amendments with *n*-alkanes and *n*-fatty acids depicting relative abundance of genera are depicted in Figures A1 and A2. *Roseovarius* (5.0%, Alphaproteobacteria), *Shewanella* (13.5%, Gammaproteobacteria), *Halomonas* (75.9%, Gammaproteobacteria), and *Marinobacter* (2.4%, Gammaproteobacteria) appear to increase the most in relative abundance after three transfers amended with *n*-alkanes (Table 7). Similarly, *Halomonas* (75.1%, Gammaproteobacteria), *Marinobacter* (2.4%, Gammaproteobacteria), *Dethiosulfatibacter* (7.3%, Clostridia), and *Acholeplasma* (20.8%, Mollicutes) appear to increase the most after progressive rounds of amendment with *n*-fatty acids. The aerobic conditions were not permissive for growth for *Desulfotignum* or *Desulfobacter* (Deltaproteobacteria), both decreasing to 0.0% of the relative abundance under amendment with either *n*-alkanes or *n*-fatty acids. *Geotoga* (Thermotogae) increased in abundance during initial rounds of amendment with both *n*-alkanes (64.5%) and *n*-fatty acids (63.3%) but decreased during the progressive enrichments. The initial amendment with *n*-alkanes appear to increase the relative abundance *Thalassospira* (0.2%, Alphaproteobacteria), *Arcobacter* (9.9%, Epsilonproteobacteria), and *Oleibacter* (2.5% Gammaproteobacteria) (Table 7) from

that seen in the production water but all had decreased by the end of the progressive enrichments.

Overall, amendment with *n*-alkanes and *n*-fatty acids selected for the Gammaproteobacteria *Halomonas* and *Marinobacter*. Significant differences in selection were that *Roseovarius* (Alphaproteobacteria) and *Shewanella* (Gammaproteobacteria) increased with *n*-alkane but not with *n*-fatty acid amendment. As well as *Thalassospira* (11.6% in initial *n*-fatty acid amendment, Alphaproteobacteria), *Oleibacter* (4.3% in initial *n*-fatty acid amendment, Gammaproteobacteria), *Dethiosulfatibacter* (7.3%, Clostridia), and *Acholeplasma* (20.8%, Mollicutes) increased with *n*-fatty acid but not *n*-alkane amendment (Table 7).

3.3 Isolation of Halomonas strains

Additional progressive amendment with *n*-fatty acids and *n*-alkanes aimed to enrich for the dominant *n*-fatty acid and *n*-alkane-degrading organisms in production water. The incubations containing *n*-fatty acids grew more quickly than those containing *n*-alkane, as assessed visually. Nine isolates were obtained from various rounds of the *n*-alkane (C_5-C_{10}) (7 isolates) and *n*-fatty acid (C_5-C_{10}) (2) amendments. The A11A isolate was amplified with 4 primers: fd1, rp2, P1, and D907R to yield a 1432 bp product. The other 8 isolates were amplified with D907R and P1 to obtain an approximately 550 bp product. All nine isolates were identified as 96-99% identical to 16S rRNA gene sequences of *Halomonas* species (data not shown).

3.3.1 Genomic and phylogenetic analysis of Halomonas A11A

Isolate A11A was obtained from the 10^{-4} dilution of the first progressive transfer with *n*-alkanes (illustrated in Figure 1). Phylogenetic analysis of the 1432 bp 16S rRNA gene sequence of the *Halomonas* A11A isolate is represented by a neighbor-joining phylogenetic tree depicting its relationship to *Halomonas* species and selected other known marine hydrocarbon degrading bacteria (Fig. 4). Based on multiple alignments (Clustal W and MUSCLE) and tree reconstruction of sequence-similarity calculations (neighbor-joining and maximum-likelihood) the 16S rRNA gene sequence of *Halomonas* A11A is most similar to that of several unclassified *Halomonas* strains: 99 % (1426/1432 bp) identical to *Halomonas* sp. HB.br (GU228481.1), 99 % (1425/1432 bp) identical to *Halomonas* sp. IB-559, and 99 % (1423/1429 bp) identical to *Halomonas* sp. JA6. The most closely related described species is *Halomonas alkalicola* CICC11012s (KU530128.1) which is 99 % (1418/1432 bp) identical. *Halomonas campisalis* LL1 was 97 % (1398/1432 bp) identical to the A11A isolate.

The assembled *Halomonas* A11A genome (3,264,921 bp) was screened for functional genes relevant to hydrocarbon degradation. KEGG KOBAS (KEGG Orthology Based Annotation System) 3.0 was used to detect functional genes in the *Halomonas* A11A genome (Xie et al., 2011). Aerobic hydrocarbon degradation genes specific for *n*-alkane and aromatic hydrocarbon degradation were detected in *Halomonas* A11A (Table 8). Enzyme sequences for alkane monooxygenase, *alk*B were detected but cytochrome p450 CYP 153 was not. Several dioxygenase enzymes were also detected: benzene dioxygenase (*bed)*, toluene dioxygenase (*tod*) catechol 2,3-dioxygenase (*dmp, xyl, cat*) a phenol dioxygenase and phenol hydroxylase (*dmp,*

pox). No enzymes for a xylene monooxygenase (*xylMA*), ethylbenzene dioxygenase (*etbA*), naphthalene 1,2-dioxygenase (*nah, nda, nhz, dnt*) or catechol 1,2-dioxygenase (*cat*) were detected in the genome (Table 8). The detection of these enzymes is limited to what is annotated in databases, we acknowledge that some enzymes are not well annotated and additional support for detection of some of these enzymes is necessary.

To more fully illustrate the functional metabolic pathways of interest in *Halomonas* A11A, fatty acid, alkane, and BTEX metabolic pathways were reconstructed using KEGG Mapper. Fatty acid metabolism in *Halomonas* A11A is illustrated in Figure 5 and 6; genes present in the genome are indicated in green and suggest several complete pathways for fatty acid metabolism are present. Not found in this genome assembly are genes coding for three acyl-CoA dehydrogenases (1.3.3.6, 1.3.8.9, 1.1.1.211), an enoyl-CoA dehydrogenase (4.2.1.17), one ligase enzyme (6.2.1.6), and an acyltransferase (6.2.1.20) used in long-chain (C_{13}-C_{22}) fatty acid metabolism. Figure 7 depicts alkane metabolism; here, nearly all enzymes are present for complete oxidation of alkanes, it has a rubredoxin reductase (1.18.1.1) but does not have rubredoxin reductase (1.18.1.4) and ferredoxin reductase (1.18.1.3). Genes for benzene metabolism are illustrated in Figure 8; the genome appears to contain sequences for several enzymes used in the initial oxidation reactions, such as benzene dioxygenase (1.14.12.3), phenol hydroxylase (1.14.13.-), phenol 2-monooxygenase (1.14.13.7), catechol 2,3-dioxygeanse (1.13.11.2) but is missing several down-stream enzymes. Similarly, the pathway for toluene degradation (Figure 9) features several of the same initial enzymes, such as toluene dioxygenase (1.14.12.3, 1.14.12.11) enzymes and aryl-alcohol dehydrogenase (1.1.1.90) and 4-cresol dehydrogenase

enzymes; however, the genome lacks several downstream genes involved in toluene degradation. The pathway for ethylbenzene degradation (Figure 10) in *Halomonas* A11A only features 2-hydroxy-6-oxo-octa-2,4-dienoate hydrolase (3.7.1.-) and acetyl-CoA transferase (2.3.1.16) enzymes. The putative pathway for xylene metabolism (Figure 11) in *Halomonas* A11A lacks the initial xylene monooxygenase (1.14.13.-) but contains the aryl-alcohol dehydrogenase (1.1.1.90) enzymes and additional enzymes similar to those for toluene and benzene metabolism. Table A2 lists the genes of interest in the fatty acid, alkane, and BTEX metabolic pathways.

3.3.2 Physiological analysis of Halomonas A11A

Halomonas A11A showed hydrocarbon degradation ability based on the preliminary screening in liquid medium and was chosen for additional investigation using oxygen respirometry. The isolate showed growth, as judged by turbidity, after three transfers with all tested *n*-alkane substrates and toluene but did not grow with BTEX or in the unamended medium. Based on the successful screening results, *Halomonas* A11A was chosen for additional testing with oxygen respirometry to quantify the rate of aerobic hydrocarbon degradation via oxygen consumption. Prior to inoculation and incubation the cells, were quantified via optical density and CFU (colony-forming units). Based on CFU calculations and OD measurements a *Halomonas* A11A culture of 0.5 OD equals 4.6×10^6 cells/mL.

3.3.3 Oxygen respirometry and hydrocarbon degradation by Halomonas A11A

Based on peak rates of oxygen uptake achieved with different inoculum size, 10% (0.21 μM/min) vs 5% (0.019 μM/min), a 10% (v/v) inoculum size from a 0.5

OD_{610} culture (e.g. 3.5 x 10^5 cells/mL) was determined to be the most appropriate for oxygen respirometry (Figure A3). Further optimization of conditions for oxygen respirometry tested 2,2,4,4,6,8,8-heptamethylnonane (HMN) as an inert carrier for the hydrocarbons to avoid possible toxic effects, solubility limitations, and volatilization of the hydrocarbons. The use of HMN was determined to be appropriate since it did not affect oxygen consumption values or rates of oxygen uptake when comparing triplicate cultures containing *n*-pentane versus cultures containing *n*-pentane in HMN (Figure 12). Likewise, substrate unamended controls with HMN added did not differ from previously observed unamended values without HMN (Figure 12). Cells were not washed prior to inoculation, instead, the carry-over of hydrocarbons from transfer to the unamended control was subtracted from the totals and not included in the calculations.

Initial screening of hydrocarbon degradation using individual C_5-C_{10}, C_{16} *n*-alkanes and toluene showed that all aliphatic hydrocarbons (C_5-$C_{10,}$ C_{16} *n*-alkanes) and the aromatic hydrocarbon, toluene, resulted in higher oxidation uptake (Figure 13) and total oxygen consumption (Figure 14) compared to the unamended plus heptamethylnonane (HMN) and sterile controls. All hydrocarbons appear to peak in rate of oxygen uptake between 68 and 76 hours after inoculation (Figure 13). Incubation with C_6 *n*-hexane and C_7 *n*-heptane yield the highest rates of oxygen uptake of 0.327 and 0.368 μM/min, respectively and the highest total oxygen consumption at 669.587 and 701.979 μM O_2, respectively at 80 hours after inoculation (Figure 14). The isolate did not consume oxygen in response to 0.1 M BTEX; oxygen rates and consumption were similar to the sterile heat-killed control (data not shown).

Molar O_2 consumption calculations to infer hydrocarbon oxidation rates were obtained from oxygen respirometry yields of total oxygen consumption and oxygen uptake rates calculated from Figures 13 and 14 and Table A3. Complete (> 75%) or partial oxidation was determined by calculating the percent of theoretical molar O_2 consumption derived from the experimental oxygen consumption versus the theoretical molar O_2 concentration required to completely oxidize the hydrocarbons (Table 9). Different concentrations of each hydrocarbon were used to have similar amounts of carbon in each experimental unit. The most complete or highest percent of theoretical oxygen consumption occurred with amendments of C_9 *n*-nonane (118%), followed by C_7 *n*-heptane (92.9%), C_6 *n*-hexane (82.4%), and C_5 *n*-pentane (76.2%) (Table 9). The lowest percent of theoretical oxygen consumption occurred when coupled with amendments of C_{10} *n*-decane (67.9%), toluene (64.5%), C_{16} *n*-hexadecane (56.6%), and C_8 *n*-octane (49.1%) (Table 9).

Following hydrocarbon degradation screening and optimization experiments the *Halomonas* A11A isolate was used to obtain average experimental yields for oxygen consumption during hydrocarbon oxidation inoculated from cells grown on n-pentane versus grown on n-decane. All rates were obtained by comparison to triplicate unamended HMN controls and heat-killed controls. In the first experiment, cells grown on C_5 *n*-pentane were used as an inoculum and inoculated in bottles amended with 20 μM C_5 *n*-pentane and C_{10} *n*-decane (Figures 15). The total oxygen consumption over 68 hours for the C_5 *n*-pentane amended bottles and the C_{10} *n*-decane amended bottles were 300.8 and 272.5 μM, respectively (Figure 15). Cells grown on C_5 *n*-pentane and amended with C_{10} *n*-decane appear to lag in oxygen uptake

compared to the cells amended with C_5 *n*-pentane. The C_5 *n*-pentane-amended bottles increased steadily in oxygen uptake and peaked at 0.151 μM/min at 60 hours; whereas, the C_{10} *n*-decane-amended bottles increased steadily in oxygen uptake rate until 37 hours then slowed, peaked at hour 60 at 0.107 μM/min, and was sustained for the remainder of the experiment (Figure A4). In both cases, the rates of oxygen uptake and total oxygen consumption were greater than what was observed with the unamended HMN control. The total O_2 consumption and molar calculations suggest the C_5-grown *Halomonas* A11A cells completely oxidized *n*-pentane C_5 (94.5% of theoretical) but did not completely oxidize the *n*-decane C_{10} (60.6% of theoretical) (Table 10).

The test was repeated with cells grown on C_5 *n*-pentane as an inoculum and inoculated into bottles amended with 20 μM C_{16} *n*-hexadecane and toluene (Figure 16) to contrast extremes of the hydrocarbon range tested (e.g. C_5 versus C_{16}) and screen for induction of a different enzyme system (e.g. alkane monooxygenase versus toluene dioxygenase). The total oxygen consumption over 72 hours for the C_{16} *n*-hexadecane amended and the toluene amended bottles was 289 μM and 337 μM O_2, respectively (Figure 16). Oxygen uptake rates for C_{16} *n*-hexadecane and toluene were very similar; the oxygen uptake rate for C_{16} *n*-hexadecane peaked at 0.2 μM/min at 68 hours and the uptake rates for toluene peaked at 0.177 at 60 hours (Figure A6). The rates in oxygen uptake and total oxygen consumption for C_{16} *n*-hexadecane and toluene are greater than what was observed with the unamended HMN control. Here, the C_5-grown *Halomonas* A11A cells did not appear to completely oxidize C_{16} *n*-hexadecane

(72.3%) or toluene (37.0%) based on total oxygen consumption and molar calculations (Table 10).

The test was repeated with cells grown on C_{10} *n*-decane as an inoculum and inoculated in bottles amended with 20 μM C_5 *n*-pentane or C_{10} *n*-decane (Figure 17). Here, the C_5 *n*-pentane amended bottles peaked in oxygen uptake at 0.218 μM/min at 72 hours and the C_{10} *n*-decane amended bottles peaked in oxygen uptake at 0.239 μM/min at 92 hours; these peak rates occurred later than the previous experiment with cells grown on C_5 *n*-pentane (Figure A5). The total oxygen consumption over 80 hours for the C_5 *n*-pentane amended bottles and C_{10} *n*-decane amended bottles is 547.5 and 611.8 μM, respectively (Figure 17). The rates of oxygen uptake and total oxygen consumption observed with the C_{10} *n*-decane-grown cells amended with C_5 *n*-pentane and C_{10} *n*-decane are higher than those observed in the unamended HMN controls. Cells grown on C_{10} *n*-decane appear to generate complete oxidation of C_5 *n*-pentane (99.2%) and C_{10} *n*-decane (104.4%) (Table 10).

Collectively, cells grown on C_{10} *n*-decane appear to generate the most complete oxidation and the highest percent of theoretical for C_5 *n*-pentane (99.2%) and C_{10} *n*-decane (104.4%) (Table 10). Cells grown on C_5 *n*-pentane appear to generate complete oxidation when amended with C_5 *n*-pentane (94.5%) but suggest incomplete oxidation when amended with C_{10} *n*-decane (60.6%) (Table 10). The lowest experimental yields and percent of theoretical were obtained from C_5 *n*-pentane-grown cells amended with C_{16} *n*-hexadecane (72.3%) and toluene (37.0%), suggesting incomplete oxidation of the hydrocarbons.

4.0 Discussion

Gammaproteobacteria were the most abundant class of bacteria after amendment with *n*-alkanes and *n*-fatty acids in production water. More specifically, *Halomonas* dominated all of the progressive enrichments amended with *n*-alkanes and *n*-fatty acids. Isolation of *Halomonas* strain A11A allowed the direct testing of *n*-alkanes C_5-C_{10}, C_{16}, and toluene degradation and quantification of hydrocarbon oxidation rate. These results suggest that Gammaproteobacteria such as *Halomonas* play an important role in aerobic hydrocarbon degradation in petroleum production facilities.

Aerobic hydrocarbon biodegradation in the production water is supported by additional observation. Organic extractions of the production water (Table 4) provided evidence for aerobic hydrocarbon degradation; the detection of aerobic intermediates including, cresols, phenols, catechols, cyclohexanols, and various fatty acids indicate active aerobic hydrocarbon degradation occurs in the production water (Gibson et al., 1968; 1984; Smith et al., 1991). Conversely, some key indicators of anaerobic hydrocarbon degradation were absent; no benzyl- or alkylsuccinates or *e*-phenylitaconate were detected (Leuthner et al., 1998; Beller et al., 2000; Callaghan et al., 2010; Aktas et al., 2012). The absence of alkylsuccinates could either suggest very little anaerobic aliphatic hydrocarbon degradation occurs in the tank or possibly anaerobic alkane degradation does not occur through addition to fumarate but through a different mechanism. Similarly, the absence of signature metabolites for anaerobic aromatic hydrocarbon degradation, benzylsuccinates, suggests either very little or only transient anaerobic aromatic hydrocarbon degradation occurs or that the aromatic

hydrocarbons are activated through another mechanism. Considering the presence of many different aerobic hydrocarbon intermediates and the absence of anaerobic hydrocarbon degradation signature metabolites, we assume aerobic oxidation of the hydrocarbons was an important degradation pathway in this production water tank.

A number of methods examined aerobic hydrocarbon degradation potential in the production water. Several isolates were obtained through amendment with hydrocarbons. Based on the1432 bp 16S rRNA gene sequence, isolate A11A, enriched through amendment with hydrocarbons, was identified as most closely related to *Halomonas* (*Oceanospirillales*). The closest characterized species is *Halomonas alkalicola* CICC11012s (99 %). *H. alkalicola* CICC11012s was originally isolated from a household product plant in China (Tang et al., 2017). Hydrocarbon degradation ability has not been tested on this *H. alkalicola* CICC11012s. The most closely related *Halomonas* strain to *Halomonas* A11A is *Halomonas* HB.br (GU228481.1, 99%) (McSweeney et al., 2011). *Halomonas* HB.br was isolated from an oxalate degradation bioreactor; here, the authors detected *Halomonas* sp. in three separate bioreactors and reasoned that these microorganisms play a role in degradation of oxalate and other organic acids.

Halomonas A11A demonstrates the potential to oxidize a wide range of hydrocarbons such as, *n*-alkanes C_5-C_{10} and C_{16} as well as toluene (Tables 9 and 10) and to play an important role in hydrodcarbon degradation. Upon initial examination, *Halomonas* A11A appears to oxidize C_5-C_{10} *n*-alkanes more completely rather than longer C_{16} *n*-alkane or aromatic toluene hydrocarbons; these findings are comparable

to the range of *n*-alkanes detected in the original production water (Table 4). The most complete oxidation (>75 % of theoretical) was achieved with *Halomonas* A11A cells grown on C_{10}, followed by amendments with C_5 or C_{10} (Table 10). The reconstructed pathways depicting alkane metabolism also support these findings; the *Halomonas* A11A genome appears to contain nearly all of the gene sequences coding for enzymes required for complete hydrocarbon oxidation; however, no gene sequences for the ferredoxin or rubredoxin reductase were identified. This could be due to differences in the enzyme sequence compared to those in the KEGG database or to an incomplete genome assembly The highest oxidation rates and most complete oxidation occurred with *n*-pentane C_5 (99.2 %) and *n*-decane C_{10} (104.4 %) when grown previously with *n*-decane C_{10} (Table 10). The experimental yields that were >100 % are likely caused by carry-over of *n*-decane C_{10} in the inoculum. These experiments also allowed us to hypothesize if a different enzyme system would be induced by growing cells on a short C_5 *n*-alkane and then amending them with longer C_{10} or C_{16} *n*-alkanes. We can see that when cells are grown on C_5 *n*-pentane and amended with C_{10} *n*-decane (60.6%) they are not as efficient compared to when the cells are grown on C_{10} *n*-decane and amended with C_{10} *n*-decane (104.4%) (Table 10). This may suggest a difference in affinity for the alkane monooxygenase enzyme or more than one copy of the gene.

Conversely, *Halomonas* A11A cells grown on C_5 and amended with C_{16} (72.3 %) and toluene (37 %) generated the lowest experimental percentages, suggesting less efficient or incomplete oxidation (Table 10) or insufficient time for induction of a different enzyme system. Moreover, the reconstructed pathways for benzene, toluene,

ethylbenzene, and xylene were far from complete. Collectively, these findings could suggest *Halomonas* A11A lacks the full suite of enzymes for complete oxidation of aromatic compounds or may imply that the enzyme systems used to activate C_{16} and toluene are different than those used to activate C_5-C_{10} *n*-alkanes and were not active in the cell resulting in less efficient oxidation as enzyme systems were switched. Further, the enzyme kinetics for long chain alkanes and for aromatic toluene have different kinetic affinities and specific activities than short- and mid-chain length *n*-alkanes. While very little data exists for mono- and dioxygenase enzyme kinetics for *Halomonas* sp. some reference material exists for *P. oleovorans* and *P. putida* and may be applicable due to the phylogenetic similarity between *Pseudomonas* and *Halomonas*. The K_m values for hexane, octane, and decane were determined in *P. oleovorans* and used to examine oxidation rates catalyzed by a monooxygenase (Peterson et al., 1967). At saturating concentrations (hexane (K_m = 6.0 mM), octane (K_m = 0.77 mM), and decane (K_m = 0.58 mM)) they determined octane was oxidized most rapidly (3.3 μmoles/min/mg protein) followed by hexane (2.5 μmoles/min/mg protein) then decane (1.3 μmoles/min/mg protein). They also note that the K_m for hexane hydroxylation is much higher than those for octane and decane, suggesting the purified monooxygenase enzyme in their study has a lower affinity for hexane. In a separate study, the specific activity of a monooxygenase, also described as an alkane and fatty acid ω-hydroxylase, in *P. oleovorans* was examined with octane as the substrate (Ruettinger et al., 1977). Here, they report the specific activity of the monooxygense to be 1.23 μmol/min/mg, which is three times greater than these authors previously reported (McKenna et al., 1970). More recently, the specific

activities of benzene and toluene dioxygenases have been investigated in *P. putida* and may provide insight on the kinetics of BTEX oxidation in *Halomonas* A11A (Bagnéris et al., 2005). Here, they find significant differences in substrate specificity between the benzene and toluene dioxygenases despite the similarities in catalytic properties, structure, and gene organization; the specific activity (μmol/min/mg) of the toluene dioxygenase with benzene as a substrate was 0.045, with ethylbenzene as a substrate was 0.054, and with toluene as the substrate was 0.056; all of which are significantly lower than the specific activity recorded for octane oxidation in *P. putida*. It is not surprising that mid-length *n*-alkanes have a higher specific activity than aromatic hydrocarbons; taken together, these findings combined with the results obtained in our study may suggest that *Halomonas* A11A is capable of degrading aromatic hydrocarbons, such as toluene, but the enzyme system was not induced until the cells were grown with toluene, resulting in less efficient oxidation. Exposure of the cells on toluene would likely result in more efficient oxidation. A more thorough investigation of mono- and dioxygenase kinetics in *Halomonas* and degradation studies with C_{16}- or toluene-grown cells may provide insight on these questions.

Complete oxidation of alkanes leads to the production of CO_2 while generating cell growth and proliferation (van Beilen et al., 2003). Alternatively, the incomplete oxidation of *n*-alkanes and other hydrocarbons and formation of these partially oxidized by-products could lead to them serving as substrates for other bacteria. Short-chain ($<C_{12}$) *n*-alkanes are notably difficult targets for degradation by bacteria due to their high water solubility and toxicity (van Beilen et al., 1994; Belhaj et al., 2002; Smits et al., 2002). This is one of the first reports of a *Halomonas* isolate to degrade a

short-chain (C_5) *n*-alkane and toluene hydrocarbons in pure culture. Based on the data shown here, the ability to oxidize a wide range of hydrocarbons including short-chain *n*-alkanes, *Halomonas* A11A may be a candidate for use as a bioremediation tool.

One previous study estimated *n*-alkane degradation potential by isolates, including two *Halomonas* strains, from an oil-contaminated site through comparative gas chromatography of *n*-alkane-amended and unamended controls (Hassanshahian et al., 2012). Here, they note medium length (C_{12}-C_{18}) *n*-alkanes were degraded more completely (greater than 50%) than *n*-alkanes with shorter (C_9-C_{11}) or longer (C_{19}-C_{25}) chain lengths. The authors in this study determined the *alkB* group III genes, characterized to catalyze the degradation of long-chain *n*-alkanes (>C_{16}) were present in the two *Halomonas* strains (Kohno et al., 2002; Hassanshahian et al., 2012). Our study adds to the list of possible targets for aerobic *n*-alkane degradation for *Halomonas* sp. In a separate study, Feknous and others measured *n*-alkane degradation by *Halomonas venusta* NY-8 using turbidity measurements (Feknous et al., 2017). Here, they noted a lag period in the presence of *n*-hexane, but turbidity increased from an OD of 0.04 to 0.277 after 216 hours. In the presence of *n*-heptane growth was stimulated within 48 hours, ODs increasing from 0.107 to 0.248 within 96 hours. The most significant growth was observed in the presence of *n*-decane, where they observed ODs to increase from 0.135 to 0.902 within 24 hours. Based on growth kinetics determined by turbidity measurements they conclude *H. venusta* NY-8 can degrade a wide range of hydrocarbons at different rates, with the maximum rate occurring at 3.12 mg/L *n*-heptane. Long chain alkane hydroxylases, *almA* (>C_{32}) and *ladA* (C_{15} to C_{36}) (Feng et al., 2007; Throne-Holst et al., 2007) were not detected in the

genome of A11A. While this information suggested *Halomonas* sp. can grow in the presence of lower molecular weight *n*-alkanes our study aimed to provide a more quantitative measurement of *n*-alkane oxidation.

Compared to other hydrocarbon-degrading microorganisms, such as *Pseudomonas* and *Marinobacter,* very little is known about the role of *Halomonas* sp. in aerobic hydrocarbon degradation. *Halomonas* represented a small percentage (2.2%) of the relative abundance in the original production water but may play a significant role in aerobic hydrocarbon degradation. Several studies have detected *Halomonas* sp. in hydrocarbon-impacted environments (Wang et al., 2007; Mnif et al., 2009; Hassanshahian et al., 2012; Feknous et al., 2017). Others have quantified surfactant production by *Halomonas* sp. and observed strains to degrade (non-hydrocarbon) aromatic compounds (Garcia et al., 2004; Mnif et al., 2009). However, few have directly isolated strains capable of hydrocarbon degradation or quantified the hydrocarbon degradation rates; even fewer have identified genes responsible for hydrocarbon degradation (Garcia et al., 2004; Hassanshahian et al., 2012; Feknous et al., 2017).

Oxygenases initiate the aerobic oxidation of alkanes by introducing oxygen atoms derived from molecular oxygen into the carbon-hydrogen chain to produce primary alcohols and later fatty acids that may enter downstream metabolic pathways (Hayaishi et al., 1955; van Beilen et al., 2003). For aliphatic hydrocarbons, such as *n*-alkanes, the most common and well-characterized oxygenases or monooxygenases include the *alkB* alkane hydroxylase system. This membrane-bound alkane hydroxylase is well described in *Pseudomonas putida* (*oleovorans*) GPo1 and

Pseudomonas aeruginosa (Kok et al., 1989ab; van Beilen et al., 1994, Das et al., 2015). In *P. putida* GPo1 the proteins are encoded on a large plasmid, known as the OCT plasmid (Chakrabarty et al., 1973). Two *alk* clusters are encoded on the plasmid; the *alkBFGHJKL* includes the *alkB* promoter, a membrane-bound soluble electron transport system with two rubredoxins (*alkF* and *alkG*), a NAD-dependent aldehyde dehydrogenase (*alkH*), an alcohol dehydrogenase (*alkJ*), an acyl-coA synthetase (*alkK*), and an outer-membrane protein (*alkL*) (Kok et al., 1989a; van Beilen et al., 1992; 1994). The other *alk* cluster (*alkST*) encodes for a rubedoxin reductase (*alkT*) and the positive regulator for both *alk* clusters (*alkS*) (Kok et al., 1989b). In general, the substrate range for *alkB* is reported to range from C_5-C_{16} and may oxidize at the terminal, sub-terminal, or biterminal positions (Smits et al., 2002; van Beilen et al., 2003). Others have described the *alkB* system in three groups based on phylogenetic comparisons (Kohno et al., 2002; Hassanshahian et al., 2012). The *alkB* system also shows promise as a gene marker for detecting *n*-alkane-degrading bacteria in contaminated environments (van Beilen et al., 2003).

To date, few studies have characterized the alkane hydroxylase systems in *Halomonas*. Hassanshahian and others detected *alkB* genes in several isolates including two *Halomonas* strains, using several *alkB*-specific primers (Kohno et al, 2002; Hassanshahian et al., 2010; 2012). The lack of available annotated *alkB* sequences for *Halomonas* sp. made detection and comparison difficult; we detected *alkB* in *Halomonas* A11A through KEGG KOBAS gene sequence matches; however we do not know how many copies of *alkB* are in the *Halomonas* A11A genome.

Bacterial cytochrome p450 systems have also been reported to catalyze *n*-alkane oxidation. Cytochrome p450s are ubiquitous catalysts found in all domains of life able to hydroxylate and oxidize a wide range of compounds (van Beilen et al., 2005). The p450-like enzyme responsible for *n*-alkane oxidation was characterized in *Acinetobacter* and classified as class I p450 family CYP153 (Asperger et al., 1981; Maier et al., 2001). To date, few have identified or detected cytochrome class I CYP153 enzymes in any *Halomonas* sp. One study detected one CYP153 in a strain of *Halomonas ventosae* (AY268080) (Wang et al., 2010). Here, they amplified to detect both CYP153 p450 and *alkB* genes using degenerate primers of 800 and 550 bp products, respectively, but no PCR products were verified by sequencing. Although they did not detect an *alkB* alkane monooxygenase they did detect, for the first time, one copy of the CYP153 gene in a *Halomonas* sp. They also imply that *Halomonas* and the other bacteria in their study, related to *Alcanivorax dieselolei* B-5^{T}, may have acquired the CYP153 through horizontal gene transfer based on the high similarity in phylogeny among the isolates based on alignment of the putative cytochrome p450 CYP153 family. In our study, we did not detect a cytochrome p450 CYP153 family alkane hydroxylase gene; and no other alkane monooxygenases (*ladA*, almA) were detected, suggesting *Halomonas* A11A degrades *n*-alkanes via the *alkB* alkane hydroxylase gene system.

Besides aliphatic hydrocarbons, aromatic and polycyclic aromatic hydrocarbons are of great concern as pollutants in oil-impacted environments. *Halomonas* sp. are not the most abundant species of bacteria associated with these environments but some studies have characterized *Halomonas* sp. to degrade or grow

with a range of aromatic compounds or cleave aromatic rings (Romano et al., 1996; Ventosa et al., 1998; Melcher et al., 2002; Alva et al., 2003). More than one study has shown that *Halomonas* sp. can degrade phenol and benzoate (Alva et al., 2003; Garcia et al., 2005; Sei et al., 2009; Lu, et al., 2015). Compared to aliphatic hydrocarbons, even less is known about the degradation of BTEX compounds by *Halomonas* sp. In one study, *Halomonas* sp. were associated with an enrichment culture composed largely of Gammaproteobacteria obtained from a hypersaline environment that degraded benzene and toluene in up to 29% NaCl; however no toluene- or benzene-degrading *Halomonas* isolates were obtained or tested directly (Sei et al., 2009).

Aromatic hydrocarbons are activated by mono- and dioxygenases in aerobic environments (El-Naas et al., 2014). The initial oxidation produces catechol compounds, which are then targeted by a series of ring cleavage reactions to ultimately produce pyruvate and enter the Krebs cycle (Smith et al., 1990; Cozzarelli et al., 2003). Several studies have detected catabolic genes involved in aromatic compound degradation in *Halomonas* sp. Specifically, one group detected sequences for catechol 1,2-dioxygenase (FN997643), 83% sequence similarity to the *Halomonas organivorans* partial (*catA*) gene sequences, and phenol hydroxylase (GQ281096), 79% similar to the *Pseudomonas* sp. DHS3Y phenol hydroxylase alpha subunit gene sequence, enzymes in a *Halomonas* isolate capable of phenol degradation (94% of 500 mg/L phenol) (Lu et al., 2015). Others have isolated catechol 1,2-dioxygenase (*catRBCA*) gene cluster as well as the benzoate catabolic gene cluster (*benAB*) from *Halomonas organivorans* G-16.1 when grown with phenol and benzoic acid (de Lourdes Moreno et al., 2011). Few studies have isolated *Halomonas* sp. able to

degrade BTEX compounds in pure culture; in our study we obtained quantitative evidence for toluene oxidation by *Halomonas* A11A as well as genomic evidence for a range of aromatic hydrocarbon degradation genes. We detected sequences for benzene (*bed*) and toluene (*tod*) dioxygenases, which catalyze the initial oxidation of benzene and toluene aromatic rings, and phenol hydroxylase P0 protein (*dmp, pox*), phenol 2-monooxygenase, and phenol dioxygenase enzymes, which are used in the initial attack of aromatic hydrocarbons (Fong et al., 1996). Catechol 2,3-dioxygenase (*dmp, xyl, cat*) enzyme sequences and enzymes for complete degradation to produce formate, pyruvate, and acetyl-CoA were also detected (Figure 8, Table 8, Table A2). No evidence for gene sequences encoding for xylene monooxygenase (*xylMA*), ethylbenzene dioxygenase (*etbA*), naphthalene 1,2-dioxygenase (*nah, ndo, nhz, dnt*), or catechol 1,2-dioxygenase were detected. While some of these enzymes and others were not detected, it may be due to a lack of available annotated sequences for *Halomonas* sp. and/or an incomplete genome for A11A. The presence of gene sequences coding for these enzymes in *Halomonas* A11A suggests the isolate may be capable of complete oxidation of several aromatic hydrocarbons, including benzene and toluene, and is supported by the toluene oxidation observed in our study. To more fully support this hypothesis, comparative BLAST analysis of nucleotide sequences for the genes of interest and parallel degradation studies are necessary.

Many other microorganisms were enriched with *n*-alkane amendment and may play an important role in the production water. *Marinobacter* and *Marinobacterium* (*Alteromonadales*) are commonly associated with hydrocarbon and petroleum-contaminated environments and were present at 0.1% and 7.4%, respectively, of the

relative abundance in the original production water (Huu et al., 1999). *Marinobacter* species are one of the most common aerobic marine microorganisms associated with laboratory and *in-situ* hydrocarbon degradation (Gauthier et al., 1992; Huu et al., 1999; Striebich et al., 2014). Like many other hydrocarbonoclastic bacteria, *Marinobacter* employs the *alkB* alkane monooxygenase for alkane degradation but has shown potential to degrade a wide variety of hydrocarbons such as diesel fuels, jet fuels, and polycyclic aromatic hydrocarbons (PAH); some studies exhibit *Marinobacter* growing on hexadecane or single PAHs as sole carbon sources (Vila et al., 2010; Smith et al., 2013; Striebich et al., 2014). The relative abundance of *Marinobacter* did not significantly increase with amendment with *n*-alkanes or *n*-fatty acids and ended at 2.4% with both after the progressive enrichments were complete. This slight increase in abundance of *Marinobacter* in both types of enrichment may suggest that *Marinobacter* is a participant in the degradation of complex organics such as *n*-alkanes and *n*-fatty acids. A close relative of *Marinobacter*, *Marinobacterium* was present in the production water at 7.4% and has been associated with hydrocarbon-contaminated environments, oil-water separator tanks, hypersaline oil-impacted environments, and shown potential to degrade some hydrocarbons even under sulfate-reducing conditions (Yakimov et al., 2005; Yuehui et al., 2008; van der Krann et al., 2010; Sherry et al., 2013). *Marinobacterium* was under the limit of detection (0.0%) at the end of the progressive enrichments amended with both *n*-alkanes and *n*-fatty acids but two strains were isolated using a different enrichment protocol and discussed in more detail in Appendix 2.

Some microorganisms commonly associated with hydrocarbon-impacted environments or known to degrade hydrocarbons were below the limit of detection in the original production water but appear as isolates or in later enrichments. *Oleibacter* sp., another Gammaproteobacterium, (*Oceanospirillales*) exhibits *n*-alkane-degradation ability (Teramoto et al., 2011). *Oleibacter marinus* 2O1 was isolated from seawater after amendment from crude oil. In the initial round of enrichments with *n*-alkane amendments, *Oleibacter* increased from below the limit of detection (0.0 %) in the original water to 2.4 % but is not detected after the final rounds of enrichments *Roseovarius,* an Alphaproteobacterium, has been associated with oil-impacted environments and identified to degrade a range of hydrocarbons including crude oil, aliphatic hydrocarbons (C_{10}-C_{35}), and polycyclic aromatic hydrocarbons (Harwati et al., 2007; Lai et al., 2011; Kimes et al., 2013; Gallego et al., 2014; Kappell et al., 2014). *Roseovarius* was not isolated from the production water but did increase from below detection (0.0 %) in the original production water to 5.0 % after amendment with *n*-alkanes. Since *Roseovarius* was not detected in the *n*-fatty acid-amended enrichments we propose that *Roseovarius* may have a more direct role in the oxidation of hydrocarbons. *Shewanella,* another Gammaproteobacterium (*Alteromonadales*), is recognized as iron-reducing bacterium (Caccavo et al., 1992; Myers et al., 1994; Heidelberg et al., 2002). This problematic microorganism is a facultative anaerobe with a versatile metabolism capable of dissimilatory iron reduction, sulfite reduction, and iron sulfide production, all of which have been linked to biocorrosion events in pipelines or storage tanks (Gerdes et al., 2005; Beech et al., 2010; Lutterbach et al., 2009). In the original production water *Shewanella* was below the limit of detection

(0.0 %) but increased to 13.5 % after amendment with *n*-alkanes; *Shewanella* may play a major role as an *n*-alkane degrader in the production water as well as participate in the biocorrosion process. These microorganisms, as well as others in the production water, are part of a larger community that contributes to aerobic hydrocarbon degradation and may participate in downstream biocorrosion events.

A few microorganisms were only detected in the *n*-fatty acid-amended enrichments. These microorganisms, *Dethiosulfatibacter* (7.3%, Clostridia) and *Acholeplasma* (20.8%, Mollicutes) most likely play a larger role in cycling fatty acids and partially oxidized organics in the production water instead of directly oxidizing hydrocarbons. We also observed *Geotoga* (*Thermotogae*), an anaerobe tolerant to some oxygen (Davey et al., 1993), to increase in relative abundance during the initial enrichments but this was likely due to enrichment bottles becoming anoxic and permitting the growth of surviving anaerobes rather than enriching for *n*-alkane or *n*-fatty acid degraders; *Geotoga* is not known to degrade *n*-alkanes but is detected in hydrocarbon-impacted environments (Grassia et al., 1996; Magot et al., 2000) and was present (9.0%) in the original production water.

Our hypothesis, that Gammaproteobacteria in the production water degrade *n*-alkanes using a monooxygenase such as the *alkB* or cytochrome p450 systems, has been supported by our findings. Considering the enrichment of *Halomonas* with *n*-alkane amendment, the complete oxidation of a wide range of *n*-alkanes, and the detection of an *alkB* in *Halomonas* A11A, we report here that *Halomonas* A11A, representing Gammaproteobacteria, degrades *n*-alkanes in the production water via

monooxygenase gene systems. This study is among the first to report *Halomonas* as a hydrocarbonoclastic microorganism capable of degrading short-chain (C_5-C_{10}) and long-chain (C_{16}) *n*-alkanes as well as aromatic hydrocarbons like toluene. We propose that *Halomonas* and other Gammaproteobacteria oxidize *n*-alkanes and other hydrocarbons both completely and incompletely in the production water and contribute to biocorrosion by supplying corrosive species, such as sulfidogenic and fermentative microorganisms, with partially oxidized substrates. Therefore, we propose that monitoring the aerobic microbial community in production water tanks and other sites where oxygen intrusion is common is equally as important as monitoring for sulfidogenic and fermentative species. Methods to monitor for aerobic hydrocarbon degradation could include detection of mono- and dioxygenase gene targets (*alkB*, CYP153, *tod*, *bed*, catechol dioxygenases etc.) and/or signature metabolites indicative of aerobic hydrocarbon degradation. Further, we propose that production water tanks and other production equipment be kept anoxic when possible; preventing oxygen exposure to production water will slow down the hydrocarbon degradation process and slow the proliferation of sulfide and acid production.

Many production water tanks experience chronic corrosion enhanced by microbial activity. Identifying microorganisms in the tank and how they contribute to the corrosion and degrade the hydrocarbons, is valuable to the oil and gas industry and for human health. Characterizing the microorganisms and their physiology promotes further understanding in methods to mitigate corrosion events and ultimately prevent the release of petroleum products. The majority of literature focuses on the impact of

sulfur cycling in biocorroded environments but hydrocarbon-degrading and heterotrophic microorganisms remain largely uncharacterized in their role in biocorrosion. *Halomonas* spp. may also contribute more directly to biocorrosion through cycling sulfur or through surfactant production but this was not the focus of this study (Ventosa et al., 1998; Mnif et al., 2009). Further, we only examined one isolate; in the production water system the competition with other organisms and syntrophic behavior of the microbial community may result in more partially oxidized metabolites rather than complete oxidation of the hydrocarbons. Together, the detection, isolation, and enrichment of a wide range of bacteria including Gamma-, Alpha-, Epsilon-, and Deltaproteobacteria allow us to better understand how they function in the production water and continue to contribute knowledge regarding the interactions that occur in production water and petroleum systems. Aerobic heterotrophs and hydrocarbonoclastic bacteria, lithotrophic sulfide oxidizers, chemolithotrophic iron-reducers, and anaerobic sulfate-reducing bacteria are all present in the production water; together, under the right conditions, these microorganisms may contribute to biocorrosion in the production water tanks and other production equipment.

References

Abed RM, & Köster J. (2005). The direct role of aerobic heterotrophic bacteria associated with cyanobacteria in the degradation of oil compounds. *Int Biodeter Biodegr*, *55*(1), 29-37.

Aeckersberg F, Bak F, Widdel F. (1991). Anaerobic oxidation of saturated hydrocarbons to CO2 by a new type of sulfate-reducing bacterium. *Arch Microbiol* 156:5–14

Aeckersberg F, Rainey FA, & Widdel F. (1998). Growth, natural relationships, cellular fatty acids and metabolic adaptation of sulfate-reducing bacteria that utilize long-chain alkanes under anoxic conditions. *Arch Microbiol*, *170*(5), 361-369.

Aeckersberg F, Rainey FA, Widdel F. (1998). Growth, natural relationships, cellular fatty acids and metabolic adaptation of sulfate-reducing bacteria that utilize long-chain alkanes underanoxic conditions. *Arch Microbiol* 170:361–369

Adams JM, Constable JV, Guenther AB, & Zimmerman P. (2001). An estimate of natural volatile organic compound emissions from vegetation since the last glacial maximum. *Chemosphere 3*(1), 73-91.

Adkins JP, Cornell LA & Tanner RS (1992) Microbial composition of carbonate petroleum reservoir fluids. *Geomicrobiol J* 10: 87–97.

Aitken CM, Jones DM, & Larter SR (2004). Anaerobic hydrocarbon biodegradation in deep subsurface oil reservoirs. *Nature*, *431*(7006), 291.

Aitken CM, Jones DM, Maguire MJ, Gray ND, Sherry A, Bowler BFJ, ... & Head IM. (2013). Evidence that crude oil alkane activation proceeds by different mechanisms under sulfate-reducing and methanogenic conditions. *Geochim Cosmochimica Ac*, *109*, 162-174.

Aktas DF, Lee JS, Little BJ, Duncan KE, Perez-Ibarra BM, & Suflita JM (2013). Effects of oxygen on biodegradation of fuels in a corroding environment. *Int Biodeter Biodegr*, *81*, 114-126.

Aktas DF, Lee JS, Little BJ, Ray RI, Davidova IA, Lyles CN, Suflita JM, (2010). Anaerobic metabolism of biodiesel and its impact on metal corrosion. *Energy Fuels* 24, 2924-2928.

Albaigés J. (1989). Marine pollution: an introduction. *Mar Pollu* (Albaiges J, ed), pp 1-10. Hemisphere Publishing Corp., New York.

Alfreider A & Vogt C. (2007). Bacterial diversity and aerobic biodegradation potential in a BTEX contaminated aquifer. *Water Air Soil Poll* 183, 415–426.

Altenschmitd U and Fuchs G. (1991). Anaerobic degradation of toluene in denitrifying *Pseudomonas* sp.: indication for toluene methylhydroxylation and benzoyl-CoA as central aromatic intermediate. *Arch Microbiol 156*: 152-158.

Altschul SF, Madden TL, Schafferetal AA. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25(17):3389–3402.

Alva VA, & Peyton BM (2003). Phenol and catechol biodegradation by the haloalkaliphile *Halomonas campisalis*: influence of pH and salinity. *Environ Sci Technol*, *37*(19), 4397-4402.

Andreoni, & Gianfreda, L. (2007). Bioremediation and monitoring of aromatic-polluted habitats. *Appl Microbiol Biot*, *76*(2), 287-308.

Ananina LN, Plotnikova EG, Gavrish EY, Demakov VA, & Evtushenko LI (2007). *Salinicola socius* gen. nov., sp. nov., a moderately halophilic bacterium from a naphthalene-utilizing microbial association. *Microbiology*, *76*(3), 324-330.

Arp DJ. (1999). Butane metabolism by butane-grown *Pseudomonas butanovora. Microbiology.* 145: 1173–1180.

Ashraf W, Mihdhir A, Murrell JC (1994) Bacterial oxidation of propane. *FEMS Microbiol Lett* 122: 1–6.

Asperger O, Naumann A, & Kleber HP. (1981). Occurrence of cytochrome P-450 in *Acinetobacter* strains after growth on n-hexadecane. *FEMS Microbiol Lett*, *11*(4), 309-312.

Atlas RM. (1981). Microbial degradation of petroleum hydrocarbons: an environmental perspective. *Microbiol Rev*, vol. 45, no. 1, pp. 180–209.

Attaway HH, & Schmidt MG. (2002). Tandem biodegradation of BTEX components by two *Pseudomonas* sp. *Curr Opin Microbiol*, *45*(1), 30-36

Aüllo T, Ranchou-Peyruse A, Ollivier B, & Magot M. (2013). *Desulfotomaculum* spp. and related gram-positive sulfate-reducing bacteria in deep subsurface environments. *Front Microbiol*, *4*.

Austin RN, Chang HK, Zylstra GJ, Groves JT. (2000). The non-heme diiron alkane monooxygenase of *Pseudomonas oleovorans* (AlkB) hydroxylates via a substrate radical intermediate. *J Am Chem Soc* 122: 11747–11748.

Austin RN & Groves JT. (2011). Alkane-oxidizing metalloenzymes in the carbon cycle. *Metallomics*, *3*(8), 775-787.

Avanzi IR, Gracioso LH, Baltazar MDPG Karolski, B Perpetuo EA & Nascimento CA. (2015). Aerobic biodegradation of gasoline compounds by bacteria isolated from a hydrocarbon-contaminated soil. *Environ Eng Sci*, *32*(12), 990-997.

Bagnéris C, Cammack R, & Mason JR. (2005). Subtle difference between benzene and toluene dioxygenases of *Pseudomonas putida*. *Appl Environ Microbiol*, *71*(3), 1570-1580.

Baker PW, Ito K, Watanabe K. (2003). Marine prosthecate bacteria involved in the ennoblement of stainless steel. *Environ Microbiol*. 5:925-932.

Ball HA, Johnson HA, Reinhard M, & Spormann AM. (1996). Initial reactions in anaerobic ethylbenzene oxidation by a denitrifying bacterium, strain EB1. *J Bacteriol*, *178*(19), 5755-5761.

Baptist JN, Gholson RK, Coon MJ. (1963). Hydrocarbon oxidation by a bacterial enzyme system. I. Products of octane oxidation. *Biochim Biophys Acta* 69:40–47.

Barona, A, Elías, A, Arias, R, Acha, E, & Cano, I. (2007). Desorption and Biofiltration for the Treatment of Residual Organic Gases Evolved in Soil Decontamination Processses. *Chem Eng Technol*, *30*(11), 1499-1505.

Beech IB. (2002). Biocorrosion: Role of sulfate reducing bacteria. *Encyclo Environ Microbiol*.

Beech IB, & Coutinho CLM. (2003). Biofilms on corroding materials. *Biofilms in Medicine, Industry and Environmental Biotechnology-Characteristics, Analys Contr*, 115-131.

Beech IB, and Gaylarde CC. (1999). Recent advances in the study of biocorrosion: an overview. *Rev Microbiol*, 30(3):117-190.

Beech IB, & Sunner J. (2004). Biocorrosion: towards understanding interactions between biofilms and metals. *Curr Opin Biotechnol*, *15*(3), 181-186.

Beech IB, Sunner JA, & Hiraoka K. (2010). Microbe-surface interactions in biofouling and biocorrosion processes. *Int Microbiol*, *8*(3), 157-168.

Belhaj A, Desnoues N, & Elmerich C. (2002). Alkane biodegradation in *Pseudomonas aeruginosa* strains isolated from a polluted zone: identification of alkB and alkB-related genes. *Res Microbiol*, *153*(6), 339-344.

Beller HR (2000). Metabolic indicators for detecting in situ anaerobic alkylbenzene degradation. *Biodeg*, *11*(2-3), 125-139.

Beller H, Kane SR, Legler TC, & Alvarez PJ. (2002). A real-time polymerase chain reaction method for monitoring anaerobic, hydrocarbon-degrading bacteria based on a catabolic gene. *Environ Sci Technol*, *36*(18), 3977-3984.

Beller HR, Reinhard M, Grbic-Galic D (1992) Metabolic by-products of anaerobic toluene degradation by sulfate-reducing enrichment cultures. *Appl Environ Microbiol* 58: 3192–3195.

Beller HR, & Spormann AM (1997). Anaerobic activation of toluene and o-xylene by addition to fumarate in denitrifying strain T. *J Bacteriol*, *179*(3), 670-676.

Benson DA, Boguski MS, Lipman DJ. (1999). GenBank, *Nucleic Acids Res*. 27(1):12–17.

Bermont-Bouis D, Janvier M, Grimont PAD, Dupont I, & Vallaeys T. (2007). Both sulfate-reducing bacteria and *Enterobacteriaceae* take part in marine biocorrosion of carbon steel. *J Appl Microbiol*, *102*(1), 161-168.

Bernhardt R. (2006). Cytochromes P450 as versatile biocatalysts. *J Biotechnol*, *124*(1), 128-145.

Bertrand E, Sakai R, Rozhkova-Novosad E, Moe L, Fox BG, Groves JT, Austin RN (2005) Reaction mechanisms of non-heme diiron hydroxylases characterized in whole cells. *J Inorg Biochem* 99: 1998–2006.

Boetius A, Ravenschlag K, Schubert CJ, Rickert D, Widdel F, Gieseke A, & Pfannkuche O. (2000). A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature*, *407*(6804), 623-626.

Boisvert S, Laviolette F, & Corbeil J. (2010). Ray: simultaneous assembly of reads from a mix of high-throughput sequencing technologies. *J Comput Biol*, *17*(11), 1519-1533.

Boisvert S, Raymond, F Godzaridis, É Laviolette F, & Corbeil J. (2012). Ray Meta: scalable de novo metagenome assembly and profiling. *Genome Biol*, *13*(12), R122.

Bolger AM, Lohse M, & Usadel B. (2014). Trimmomatic: A flexible trimmer for Illumina Sequence Data. *Bioinformatics*, 170.

Boll M & Heider J. (2010). Anaerobic degradation of hydrocarbons: mechanisms of C–H-bond activation in the absence of oxygen. In *Handbook of Hydrocarbon and Lipid Microbiology* (pp. 1011-1024). Springer Berlin Heidelberg.

Bombach P, Richnow HH, Kästner M, & Fischer A. (2010). Current approaches for the assessment of in situ biodegradation. *Appl Microbiol Biot*, *86*(3), 839-852.

Boulton CA, & Ratledge C. (1984). The physiology of hydrocarbon-utilizing microorganisms. *T Enz Ferment Biotechnol*, *9*, 11-77.

Bowker KA. (2007). Barnett Shale gas production, Fort Wort Basin: issues and discussion. *AAPG Bull* 91(4):523–533.

Boylan DB, & Tripp BW. (1971). Determination of hydrocarbons in seawater extracts of crude oil and crude oil fractions. *Nature*, *230*(5288), 44-47.

Britton LN. (1984). Microbial degradation of aliphatic hydrocarbons. *Microbial degradation of organic compounds.*, 89-129.

Brooijmans RJW, Pastink MI, and Siezen RJ. Hydrocarbon-degrading bacteria: the oil-spill clean-up crew. *Microbial Biot*, vol. 2, no. 6, pp. 587–594, 2009.

Brysch K, Schneider C, Fuchs G, & Widdel F. (1987). Lithoautotrophic growth of sulfate-reducing bacteria, and description of *Desulfobacterium autotrophicum* gen. nov., sp. nov. *Arch Microbiol*, *148*(4), 264-274.

Caccavo F, Blakemore RP, & Lovley DR. (1992). A hydrogen-oxidizing, Fe (III)-reducing microorganism from the Great Bay Estuary, New Hampshire. *Appl Environ Microbiol*, *58*(10), 3211-3216.

Caldwell ME, & Suflita JM. (2000). Detection of phenol and benzoate as intermediates of anaerobic benzene biodegradation under different terminal electron-accepting conditions. *Environ Sci Technol*, *34*(7), 1216-1220.

Callaghan AV. (2013). Metabolomic investigations of anaerobic hydrocarbon-impacted environments. *Curr Opin Biot*, *24*(3), 506-515.

Callaghan AV, Davidova IA, Savage-Ashlock K, Parisi VA, & Gieg LM. (2010). Diversity of benyzl-and alkylsuccinate synthase genes in hydrocarbon-impacted environments and enrichment cultures. *Envirno Sci Technol.* 44: 7287–7294.

Callaghan AV, Gieg LM, Kropp KG, Suflita JM, & Young LY. (2006). Comparison of mechanisms of alkane metabolism under sulfate-reducing conditions among two bacterial isolates and a bacterial consortium. *Appl Environ Microbiol*, *72*(6), 4274-4282.

Callaghan AV, Tierney M, Phelps CD, & Young LY. (2009). Anaerobic biodegradation of n-hexadecane by a nitrate-reducing consortium. *Appl Environ Microbiol*, *75*(5), 1339-1344.

Callaghan AV, Wawrik B, Chadhain SMN, Young LY, & Zylstra GJ. (2008). Anaerobic alkane-degrading strain AK-01 contains two alkylsuccinate synthase genes. *Biochem Biophys Res Co*, *366*(1), 142-148.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, & Huttley GA. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat Methods*, *7*(5), 335-336.

Cardini G, & Jurtshuk P. (1968). Cytochrome P-450 involvement in the oxidation of n-octane by cell-free extracts of *Corynebacterium* sp. strain 7E1C. *J Biolog Chem*, *243*(22), 6070-6072.

Castorena G, Mugica V, Le Borgne S, Acuña ME, Bustos-Jaimes I & Aburto J. (2006). Carbazole biodegradation in gas oil/water biphasic media by a new isolated bacterium *Burkholderia* sp. strain IMP5GC. *J Appl Microbiol*, *100*(4), 739-745.

Chakrabarty AM, Chou G, & Gunsalus IC. (1973). Genetic regulation of octane dissimilation plasmid in *Pseudomonas*. *Proc Nationl Acad Sci*, *70*(4), 1137-1140.

Chakraborty R, & Coates JD. (2004). Anaerobic degradation of monoaromatic hydrocarbons. *Appl Microbiol Biot*, *64*(4), 437-446.

Chevenet F, Brun C, Bañuls AL, Jacq B & Christen R. (2006). TreeDyn: towards dynamic graphics and annotations for analyses of trees. *BMC Bioinformatics*, *7*(1), 439.

Cluff MA, Hartsock A, MacRae JD, Carter K, Mouser PJ. (2014). Temporal changes in microbial ecology and geochemistry in produced water from hydraulically fractured Marcellus Shale gas wells. *Environ Sci Technol*. 48:6508-6517.

Cole JR, Wang Q, Cardenas E. et al. (2009). The ribosomal database project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res*. 37(1):141–145.

Cord-Ruwisch R, Kleinitz W, & Widdel F. (1987). Sulfate-reducing bacteria and their activities in oil production. *J Petro Technol*, *39*(01), 97-106.

Coates JD, Anderson RT, Woodward JC, Phillips EJ, & Lovley DR. (1996). Anaerobic hydrocarbon degradation in petroleum-contaminated harbor sediments under sulfate-reducing and artificially imposed iron-reducing conditions. *Environ Sci Technol*, *30*(9), 2784-2789.

Coates JD, Chakraborty R, McInerney MJ. (2002). Anaerobic benzene biodegradation—a new era. *Res Microbiol* 153:621–628

Coates JD, Michaelidou U, Bruce RA, O'Connor SM, Crespi JN, & Achenbach LA. (1999). Ubiquity and diversity of dissimilatory (per) chlorate-reducing bacteria. *Appl Environ Microbiol*, *65*(12), 5234-5241.

Coates JD, Woodward J, Allen J, Philp P. & Lovley DR. (1997). Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments. *Appl Envirno Microbiol*, *63*(9), 3589-3593.

Cozzarelli IM, & Baehr AL. (2003). Volatile fuel hydrocarbons and MTBE in the environment. *Treatise Geochem*, *9*, 612.

Cravo-Laureau C, Grossi V, Raphel D, Matheron R, & Hirschler-Réa A. (2005). Anaerobic n-alkane metabolism by a sulfate-reducing bacterium, Desulfatibacillum aliphaticivorans strain CV2803T. *Appl Envirno Microbiol*, *71*(7), 3458-3467.

Cui Z, Lai Q, Dong C, & Shao Z. (2008). Biodiversity of polycyclic aromatic hydrocarbon-degrading bacteria from deep sea sediments of the Middle Atlantic ridge. *Environ Microbiol*, *10*(8), 2138-2149.

Dagley S.1984. Microbial degradation of aromatic compounds. *Dev Ind Microbiol.* 25:53-65.

Dahle H, Garshol F, Madsen M, & Birkeland NK. (2008). Microbial community structure analysis of produced water from a high-temperature North Sea oil-field. *Ant van Leeuwen*, *93*(1-2), 37-49.

Dalsgaard T, & Bak F. (1994). Nitrate reduction in a sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, isolated from rice paddy soil: sulfide inhibition, kinetics, and regulation. *Appl Environ Microbiol*, *60*(1), 291-297.

Dalvi S, Azetsu S, Patrauchan MA, Aktas, DF, & Fathepure BZ. (2012). Proteogenomic elucidation of the initial steps in the benzene degradation pathway of a novel halophile, *Arhodomonas* sp. strain Rozel, isolated from a hypersaline environment. *Appl Environ Microbiol*, *78*(20), 7309-7316.

Das D, Baruah R, Roy AS, Singh AK, Boruah HPD, Kalita J, & Bora TC. (2015). Complete genome sequence analysis of *Pseudomonas aeruginosa* N002 reveals its genetic adaptation for crude oil degradation. *Genomics*, *105*(3), 182-190.

Daumas S, Cord-Ruwisch R, & Garcia JL (1988). *Desulfotomaculum geothermicum* sp. nov., a thermophilic, fatty acid-degrading, sulfate-reducing bacterium isolated with H 2 from geothermal ground water. *Antonie van leeuwenhoek*, *54*(2), 165-178.

Davey ME, Wood WA, Key R, Nakamura K, & Stahl DA. (1993). Isolation of three species of *Geotoga* and *Petrotoga*: two new genera, representing a new lineage in the bacterial line of descent distantly related to the "Thermotogales". *Syst Appl Microbiol*, *16*(2), 191-200.

Davidova IA, Callaghan AV, Duncan KE, Sunner J, Biri B, & Wawrik B. (2011). Long-chain paraffin metabolism by a methanogenic bacterial consortium enriched from marine sediments. In *8th International Symposium of Subsurface Microbiology*. (Garmisch-Partenkirchen: International Symposium of Subsurface Microbiology.

Davidova IA, Gieg LM, Nanny M, Kropp KG, & Suflita JM. (2005). Stable isotopic studies of n-alkane metabolism by a sulfate-reducing bacterial enrichment culture. *Appl Environ Microbiol*, *71*(12), 8174-8182.

Davis JP, Struchtemeyer CG, & Elshahed MS. (2012). Bacterial communities associated with production facilities of two newly drilled thermogenic natural gas wells in the Barnett Shale (Texas, USA). *Microbial Ecolo*, *64*(4), 942-954.

de Lourdes Moreno M, Sánchez-Porro C, Piubeli F, Frias L, García MT, & Mellado E. (2011). Cloning, characterization and analysis of cat and ben genes from the phenol degrading halophilic bacterium *Halomonas organivorans*. *PloS One*, *6*(6), e21049.

Dean JA (1990). Lange's handbook of chemistry. *Material Manufact Proc*, *5*(4), 687-688.

Dereeper A, Audic S, Claverie JM, & Blanc G. (2010). BLAST-EXPLORER helps you building datasets for phylogenetic analysis. *BMC Evol Biol*, *10*(1), 8.

Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, & Claverie JM. (2008). Phylogeny. fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res*, *36*(suppl_2), W465-W469.

Dexter SC, Duquette DJ, Siebert OW, & Videla HA. (1991). Use and limitations of electrochemical techniques for investigating microbiological corrosion. *Corrosion*, *47*(4), 308-318.

Dinh HT, Kuever J, MussBmann M, & Hassel AW. (2004). Iron corrosion by novel anaerobic microorganisms. *Nature*, *427*(6977), 829.

Doherty VF, & Otitoloju AA. (2016). Occurrence and distribution of monocyclic aromatic hydrocarbons (BTEX) and the impact on macrobenthic community structure in Lagos lagoon, Nigeria. *Environ Monit Assess*, *188*(10), 571.

Duncan KE, Gieg LM, Parisi VA, Tanner RS, Tringe SG, Bristow J, & Suflita JM. (2009). Biocorrosive thermophilic microbial communities in Alaskan North Slope oil facilities. *Environ Sci Technol*, *43*(20), 7977-7984.

Edgar RC. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res*, *32*(5), 1792-1797.

Edgar R.C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460-2461.

El-Naas MH, Acio JA, & El Telib AE. (2014). Aerobic biodegradation of BTEX: progresses and prospects. *J Environ Chem Eng*, *2*(2), 1104-1122.

Elias I, & Lagergren J. (2007). Fast computation of distance estimators. *BMC Bioinformatics*, *8*(1), 89.

Ellis WA, Neill SD, O'brien JJ, Ferguson HW, & Hanna J. (1977). Isolation of Spirillum/Vibrio-like organisms from bovine fetuses. *Vet Rec*, *100*(21), 451-452.

Enning D, & Garrelfs J. (2014). Corrosion of iron by sulfate-reducing bacteria: new views of an old problem. *Appl Environ Microbiol*, *80*(4), 1226-1236.

Enning D, Venzlaff H, Garrelfs J, Dinh HT, Meyer V, Mayrhofer K, & Widdel F. (2012). Marine sulfate-reducing bacteria cause serious corrosion of iron under electroconductive biogenic mineral crust. *Environ Microbiol*, *14*(7), 1772-1787.

Evans PJ, Ling W, Goldschmidt B, Ritter ER, Young LY. (1992). Metabolites formed during anaerobic transformation of toluene and o-xylene and their proposed relationship to the initial steps of toluene mineralization. *Appl Environ Microbiol* 58: 496–501.

Fathepure BZ. (2014). Recent studies in microbial degradation of petroleum hydrocarbons in hypersaline environments. *Front Microbiol*, *5*.

Feknous N, Branes Z, Rouabhia K, Batisson I, & Amblard C. (2017). Isolation characterization and growth of locally isolated hydrocarbonoclastic marine bacteria (eastern Algerian coast). *Environ Monit Assess*, *189*(2), 49.

Felsenstein J. (1989). *PHYLIP - Phylogeny Inference Package (Version 3.2). Cladistics* 5: 164-166

Feng X, Pisula W, & Müllen K. (2009). Large polycyclic aromatic hydrocarbons: synthesis and discotic organization. *Pure Appl Chem*, *81*(12), 2203-2224.

Feng L, Wang W, Cheng J, Ren Y, Zhao G, Gao C, & Liu R. (2007). Genome and proteome of long-chain alkane degrading *Geobacillus thermodenitrificans* NG80-2 isolated from a deep-subsurface oil reservoir. *Proc Natl Acad Sci*, *104*(13), 5602-5607.

Fichter JK, Johnson K, French K, & Oden R. (2009). Biocides control Barnett shale fracturing fluid contamination. *Oil Gas J*, *107*(19), 38-44.

Fiehn O. (2001). Combining genomics, metabolome analysis, and biochemical modelling to understand metabolic networks. *Comp Funct Genom*, *2*(3), 155-168.

Figueras MJ, Collado L, Levican A, Perez J, Solsona MJ, Yustes C. 2011_a. *Arcobacter molluscorum* sp. nov., a new species isolated from shellfish. *Syst Appl Microbiol*. 34(2):105-109.

Figueras MJ, Levican A, Collado L, Inza MI, Yustes C. 2011_b. *Arcobacter ellisii* sp. nov., isolated from mussels. *Syst Appl Microbiol*, 34(6):414-418.

Funhoff EG, Bauer U, García-Rubio I, Witholt B, & van Beilen JB. (2006). CYP153A6, a soluble P450 oxygenase catalyzing terminal-alkane hydroxylation. *J Bacteriology*, *188*(14), 5220-5227.

Gadd GM (2009). Biosorption: critical review of scientific rationale, environmental importance and significance for pollution treatment. *J Chem Biot*, *84*(1), 13-28.

Gallego S, Vila J, Tauler M, Nieto JM, Breugelmans P, Springael D, & Grifoll M. (2014). Community structure and PAH ring-hydroxylating dioxygenase genes of a marine pyrene-degrading microbial consortium. *Biodegradation*, *25*(4), 543-556.

García MT, Mellado E, Ostos JC, & Ventosa A. (2004). *Halomonas organivorans* sp. nov., a moderate halophile able to degrade aromatic compounds. *Int J Syst Evol Microbiol*, *54*(5), 1723-1728.

García MT, Ventosa A, & Mellado E. (2005). Catabolic versatility of aromatic compound-degrading halophilic bacteria. *FEMS Microbiol Ecol*, *54*(1), 97-109.

Gauthier MJ, Lafay B, Christen R, Fernandez L, Acquaviva M, Bonin P & Bertrand JC. (1992). *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremely halotolerant, hydrocarbon-degrading marine bacterium. *Int J Syst Bacteriol*. 42, 568-576.

George SC, Boreman CJ, Minifie SA and Teerman SC. (2002). The effect of minor to moderate biodegradation on C_5 to C_9 hydrocarbons in crude oils. *Org Geochem* 33: 1293-1317.

Gerdes B, Brinkmeyer R, Dieckmann G, & Helmke E. (2005). Influence of crude oil on changes of bacterial communities in Arctic sea-ice. *FEMS Microbiol Ecol*, *53*(1), 129-139.

Gevertz D, Telang AJ, Voordouw G, & Jenneman GE. (2000). Isolation and characterization of strains CVO and FWKO B, two novel nitrate-reducing, sulfide-

oxidizing bacteria isolated from oil field brine. *Appl Environ Microbiol*, *66*(6), 2491-2501.

Gibson DT, Koch JR, & Kallio RE. (1968). Oxidative degradation of aromatic hydrocarbons by microorganisms. I. Enzymic formation of catechol from benzene. *Biochemistry*, *7*(7), 2653-2662.

Gibson DT, & Subramanian V. (1984). Microbial Degradation of Organic Compounds (DT Gibson, ed.).

Gieg LM, Alumbaugh RE, Field J, Jones J, Istok JD, & Suflita JM. (2009). Assessing in situ rates of anaerobic hydrocarbon bioremediation. *Microbial Biot*, *2*(2), 222-233.

Golyshin PN, Martins Dos Santos VA, Kaiser O, Ferrer M, Sabirova YS, Lunsdorf H, et al. (2003) Genome sequence completed of *Alcanivorax borkumensis*, a hydrocarbon-degrading bacterium that plays a global role in oil removal from marine systems. *J Biotechnol.* 106:215–220.

Grabowski A, Nercessian O, Fayolle F, Blanchet D, & Jeanthon C. (2005). Microbial diversity in production waters of a low-temperature biodegraded oil reservoir. *FEMS Microbiol Ecol*, *54*(3), 427-443.

Grassia GS, McLean KM, Glénat P, Bauld J, & Sheehy AJ. (1996). A systematic survey for thermophilic fermentative bacteria and archaea in high temperature petroleum reservoirs. *FEMS Microbiol Ecol*, *21*(1), 47-58.

Gray ND, Sherry A, Hubert C, Dolfing J, & Head IM. (2010). Methanogenic degradation of petroleum hydrocarbons in subsurface environments: remediation, heavy oil formation, and energy recovery. *Adv Appl Microbiol*, *72*, 137-161.

Gregoire P, Engelbrektson A, Hubbard CG, Metlagel Z, Csencsits R, Auer M, & Coates JD. (2014). Control of sulfidogenesis through bio-oxidation of H2S coupled to (per) chlorate reduction. *Environ Microbiol Rep*, *6*(6), 558-564.

Grundmann O, Behrends A, Rabus R, Amann J, Halder T, Heider J, & Widdel F. (2008). Genes encoding the candidate enzyme for anaerobic activation of n-alkanes in the denitrifying bacterium, strain HxN1. *Environ Microbiol*, *10*(2), 376-385.

Guindon S, & Gascuel O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol*, *52*(5), 696-704.

Hall BG. (2013). Building phylogenetic trees from molecular data with MEGA. *Mol. Biol. Evol*. 30(5):1229-1235.

Hamilton WA. (2003). Microbially influenced corrosion as a model system for the study of metal microbe interactions: a unifying electron transfer hypothesis. *Biofouling*, *19*(1), 65-76.

Hansen TA. (1993). Carbon metabolism of sulfate-reducing bacteria. In *The sulfate-reducing bacteria: contemporary perspectives* (pp. 21-40). Springer New York.

Hara A, Syutsubo K, & Harayama S. (2003). *Alcanivorax* which prevails in oil-contaminated seawater exhibits broad substrate specificity for alkane degradation. *Environ Microbiol*, *5*(9), 746-753.

Harayama S, Kasai Y. & Hara A. (2004). Microbial communities in oil-contaminated seawater. *Curr Opin Biot*, *15*(3), 205-214.

Haritash AK, & Kaushik CP. (2009). Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. *J Hazard Mater*, *169*(1), 1-15.

Harris SH, Smith RL, & Suflita JM. (2007). In situ hydrogen consumption kinetics as an indicator of subsurface microbial activity. *FEMS Microbiol Ecol*, *60*(2), 220-228.

Harwati TU, Kasai Y, Kodama Y, Susilaningsih D, & Watanabe K. (2007). Characterization of diverse hydrocarbon-degrading bacteria isolated from Indonesian seawater. *Microb Environ*, *22*(4), 412-415.

Hassanshahian M, Emtiazi G, & Cappello S. (2012). Isolation and characterization of crude-oil-degrading bacteria from the Persian Gulf and the Caspian Sea. *Mar Poll Bull*, *64*(1), 7-12.

Hassanshahian M, Emtiazi G, Kermanshahi RK, & Cappello S. (2010). Comparison of oil degrading microbial communities in sediments from the Persian Gulf and Caspian Sea. *Soil Sediment Contam*, *19*(3), 277-291.

Hayaishi O, Katagiri M, & Rothberg S. (1955). Mechanism of the pyrocatechase reaction. *J Am Chem Soc*, *77*(20), 5450-5451.

Head IM, Jones DM, & Larter SR. (2003). Biological activity in the deep subsurface and the origin of heavy oil. *Nature*, *426*(6964), 344-352.

Heidelberg JF, IT Paulsen, KE Nelson, EJ Gaidos, WC Nelson, TD Read, JA Eisen, R Seshadri, N Ward, B Methe, RA Clayton, T Meyer, A Tsapin, J Scott, M Beanan, L Brinkac, S Daugherty, RT DeBoy, RJ Dodson, AS Durkin, DH Haft, JF Kolonay, R Madupu, JD Peterson, LA Umayam, O White, AM Wolf, J Vamathevan, J Weidman, M Impraim, K Lee, K Berry, C Lee, J Mueller, H Khouri, J Gill, TR Utterback, LA McDonald, TV Feldblyum, HO Smith, JC Venter, KH Nealson, and

CM Fraser. (2002). Genome sequence of the dissimilatory metal ion-reducing bacterium *Shewanella oneidensis*. *Nat Biotechnol*. 20:1118–1123.

Heider J, Spormann AM, Beller HR., & Widdel F. (1998). Anaerobic bacterial metabolism of hydrocarbons. *FEMS Microbiol Rev*, *22*(5), 459-473.

Heider J, Szaleniec M, Martins BM, Seyhan D, Buckel W, & Golding BT. (2016). Structure and function of benzylsuccinate synthase and related fumarate-adding glycyl radical enzymes. *J Mol Microb Biotech 26*(1-3), 29-44.

Heidersbach R. (2010). *Metallurgy and corrosion control in oil and gas production* (Vol. 14). John Wiley & Sons.

Herath A, Wawrik B, Qin Y, Zhou J, & Callaghan AV. (2016). Transcriptional response of *Desulfatibacillum alkenivorans* AK-01 to growth on alkanes: insights from RT-qPCR and microarray analyses. *FEMS Microbiol Ecol*, *92*(5).

Hoehler TM, Alperin MJ, Albert DB, & Martens CS. (1994). Field and laboratory studies of methane oxidation in an anoxic marine sediment: Evidence for a methanogen-sulfate reducer consortium. *Global Biogeochem Cy*, *8*(4), 451-463.

Howard PH. (1991). Handbook of environmental fate and exposure data: for organic chemicals*, Volume III Pesticides* (Vol. 3). CRC press.

Huang Y, & Li L. (2014). Biodegradation characteristics of naphthalene and benzene, toluene, ethyl benzene, and xylene (BTEX) by bacteria enriched from activated sludge. *Water Environ Res*, *86*(3), 277-284.

Hubert C, Nemati M, Jenneman G, & Voordouw G. (2005). Corrosion risk associated with microbial souring control using nitrate or nitrite. *Appl Environ Microbiol 68*(2), 272-282.

Hutchins SH. (1991). Optimizing BTEX biodegradation under denitrifying conditions. *Environ Toxicol Chem*, *10*(11), 1437-1448.

Huu NB, Denner EB, Ha DT, Wanner G, & Stan-Lotter H. (1999). *Marinobacter aquaeolei* sp. nov., a halophilic bacterium isolated from a Vietnamese oil-producing well. *Int J Syst Evol Microbiol*, *49*(2), 367-375.

Hyatt D, LoCascio PF, Hauser LJ, & Uberbacher EC. (2012). Gene and translation initiation site prediction in metagenomic sequences. *Bioinformatics*, *28*(17), 2223-2230.

Inoue K, Habe H, Yamane H, Omori T, & Nojiri H. (2005). Diversity of carbazole-degrading bacteria having the car gene cluster: isolation of a novel gram-positive carbazole-degrading bacterium. *FEMS Microbiol Lett*, *245*(1), 145-153.

Jin HM, Choi EJ, & Jeon CO. (2013). Isolation of a BTEX-degrading bacterium, *Janibacter* sp. SB2, from a sea-tidal flat and optimization of biodegradation conditions. *Bioresource Technol*, *145*, 57-64.

Jindrova E, Chocova M, Demnerova K, & Brenner V. (2002). Bacterial aerobic degradation of benzene, toluene, ethylbenzene and xylene. *Folia Microbiol*, *47*(2), 83-93.

Jones DM, Head IM, Gray ND, Adams JJ, Rowan AK, Aitken CM, ... & Oldenburg T. (2008). Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs. *Nature*, *451* (7175), 176.

Kanehisa M, Goto S, Sato Y, Furumichi M, & Tanabe M. (2011). KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Res*, *40*(D1), D109-D114.

Kappell AD, Wei Y, Newton RJ, Van Nostrand JD, Zhou J, McLellan SL, & Hristova KR. (2014). The polycyclic aromatic hydrocarbon degradation potential of Gulf of Mexico native coastal microbial communities after the Deepwater Horizon oil spill. *Front Microbiol*, *5*.

Karigar CS, & Rao SS. (2011). Role of microbial enzymes in the bioremediation of pollutants: a review. *Enzyme Res*, *2011*.

Kasai Y, Kishira H, Sasaki T, Syutsubo K, Watanabe K, & Harayama S. (2002). Predominant growth of *Alcanivorax* strains in oil-contaminated and nutrient-supplemented sea water. *Environ Microbiol*, *4*(3), 141-147.

Keith SM, & Herbert RA. (1983). Dissimilatory nitrate reduction by a strain of *Desulfovibrio desulfuricans*. *FEMS Microbiol Lett*, *18*(1-2), 55-59.

Keith LH, & Telliard WA. (1979). Priority pollutants: I. A perspective view. *Environ Sci Technol*, 13, 416–423.

Kim H, Choo YJ, Song J, Lee JS, Lee KC, & Cho JC. (2007). *Marinobacterium litorale* sp. nov. in the order *Oceanospirillales*. *Int J Syst Evol Microbiol*, *57*(7), 1659-1662.

Kim HM, Hwang CY, & Cho BC. (2010). *Arcobacter marinus* sp. nov. *Int J Syst Evol Microbiol*, *60*(3), 531-536.

Kim SJ, Park SJ, Yoon DN, Park BJ, Choi BR, Lee DH, & Rhee SK. (2009). *Marinobacterium maritimum* sp. nov., a marine bacterium isolated from Arctic sediment. *Int J of Syst Evol Microbiol*, *59*(12), 3030-3034.

Kimes NE, Callaghan AV, Aktas DF, Smith WL, Sunner J, Golding BT, & Morris PJ. (2013). Metagenomic analysis and metabolite profiling of deep–sea sediments from the Gulf of Mexico following the Deepwater Horizon oil spill. *Front Microbiol*, *4*, 50.

Kjellerup BV, Olesen BH, Nielsen JL, Frolund B, Odum S, Nielsen PH. (2003). Monitoring and characterisation of bacteria in corroding district heating systems using fluorescence in situ hybridisation and microautoradiography. *Water Sci Techno*l, 47:117-122.

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, & Glöckner FO. (2012). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res*, *41*(1), e1-e1.

Kloos K, Munch JC, & Schloter M. (2006). A new method for the detection of alkane-monooxygenase homologous genes (alkB) in soils based on PCR-hybridization. *J Microbiol Meth*, *66*(3), 486-496.

Kniemeyer O, Musat F, Sievert SM, Knittel K, Wilkes H, Blumenberg M & Widdel F. (2007). Anaerobic oxidation of short-chain hydrocarbons by marine sulphate-reducing bacteria. *Nature*, *449*(7164), 898-901.

Kodama Y, Stiknowati LI, Ueki A, Ueki K, & Watanabe K. (2008). *Thalassospira tepidiphila* sp. nov., a polycyclic aromatic hydrocarbon-degrading bacterium isolated from seawater. *Int J of Syst Evol Microbiol*, *58*(3), 711-715.

Kok M, Oldenhuis R, van der Linden MP, Meulenberg CH, Kingma J, & Witholt B. (1989a). The *Pseudomonas oleovorans* alkBAC operon encodes two structurally related rubredoxins and an aldehyde dehydrogenase. *J Biol Chem*, *264*(10), 5442-5451.

Kok M, Oldenhuis R, Van Der Linden MP, Raatjes P, Kingma J, van Lelyveld PH, & Witholt B. (1989b). The *Pseudomonas oleovorans* alkane hydroxylase gene. Sequence and expression. *J Biolog Chem*, *264*(10), 5435-5441.

Kohno T, Sugimoto Y, Sei K, & Mori K. (2002). Design of PCR primers and gene probes for general detection of alkane-degrading bacteria. *Microb Environ*, *17*(3), 114-121.

Kotani T, Yamamoto T, Yurimoto H, Sakai Y, Kato N (2003) Propane monooxygenase and NAD+ -dependent secondary alcohol dehydrogenase in propane metabolism by *Gordonia* sp. strain TY-5. *J Bacteriol* 185: 7120–7128

Krieger CJ, Roseboom W, Albracht SP, & Spormann AM. (2001). A stable organic free radical in anaerobic benzylsuccinate synthase of *Azoarcus* sp. strain T. *J Biol Chem*, *276*(16), 12924-12927.

Kropp KG, Davidova IA, & Suflita JM. (2000). Anaerobic oxidation of n-dodecane by an addition reaction in a sulfate-reducing bacterial enrichment culture. *Appl Environ Microbiol. 66*(12), 5393-5398.

Kumar S, Tamura K, Nei M. (2004). Mega3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. *Bioinformatics*. 5:150–163.

Kvenvolden KA and CK Cooper. (2003). Natural seepage of crude oil into the marine environment. *Geo-Mar Lett*, vol. 23, no. 3-4, pp. 140–146.

Ladygina N, Dedyukhina EG, & Vainshtein MB. (2006). A review on microbial synthesis of hydrocarbons. *Proc Biochem*, *41*(5), 1001-1014.

Lai Q, Zhong H, Wang J, Yuan J, Sun F, Wang L, & Shao Z. (2011). *Roseovarius indicus* sp. nov., isolated from deep-sea water of the Indian Ocean. *Int J Syst Evol Microbiol*, *61*(9), 2040-2044.

Larsen NGJ, Olsen BL, Maidak MJ, McCaughey R, Overbeek TJ, Macke TL, Marsh and Woese CR. (1993). The Ribosomal Database Project. *Nucleic Acids Res*. 21:3021-3023.

Leahy JG and RR Colwell. (1990). Microbial degradation of hydrocarbons in the environment. *Microbiol Rev*, vol. 54, no. 3, pp. 305–315.

Leuthner B, et al. (1998) Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica*: a new glycyl radical enzyme catalysing the first step in anaerobic toluene metabolism. *Mol Microbiol* 28: 615–628.

Leuthner B, Leutwein C, Schulz H, Hörth P, Haehnel W, Schiltz E, ... & Heider J. (1998). Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica*: a new glycyl radical enzyme catalysing the first step in anaerobic toluene metabolism. *Mol Microbiol*, *28*(3), 615-628.

Levican A, Collado L, Aguilar C, Yustes C, Diéguez AL, Romalde JL, Figueras MJ. (2012). *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov., new species isolated from shellfish. *Syst Appl Microbiol*, 35(3):133-138.

Li Y, Li B, Wang CP, Fan JZ, & Sun HW. (2014). Aerobic degradation of trichloroethylene by co-metabolism using phenol and gasoline as growth substrates. *Int J Mol Sci*, *15*(5), 9134-9148.

Liang R, Duncan KE, Le Borgne S, Davidova I, Yakimov MM, & Suflita JM. (2017). Microbial activities in hydrocarbon-laden wastewaters: Impact on diesel fuel stability and the biocorrosion of carbon steel. *J Biotechnol*.

Liu Y, Karnauchow TM, Jarrell KF, Balkwill DL, Drake GR, Ringelberg D, ... & Boone DR. (1997). Description of two new thermophilic *Desulfotomaculum* spp., *Desulfotomaculum putei* sp. nov., from a deep terrestrial subsurface, and *Desulfotomaculum luciae* sp. nov., from a hot spring. *Int J Syst Evol Microbiol*, *47*(3), 615-621.

López MA, Díaz de la Serna FJZ, Jan-Roblero J, Romero JM, & Hernández-Rodríguez C. (2006). Phylogenetic analysis of a biofilm bacterial population in a water pipeline in the Gulf of Mexico. *FEMS Microbial Ecol*, *58*(1), 145-154.

Lopez-Cortes A, Fardeau ML, Fauque G, Joulian C, & Ollivier B. (2006). Reclassification of the sulfate-and nitrate-reducing bacterium *Desulfovibrio vulgaris* subsp. oxamicus as *Desulfovibrio oxamicus* sp. nov., comb. nov. *Int J Syst Evol Microbiol*, *56*(7), 1495-1499.

Lovley DR, & Goodwin S. (1988). Hydrogen concentrations as an indicator of the predominant terminal electron-accepting reactions in aquatic sediments. *Geochimica Cosmochimica Acta*, *52*(12), 2993-3003.

Lovley DR & Lonergan DJ. (1990). Anaerobic oxidation of toluene, phenol, and p-cresol by the dissimilatory iron-reducing organism, GS-15. *Appl Environ Microbiol*, *56*(6), 1858-1864.

Lu ZY, Guo XJ, Li H, Huang ZZ, Lin KF, & Liu YD. (2015). High-throughput screening for a moderately halophilic phenol-degrading strain and its salt tolerance response. *Int J Mol Sci*, *16*(6), 11834-11848.

Luo C, Rodriguez-R LM, Johnston ER, Wu L, Cheng L, Xue K, & Yuan MM. (2014). Soil microbial community responses to a decade of warming as revealed by comparative metagenomics. *Appl Environ Microbiol*, *80*(5), 1777-1786.

Luo YR, Tian Y, Huang X, Yan CL, Hong HS, Lin GH, & Zheng TL. (2009). Analysis of community structure of a microbial consortium capable of degrading benzo (a) pyrene by DGGE. *Mar Pollut Bull*, *58*(8), 1159-1163.

Lutterbach MT, Contador LS, Oliveira AL, Galvão MM, de França FP, & de Souza Pimenta G. (2009, January). Iron sulfide production by Shewanella strain isolated from black powder. In *CORROSION 2009*. NACE International.

Lyles CN, Aktas DF, Duncan KE, Callaghan AV, Stevenson BS, & Suflita JM. (2013). Impact of organosulfur content on diesel fuel stability and implications for carbon steel corrosion. *Environ Sci Technol*, *47*(11), 6052-6062.

Magot M, Ollivier B, & Patel BK. (2000). Microbiology of petroleum reservoirs. *Ant van Leeuwen*, *77*(2), 103-116.

Maier T, Förster HH, Asperger O, & Hahn U. (2001). Molecular characterization of the 56-kDa CYP153 from *Acinetobacter* sp. EB104. *Biochem Biophy Res Co*, *286*(3), 652-658.

Mand J, Park HS, Jack TR, Voordouw G, Hoffmann H. 2014. Use of molecular methods (pyrosequencing) for evaluating MIC potential in water systems for oil production in the North Sea. In SPE International Oilfield Corrosion Conference and Exhibition. Society of Petroleum Engineers.

Mand J, Yin Shen, Heike Hoffmann and Gerrit Voordouw. 2017. Ch. 15 MIC under Conditions of Oxygen or Nitrate Ingress. *Microbiol Influenced Corrosion Up Oil Gas Industustry*, 309-324

Mao X, Cai T, Olyarchuk JG, & Wei L. (2005). Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. *Bioinformatics*, *21*(19), 3787-3793.

Marın MM, Smits TH, van Beilen JB, Rojo F. (2001). The alkane hydroxylase gene of *Burkholderia cepacia* RR10 is under catabolite repression control. *J Bacteriol* 183: 4202–4209.

Marın MM, Yuste L, Rojo F (2003) Differential expression of the components of the two alkane hydroxylases from *Pseudomonas aeruginosa. J Bacteriol* 185: 3232–3237.

Martin M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*, *17*(1), pp-10.

Masterson WD, LIP Dzou, AG Holba, AL Fincannon, and L Ellis. (2001). Evidence of biodegradation and evaporative fractionation in West Sak, Kuparuk and Prudhoe Bay field areas, North Slope, Alaska. *Org Geochem.* **32:**411-441.

McKew BA, Coulon F, Osborn AM, Timmis KN, & McGenity TJ. (2007). Determining the identity and roles of oil-metabolizing marine bacteria from the Thames estuary, UK. *Environ Microbiol*, *9*(1), 165-176.

McKew BA, Coulon F, Yakimov MM, Denaro R, Genovese M, Smith CJ, & McGenity TJ. (2007). Efficacy of intervention strategies for bioremediation of

crude oil in marine systems and effects on indigenous hydrocarbonoclastic bacteria. *Environ Microbiol*, *9*(6), 1562-1571.

McKenna EJ & Coon MJ. (1970). Enzymatic ω-oxidation IV. Purification and properties of the ω-hydroxylase of *Pseudomonas oleovorans*. *J Biol Chem*, *245*(15), 3882-3889.

McSweeney NJ, Plumb JJ, Tilbury AL, Nyeboer HJ, Sumich ME, McKinnon AJ, & Kaksonen AH. (2011). Comparison of microbial communities in pilot-scale bioreactors treating Bayer liquor organic wastes. *Biodegradation*, *22*(2), 397-407.

Meckenstock RU & Mouttaki H. (2011). Anaerobic degradation of non-substituted aromatic hydrocarbons. *Curr Opin Biot*, *22*(3), 406-414.

Medina-Bellver JI, Mar´ın P, Delgado A et al., "Evidence for in situ crude oil biodegradation after the Prestige oil spill," *Environ Microbiol*, vol. 7, no. 6, pp. 773–779, 2005.

Melcher RJ, Apitz SE, & Hemmingsen BB. (2002). Impact of irradiation and polycyclic aromatic hydrocarbon spiking on microbial populations in marine sediment for future aging and biodegradability studies. *Appl Environ Microbiol*, *68*(6), 2858-2868.

Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, Kubal M, & Wilkening J. (2008). The metagenomics RAST server–a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics*, *9*(1), 386.

Miller WG, Parker CT, Rubenfield M, Mendz GL, Wösten MMSM, et al. (2007). The complete genome sequence and analysis of the Epsilonproteobacterium *Arcobacter butzleri*. *PLoS ONE* 2(12).

Mnif S, Chamkha M, Labat M, & Sayadi S. (2011). Simultaneous hydrocarbon biodegradation and biosurfactant production by oilfield-selected bacteria. *J Appl Microbiol*, *111*(3), 525-536.

Mnif S, Chamkha M, & Sayadi S. (2009). Isolation and characterization of *Halomonas* sp. strain C2SS100, a hydrocarbon-degrading bacterium under hypersaline conditions. *J Appl Microbiol*, *107*(3), 785-794.

Morlett-Chávez JA, Ascacio-Martínez JÁ, Rivas-Estilla AM, Velázquez-Vadillo JF, Haskins WE, Barrera-Saldaña HA, & Acuña-Askar K. (2010). Kinetics of BTEX biodegradation by a microbial consortium acclimatized to unleaded gasoline and bacterial strains isolated from it. *Int. Biodeter. Biodegr*, 64, 581–587.

Moura I, Bursakov S, Costa C, & Moura JJ. (1997). Nitrate and nitrite utilization in sulfate-reducing bacteria. *Anaerobe*, *3*(5), 279-290.

Mukherjee AK, & Bordoloi NK. (2012). Biodegradation of benzene, toluene, and xylene (BTX) in liquid culture and in soil by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains and a formulated bacterial consortium. *Environ Sci Pollut R*, *19*(8), 3380-3388.

Munoz JA, Perez-Esteban B, Esteban M, de la Escalera S, Gomez MA, Martı´nez-Toledo MV & Gonzalez-Lopez J. (2001). Growth of moderately halophilic bacteria isolated from sea water using phenol as the sole carbon source. *Folia Microbiol* (Praha) 46, 297–302.

Munro AW, & Lindsay JG. (1996). Bacterial cytochromes P-450. *Mol Microbiol*, *20*(6), 1115-1125.

Muyzer G, De Waal E, Uitterlinden AG. (1993). Profiling of complex microbial poplulations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol*. 59:695-700.

Muyzer G, & Stams AJ. (2008). The ecology and biotechnology of sulphate-reducing bacteria. *Nature Rev Microbiol*, *6*(6), 441-454.

Myers, CR, and JM Myers. (1994). Ferric iron reduction-linked growth yields of *Shewanella putrefaciens* MR-1. *J App. Bacteriol*. 76:254–258.

NACE Standard TM0212. (2012). Detection, Testing, and Evaluation of Microbiologically Influenced Corrosion on Internal Surfaces of Pipelines. NACE International: Houston, TX, 2012; Item No. 21260.

NACE Standard 1C184. (2008). Oil and Gas "Rust": An Evil Worse Than Depletion. Offshore Technology Conference (OTC) Presentation. NACE International: Houston, TX

Nauhaus K, Boetius A, Krüger M, & Widdel F. (2002). In vitro demonstration of anaerobic oxidation of methane coupled to sulphate reduction in sediment from a marine gas hydrate area. *Environ Microbiol*, *4*(5), 296-305.

Nie Y, Chi CQ, Fang H, Liang JL, Lu SL, Lai GL, ... & Wu XL. (2014). Diverse alkane hydroxylase genes in microorganisms and environments. *Sci Rep-UK*, *4*.

Ollivier B, & Magot M. (2005). *Petroleum microbiology*. ASM Press.

Orphan VJ, Hinrichs KU, Ussler WIII, Paull CK, Taylor LT, Sylva SP, & DeLong EF. (2001). Comparative analysis of methane-oxidizing archaea and sulfate-reducing bacteria in anoxic marine sediments. *Appl Environ Microbiol*, *67*(4), 1922-1934.

Parshina SN, Sipma J, Nakashimada Y, Henstra AM, Smidt H, Lysenko AM, ... & Stams AJ. (2005). *Desulfotomaculum carboxydivorans* sp. nov., a novel sulfate-reducing bacterium capable of growth at 100% CO. *Int J Syst Evol Microbiol*, *55*(5), 2159-2165.

Pepi M, Cesa`ro A, Liut G and Baldi F. (2005) An Antarctic psychrotrophic bacterium *Halomonas* sp. ANT-3b, growing on n-hexadecane, produces a new emulsifying glycolipid. *FEMS Microbiol Ecol* 53, 157–166.

Pérez-de-Mora A, Engel M, & Schloter M. (2011). Abundance and diversity of n-alkane-degrading bacteria in a forest soil co-contaminated with hydrocarbons and metals: a molecular study on alkB homologous genes. *Microbial Ecol*, *62*(4), 959-972.

Peterson, JA, D Basu, and MJ Coon. (1966). Enzymatic-oxidation. I. Electron carriers in fatty acid and hydrocarbon hydroxylation. *J Biol Chem* 241:5162–5164.

Peterson JA, Kusunose M, Kusunose E, & Coon MJ. (1967). Enzymatic ω-oxidation II. Function of rubredoxin as the electron carrier in ω-hydroxylation. *J Biolog Chem*, *242*(19), 4334-4340.

Phelps CD, Zhang X, & Young LY. (2001). Use of stable isotopes to identify benzoate as a metabolite of benzene degradation in a sulphidogenic consortium. *Environ Microbiol*, *3*(9), 600-603.

Pimenov NV, Kuranov GV, Bryukhanov AL, Veslopolova EF, Koryukina IP, and Maslov YN. (2012). The sulfate-reducing bacterial community of sulfide-rich water of the Ust'-Kachka Resort Spring, Perm Krai, Russia. *Microbiology*, *81*(6), 721-726.

Pollastro RM. (2007). Total petroleum system assessment of undiscovered resources in the giant Barnett Shale continuous (unconventional) gas accumulation, Fort Worth Basin, Texas. *AAPG bulletin*, *91*(4), 551-578.

Prabagaran SR, Manorama R, Delille D, & Shivaji S. (2007). Predominance of *Roseobacter, Sulfitobacter, Glaciecola* and *Psychrobacter* in seawater collected off Ushuaia, Argentina, Sub-Antarctica. *FEMS Microbiol Ecol*, *59*(2), 342-355.

Prenafeta-Boldú FX, Ballerstedt H, Gerritse J, & Grotenhuis JTC. (2004). Bioremediation of BTEX hydrocarbons: effect of soil inoculation with the toluene-growing fungus *Cladophialophora* sp. strain T1. *Biodegradation*, *15*(1), 59-65.

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, & Glöckner FO. (2007). SILVA: a comprehensive online resource for quality checked and aligned

ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res*, *35*(21), 7188-7196.

Rabus R, Boll M, Heider J, Meckenstock RU, Buckel W, Einsle O, ... & Krüger M. (2016). Anaerobic microbial degradation of hydrocarbons: from enzymatic reactions to the environment. *J Mol Microb Biotech*, *26*(1-3), 5-28.

Rabus R, Nordhaus R, Ludwig W, & Widdel F. (1993). Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. *Appl Environ Microbiol*, *59*(5), 1444-1451.

Rabus R, & Widdel F. (1995). Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. *Arch Microbiol*, *163*(2), 96-103.

Rabus R, Wilkes H, Behrends A, Armstroff A, Fischer T, Pierik AJ, & Widdel F. (2001). Anaerobic initial reaction of n-alkanes in a denitrifying bacterium: evidence for (1-methylpentyl) succinate as initial product and for involvement of an organic radical in n-hexane metabolism. *J Bacteriol*, *183*(5), 1707-1715.

Rahman KSM, Rahman TJ, Kourkoutas Y, Petsas I, Marchant R, & Banat IM (2003). Enhanced bioremediation of n-alkane in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients. *Bioresourse Technol*, *90*(2), 159-168.

Rapp P, & Gabriel-Jürgens LH. (2003). Degradation of alkanes and highly chlorinated benzenes, and production of biosurfactants, by a psychrophilic *Rhodococcus* sp. and genetic characterization of its chlorobenzene dioxygenase. *Microbiology*, *149*(10), 2879-2890.

Raton FL, pp. 221–240. Watkinson RJ, Morgan P (1990) Physiology of aliphatic hydrocarbon-degrading microorganisms. *Biodegradation* 1: 79–92.

Rehm HJ, Reiff I (1981) Mechanisms and occurrence of microbial oxidation of long-chain alkanes. *Adv Biochem Eng Biot* 19: 175–215.

Roalkvam I, Drønen K, Stokke R, Daae FL, Dahle H, & Steen IH. (2015). Physiological and genomic characterization of *Arcobacter anaerophilus* IR-1 reveals new metabolic features in Epsilonproteobacteria. *Front Microbiol*, *6*.

Rojo F. (2009). Degradation of alkanes by bacteria. *Environ Microbiol*, *11*(10), 2477-2490.

Romano I, Nicolaus B, Lama L, Manca MC, & Gambacorta A. (1996). Characterization of a haloalkalophilic strictly aerobic bacterium, isolated from Pantelleria island. *Syst Appl Microbiol*, *19*(3), 326-333.

Rueter P, Rabus R, Wilkest H, Aeckersberg F, Rainey FA, Jannasch HW, & Widdel F. (1994). Anaerobic oxidation of hydrocarbons in crude oil by new types of sulphate-reducing bacteria. *Nature*, *372*(6505), 455.

Ruettinger RT, Griffith GR, & Coon MJ. (1977). Characterization of the ω-hydroxylase of *Pseudomonas oleovorans* as a nonheme iron protein. *Arch Biochem Biophys*, *183*(2), 528-537.

Santegoeds CM, LR Damgaard, G Hesselink, J Zopfi, P Lens, G. Muyzer, and D de Beer. (1999). Distribution of sulfate-reducing and methanogenic bacteria in anaerobic aggregates determined by microsensor and molecular analyses. *Appl. Environ Microbiol* 65:4618-4629.

Santegoeds CM, Ferdelman TG, Muyzer G and Beer D. (1998). Structural and functional dynamics of sulfate-reducing poplulations in bacterial biofilms. *Appl Environ Microbiol*. 64:3731-3739.

Savage KN, Krumholz LR, Gieg LM, Parisi VA, Suflita JM, Allen J & Elshahed MS. (2010). Biodegradation of low-molecular-weight alkanes under mesophilic, sulfate-reducing conditions: metabolic intermediates and community patterns. *FEMS Microbiol Ecol*, *72*(3), 485-495.

Scheps D, Malca SH, Hoffmann H, Nestl BM, & Hauer B. (2011). Regioselective ω-hydroxylation of medium-chain n-alkanes and primary alcohols by CYP153 enzymes from *Mycobacterium marinum* and *Polaromonas* sp. strain JS666. *Org Biomol Chem*, *9*(19), 6727-6733.

Schneiker S, dos Santos VAM, Bartels D, Bekel T, Brecht M, Buhrmester J & Goesmann, A. (2006). Genome sequence of the ubiquitous hydrocarbon-degrading marine bacterium *Alcanivorax borkumensis*. *Nat Biotechnol*, *24*(8), 997-1004.

Schönheit P, Kristjansson JK, & Thauer RK. (1982). Kinetic mechanism for the ability of sulfate reducers to out-compete methanogens for acetate. *Arch Microbiol*, *132*(3), 285-288.

Sei A, & Fathepure BZ. (2009). Biodegradation of BTEX at high salinity by an enrichment culture from hypersaline sediments of Rozel Point at Great Salt Lake. *J Appl Microbiol*, *107*(6), 2001-2008.

Shanklin J, Achim C, Schmidt H, Fox BG, Munck E. (1997). Mossbauer studies of alkane omegahydroxylase: evidence for a diiron cluster in an integral- membrane enzyme. *Proc Natl Acad Sci USA* 94: 2981–2986.

Shadi AM, Yaghmaei S, Vafaei F, Khataee AR, & Hejazi MS. (2015). Degradation of benzene, toluene, and xylene (BTX) from aqueous solution by isolated bacteria from contaminated sites. *Res Chem Intermediat. 41*(1), 265-275.

Sherry A, Gray ND, Ditchfield AK, Aitken CM, Jones DM, Röling WFM & Head, IM. (2013). Anaerobic biodegradation of crude oil under sulphate-reducing conditions leads to only modest enrichment of recognized sulphate-reducing taxa. *Int Biodeter Biodegr*, *81*, 105-113.

Shim H, Shin E, & Yang ST. (2002). A continuous fibrous-bed bioreactor for BTEX biodegradation by a co-culture of *Pseudomonas putida* and *Pseudomonas fluorescens*. *Adv Environ Res*, *7*(1), 203-216.

Sievert SM, Wieringa E, Wirsen CO, & Taylor CD. (2007). Growth and mechanism of filamentous-sulfur formation by *Candidatus Arcobacter sulfidicus* in opposing oxygen-sulfide gradients. *Environ Microbiol*, *9*(1), 271-276.

Singh R, & Celin SM. (2010). Biodegradation of BTEX (benzene, toluene, ethyl benzene and xylene) compounds by bacterial strain under aerobic conditions. *J Ecobiotechnol*, *2*(4).

Sluis MK, Sayavedra-Soto LA, Arp DJ. (2002). Molecular analysis of the soluble butane monooxygenase from ‘‘*Pseudomonas butanovora.*’’ *Microbiology* 148: 3617–3629.

Smith CB, BB Tolar, JT Hollibaugh, GM King. (2013). Alkane hydroxylase gene (alkB) phylotype composition and diversity in northern Gulf of Mexico bacterioplankton. *Front. Microbiol*., 4., pp. 370–378

Smith DW. (1993). Ecological actions of sulfate - reducing bacteria. In: Odom JM (Ed) The Sulfate -Reducing Bacteria: *Contemporary Perspectives*. Springer-Verlag, New York, pp. 161–187.

Smith MR. (1991). The biodegradation of aromatic hydrocarbons by bacteria. In *Physiology of Biodegradative Microorganisms* (pp. 191-206). Springer Netherlands.

Smits TH, Balada SB, Witholt B, & van Beilen JB. (2002). Functional analysis of alkane hydroxylases from gram-negative and gram-positive bacteria. *J Bacteriol*, *184*(6), 1733-1742.

Smits THM, Rothlisberger M, Witholt B, van Beilen JB (1999) Molecular screening for alkane hydroxylase genes in Gram-negative and Gram-positive strains. *Environ Microbiol* 1: 307–317.

Smits TH, Witholt B, van Beilen JB. (2003). Functional characterization of genes involved in alkane oxidation by *Pseudomonas aeruginosa*. *Anton van Leeuwen* 84: 193–200.

So CM, Phelps CD, & Young LY. (2003). Anaerobic transformation of alkanes to fatty acids by a sulfate-reducing bacterium, strain Hxd3. *Appl Environ Microbiol*, *69*(7), 3892-3900.

So CM, & Young LY. (1999). Isolation and characterization of a sulfate-reducing bacterium that anaerobically degrades alkanes. *Appl Environ Microbiol*, *65*(7), 2969-2976.

Sooknah R, Papavinasam S, & Revie RW. (2007, January). Monitoring microbiologically influenced corrosion: a review of techniques. In *CORROSION 2007*. NACE International.

Sotsky JB, CW Greer and RM Atlas. (1994). Frequency of genes in aromatic and aliphatic hydrocarbon biodegradation pathways within bacterial populations from Alaskan sediments. *Can J Microbiol*. 40:981–985.

Stams AJM, Elferink SO & Westermann P. (2003). Metabolic interactions between methanogenic consortia and anaerobic respiring bacteria. In *Biomethanation I* (pp. 31-56). Springer Berlin Heidelberg.

Stefanie JOE, Visser A, Pol LWH & Stams AJ. (1994). Sulfate reduction in methanogenic bioreactors. *FEMS Microbiol Rev*, *15*(2-3), 119-136.

Stevenson BS, Drilling HS, Lawson PA, Duncan KE, Parisi VA, & Suflita JM. (2011). Microbial communities in bulk fluids and biofilms of an oil facility have similar composition but different structure. *Environ Microbiol*, *13*(4), 1078-1090.

Striebich RC, Smart CE, Gunasekera TS, Mueller SS, Strobel EM, McNichols BW, & Ruiz ON. (2014). Characterization of the F-76 diesel and Jet-A aviation fuel hydrocarbon degradation profiles of *Pseudomonas aeruginosa* and *Marinobacter hydrocarbonoclasticus*. *Int Biodeter Biodegr*, *93*, 33-43.

Suflita JM, Lyles CN, Aktas DF, & Sunner J. (2014). Biocorrosion issues associated with the use of ultra-low sulfur diesel and biofuel blends in the energy infrastructure. *Understanding Biocorrosion: Fundamentals and Applications*, *313*.

Suflita JM, Phelps TJ, & Little B. (2008). Carbon dioxide corrosion and acetate: a hypothesis on the influence of microorganisms. *Corrosion*, *64*(11), 854-859.

Tang X, Zhai L, Lin Y, Yao S, Wang L, Ge Y, & Liu J. (2017). *Halomonas alkalicola* sp. nov., isolated from a household product plant. *Inter J Syst Evol Microbiol*, *67*(5), 1546-1550.

Tanner RS. (1989). Monitoring sulfate-reducing bacteria: comparison of enumeration media. *J Microbiol Method*, *10*(2), 83-90.

Telang AJ, Ebert S, Foght JM, Westlake D, Jenneman GE, Gevertz D, & Voordouw G. (1997). Effect of nitrate injection on the microbial community in an oil field as monitored by reverse sample genome probing. *Appl Environ Microbiol*, *63*(5), 1785-1793.

Telang AJ, Jenneman GE, & Voordouw G. (1999). Sulfur cycling in mixed cultures of sulfide-oxidizing and sulfate-or sulfur-reducing oil field bacteria. *Can J Microbiol*, *45*(11), 905-913.

Teramoto M, Ohuchi M, Hatmanti A, Darmayati Y, Widyastuti Y, Harayama S, & Fukunaga Y. (2011). *Oleibacter marinus* gen. nov., sp. nov., a bacterium that degrades petroleum aliphatic hydrocarbons in a tropical marine environment. *Inter J Syst Evol Microbiol*, *61*(2), 375-380.

Teske A, Sigalevich P, Cohen, Y & Muyzer G. (1996). Molecular identification of bacteria from a coculture by denaturing gradient gel electrophoresis of 16S ribosomal DNA fragments as a tool for isolation in pure cultures. *Appl Environ Microbiol*, *62*(11), 4210-4215.

Thauer RK, & Shima S. (2008). Methane as fuel for anaerobic microorganisms. *Annals of the New York Academy of Sciences*, *1125*(1), 158-170.

Thompson JD. (1994). CLUSTAL W: improving the sensitivity of progressive sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, *22*, 4553-4559.

Throne-Holst M, Wentzel A, Ellingsen TE, Kotlar HK, & Zotchev SB. (2007). Identification of novel genes involved in long-chain n-alkane degradation by *Acinetobacter* sp. strain DSM 17874. *Appl Environ Microbiol*, *73*(10), 3327-3332.

Tsao, CW, Song HG, & Bartha R. (1998). Metabolism of benzene, toluene, and xylene hydrocarbons in soil. *Appl Environ Microbiol*, *64*(12), 4924-4929.

Ukpaka CP, Amadi SA, and Odharo IAJ. 2011. Modeling the rate of biocorrosion and the effects of redox-reactions of metals in water environment. *J Eng Technol Res*. 3(13):371-380.

van Beilen JB, Li Z, Duetz WA, Smits THM, & Witholt B. (2003). Diversity of alkane hydroxylase systems in the environment. *Oil Gas Sci Technol*, *58*(4), 427-440.

van Beilen JB, Eggink G, Enequist H, Bos R, & Witholt B. (1992). DNA sequence determination and functional characterization of the OCT-plasmid-encoded alkJKL genes of *Pseudomonas oleovorans*. *Mol Microbiol*, *6*(21), 3121-3136.

van Beilen JB, & Funhoff EG. (2007). Alkane hydroxylases involved in microbial alkane degradation. *Appl Environ Microbiol*, *74*(1), 13-21.

van Beilen JB, Holtackers R, Lüscher D, Bauer U, Witholt B, & Duetz WA. (2005). Biocatalytic production of perillyl alcohol from limonene by using a novel *Mycobacterium* sp. cytochrome P450 alkane hydroxylase expressed in *Pseudomonas putida*. *Appl Environ Microbio*, *71*(4), 1737-1744.

van Beilen JB, Marín MM, Smits TH, Röthlisberger M, Franchini AG, Witholt B, & Rojo F. (2004). Characterization of two alkane hydroxylase genes from the marine hydrocarbonoclastic bacterium *Alcanivorax borkumensis. Environ Microbiol*, *6*(3), 264-273.

van Beilen JB, S Panke, S Lucchini, AG Franchini, M Rothlisberger and B Witholt. (2001). Analysis of *Pseudomonas putida* alkane degradation gene clusters and flanking insertion sequences: evolution and regulation of the alk genes. *Microbiology* 147:1621–1630.

van Beilen JB, Wubbolts MG, & Witholt B. (1994). Genetics of alkane oxidation by *Pseudomonas oleovorans*. *Biodegradation*, *5*(3-4), 161-174.

van der Kraan G, J Bruining BP Lomans, MCM van Loosdrecht, G Muyzer. (2010). Microbial diversity of an oil-water processing site and its associated oil field: the possible role of microorganisms as information carriers from oil-associated environments. *FEMS Microbiol Ecol*, 71, pp. 428-443

van Hamme JD, Singh A, Ward OP (2003) Recent advances in petroleum microbiology. *Microbiol Mol Biol Rev* 67: 503–549.

Vandamme P, Falsen E, Rossau R, Hoste B, Segers P, Tytgat R, & De Ley J. (1991). Revision of *Campylobacter, Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. *Intern J Syst Evol Microbiol*, *41*(1), 88-103.

Ventosa A, Nieto JJ, & Oren A. (1998). Biology of moderately halophilic aerobic bacteria. *Microbiol Molec Biol Rev*, *62*(2), 504-544.

Verfürth K, Pierik AJ, Leutwein C, Zorn S, & Heider J. (2004). Substrate specificities and electron paramagnetic resonance properties of benzylsuccinate synthases in anaerobic toluene and m-xylene metabolism. *Arch Microbiol*, *181*(2), 155-162.

Venzlaff H, Enning D, Srinivasan J, Mayrhofer KJ, Hassel AW, Widdel F, & Stratmann M. (2013). Accelerated cathodic reaction in microbial corrosion of iron due to direct electron uptake by sulfate-reducing bacteria. *Corrosion Sci*, *66*, 88-96.

Videla HA. (1996). *Manu Biocorrosion*. CRC Lewis Publishers, Boca

Videla HA. (2002). Prevention and control of biocorrosion. *Int Biodeter Biodegr 49*(4), 259-270.

Vigneron A, Alsop EB, Chambers B, Lomans BP, Head IM, & Tsesmetzis N. (2016). Complementary microorganisms in highly corrosive biofilms from an offshore oil production facility. *Appl Environ Microbiol*, *82*(8), 2545-2554.

Vila J, JM Nieto, J Mertens, D Springael, M Grifoll. (2010). Microbial community structure of a heavy fuel oil-degrading marine consortium: linking microbial dynamics with polycyclic aromatic hydrocarbon utilization. *FEMS Microbiol Ecol*, 73., pp. 349–362

Voordouw G, Armstrong SM, Reimer MF, Fouts B, Telang AJ, Shen YIN, & Gevertz D. (1996). Characterization of 16S rRNA genes from oil field microbial communities indicates the presence of a variety of sulfate-reducing, fermentative, and sulfide-oxidizing bacteria. *Appl Environ Microbiol*, *62*(5), 1623-1629.

Watkinson RJ, and Morgan P. 1990. Physiology of aliphatic hydrocarbon-degrading microorganisms. *Biodegradation*. 1: 79–92.

Wang YN, Cai H, Chi CQ, Lu AH, Lin XG, Jiang ZF, & Wu XL. (2007a). *Halomonas shengliensis* sp. nov., a moderately halophilic, denitrifying, crude-oil-utilizing bacterium. *Intern J Syst Evol Microbiol*, *57*(6), 1222-1226.

Wang Q, Garrity GM, Tiedje JM, & Cole JR. (2007b). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol*, *73*(16), 5261-5267.

Wang L, Wang W, Lai Q, & Shao Z. (2010). Gene diversity of CYP153A and AlkB alkane hydroxylases in oil-degrading bacteria isolated from the Atlantic Ocean. *Environ Microbiol*, *12*(5), 1230-1242.

Wawrik B, Marks CR, Davidova IA, McInerney MJ, Pruitt S, Duncan KE, ... & Callaghan AV. (2016). Methanogenic paraffin degradation proceeds via alkane addition to fumarate by '*Smithella*'spp. mediated by a syntrophic coupling with hydrogenotrophic methanogens. *Environ Microbiol*, *18*(8), 2604-2619.

Wawrik B, Mendivelso M, Parisi VA, Suflita JM, Davidova IA, Marks CR, & Strąpoć D. (2012). Field and laboratory studies on the bioconversion of coal to methane in the San Juan Basin. *FEMS Microbiol Ecol*, *81*(1), 26-42.

Weijma J, Gubbels F, Pol LH, Stams AJM, Lens P, & Lettinga G. (2002). Competition for H2 between sulfate reducers, methanogens and homoacetogens in a gas-lift reactor. *Water Sci Technol*, *45*(10), 75-80.

Weisburg WG, Barns SM, Pelletier DA, Lane DJ. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol*. Jan;173(2):697-703.

Wentzel A, Ellingsen TE, Kotlar HK, Zotchev SB, Throne-Holst M. (2007). Bacterial metabolism of long-chain n-alkanes. *Appl Microbiol Biot* 76: 1209–1221.

Widdel F. (1988). Microbiology and ecology of sulfate-and sulfur-reducing bacteria. *Biol Anaerob Microorg*, 469-585.

Widdel F, & Bak F. (1992). Gram-negative mesophilic sulfate-reducing bacteria. In *The prokaryotes* (pp. 3352-3378). Springer New York.

Widdel F, Hansen TA, Balows A, Truper HG, Dworkin M, Harder W, and Schleifer KH. (1992). The dissimilatory sulfate-and sulfur-reducing bacteria. *The prokaryotes: a handbook on the biology of bacteria: ecophysiology, isolation, identification, applications.,* 1(2): 582-624.

Widdel F, & Rabus R. (2001). Anaerobic biodegradation of saturated and aromatic hydrocarbons. *Curr Opin Biot*, *12*(3), 259-276.

Wilkes H, Rabus R, Fischer T, Armstroff A, Behrends A, & Widdel F. (2002). Anaerobic degradation of n-hexane in a denitrifying bacterium: further degradation of the initial intermediate (1-methylpentyl) succinate via C-skeleton rearrangement. *Arch Microbiol*, *177*(3), 235-243.

Wirsen CO, Sievert SM, Cavanaugh CM, Molyneaux SJ, Ahmad ATLT, Taylor LT, Taylor CD. (2002). Characterization of an autotrophic sulfide-oxidizing marine *Arcobacter* sp. that produces filamentous sulfur. *Appl Environ Microbol*, 68(1):316-325.

Wu YW, Tang YH, Tringe SG, Simmons BA, & Singer SW. (2014). MaxBin: an automated binning method to recover individual genomes from metagenomes using an expectation-maximization algorithm. *Microbiome*, *2*(1), 26.

Wu J, Mao X, Cai T, Luo J, & Wei L. (2006). KOBAS server: a web-based platform for automated annotation and pathway identification. *Nucleic Acids Res*, *34*(suppl_2), W720-W724.

Xie C, Mao X, Huang J, Ding Y, Wu J, Dong S, Wei L. (2011). KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Res*, *39*(suppl_2), W316-W322.

Xue Y, & Voordouw G. (2015). Control of microbial sulfide production with biocides and nitrate in oil reservoir simulating bioreactors. *Front Microbiol*, *6*, 1387.

Yakimov MM, Timmis KN, & Golyshin PN. (2007). Obligate oil-degrading marine bacteria. *Curr Opi Biot*, *18*(3), 257-266.

Yakimov M, R Denaro, M Genovese, S Cappelo, G D'Auria, TN Chernikova, KN Timmis, PN Golyshin, L Giluliano. (2005). Natural microbial diversity in superficial sediments of Milazzo Harbor (Sicily) and community successions during microcosm enrichment with various hydrocarbons. *Environ Microbiol*, 7., pp. 1426-1441

Yuehui S, Z Beiwen, Z Fan, W Zhengliang, S Fuchang, Z Lingge, X Tingsheng, Y Longjiang (2008). Analysis on the microbial diversity of Qinghai high salt content oil reservoirs. *The 2nd International Conference on Bioinformatics and Biomedical Engineering.* pp. 780-783

Zhao HP, Wang L, Ren JR, Li Z, Li M, & Gao HW. (2008). Isolation and characterization of phenanthrene-degrading strains *Sphingomonas* sp. ZP1 and *Tistrella* sp. ZP5. *J Hazard Mater*, *152*(3), 1293-1300.

Zhang T, Fang HHP, Ko BCB. (2003). Methanogen population in a marine biofilm corrosive to mild steel. *Appl Microbiol Biot*, 63:101-106.

Zhang L, Zhang C, Cheng Z, Yao Y, & Chen J. (2013). Biodegradation of benzene, toluene, ethylbenzene, and o-xylene by the bacterium Mycobacterium cosmeticum byf-4. *Chemosphere*, *90*(4), 1340-1347.

Zhou Y, Huang H, & Shen D. (2016). Multi-substrate biodegradation interaction of 1, 4-dioxane and BTEX mixtures by *Acinetobacter baumannii* DD1. *Biodegradation*, *27*(1), 37-46.

Zengler K, Richnow HH, Rosselló-Mora R, Michaelis W, & Widdel F. (1999). Methane formation from long-chain alkanes by anaerobic microorganisms. *Nature*, *401*(6750), 266-269.

Zerbino DR, & Birney E. (2008). Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res*, *18*(5), 821-829.

Zobell CE. (1946). Action of microorganisms on hydrocarbons. *Bacteriol Rev*, vol. 10, pp. 1–49.

Tables

Chapter 2: The role of Gammaproteobacteria in aerobic alkane degradation in oilfield production water from the Barnett Shale

Table 1. Hydrocarbon and heterotrophic substrate amendments used for enrichment of microorganisms in production water.

Substrate	Volume per 30 ml
n-alkanes (C_5-C_{10})[a]	100 µl of 0.1 M solution
BTEX (benzene, toluene, ethylene, xylene)[a]	100 µl of 0.1 M solution
n-fatty acids (C_5-C_{10})[b]	300 µl of 0.1 M solution
Oxidized BTEX (*p*-cresol, phenol, catechol)[a]	400 µl of 0.1 M solution
Yeast Extract[c,d]	0.3 ml of 10 mg/ml
Tryptone[c,d]	0.2 ml of 20 mg/ml

[a]All hydrocarbon substrates were from Sigma-Aldrich (St. Louis, MO, USA): C_5, *n*-pentane, anhydrous >99% CAS 109-66-0; C_6, *n*-hexane, anhydrous >99% CAS 110-54-3; C_7 , *n*-heptane, anhydrous >99% CAS 142-82-5; C_8, *n*-octane, anhydrous >99% CAS 111-65-9; C_9 *n*-nonane, anhydrous >99% CAS 111-84-2; C_{10} *n*-decane, anhydrous >99% CAS 124-18-5; Benzene, for HPLC, 99.9%, CAS 71-43-2; Toluene, anhydrous >99.8, CAS 108-88-3, Ethylbenzene, analytical standard, CAS 100-41-4; -xylene, analytical standard, CAS 95-47-6; *p*-cresol, >99%, FG CAS 106-44-5; phenol, GR for analysis ACS, Reag, CAS 108-95-2; catechol/ 1,2-dihydroxybenzene, *ReagentPlus*, >99%, CAS 120-80-9

[b]All *n*-fatty acids were Sigma-Aldrich (St. Louis, MO, USA): C_5, pentanoic acid, analytical standard, CAS 109-52-4; C_6, hexanoic acid, >99%, CAS 142-62-1; C_7, heptanoic acid, GC grade, CAS 111-14-8; C_8, octanoic acid, FG grade, CAS 24-07-2; C_9, nonanoic acid >96% FG grade, CAS 112-05-0; C_{10}, decanoic acid, >98% FCC, FG grade, CAS 334-48-5

[c]Difco, Detroit, MI, USA

[d]Positive control substrates for heterotrophic microorganisms

Table 2. Balanced stoichiometry for complete oxidation of various hydrocarbons.

n-alkane	Balanced stoichiometry	Carbon:Oxygen ratio for 100% theoretical yield
C_5 *n*-pentane	$C_5H_{12} + 8O_2 \rightarrow 5CO_2 + 6H_2O$	1:8
C_6 *n*-hexane	$C_6H_{14} + 9O_2 \rightarrow 6CO_2 + 6H_2O$	1:9
C_7 *n*-heptane	$C_7H_{16} + 11O_2 \rightarrow 7CO_2 + 8H_2O$	1:11
C_8 *n*-octane	$C_8H_{18} + 12O_2 \rightarrow 8CO_2 + 8H_2O$	1:12
C_9 *n*-nonane	$C_9H_{20} + 14O_2 \rightarrow 9CO_2 + 10H_2O$	1:14
C_{10} *n*-decane	$C_{10}H_{22} + 15.5O_2 \rightarrow 10CO_2 + 11H_2O$	1:15.5
C_{16} *n*-hexadecane	$C_{16}H_{36} + 25O_2 \rightarrow 16CO_2 + 18H_2O$	1:25
Toluene	$C_7H_5CH_3 + 10O_2 \rightarrow 8CO_2 + 4H_2O$	1:10

Stoichiometric calculations made using http://www.webqc.org/balance.php

Table 3. Environmental parameters of Barnett Shale production water. Measurements were made in-field (pH, oxygen, sulfide, and temperature) or immediately following return to the laboratory (sulfate, thiosulfate, salinity, and direct cell counts).

Water chemistry parameter	Result
pH	6.9
Oxygen	0 ppm
Sulfide	Undetectable
Thiosulfate	6.5 mg/L
Sulfate	150 uM
Salinity	2.7%
Temperature	30 °C
Direct cell counts	≤ 1.68e +6 cells/ mL

Table 4. Organic extractions of Barnett Shale production water identifying putative polar metabolites, intermediates, and other compounds. Production water extractions were concentrated, derivatized, and analyzed by Gas Chromatography-Mass Spectroscopy (GC-MS). Metabolite identities were made by comparing GC retention times and fragmentation by MS profiles of commercially available standards.

Class	**Species detected in production water**
n-alkane	C_5-C_{12}
Iso-alkane	C_7-C_{17}
n-fatty acid	C_4-C_6
Alkanoic acids	C_5-C_{10}, C_{16}, C_{18} and unsaturated C_{18}
Aromatic	Alkylated benzene, toluene, alkylated toluene, xylene, alkylated xylene, phenol, alkylated phenol, *p*-cresol, *o*-cresol, benzoic acid, benzenediol
Polyaromatic	Naphthalene, alkylated naphthalene
Cycloalkane	Alkylated cyclohexane
Alcohol	Cyclohexanol
α-hydroxy acids	Glycolic acid, lactic acid, hydroxypropanoic acid, hydroxybutanoic acid
Dioic acids	Succinic acid, pentanedioic acid, hexanedioic acid
Other acids	2-butoxy acetic acid

Table 5. Summary of the dominant classes and corresponding genera of bacteria detected via 16S rRNA sequencing of original Barnett Shale production water.

Class	Relative abundance (%)	Genus*	Relative abundance (%)
Deltaproteobacteria	41.0	*Desulfotignum*	4.6
		Desulfobacter	29.1
		Desulfuromonas	1.1
Gammaproteobacteria	10.5	*Halomonas*	2.2
		Marinobacterium	7.4
Bacilli	2.0	*Bacillus*	1.1
Clostridia	10.2	*Halanaerobium*	2.4
		Dehalobacterium	4.8
Synergistia	5.3	*Dethiosulfovibrionaceae*	3.3
Thermotogae	11.4	*Geotoga*	9.0

*Only Genera with a relative abundance of >1% are listed

Table 6. Aerobic enrichment of microorganisms in Barnett Shale production water amended with hydrocarbon and heterotrophic substrates in Widdel's medium. Growth is indicated by (+/-), where (+) indicates growth, estimated by visual turbidity after 3 transfers, (-) indicates no growth after 3 transfers. All enrichments were prepared in triplicate in aerobic 160 mL sealed serum bottles. Unamended bottles, which consisted of the medium and inoculum but without the substrate, were used to check for endogenous respiration. Sterile heat-killed bottles were used as negative controls. Bottles were incubated at 25 °C.

Substrate/ Amendment	Growth
Unamended Control	-
Sterile heat-killed control	-
n-alkanes (C_5-C_{10})	+
BTEX (benzene, toluene, ethylene, xylene)	-
n-fatty acids (C_5-C_{10})	+
Oxidized BTEX (*p*-cresol, phenol, catechol)	-
Yeast Extract & Tryptone	+

Table 7. Summary of 16S rRNA sequence libraries from the original Barnett Shale production water and after the third round of the initial and progressive enrichments amended with *n*-alkanes and *n*-fatty acids.

			Relative abundance (%)			
			n-alkane amendment		*n*-fatty acid amedment	
Class	Genus*	Original	Initial	Progressive	Initial	Progressive
Alphaproteobacteria	*Thalassospira*	0.2	0.4	0.2	11.6	0.0
	Roseovarius	0.0	0.3	5.0	0.1	0.0
Deltaproteobacteria	*Desulfotignum*	4.6	0.0	0.0	0.2	0.0
	Desulfobacter	29.1	0.0	0.0	0.7	0.0
Epsilonproteobacteria	*Arcobacter*	0.7	9.9	0.0	2.0	0.0
Gammaproteobacteria	*Shewanella*	0.0	0.5	13.5	1.5	0.5
	Halomonas	2.2	0.3	75.9	0.3	75.1
	Marinobacterium	7.4	0.9	0.0	1.0	0.0
	Marinobacter	0.1	0.4	2.4	0.1	2.4
	Oleibacter	0.0	2.5	0.0	4.3	0.0
Clostridia	*Dethiosulfatibacter*	0.6	0.0	0.0	0.3	7.3
Thermotogae	*Geotoga*	9.0	64.5	9.2	63.3	9.2
Mollicutes	*Acholeplasma*	ND	0.0	0.0	0.2	20.8

* Only genera that attained a relative abundance > 1% in either the original production water or the final round of enrichments are shown.

Table 8. Evaluation of *Halomonas* A11A genome for genes involved in the aerobic degradation of alkanes and aromatics.

Class of Enzyme	Enzyme Designation	Enzyme Commission #	Gene	*Halomonas* A11A
Monooxygenase	Alkane monooxygenase	1.14.15.3	*alkB*	Yes
	Cytochrome p450	1.14.15.3	CYP 153	ND
	Alkane monooxygenase	1.14.13.-	*almA*	ND
	Alkane monooxygenase	-	*ladA*	ND
	Xylene monooxygenase	1.14.13.-	*xylMA*	ND
	Phenol 2-monooxygenase	1.14.13.7	-	Yes
Dioxygenase	Benzene dioxygenase	1.14.12.3	*bed*	Yes
	Toluene dioxygenase	1.14.12.11	*tod*	Yes
	Ethylbenzene dioxygenase	1.13.12.-	*etbA*	ND
	Naphthalene 1,2-dioxygenase	1.14.12.12	*nah, ndo, nbz, dnt*	ND
	Phenol dioxygenase	1.14.13.7	-	Yes
	Catechol 1,2-dioxygenase	1.13.11.1	*cat*	ND
Other	Catechol 2,3-dioxygenase	1.13.11.2	*dmp, xyl, cat*	Yes
	Phenol hydroxylase	1.14.13.-	*dmp, pox*	Yes

ND, not detected

Yes: Genes detected in A11A genome through the MGMIC pipeline housing the KEGG KOBAS database using the default cutoffs: *E*-value $<10^{-5}$ and rank ≤ 5 (Xei et al., 2011)

Table 9. Range of hydrocarbons oxidized by *Halomonas* A11A. Oxygen (μM) consumption during hydrocarbon oxidation ("Experimental") is compared to the consumption based on complete oxidation ("Theoretical", see Table 2). Experimental values were derived from single incubation bottles inoculated with C_5 *n*-pentane-grown inoculum cells amended with nearly-equal carbon concentrations as shown in Figures 6 & 7.

Hydrocarbon	Experimental: μM hydrocarbon	Theoretical: μM O_2 for 100% oxidation	Experimental: μM of O_2 consumed	Percent of theoretical consumed
C_5 *n*-pentane	40	320	243.8	76.2
C_6 *n*-hexane	36	324	266.9	82.4
C_7 *n*-heptane	32	352	327.1	92.9
C_8 *n*-octane	28	336	165.1	49.1
C_9 *n*-nonane	24	336	397.1	118.2
C_{10} *n*-decane	20	310	210.5	67.9
C_{16} *n*-hexadecane	12	300	169.7	56.6
Toluene	32	320	207.5	64.9

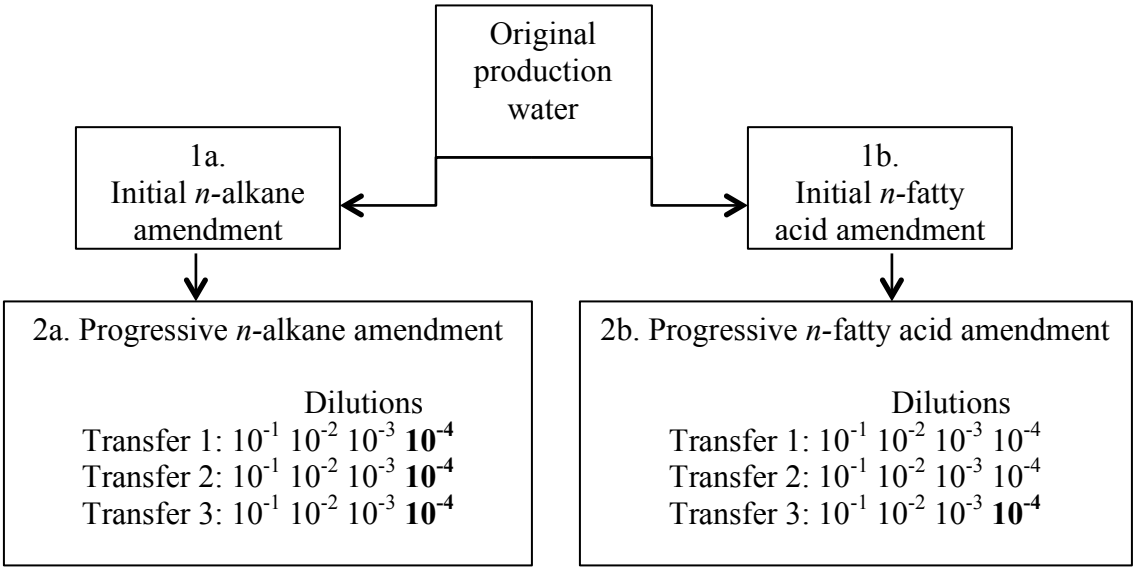
Table 10. Comparison of oxygen consumed for *n*-pentane versus *n*-decane grown *Halomonas* A11A cells. Experimental values were derived from averaged triplicate incubations for C_5 *n*-pentane-grown or C_{10} *n*-decane-grown inoculum cells amended with 20 µM of the hydrocarbon.

Hydrocarbon	Experimental: µM hydrocarbon	Theoretical: µM O_2 for 100% oxidation	Experimental: µM of O_2 consumed	Percent of theoretical consumed
C_5 *n*-pentane grown cells (Figure 8)				
C_5 *n*-pentane	20	160	151.9 ± 19.4	94.5
C_{10} *n*-decane	20	310	187.9 ± 9.6	60.6
C_5 *n*-pentane grown cells (Figure 9)				
C_{16} *n*-hexadecane	20	500	361.3 ± 17.5	72.3
Toluene	20	200	74.1 ± 13.8	37.0
C_{10} *n*-decane grown cells (Figure 10)				
C_5 *n*-pentane	20	160	158.8 ± 20.1	99.2
C_{10} *n*-decane	20	310	323.6 ± 17.9	104.4

Figures

Chapter 2: The role of Gammaproteobacteria in aerobic alkane degradation in oilfield production water from the Barnett Shale

Figure 1: Diagram of enrichment design. The original production water was subject to an initial round of amendment with *n*-alkanes (C_5-C_{10}) or *n*-fatty acids (C_5-C_{10}) conducted in triplicate and transferred three times. After initial amendment, progressive amendments with *n*-alkanes or *n*-fatty acids were made by serial dilution to 10^{-4} and transferred three times. Isolates were obtained from the 10^{-4} dilution of each *n*-alkane transfer and from the final transfer of *n*-fatty acids (bolded).



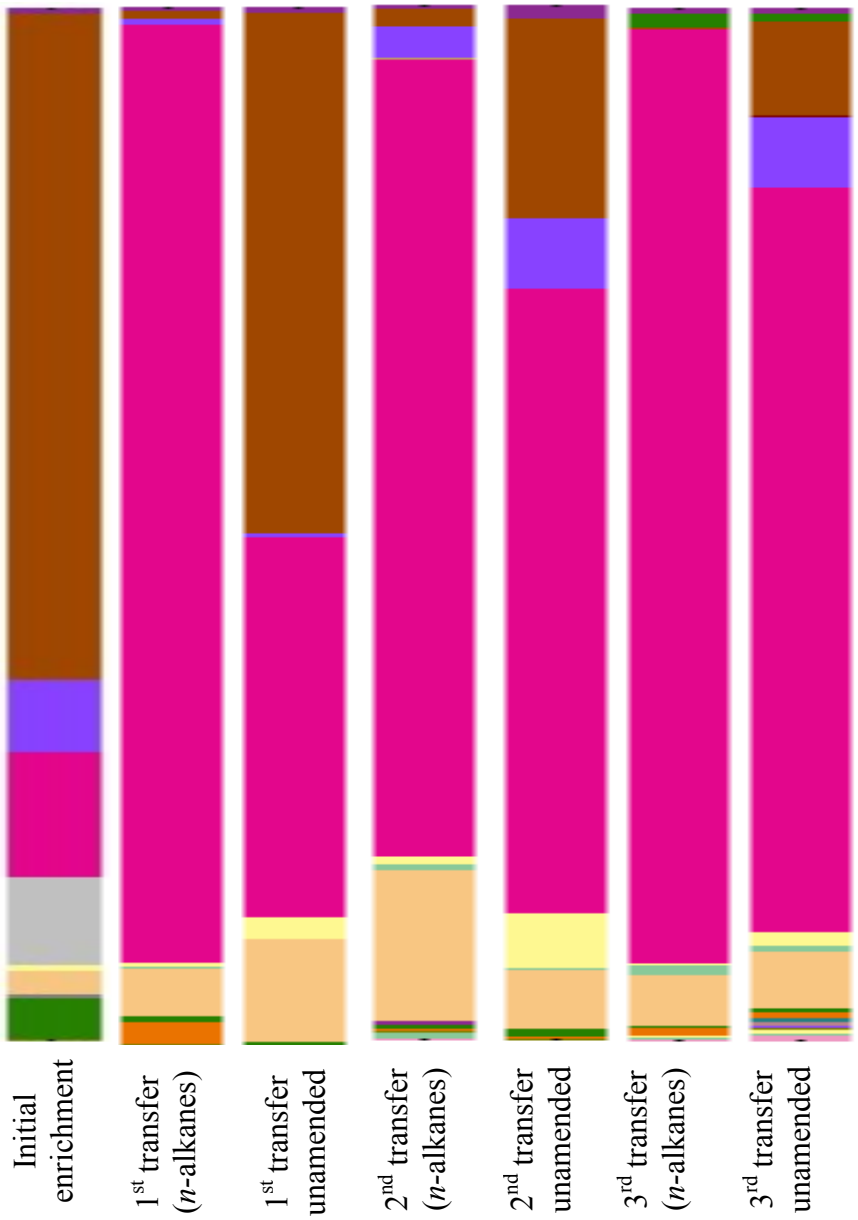


Figure 2. 16S sequence libraries representing the relative abundance (%) of different classes of bacteria in the initial and progressive rounds ("transfer") of enrichment amended with *n*-alkanes (C_5-C_{10}) compared to the corresponding unamended transfer. Each transfer is represented by the 10^{-4} dilution.

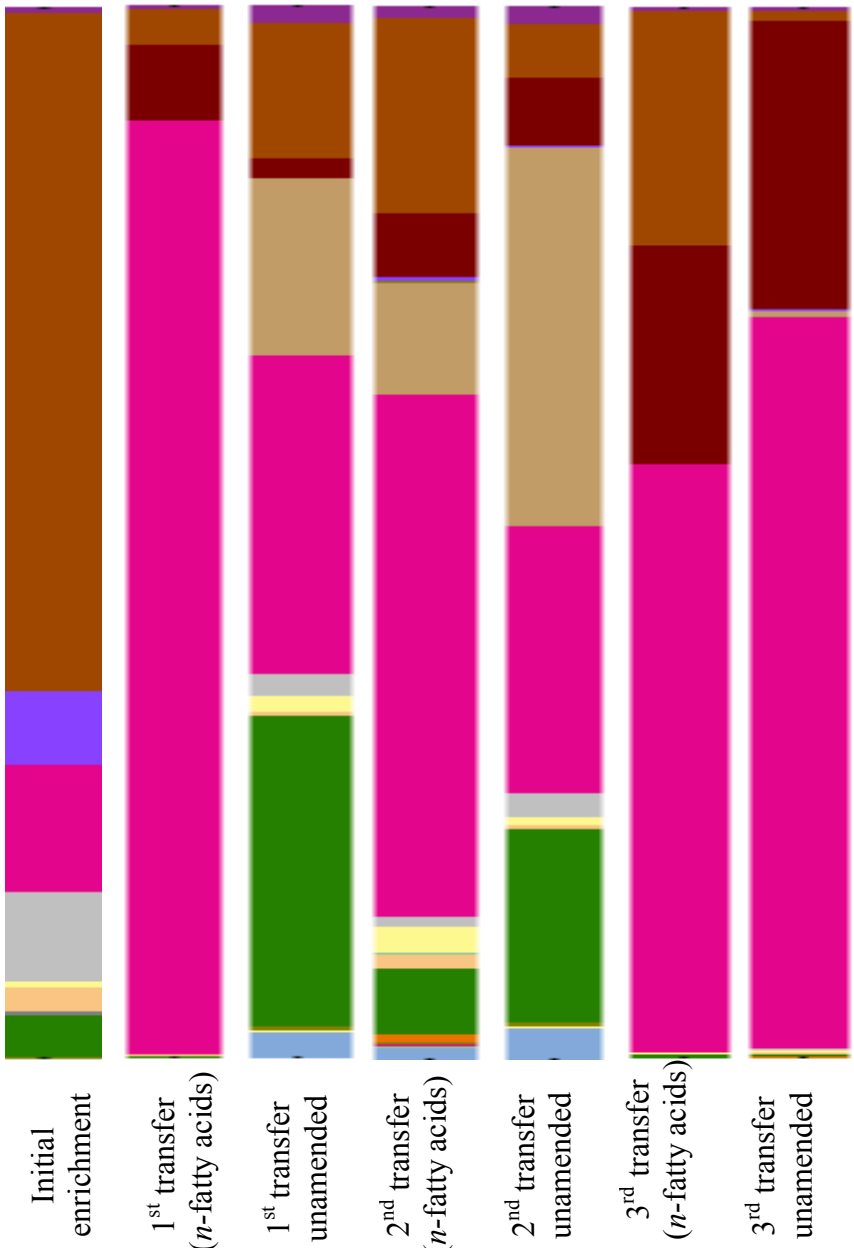


Figure 3. 16S sequence libraries representing the relative abundance (%) of different classes of bacteria in the initial and progressive rounds ("transfer") of enrichment amended with *n*-fatty acids (C_5-C_{10}) compared to the corresponding unamended transfer. Each transfer is represented by the 10^{-4} dilution.

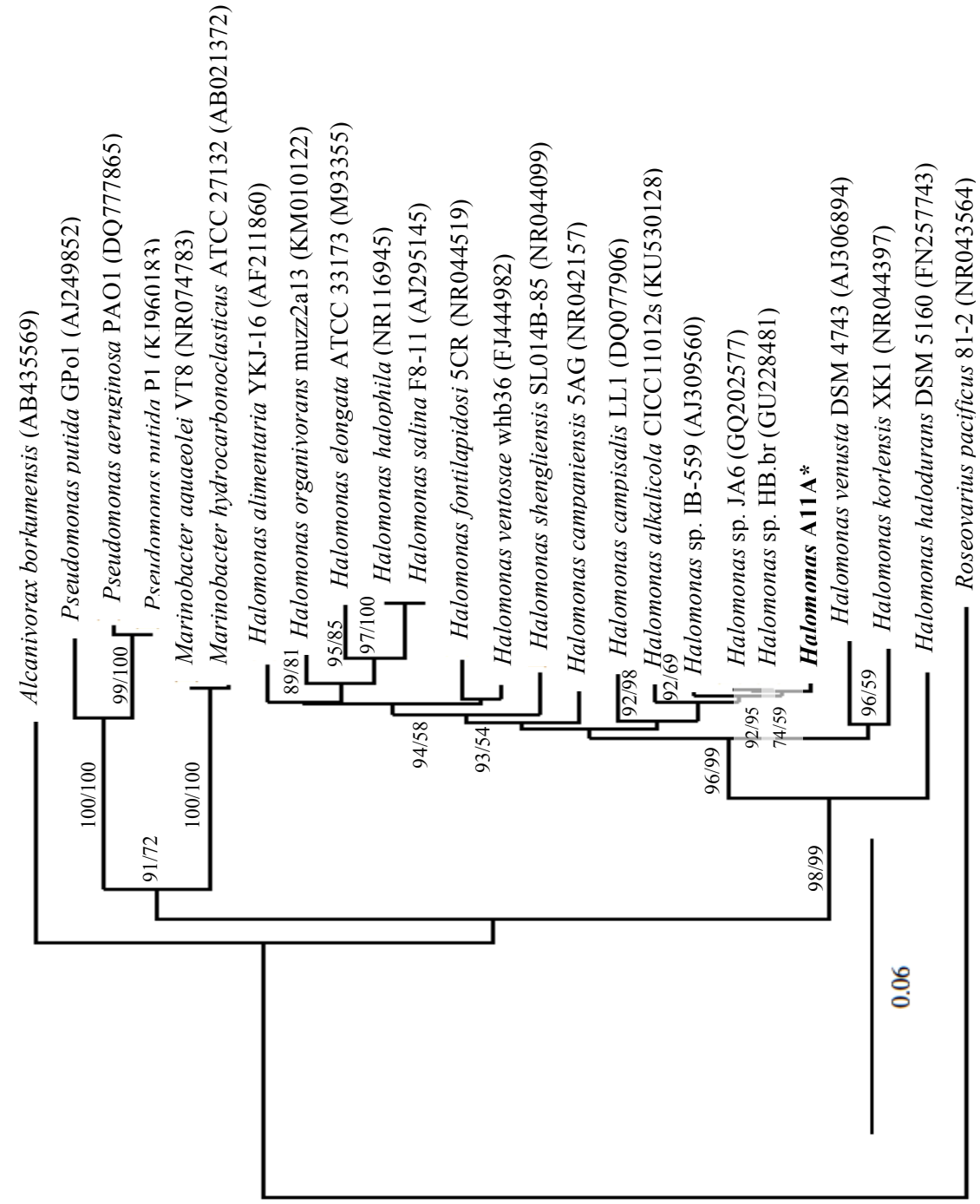
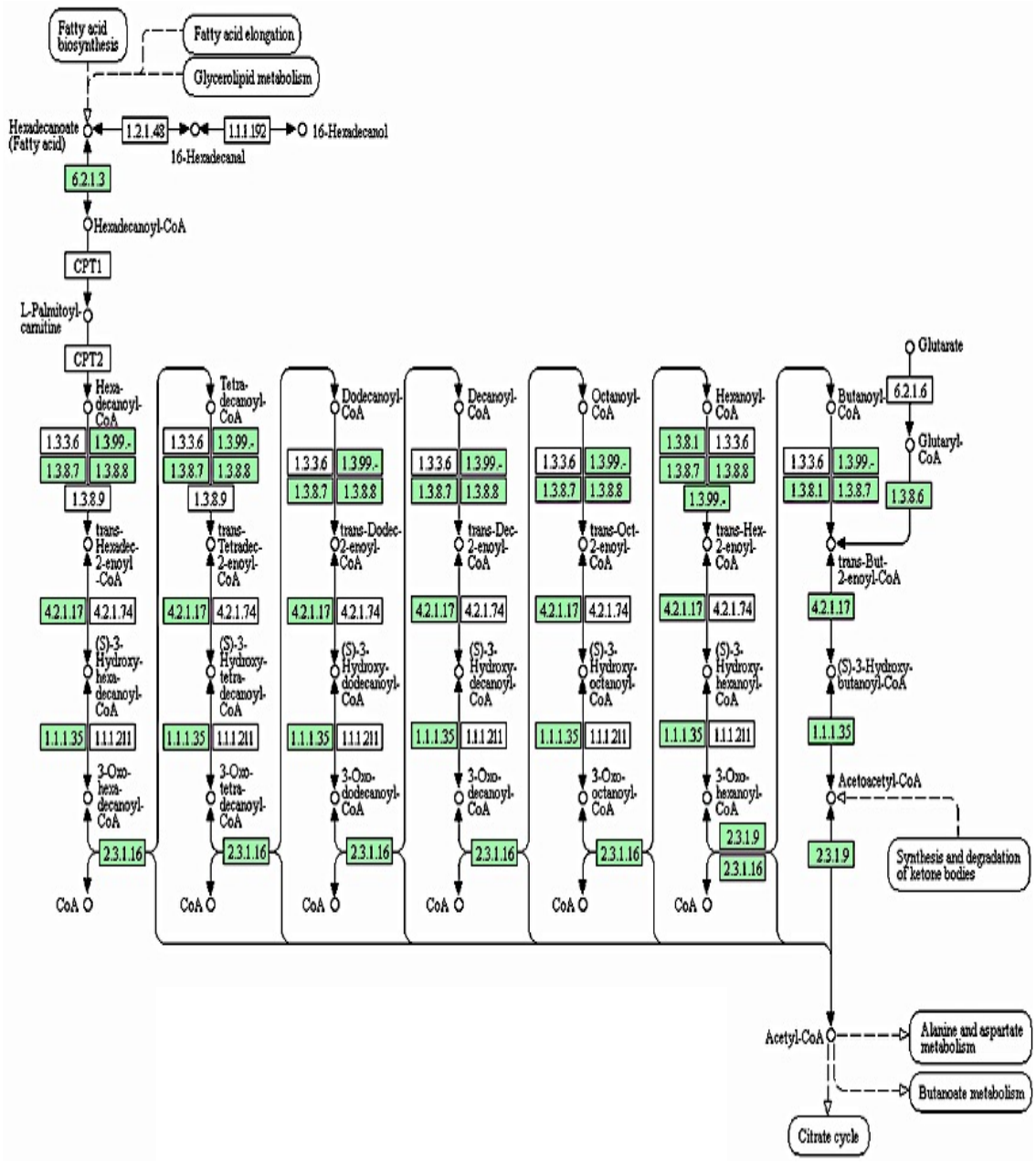


Figure 4. Phylogenetic tree of *Halomonas*, featuring strain A11A (in bold) (1432 bp), and selected other hydrocarbon-degrading Gammaproteobacteria. The phylogenetic relationship is based on multiple alignment (Clustal W) of near-complete (≥ 1411 bp)

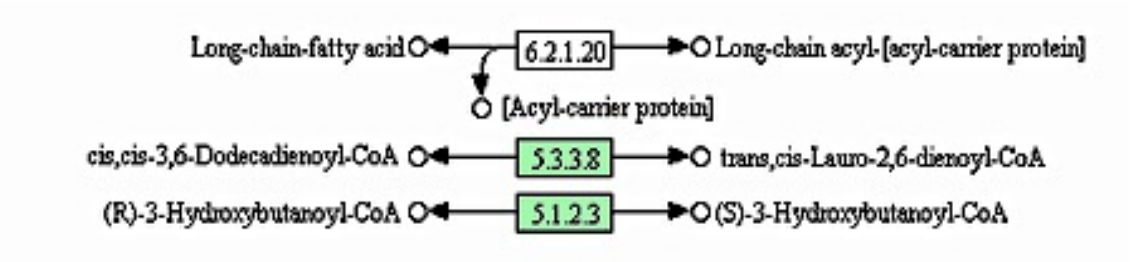
16S rRNA gene sequences using the neighbor-joining algorithm. Bootstrap values (≥ 50 %) based on 1000 replications are shown on nodes. The values represent bootstrap values for the topologies of maximum-likelihood and neighbor-joining trees, respectively. *Roseovarius pacificus* 81-2 (Alphaproteobacterium) was used as the outgroup. Bar, 0.06 substitutions per nucleotide position.

Figure 5. Fatty acid metabolism in *Halomonas* A11A.



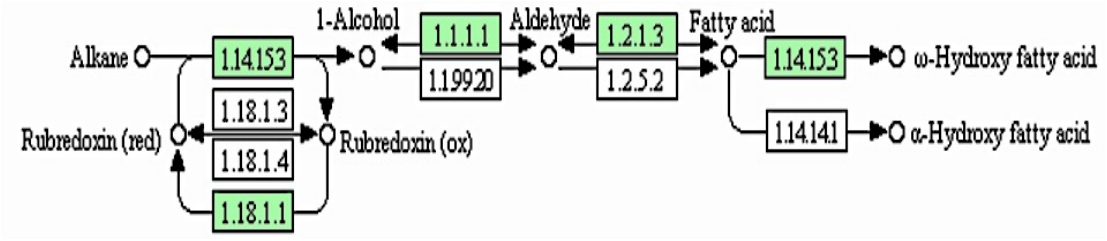
Enzymes inferred from genes present in the *Halomonas* A11A genome are indicated in green.

Figure 6. Long chain (C_{13}-C_{22}) fatty acid metabolism in *Halomonas* A11A.



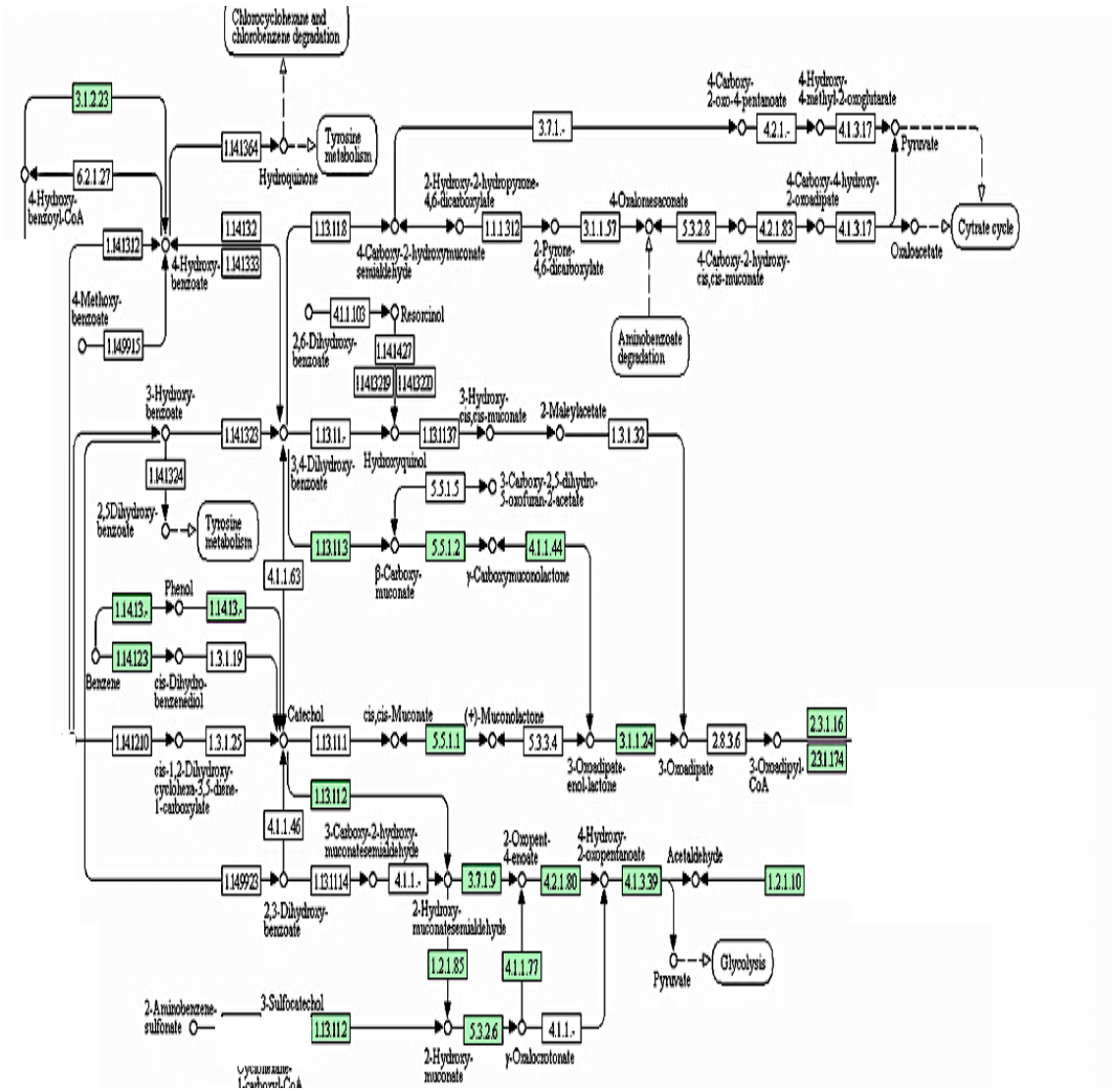
Enzymes inferred from genes present in the *Halomonas* A11A genome are indicated in green.

Figure 7. Alkane metabolism in *Halomonas* A11A.



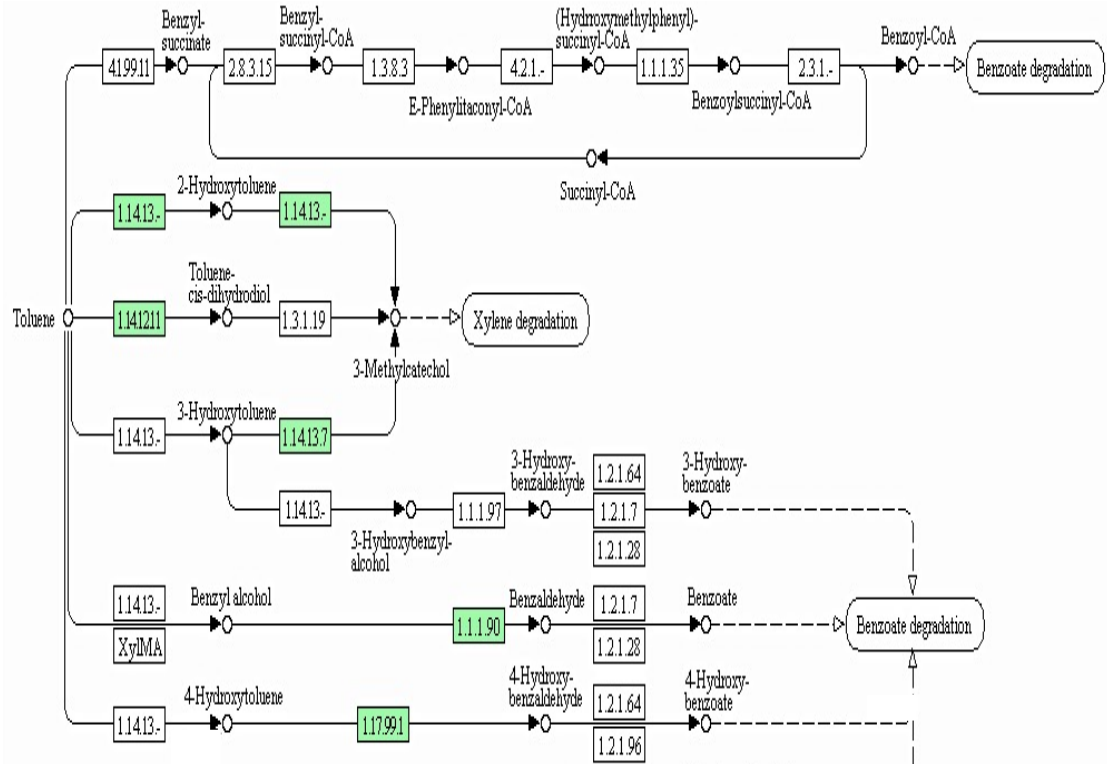
Enzymes inferred from genes present in the *Halomonas* A11A genome are indicated in green.

Figure 8. Benzene degradation in *Halomonas* A11A.



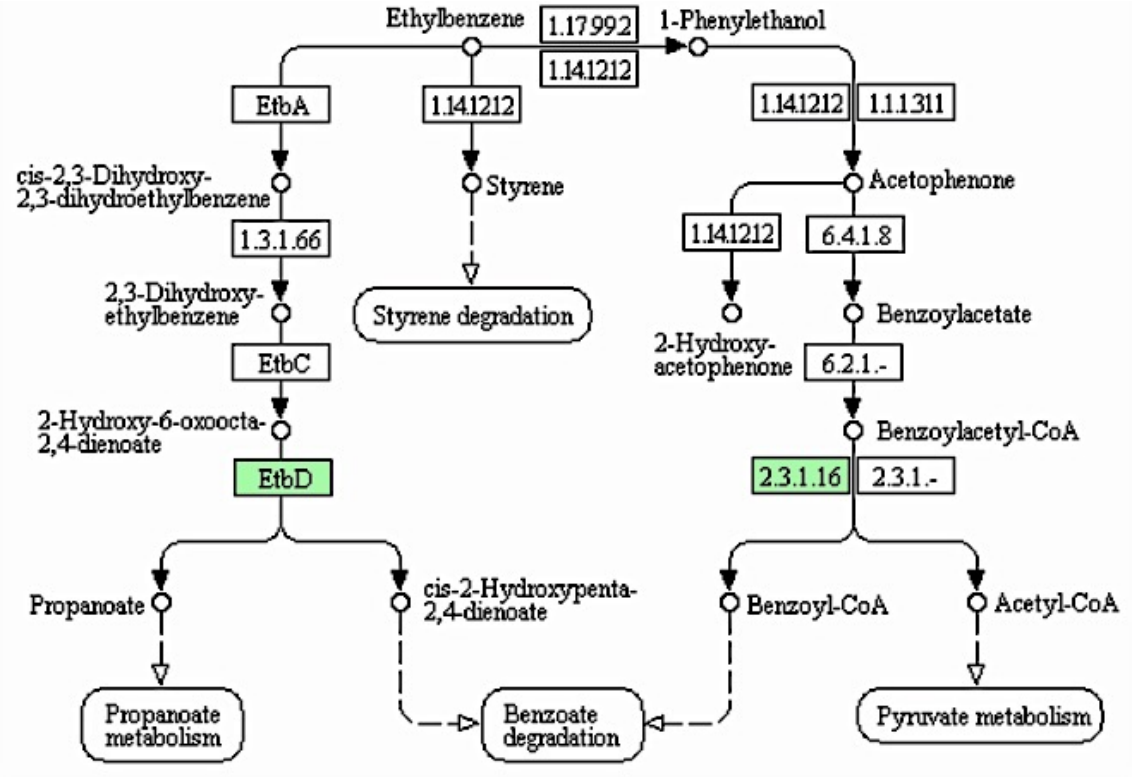
Enzymes inferred from genes present in the *Halomonas* A11A genome are indicated in green.

Figure 9. Toluene degradation in *Halomonas* A11A.



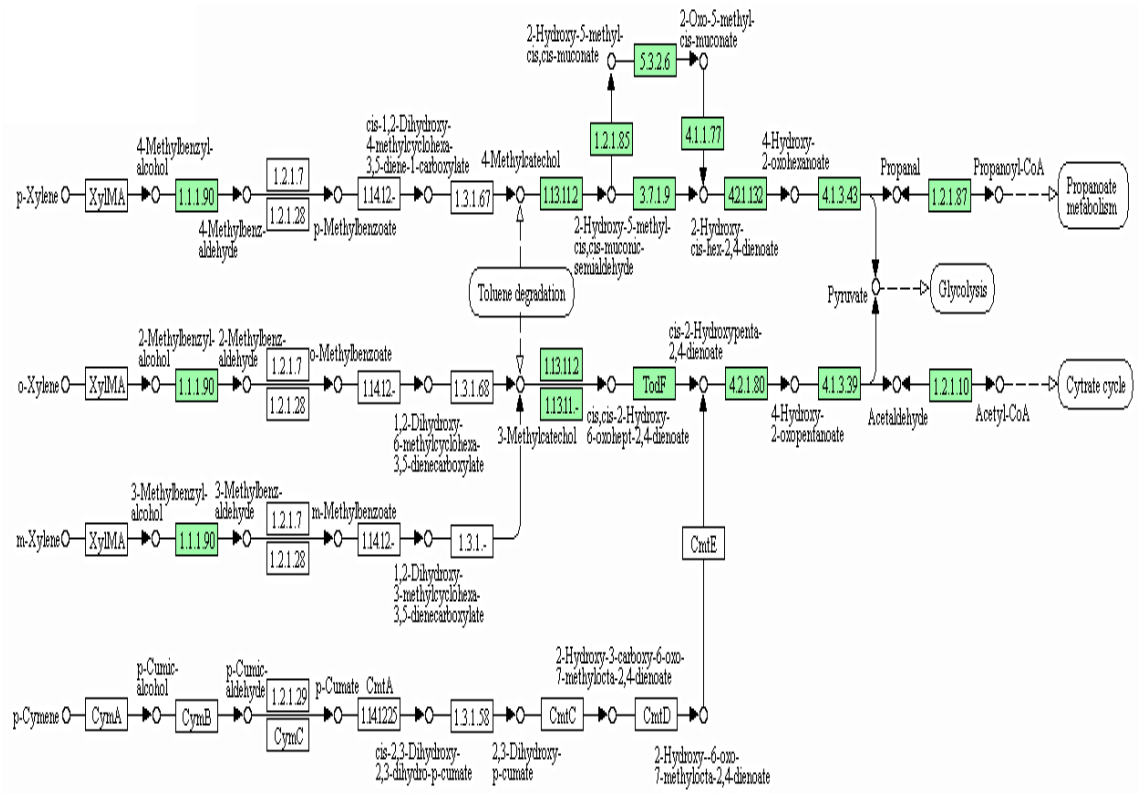
Enzymes inferred from genes present in the *Halomonas* A11A genome are indicated in green.

Figure 10. Ethylbenzene degradation in *Halomonas* A11A.



Enzymes inferred from genes present in the *Halomonas* A11A genome are indicated in green.

Figure 11. Xylene degradation in *Halomonas* A11A.



Enzymes inferred from genes present in the *Halomonas* A11A genome are indicated in green.

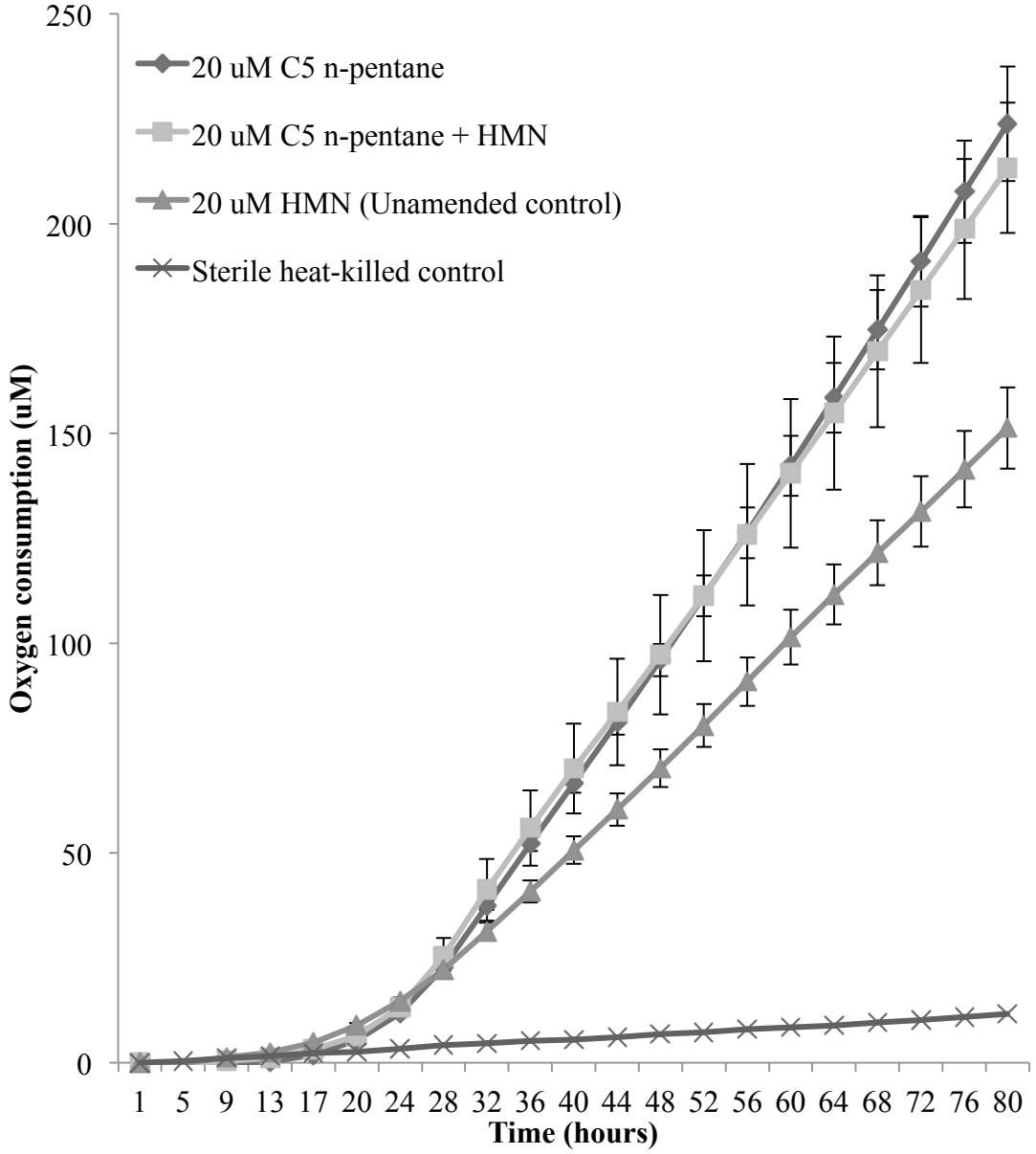


Figure 12. Effect of 2,2,4,4,6,8,8-heptamethylnonane (HMN) as a carrier for *n*-alkanes on the total consumption of oxygen (μM). Triplicate replicates containing either *n*-pentane, (diamonds) *n*-pentane plus HMN (squares), or HMN (triangles) were inoculated with 10% (v/v) of *Halomonas* A11A at 0.5 OD_{610}. A sterile heat-killed control and an unamended bottle, which consisted of the medium and inoculum but without the substrate, were used to compare the endogenous respiration. Each bottle was incubated at 25 °C. Bars represent one standard deviation of the average from the triplicate replicates.

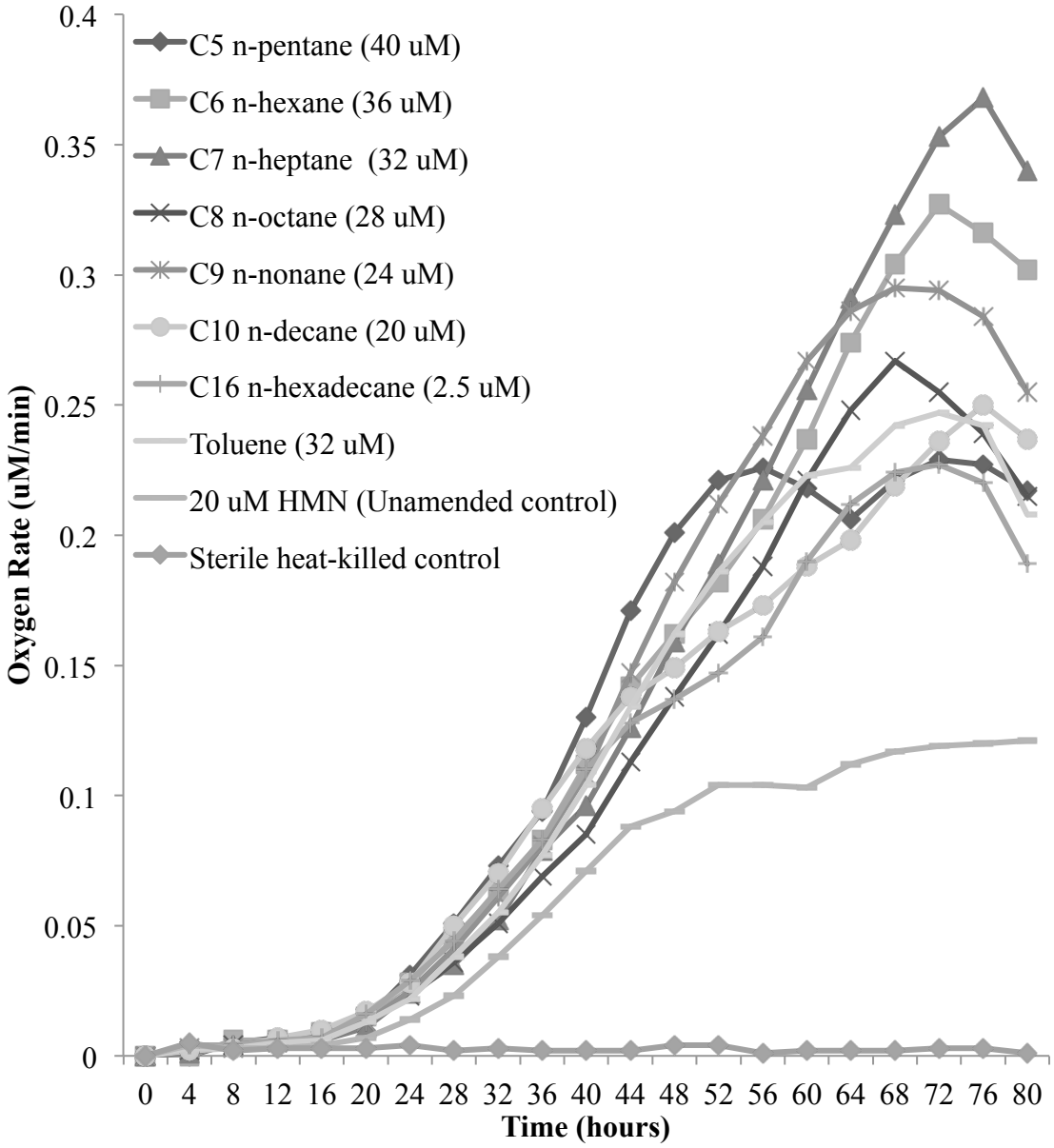


Figure 13. Rate of oxygen uptake (μM/min) by *Halomonas* A11A when grown with various hydrocarbons. Widdel's medium with hydrocarbon substrates at equal carbon number concentration served as the sole carbon source for cell growth. Bottles were inoculated with 0.5 OD_{610} 10% (v/v) of *Halomonas* A11A previously grown on 20 μM C_5 *n*-pentane. A sterile heat-killed control and an unamended bottle with heptamethylnonane (HMN) served as controls. Each bottle was incubated at 25 °C.

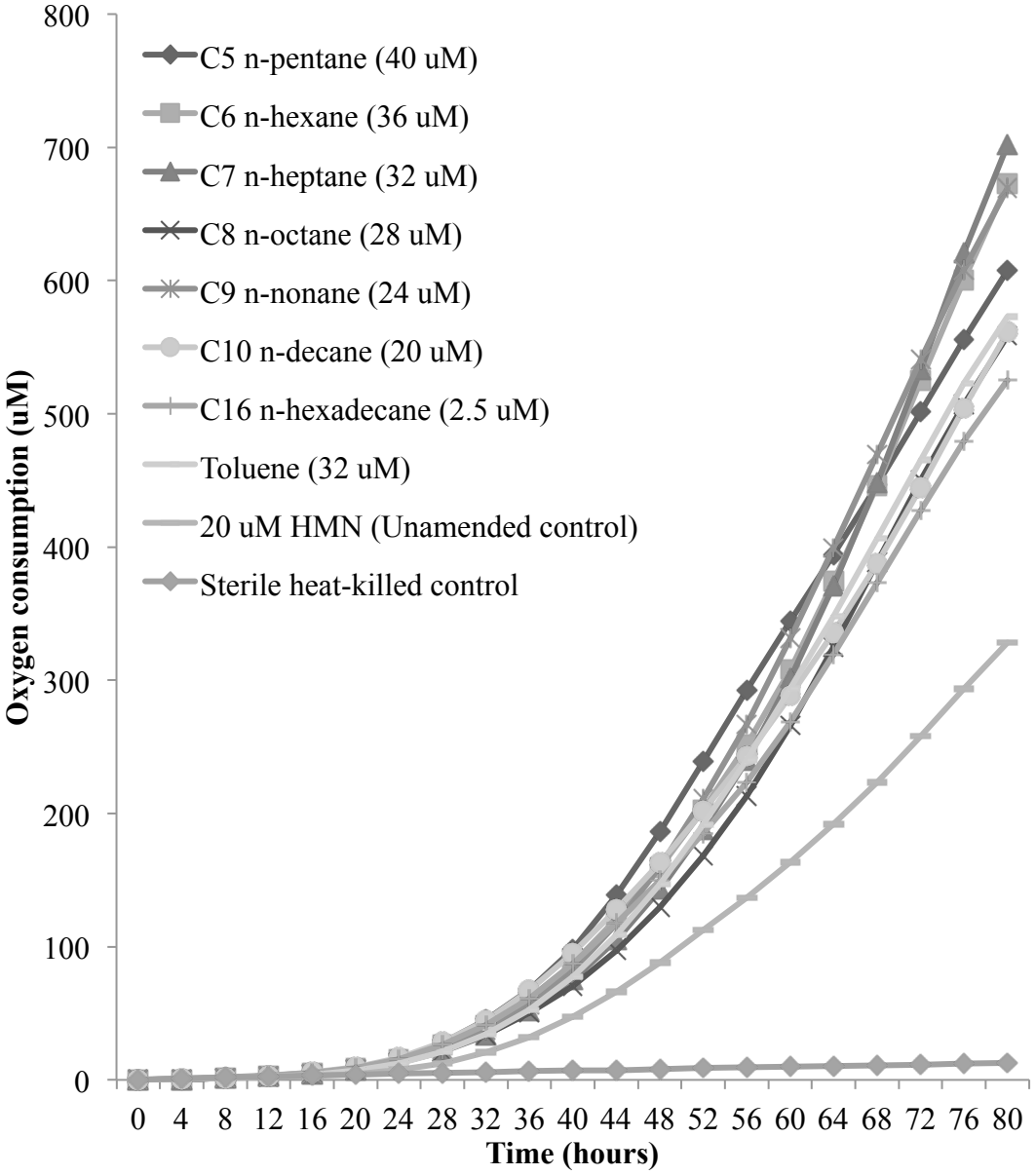


Figure 14. Total oxygen consumption (μM) by *Halomonas* A11A coupled to hydrocarbon oxidation. Widdel's medium with hydrocarbon substrates at equal carbon number concentration served as the sole carbon source for cell growth. Bottles were inoculated with 0.5 OD_{610} 10% (v/v) of *Halomonas* A11A previously grown on 20 μM C_5 *n*-pentane. A sterile heat-killed control and an unamended bottle with heptamethylnonane (HMN) served as controls. Each bottle was incubated at 25 °C.

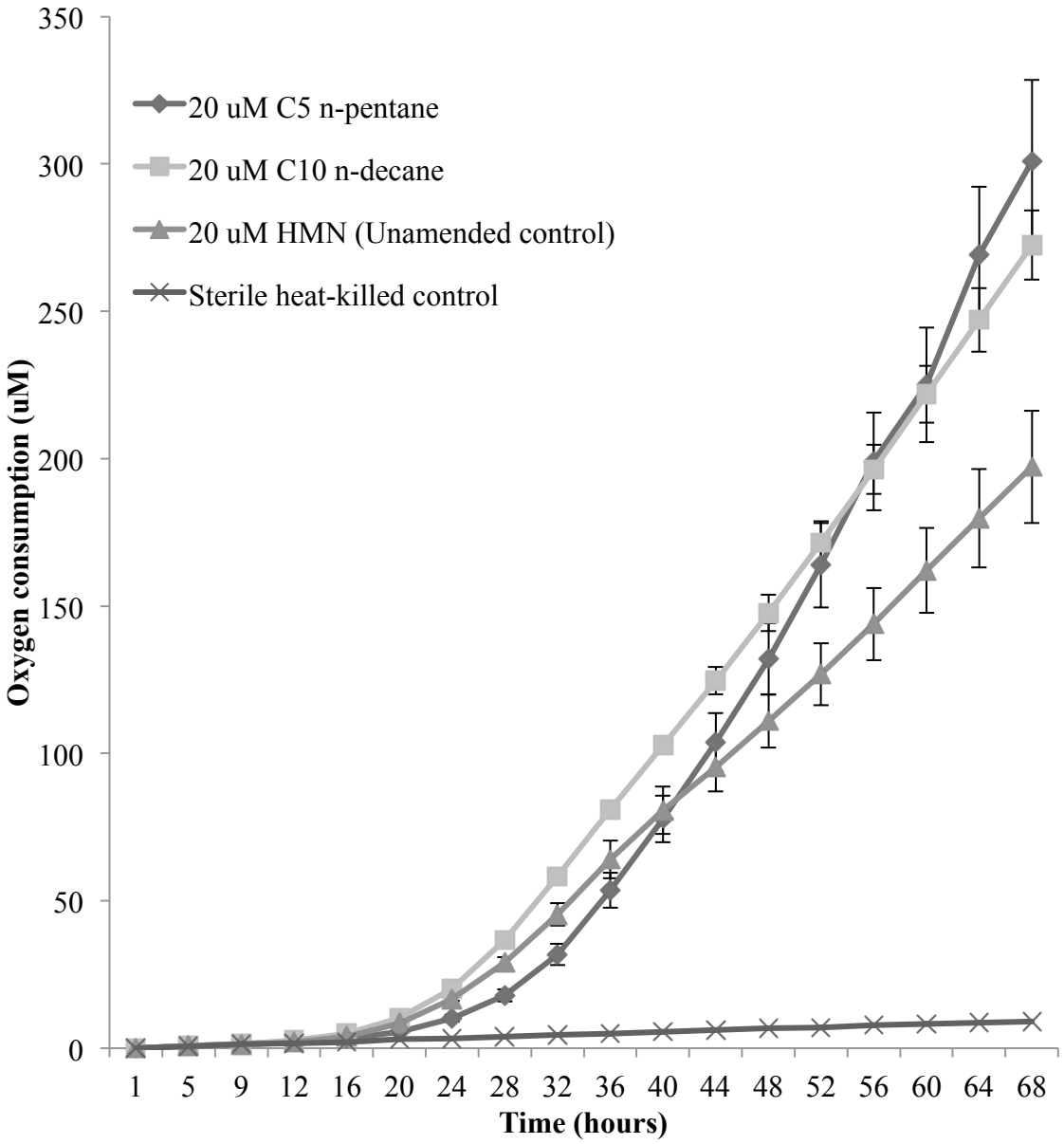


Figure 15. Total oxygen consumption (μM) coupled to *n*-pentane (diamonds) and *n*-decane (squares) oxidation by *n*-pentane-grown *Halomonas* A11A cells. Widdel's medium with 20μM *n*-pentane and 20μM *n*-decane served as the sole carbon source for cell growth. Triplicate replicates were inoculated with 0.5 OD_{610} 10% (v/v) of *Halomonas* A11A. A sterile heat-killed control and an unamended bottle with heptamethylnonane (HMN) (triangles) served as controls. Each bottle was incubated at 25 °C. Bars represent one standard deviation of the average from the triplicate replicates.

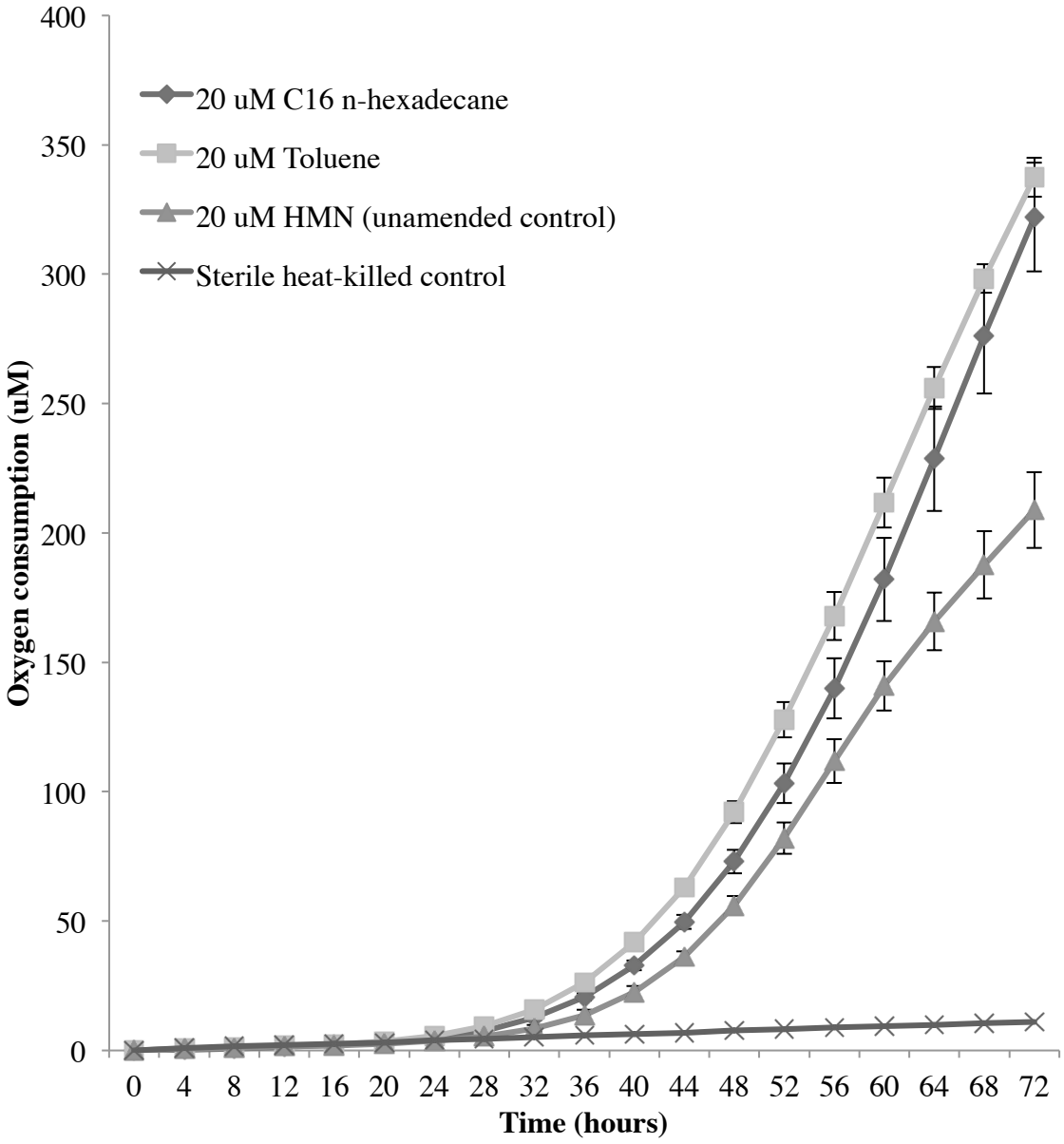


Figure 16. Total oxygen consumption (μM) coupled to *n*-hexadecane (diamonds) and toluene (squares) by *n*-pentane-grown *Halomonas* A11A cells. Widdel's medium with 20μM *n*-hexadecane and 20μM toluene served as the sole carbon source for cell growth. Triplicate replicates were inoculated with 0.5 OD_{610} 10% (v/v) of *Halomonas* A11A. A sterile heat-killed control and an unamended bottle with heptamethylnonane (HMN) (triangles) served as controls. Each bottle was incubated at 25 °C. Bars represent one standard deviation of the average from the triplicate replicates.

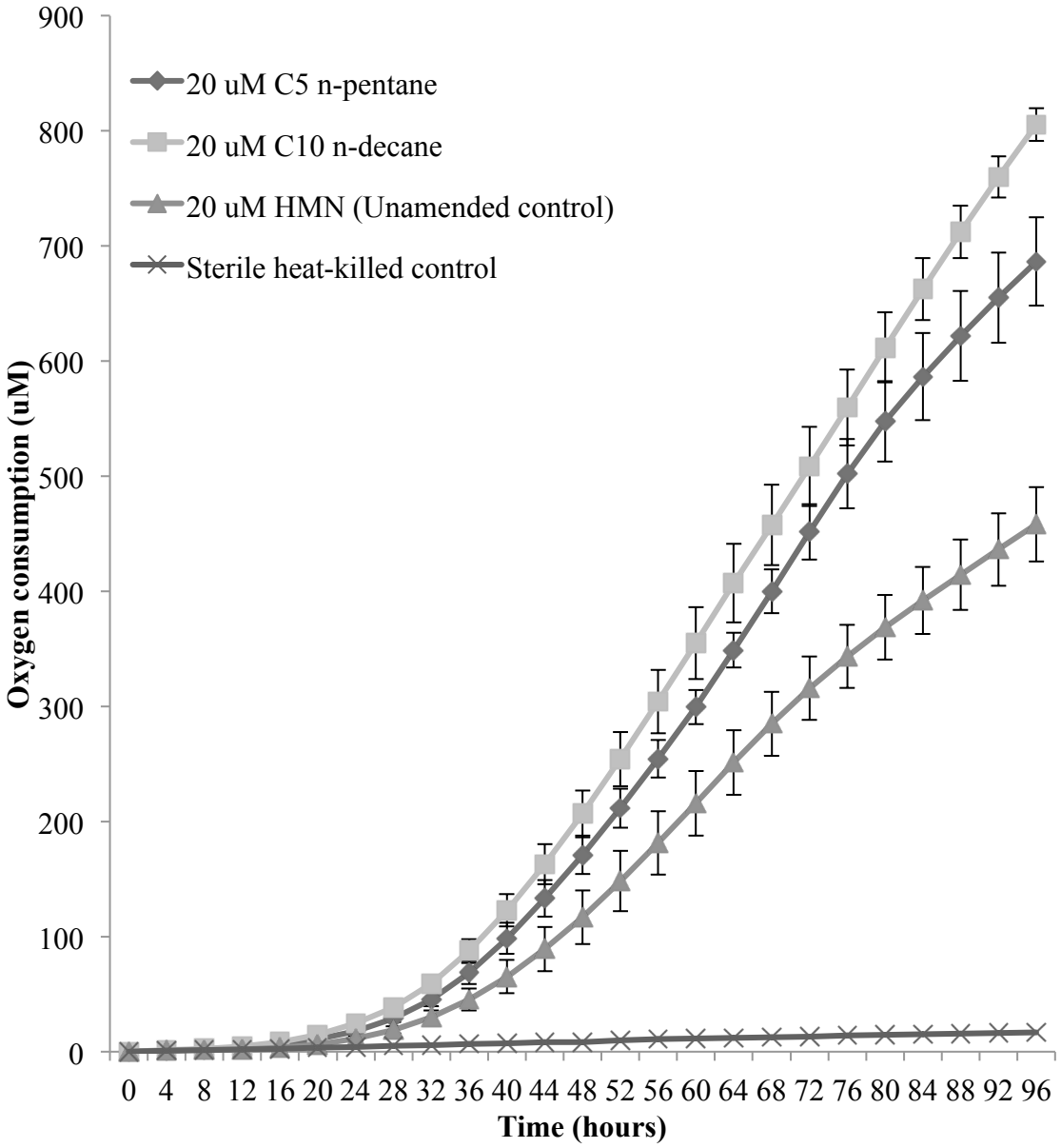


Figure 17. Total oxygen consumption (μM) coupled to *n*-pentane (diamonds) and *n*-decane (squares) oxidation by *n*-decane-grown by *Halomonas* A11A cells. Widdel's medium with 20μM *n*-pentane and 20μM *n*-decane served as the sole carbon source for cell growth. Triplicate replicates were inoculated with 0.5 OD_{610} 10% (v/v) of *Halomonas* A11A. A sterile heat-killed control and an unamended bottle with heptamethylnonane (HMN) (triangles) served as controls. Each bottle was incubated at 25 °C. Bars represent one standard deviation of the average from the triplicate replicates.

Appendix 1:

Supplemental Tables for Chapter 2: The role of Gammaproteobacteria in aerobic alkane degradation in oilfield production water from the Barnett Shale

Table A1. Additional progressive aerobic enrichment amended with *n*-alkanes (C_5-C_{10}) and *n*-fatty acids (C_5-C_{10}) in Widdel's medium. (1) First progressive transfer (2) second progressive transfer (3) third progressive transfer. Growth is indicated by (+/-), where (+) indicates minimal growth and (++++) indicates most turbid growth, estimated by visual turbidity at each transfer, (-) indicates no growth. All enrichments were prepared in triplicate (A, B, C). All amended bottles received a final concentration of 3.34 mM *n*-alkanes (C_5-C_{10}) or 10 mM *n*-fatty acids (C_5-C_{10}) respectively. All bottles were incubated at 25 °C for up to two weeks. Sterile heat-killed bottles and unamended bottles, which consisted of the medium and inoculum but without the substrate were used as controls.

1. First progressive transfer

	n-fatty acids (C_5-C_{10})				*n*-alkanes (C_5-C_{10})		
Dilution	A[1]	B[1]	C[1]		A	B	C
10^{-1}	++++	+++	++++	10^{-1}	++	+++	+++
10^{-2}	++++	+	++	10^{-2}	+	++	++
10^{-3}	++	+	++	10^{-3}	+	+	+
10^{-4}	++	+	++++	10^{-4}	-	+	+

2. Second progressive transfer

	n-fatty acids (C_5-C_{10})				*n*-alkanes (C_5-C_{10})		
Dilution	A[1]	B[1]	C[1]		A	B	C
10^{-1}	++++	+++	++++	10^{-1}	++	++	++
10^{-2}	++++	+	++	10^{-2}	+	+	+
10^{-3}	++	+	++	10^{-3}	+	+	+
10^{-4}	++	+	++++	10^{-4}	-	-	+

3. Third progressive transfer

	n-fatty acids (C_5-C_{10})				*n*-alkanes (C_5-C_{10})		
Dilution	A[1]	B[1]	C[1]		A	B	C
10^{-1}	++++	+++	++++	10^{-1}	++	++	++
10^{-2}	++++	+++	++++	10^{-2}	+	++	++
10^{-3}	++++	++	+++	10^{-3}	+	+	+
10^{-4}	++++	++	++++	10^{-4}	-	+	+

Unamended controls were prepared in triplicate for each transfer by inoculating medium with no added substrate. Growth occurring in the unamended controls never scored more than (++) and was usually (+) or (-).

Table A2. Select metabolic pathways and genes of interest in the *Halomonas* A11A genome. Detection of annotated gene sequences in *Halomonas* A11A by KEGG KOBAS using the default cutoffs: *E*-value $<10^{-5}$ and rank ≤5, and illustrated using KEGG Mapper v. 2.6 (http://www.genome.jp/kegg/mapper.html).

Metabolic pathway	Function/description	Enzyme Commission #	*Halomonas* A11A
Fatty acid	Long chain fatty acid CoA ligase	6.2.1.3	Yes
	acyl-CoA dehydrogenase	1.3.3.6	No
	acyl-CoA dehydrogenase	1.3.8.7	Yes
	acyl-CoA dehydrogenase	1.3.99.-	Yes
	acyl-CoA dehydrogenase	1.3.8.8	Yes
	acyl-CoA dehydrogenase	1.3.8.9	No
	enoyl-CoA hydratase	4.2.1.17	Yes
	enoyl-CoA hydratase	4.2.1.74	No
	acyl-CoA dehydrogenase	1.1.1.35	Yes
	acyl-CoA dehydrogenase	1.1.1.211	No
	acetyl-CoA acyltransferase	2.3.1.16	Yes
	glutaryl-CoA dehydrogenase	1.3.8.6	Yes
	acetyl-CoA acyltransferase	2.3.1.9	Yes
	ligase	6.2.1.6	No
Long chain fatty acid (C_{13}-C_{22})	Acyltransferase	6.2.1.20	No
	CoA dehydrogenase	5.3.3.8	Yes
	CoA dehydrogenase	5.1.2.3	Yes
Alkane	Alkane monooxygenase	1.14.15.3	Yes
	Ferredoxin reductase	1.18.1.3	No
	Rubredoxin reductase	1.18.1.4	No
	Rubredoxin reductase	1.18.1.1	Yes
	Alcohol dehydrogenase	1.1.1.1	Yes
	Oxidoreductase	1.1.99.20	No
	Aldehyde dehydrogenase	1.2.1.3	Yes
	Oxidoreductase	1.2.5.2	No
	Alkane monooxygenase	1.14.15.3	Yes
Aromatic degradation	Benzene/toluene dioxygenase	1.14.12.3	Yes
	phenol hydroxylase	1.14.13.-	Yes
	dehydrogenase	1.3.1.19	No
	Toluene monooxygenase	1.14.13.-	No
	Phenol 2-monooxygenase	1.14.13.7	Yes
	Xylene monooxygenase	1.14.13.-	No
	Aryl-alcohol dehydrogenase	1.1.1.90	Yes
	Hydroxybenzaldehyde dehydrogenase	1.2.1.64	No

Hydroxybenzaldehyde dehydrogenase	1.2.1.7	No
Hydroxybenzaldehyde dehydrogenase	1.2.1.28	No
Hydroxybenzyl-alcohol dehydrogenase	1.1.1.97	No
4-cresol dehydrogenase	1.17.99.1	Yes
4-hydroxybenzaldehyde dehydrogenase	1.2.1.96	No
Benzylsuccinate synthase	4.1.99.11	No
Benzylsuccinate CoA-transferase	2.8.3.15	No
Benzylsuccinyl-CoA dehydrogenase	1.3.8.3	No
Catechol 1,2-dioxygenase	1.13.11.1	No
Catechol 2,3-dioxygenase	1.13.11.2	Yes
Muconate cycloisomerase	5.5.1.1	Yes
Muconolactone D-isomerase	5.3.3.4	No
3-oxoadipate enol-lactonase	3.1.1.24	Yes
3-oxoadipate CoA-transferase	2.8.3.6	No
Acetyl-CoA transferase	2.3.1.16	Yes
3-oxoadipyl-CoA thiolase	2.3.1.174	Yes
2-hydroxymuconate-semialdehyde hydrolase	3.7.1.9	Yes
2-keto-4-pentenoate hydratase	4.2.1.80	Yes
4-hydroxy 2oxovalerate aldolase	4.1.3.39	Yes
Acetaldehyde dehydrogenase	1.2.1.10	Yes
Procatechuate 3,4-dioxygenase	1.13.11.3	Yes
3-carboxy-cis,cis-muconate cycloisomerase	5.5.1.2	Yes
4-carboxymuconolactone decarboxylase	4.1.1.44	Yes
Ethylbenzene hydroxylase	1.17.99.2	No
Naphthalene 1,2-dioxygenase	1.14.12.12	No
Phenylethanol dehydrogenase	1.1.1.311	No
Acetophenone carboxylase	6.4.1.8	No
Ethylbenzene dioxygenase	1.14.12.-	No
Cis-dihydroethylcatechol dehydrogenase	1.3.1.66	No
2-hydroxy-6-oxo-octa-2,4-dienoate hydrolase	3.7.1.-	Yes

Positive detection of enzymes in *Halomonas* A11A genome indicated by "Yes" in table.

Table A3. Molar calculations of hydrocarbon oxidation. (A) Amount of hydrocarbons used. (B) Total oxygen consumed in experimental bottles at peak oxidation rate. (C) Total oxygen rate in unamended bottles at peak oxidation rate. (D) Difference in total oxygen consumed between experimental and unamended bottles at time of peak oxidation rate. (E) Theoretical concentration of oxygen required to (100%) completely oxidize the hydrocarbon in the experimental bottle. (F) Percent of theoretical calculated by (D)/(E).

		Experimental (μM)			Theoretical	
	A	B	C	D	E	F
Hydrocarbon	μM	Total O_2	Unamended	Difference	100%	%
n-pentane C_5	20	295	143	152	160	95
n-decane C_{10}	20	331	143	188	310	60.6
n-hexadecane C_{16}	20	276	140	361.1	500	72.3
toluene $C_7H_5CH_3$	20	261	187	74.1	200	37.0

Appendix 1:

Supplemental figures for Chapter 2: The role of Gammaproteobacteria in aerobic alkane degradation in oilfield production water from the Barnett Shale

Figure A1. 16S sequence libraries representing the relative abundance (%) of different genera of bacteria in the initial and progressive rounds ("transfer") of enrichment amended with *n*-alkanes (C_5-C_{10}) compared to the corresponding unamended transfer. Each transfer is represented by the 10^{-4} dilution. The relative abundance of *Desulfobacter* is less than 1% in the enriched cultures the purple color seen in high relative abundance in lanes 2-22 refers to *Halomonas*.

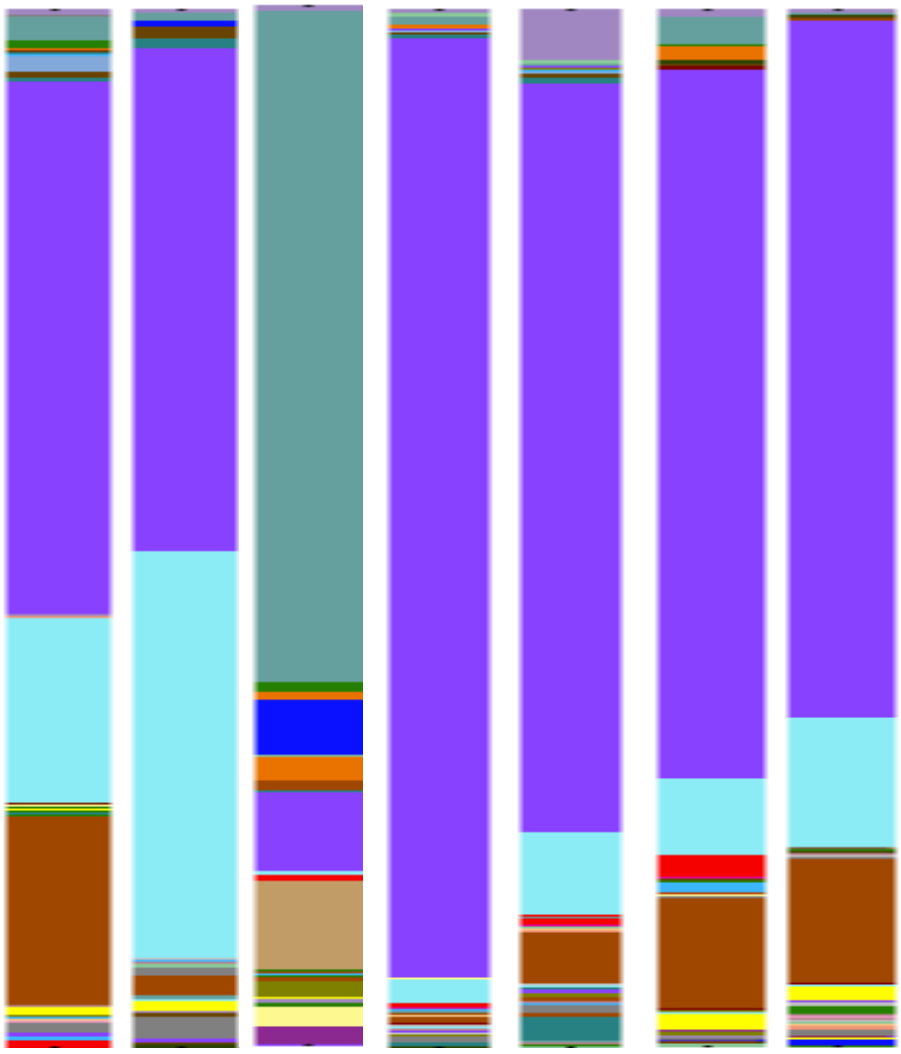
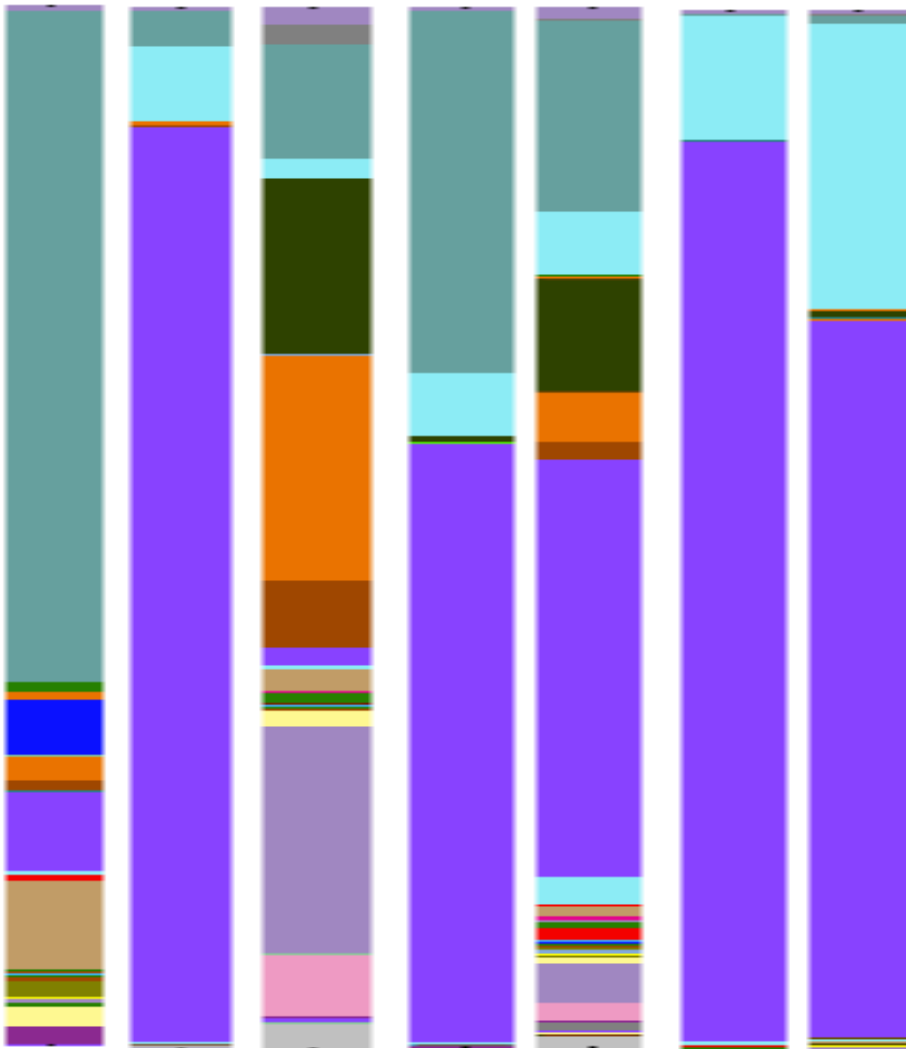

Initial enrichment
1st transfer (n-alkanes)
1st transfer unamended
2nd transfer (n-alkanes)
2nd transfer unamended
3rd transfer (n-alkanes)
3rd transfer unamended

Figure A2. 16S sequence libraries representing the relative abundance (%) of different genera of bacteria in the initial and progressive rounds ("transfer") of enrichment amended with *n*-fatty acids (C_5-C_{10}) compared to the corresponding unamended transfer. Each transfer is represented by the 10^{-4} dilution. The relative abundance of *Desulfobacter* is less than 1% in the enriched cultures, the purple color seen in high relative abundance in lanes 2-22 refers to *Halomonas*.


Initial enrichment
1st transfer (n-fatty acids)
1st transfer unamended
2nd transfer (n-fatty acids)
2nd transfer unamended
3rd transfer (n-fatty acids)
3rd transfer unamended

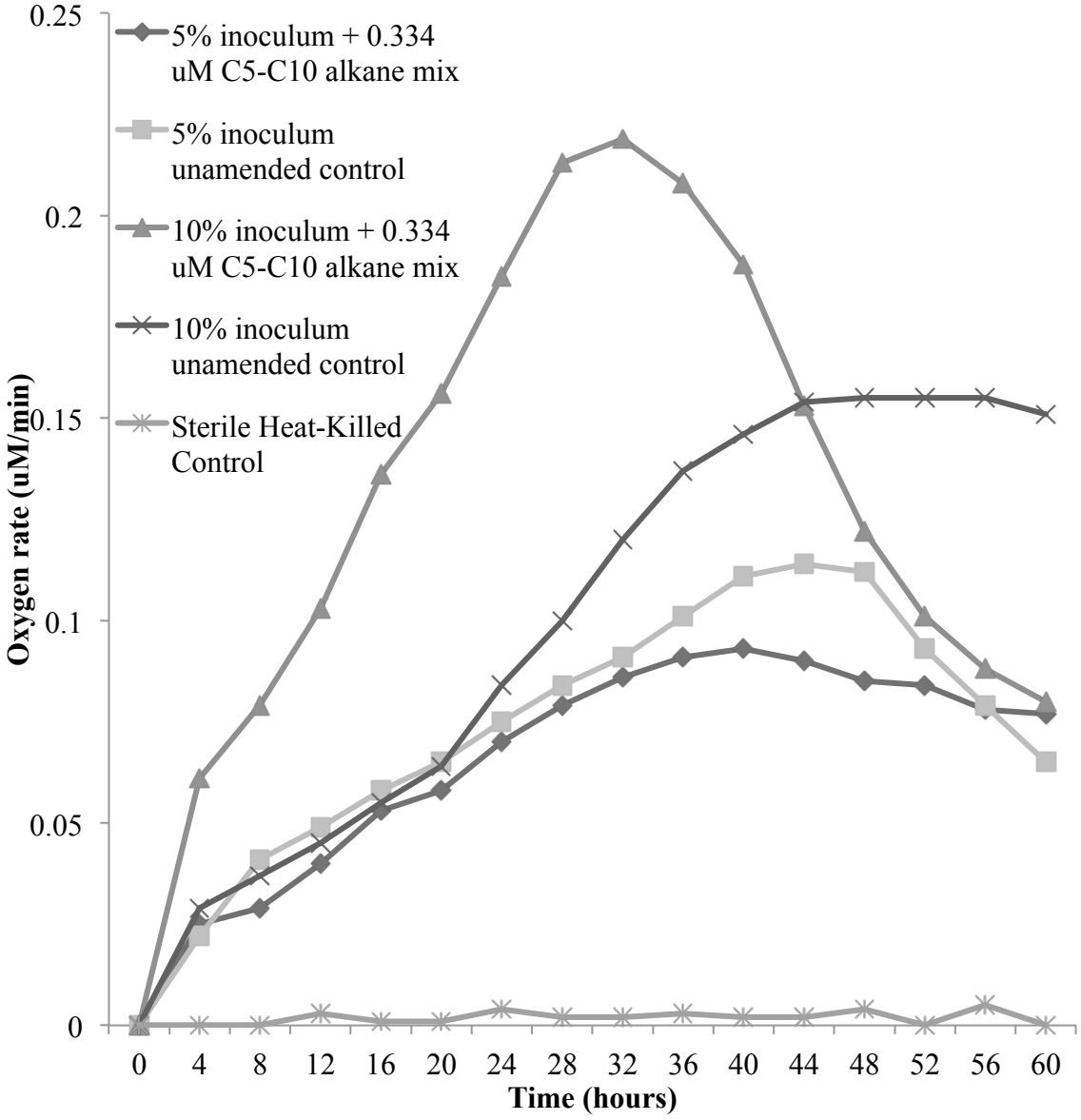


Figure A3. Effect of different inoculum sizes on oxygen uptake by *Halomonas* A11A. Widdel's medium with C_5-C_{10} *n*-alkane mix at 0.334 µM equimolar concentration served as the sole carbon source for cell growth. Bottles were inoculated with 10% (v/v) or 5% (v/v) of *Halomonas* A11A grown on the n-alkane mix at 0.5 OD_{610}. Sterile heat-killed and unamended bottles, which consisted of the medium and inoculum but without the substrate, was used to check for endogenous respiration. All bottles were incubated at 25 °C.

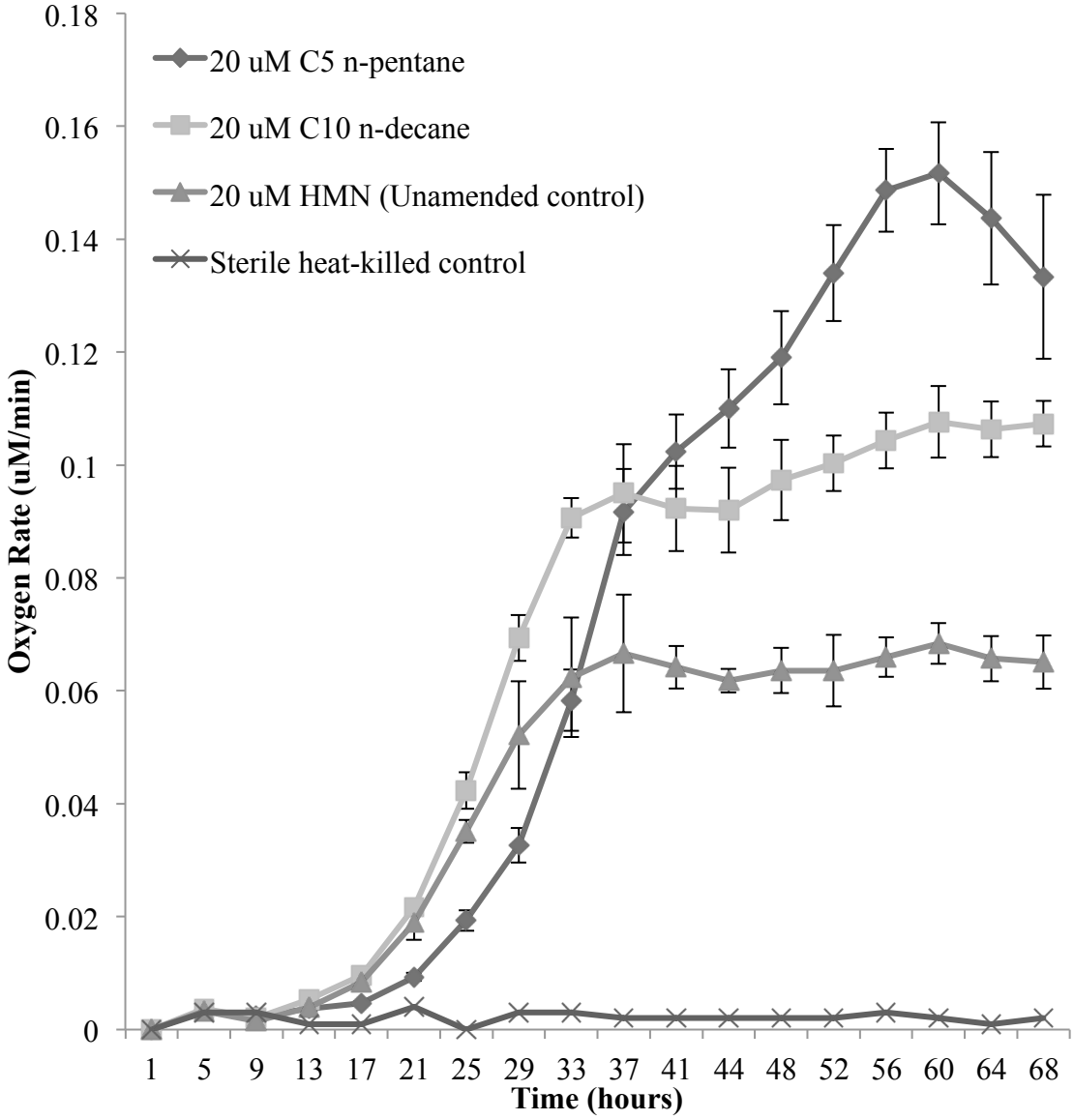


Figure A4. Rate of oxygen uptake (μM/min) of *n*-pentane and *n*-decane by *n*-pentane-grown *Halomonas* A11A cells. Widdel's medium with 20 μM *n*-pentane and 200 nM *n*-decane served as the sole carbon source for cell growth. Triplicate replicates were inoculated with 10% (v/v) of *Halomonas* A11A at 0.5 OD_{610}. A sterile heat-killed control and an unamended bottle with heptamethylnonane, which consisted of the medium and inoculum but without the substrate, were used to compare the endogenous respiration. Bars represent one standard deviation of the average from the triplicate replicates. Each bottle was incubated at 25 °C.

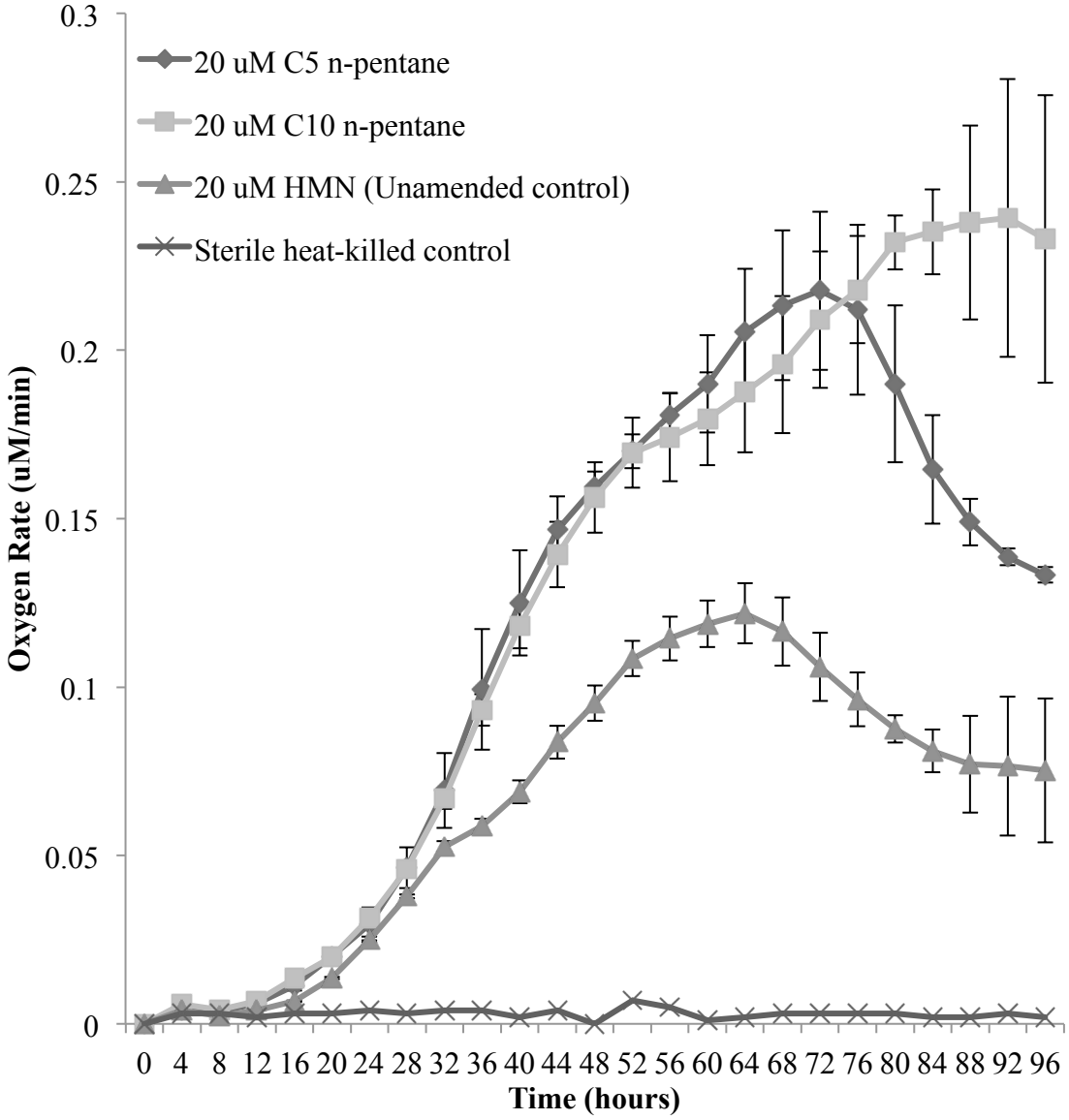


Figure A5. Rate of oxygen uptake (μM/min) of *n*-pentane and *n*-decane by *n*-decane-grown *Halomonas* A11A cells. Widdel's medium with 200 nM *n*-pentane and 200 nM *n*-decane served as the sole carbon source for cell growth. Triplicate replicates were inoculated with 10% (v/v) of *Halomonas* A11A at 0.5 OD_{610}. A sterile heat-killed control and an unamended bottle with heptamethylnonane, which consisted of the medium and inoculum but without the substrate, were used to compare the endogenous respiration. Bars represent one standard deviation of the average from the triplicate replicates. Each bottle was incubated at 25 °C.

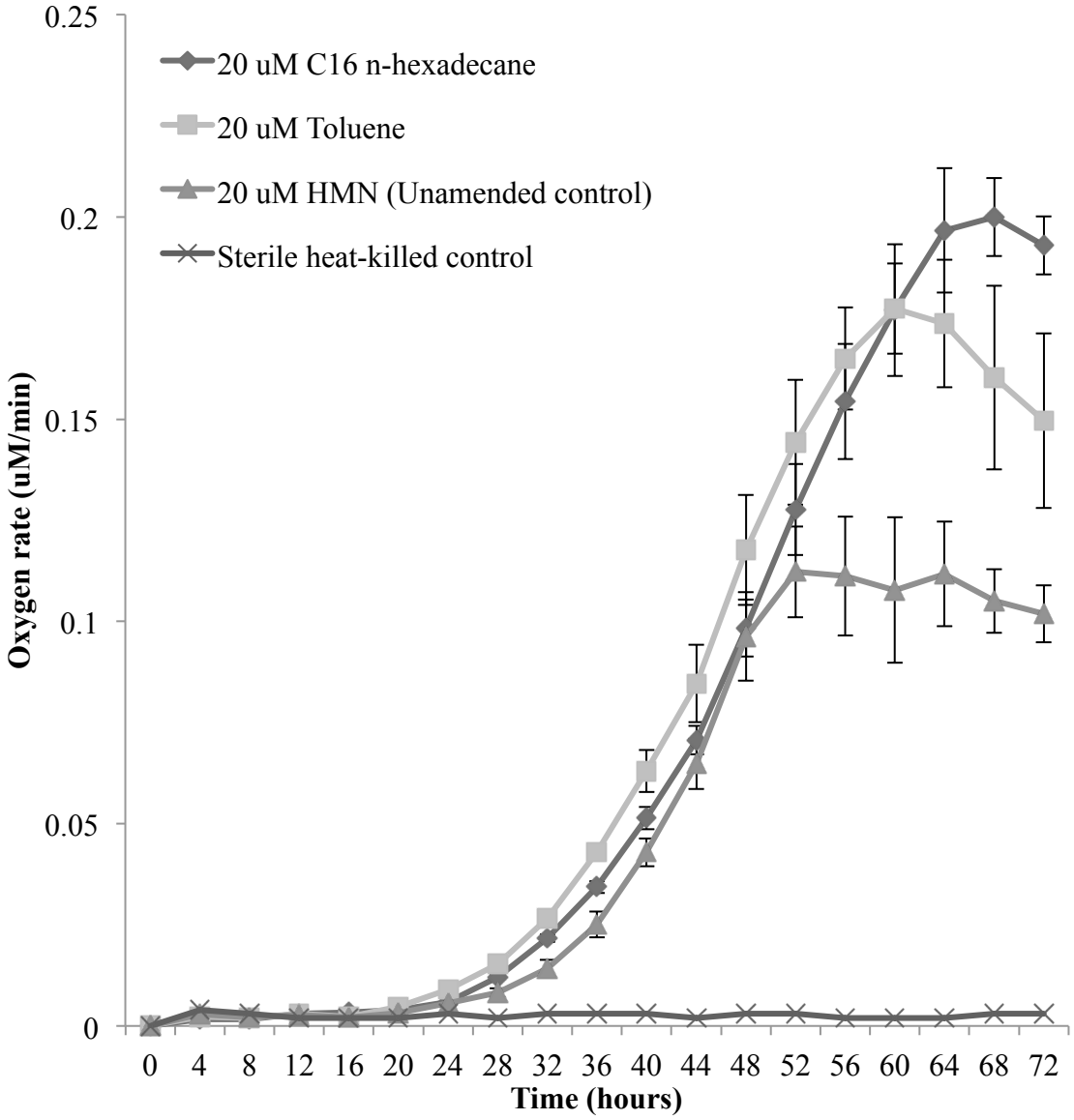


Figure A6. Rate of oxygen uptake (μM/min) of *n*-hexadecane and toluene by *n*-pentane-grown *Halomonas* A11A cells. Widdel's medium with 200 nM *n*-hexadecane and 200 nM toluene served as the sole carbon source for cell growth. Triplicate replicates were inoculated with 10% (v/v) of *Halomonas* A11A at 0.5 OD_{610}. A sterile heat-killed control and an unamended bottle with heptamethylnonane, which consisted of the medium and inoculum but without the substrate, were used to compare the endogenous respiration. Bars represent one standard deviation of the average from the triplicate replicates. Each bottle was incubated at 25 °C.

Appendix 2:

Isolation of the dominant cultivable aerobic heterotrophic bacteria from oil field production water produced from the Barnett Shale

Abstract

Aerobic heterotrophic microorganisms are commonly found in oil production waters but their contribution to biocorrosion is largely unknown. This study aimed to isolate aerobic heterotrophic bacteria from a petroleum production water tank and determine their potential to degrade petroleum hydrocarbons. Partial oxidation of hydrocarbons by aerobic bacteria could provide nutrients to foster the activity of sulfate-reducing and other biocorrosive microorganisms. Fifteen isolates obtained from heterotrophic amendment of production water were identified via 16S rRNA ribosomal gene sequencing as *Arcobacter* (7 isolates), *Thalassospira* (5*)*, *Marinobacterium* (2) and *Salinicola* (1). One strain each of *Arcobacter* and *Marinobacterium* were selected for hydrocarbon degradation testing as representatives of Epsilonproteobacteria and Gammaproteobacteria, respectively. The *Marinobacterium* strain showed moderate growth with C_5-C_{10}, C_{16} *n*-alkanes and strong growth with C_5-C_{10} *n*-fatty acids as the sole carbon source. The *Arcobacter* strain was unable to grow on any of the hydrocarbon substrates above but *Arcobacter* species are noted for contributing to biocorrosion by the oxidation of sulfide. Other studies have found that *Thalassospira* and *Salinicola* strains can degrade polyaromatic hydrocarbons. The microorganisms isolated in this study serve as representatives of the aerobic heterotrophic microbial community in the petroleum production water and indicate that aerobic heterotrophic

bacteria could indirectly contribute to biocorrosion through the partial oxidation of hydrocarbons and sulfide oxidation.

1.0 Introduction

Petroleum production facilities play host to a wide variety of microorganisms. The functions of these resident microorganisms can be deleterious: contributing to direct oxidation of steel and sulfide-driven MIC, reservoir souring, or hydrocarbon degradation leading to poor product quality (Head et al., 2003; Duncan et al., 2009). While these problems plague petroleum production and cost the industry millions of dollars annually, efforts to monitor and predict biocorrosion are met with little success. This is likely due to the limited understanding of the mechanisms of biocorrosion and the microbial communities that contribute to it (Hamilton et al., 2003; Little et al., 2006; Vigneron et al., 2016). Thus, research on the microbial populations associated with petroleum-impacted environments is invaluable. Sulfidogenic bacteria, iron-oxidizing and iron-reducing bacteria, other metal-reducing bacteria, and fermentative microorganisms are often characteristic of biocorroded systems but a significant portion of the microbial community also consists of seemingly innocuous heterotrophs (Dihn et al., 2004; Suflita et al., 2008; Stevenson et al., 2011; Vigernon et al., 2016).

The microbial physiologies represented in petroleum-impacted ecosystems are as diverse as the environments they inhabit. Petroleum and oil reservoirs are characterized as strictly anoxic environments despite playing host to a wide variety of microaerophilic and aerobic microorganisms as well as the facultative and obligate anaerobes (Adkins et al. 1992; Voordouw et al., 1996; Telang et al., 1997; Mand et al., 2017). The source of these aerobic microorganisms is not well understood but their involvement in hydrocarbon degradation and biocorrosion could provide clues about the overall microbial ecology of oily ecosystems. The role of the general heterotrophs in production water is understood to

cycle carbon, including the petroleum hydrocarbon substrates, but how these heterotrophic organisms contribute to corrosion is largely unknown and partly ignored (Abed et al., 2005).

The objective of this study was to use cultivation based methods to isolate some of the dominant cultivatable aerobic heterotrophic microorganisms from petroleum production water and to elucidate their role in the production water, especially with respect to biocorrosion. Isolates obtained from petroleum production water from a water storage tank producing from the Barnett Shale were isolated using heterotrophic media and identified via 16S rRNA gene sequences. Hydrocarbon degradation potential was examined qualitatively using liquid and solid medium amended with a range of hydrocarbons found in the production water. Comparisons of these isolates to known petroleum-related microbial communities can provide insight into what roles these cultivatable microorganisms may play in the production water microbial community.

2.0 Materials and Methods

2.1 Sample collection from Barnett Shale production water tank

Production water produced from the Barnett Shale was obtained from a production water tank in Dec. 2014 from top-water and middle-water levels. Production water samples were obtained by lowering sterilized 1 L bottles through the thief hatch; bottles were sealed anaerobically once samples were collected. On-site measurements of oxygen, pH, temperature, thiosulfate and sulfide were taken using methods outlined in Chapter 2. Measurements of NaCl via Cl^- anions were estimated in the laboratory according to methods outlined in Chapter 2. Samples were filtered through a 0.45 μm

cellulose nitrate analytical test filter funnel (145-2045, Fisher Scientific, Fair Lawn, NJ, USA) within 6 hours of sampling to collect bacterial cells for isolation and identification. Filters were preserved by storing at 4° C until January 2015.

2.2 Isolation of aerobic heterotrophic bacteria from production water

Filtered cells from the top-water samples were inoculated into 2 g/L glucose broth, Difco marine broth (2216 Difco, Detroit, MI, USA), marine broth with added 1% yeast extract, or marine broth with added 1% yeast extract and 5% tryptone, all prepared in deionized water (dH_2O). All cultures were incubated for 48 hours. Turbid cultures were streaked onto marine broth with added 1.5% agar (MA) plates amended with substrates (Table 6); top-level filtered water was used rather than dH_2O to replicate the native environment. Plates were incubated at 27° C for 72 hours. Plates were checked for growth and colony morphology; two or three isolated viable colonies were chosen from each plate and streaked onto marine agar three times for purity. Marine agar plates were incubated at 27° C for 1 week. Cultures were maintained on marine agar.

2.3 Analysis of isolate DNA

Fifteen isolated colonies of dominant aerobic heterotrophic bacteria were selected for DNA extraction and downstream molecular analysis for identification based on 16S rRNA gene sequences. Cultures were inoculated in marine broth and incubated at 27°C with 100 rpm shaking for 1 week or until turbid. Following incubation, 1 mL of the culture was transferred to a sterile screw-cap tube, containing

sterile 50% glycerol, reaching a final concentration equaling 25% (v/v), and stored at -80° C for long-term preservation. Another 1 mL of the culture was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 6000 x g for 5 minutes to pellet the cells. The supernatant was removed without dislodging the pellet then centrifuged again for 30 seconds at 6000 x g. The remaining supernatant was discarded and 50 µL DNAzol (DN127, Molecular Research Center, Cincinnati, OH, USA) was added to lyse the cells and preserve DNA. Following lysis with DNAzol and vortexing, 100 µL nuclease-free H_2O (Promega, Madison, WI) was added and the lysates were diluted 1:0 and 1:100 in nuclease-free H_2O and stored at -20° C to be used for 16S rRNA gene amplification.

Samples were analyzed via 16S rRNA gene sequencing of PCR products amplified using the primers and following the protocol outlined by Muyzer et al. (Muyzer et al., 1993). Briefly, 1:100 diluted isolate DNA was amplified using 0.5 µL of each forward and reverse primer: GM5F (5 pmol/mcl) and D907R (5 pmol/mcl) to obtain an approximately 550 bp product. PCR amplification was performed in Techne Prime Thermal Cycler (Techne, Cambridge, United Kingdom) using the DGGE Touchdown protocol (Muyzer et al., 1993). In brief, DNA was incubated for 5 minutes at 94° C; the hot start technique was used to minimize nonspecific annealing and the annealing temperature was set initially at 65° C, then decreased by 1° C every 2 cycles until reaching touchdown temperature at 55° C (e.g. 20 cycles) and then repeated fifteen times at 55° C for a total of 35 cycles. A final primer extension step occurred at 72° C for 3 minutes. Products were visualized via UV-illumination after gel electrophoresis in 1% agarose (Thermo Fisher Scientific, Fair Lawn, NJ, USA) with 0.5 TBE (Tris/Borate/EDTA) Buffer stained with 0.1% SybrSafe (Invitrogen, Carlsbad,

California, USA) and assessed against the GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific, Fair Lawn, NJ, USA). Desired PCR products were purified for DNA sequencing using Exo-SAP IT (USB Co., Cleveland, OH, USA) by transferring 5 μL of the PCR product to a sterile PCR tube and adding 1.5 μL ExoSAP-IT reagent and cycled on the Techne Prime Thermal Cycler according to manufacturer's instructions. Cleaned and amplified samples were submitted for sequencing by OMRF (Oklahoma Medical Research Foundation, Oklahoma City, OK). Bacterial isolate 16S rRNA gene sequences were manually trimmed for quality using CodonCode Aligner version 5.1.5 (CodonCode Corporation, Centerville, MA, USA). The approximately 500 bp sequences were compared to existing DNA sequences in GenBank via BLASTN and RDP (http://rdp.cme.msu.edu/, Ribosomal Database Project) (Larsen et al., 1993; Altschul et al., 1997; Benson et al., 1999) (Table A7).

2.4 Screening of hydrocarbon degradation ability

Two isolates were screened for hydrocarbon degradation potential. *Arcobacter* 1A and *Marinobacterium* 7C isolates (Table A7) were inoculated into triplicate 30 mL liquid Widdel's medium, as prepared previously in Chapter 2, and amended with the C_5-C_{10} *n*-alkane mix, individual C_5-C_{10}, C_{16} *n*-alkanes, C_5-C_{10} *n*-fatty acids, or BTEX (20 nM) to a final concentration of 20 μM. Bottles were incubated for up to two weeks at 27° C with 50 rpm shaking. Heat-killed and unamended bottles served as negative controls and Marine agar served as a positive control. Growth was estimated visually by turbidity. Incubations were transferred three times to establish growth was based on hydrocarbon degradation rather than carry-over of other growth factors.

Strains *Arcobacter* 1A, *Marinobacterium* 7C, and *Halomonas* A11A (described in Chapter 2) were plated on Widdel's medium agar supplemented by spreading 80 uL 0.1 M C_5-C_{10} *n*-alkane solution (used for amendment), 80 uL of individual 100 mM C_5-C_{10} *n*-alkanes, or C_5-C_{10} *n*-fatty acids (Figure A7) as the only carbon source. To prevent toxic concentrations of BTEX affecting the growth of the isolates, 25 uL of the 0.1 M equimolar BTEX solution was dropped on to sterile 0.45 um filter paper and placed in the lid of a glass Petri dish and incubated upside down to allow the BTEX fumes to enrich the streaked isolates, the plate was sealed in a plastic box to reduce escaping fumes and incubated in a chemical fume hood. Plates were incubated individually in sealed plastic bags to prevent and limit the loss of the volatile hydrocarbons and from contaminating other plates. The plates were incubated in the dark at 25° C for 2 weeks in an incubator. Widdel's medium plates without hydrocarbons served as a negative control, and marine agar plates served as positive control plates. *Escherichia coli* AC# 25922, which cannot degrade hydrocarbons but does grow on marine agar, was used as a negative control organism for growth with hydrocarbons.

3.0 Results

3.1 Chemical parameters of Barnett Shale production water

Production water collected from the Barnett Shale in December 2014 was assessed for chemical parameters. The salinity of the production water, as estimated by Cl^- was 3.1% NaCl, oxygen was between 0.295-0.58 ppm, the temperature of the water varied from 11.13-20.0° C, the pH was 6.9, thiosulfate equaled 51 mg/L, and sulfide was measured at 0 mg/L (Table A6).

3.2 Isolation of dominant aerobic heterotrophs from Barnett Shale production water

Isolation of aerobic microorganisms from filtered water from a production water storage tank on solid heterotrophic media yielded 15 isolates (Table A7). Isolates were identified using the 16S rRNA ribosomal gene sequence with all sequences being >500 bp in length. Seven isolates were most closely related to the 16S rRNA gene sequence of *Arcobacter marinus,* ranging from 97%-99% identity. Of the eight remaining isolates, five were identified as most closely affiliated (97-100% 16S rRNA gene sequence identity) to *Thalassospira xiamenensis*, two to *Marinobacter maritimun*, and one to *Salinicola salarius*. According to RDP (Ribosomal Database Project) (http://rdp.cme.msu.edu/) Classifier program each isolate matched the respective identified genus 100%, whereas the BLASTN match at the species level ranged mostly between 97 – 100 %.

3.2 Physiological analysis: Hydrocarbon degradation screening

Two isolates, *Arcobacter* 1A and *Marinobacterium* 7C, were selected for additional physiological analysis to test hydrocarbon degradation ability. *Halomonas* A11A, obtained from *n*-alkane-amended enrichment, was also examined for hydrocarbon degradation ability and is discussed in Chapter 2. The isolates were screened for their ability to grow in the presence of a range of short and medium length aliphatic hydrocarbons, such as *n*-alkanes C_5-C_{10} mix, individual C_5-C_{10}, C_{16} *n*-alkanes, BTEX, and C_5-C_{10} *n*-fatty acids in liquid and solid medium (Table A8). Isolates were also assessed for their ability to grow in the presence of BTEX compounds. *Marinobacterium* 7C grew in the presence of all *n*-alkanes and *n*-fatty acids but not in

the presence of BTEX or in the unamended control medium. Growth in liquid medium was strong after three transfers (as assessed by turbidity) and grew best when amended with the *n*-fatty acids, the C_5-C_{10} *n*-alkane mix or medium length C_9, C_{10}, and C_{16} *n*-alkanes (Table A8). On solid media colonies were very small, smaller than colonies grown on marine agar. *Arcobacter* 1A grew very weakly and slowly in the presence of the C_5-C_{10} *n*-alkane mix but did not grow in the presence of individual *n*-alkanes or BTEX in liquid medium (Table A8). Growth of 1A was visible in the first transfer but was not visible after three transfers. The isolate did not form visible colonies on any of the hydrocarbon-amended plates but did form visible colonies on the positive control, Marine Agar. The *Arcobacter* isolate grew weakly in the presence of *n*-fatty acids in liquid and solid media (Table A8). *Halomonas* A11A grew in the presence of all *n*-alkanes and *n*-fatty acids on solid media but did not grow in the presence of BTEX compounds or in the unamended control medium. Colonies were visible but smaller than colonies on marine agar. Similarly, in the liquid medium, A11A grew strongly with all individual *n*-alkanes as well as the C_5-C_{10} *n*-alkane mix after 1 week. Growth with C_5-C_{10} *n*-fatty acids was visible after three days. No growth was visible with BTEX hydrocarbons or in the unamended control.

4.0 Discussion

The objective of this study was to isolate and identify the dominant culturable aerobic microorganisms from petroleum production water. Four different genera of aerobic/facultatively anaerobic Proteobacteria were isolated from petroleum production water. *Arcobacter* (Epsilonproteobacteria) was the most common isolate obtained from

isolation methods using heterotrophic media but does not appear to be a hydrocarbon degrader; limited growth may be a factor of hydrocarbon toxicity and requires further investigation. *Arcobacter* is more likely to function as a chemolithotrophic sulfide-oxidizer (Vandamme et al., 1991; Roalkvam et al., 2015). For the petroleum industry *Arcobacter* has shown use as a sulfide scavenger and sulfide control agent in sour reservoirs (Telang et al., 1999; Gevertz et al., 2000; Hubert et al., 2005; Gregoire et al., 2014). All of the *Arcobacter* isolates obtained in this study were most closely related to *Arcobacter marinus*. *A marinus* was originally isolated from seawater amended with seaweed and starfish obtained from the East Sea, Korea and grew optimally with 3-5 % NaCl in aerobic and microaerophilic conditions (Kim et al., 2010). To date, no studies have investigated the hydrocarbon degradation potential of *A. marinus* but others have found *Arcobacter* sp. to be associated with hydrocarbon-impacted environments.

Previously, *Arcobacter* sequences have been detected in association with petroleum-contaminated sites in the Barnett Shale and the North Sea (Grabrowski et al., 2005; Dahle et al., 2008 David et al., 2012). In one study, sequences were closely associated with sulfur-containing compounds and linked to cycling sulfide produced by sulfidogenic bacteria in the Barnett Shale (Fitcher et al., 2009; David et al., 2012). Other studies have characterized *Arcobacter* as a microaerophilic sulfide-oxidizer and nitrate reducer (Ellis et al., 1977; Vandamme et al., 1991; Gevertz et al., 2000). *Arcobacter* has been cultivated and detected in a wide variety of environments including marine, hypersaline, and oil field brine (Teske et al., 1996; Gervertz et al., 2000; Wirsen et al., 2002; Kim et al., 2010). Filamentous sulfur produced by *Arcobacter* is recognized as an end product in many of these environments (Wirsen et

al., 2002; Sievert et al., 2007). More recently, Roalkvam and others showed for the first time that an *Arcobacter* strain could grow on complex organic substrates and oxidize acetate with elemental sulfur as the electron acceptor (Roalkvam et al., 2015). Further, they characterized *Arcobacter anaerophilus* IR-1 to grow lithoautotrophically on hydrogen and hydrogen sulfide, lithoheterotrophically on thiosulfate and elemental sulfur, and organoheterotrophically on yeast extract, peptone, tryptone, and other organic acids. These findings suggest that *Arcobacter* sp., including those isolated in our study, might be capable of using complex organic substrates (possibly hydrocarbons) as carbon sources and use a wide range of electron acceptors (sulfur, nitrate, oxygen). The physiology and metabolic potential of *Arcobacter*, as well as other Epsilonproteobacteria, may be more diverse than previously thought and will require a more complete analysis to characterize their role in production water environments. This diverse physiological and metabolic potential certainly provides clues as to how these microorganisms persist in such a harsh and dynamic environment. Additional quantitative examination of *Arcobacter* spp. may reveal the true potential of these microorganisms and their range of functions in petroleum-impacted environments.

Marinobacterium isolates, representing Gammaproteobacteria, obtained from the production water using heterotrophic media show more potential as hydrocarbon degraders. *Marinobacterium* 7C was able to grow with all of the *n*-alkanes as the sole carbon source both in liquid and on solid media and grew robustly in the presence of C_5-C_{10} *n*-fatty acids. Both of the *Marinobacterium* isolates obtained in this study were most closely related to *Marinobacterium maritimum* (NR116301) (Kim et al., 2009). This species was originally isolated from Arctic marine sediments during screening for

thiosulfate-oxidizing bacteria. Optimal growth for *M. maritimum* requires aerobic, mesophilic, and 2% NaCl conditions. Very few studies have focused on pure cultures of *M. maritimum* but other *Marinobacterium* spp. have been detected in petroleum environments.

Previous studies identified other *Marinobacterium* spp. associated with crude oil-impacted marine environments, oil-water separators, and high salt oil reservoirs (Yakimov et al., 2005; Yuehui et al., 2008; van der Krann et al., 2010; Sherry et al., 2013). One study found *Marinobacterium* and other Gammaproteobacteria were significantly enriched in oil-amended sulfate-reducing enrichments (Sherry et al., 2013). Here, they suggest that the bacterial community is driven more by the presence and degradation of the hydrocarbons rather than the sulfate and consider *Marinobacterium* may be responsible for some of the observed hydrocarbon degradation. Normally, *Marinobacterium* spp. are aerobic, halophilic, heterotrophic microorganisms often associated with marine systems but have also been shown to be facultatively anaerobic and degrade phenol, benzoate, and carbazole, which is often found in petroleum distillates (Castorena et al., 2005; Inoue et al., 2005; Kim et al., 2007). Little quantitative evidence exists to classify *Marinobacterium* as a hydrocarbon degrader; our study is a brief and incomplete examination suggesting these microorganisms may grow on hydrocarbons as a sole carbon source. Additional quantitative evidence of hydrocarbon degradation is necessary to test this hypothesis.

Of the other isolates, *Thalassospira* (Alphaproteobacteria) was the second most numerous (5/15) isolate obtained from heterotrophic amendment of the production water. No further testing was done on these isolates but previous studies have described

Thalassospira strains to degrade phenanthrene, a polyaromatic hydrocarbon (PAH) present in petroleum products (Kodama et al., 2008; Zhao et al., 2008; Vila et al., 2010). Other studies have detected *Thalassospira* sequences using molecular techniques in marine PAH-degrading microbial communities, pyrene and naphthalene amended cultures, and as part of a corrosive biofilm in a water pipeline (Cui et al., 2008; Vila et al., 2010; Lopez et al., 2013). Little evidence exists to quantify the hydrocarbon or PAH-degrading properties of *Thalassospira* sp. but it likely functions as part of the larger microbial community involved in hydrocarbon and PAH degradation in petroleum-impacted environments including production water tanks.

The least abundant isolate (1/15) obtained from the production water was identified as *Salinicola* sp. *Salinicola* is a Gammaproteobacterium and part of the *Halomonadaceae* family very closely related to *Halomonas* spp. (Ananina et al., 2007; Tang et al., 2017). *Salinicola* sp. are described as moderately halophilic chemoorganotrophs capable of naphthalene degradation (Ananina et al., 2007). No physiological analysis was performed on this isolate in this study but we consider the *Salinicola* sp. in the production water may be part of the larger microbial community involved in hydrocarbon degradation. Considering its close phylogeny with *Halomonas*, discussed in Chapter 1, it may be capable of hydrocarbon degradation in pure culture but additional experiments are necessary to support this hypothesis.

Based on previous studies and the results obtained in our study it is reasonable to expect the *Arcobacter* strains in the Barnett Shale production water are contributing to biocorrosion and the sulfur cycle by functioning as chemolithotrophic sulfide oxidizers. We also propose that *Arcobacter* may play a role in hydrocarbon degradation

or degradation of other complex organics and should be investigated more thoroughly (Roalkvam et al., 2015). Similarly, *Marinobacterium* spp. present in the production water tank likely exist dominantly as heterotrophs by degrading sugars, fatty acids, and complex organic substrates, such as hydrocarbons, present in the production water. These microorganisms can serve as representatives of the more diverse microbial community in the petroleum production water that can be targets of additional cultivation measures to examine their role in biocorrosion or hydrocarbon degradation. In this study we see the dominant cultivatable aerobic population of microorganisms, representing Gamma- and Epsilonproteobacteria, have the potential to degrade petroleum hydrocarbons and may therefore provide substrates for biocorrosive microorganisms in the production water; thus, the presence of a large population of aerobic general heterotrophs in production water systems may be a matter of concern.

Appendix 2: Tables

Isolation of the dominant cultivable aerobic heterotrophic bacteria from oil field production water produced from the Barnett Shale

Table A4. Chemical parameters of Barnett Shale production water collected in December 2014. Values given are the range for three samples taken from one tank, from the bottom, middle, and top of the water layer.

Chemistry	December 2014
% NaCl	2.7-3.1
Oxygen (ppm)	0.295-0.58
Temperature (° C)	11.13-12.00
pH	6.90-6.93
Thiosulfate (mg/L)	51
Sulfide (mg/L)	0

Table A5. Isolate identity based on partial 16S rRNA gene sequences. Bacterial DNA was amplified using primers: GM5F (5 uM) and D907R (5 uM) primers. All sequences were >500 bp in length. Trimmed sequences were compared to those in Ribosomal Database Project (RDP) using the RDP Classifier Program and to the GenBank database using BLASTN.

	Ribsomal Database Project: Classifier[1]		BLAST[b]		
Isolate[c]:	% Genus match	Genus Identity	Closest Match	Accession #	% Identity
1A	100%	*Arcobacter*	*Acrobacter marinus* CL-S1	NR_116342.1	97%
3A	100%	*Arcobacter*	*Acrobacter marinus* CL-S2	NR_116342.1	98%
3B	100%	*Arcobacter*	*Acrobacter marinus* CL-S3	NR_116342.1	99%
4A	100%	*Arcobacter*	*Acrobacter marinus* CL-S4	NR_116342.1	99%
5A	100%	*Arcobacter*	*Acrobacter marinus* CL-S5	NR_116342.1	99%
6A	100%	*Arcobacter*	*Acrobacter marinus* CL-S6	NR_116342.1	100%
7A	100%	*Thalassospira*	*Thalassospira xiamenensis*	KJ658416.1	99%
7B	100%	*Thalassospira*	*Thalassospira xiamenensis*	KJ658416.1	99%
7C	100%	*Marinobacterium*	*Marinobacterium maritimum*	NR_116301.1	97%
8A	100%	*Salinicola*	*Salinicola salarius*	KC583226.1	100%
8B	100%	*Marinobacterium*	*Marinobacterium maritimum*	NR_116301.1	97%
8C	100%	*Thalassospira*	*Thalassospira xiamenensis*	KJ658416.1	99%
9A	100%	*Arcobacter*	*Acrobacter marinus* CL-S1	NR_116342.1	99%
9B	100%	*Thalassospira*	*Thalassospira xiamenensis*	KJ658416.1	99%
9C	100%	*Thalassospira*	*Thalassospira xiamenensis*	KJ658416.1	99%

[a]The RDP Classifier shows the genus match percentage of each isolate and genus level identification.
[b]BLASTN indicates closest genus and species match with percent identity to characterized species with accession numbers.
[c]Isolate numbers indicate the original plate they were isolated on (1-9), and the letter of the colony on the plate (A, B, or C).

Table A6. Hydrocarbon degradation screening by isolates obtained from Barnett Shale production water. Growth in liquid medium was estimated by visual turbidity estimates and scored based on (+) weak growth to (+++) strong growth or no growth (-) in Widdel's medium amended with various hydrocarbons at 200 μM. Turbidity scores were based on growth after three transfers. Growth on solid medium was estimated by formation of visible colonies (+) or no growth (-) on Widdel's medium plates amended with 80 μL of 100 mM of various hydrocarbons. Unamended Widdel's medium was used as a negative control, Marine agar as a positive control. Plates were incubated in individual sealed plastic bags at 25° C in the dark for 2 weeks.

	Liquid medium			Solid medium		
Substrate	1A	7C	A11A	1A	7C	A11A
C_5-C_{10} *n*-alkanes	+	++	+++	-	+	+
C_5 *n*-pentane	-	+	+++	-	+	+
C_6 *n*-hexane	-	+	+++	-	+	+
C_7 *n*-heptane	-	+	++	-	+	+
C_8 *n*-octane	-	+	++	-	+	+
C_9 *n*-nonane	-	++	++	-	+	+
C_{10} *n*-decane	-	++	+++	-	+	+
C_{16} *n*-hexadecane	-	+	++	-	+	+
C_5-C_{10} *n*-fatty acids	+	+++	+++	+	+	+
BTEX	-	-	-	-	-	-
Unamended	-	-	-	-	-	-
Marine broth	+	+	+	+	+	+

1A *Arcobacter*, 7C *Marinobacterium*, A11A *Halomonas*
E.coli AC#25922 was used as a negative control organism and did not grow on any of the hydrocarbon-amended plates. It did show growth on the positive control medium, Marine agar.

Appendix 2: Figures

Isolation of the dominant cultivable aerobic heterotrophic bacteria from oil field production water produced from the Barnett Shale

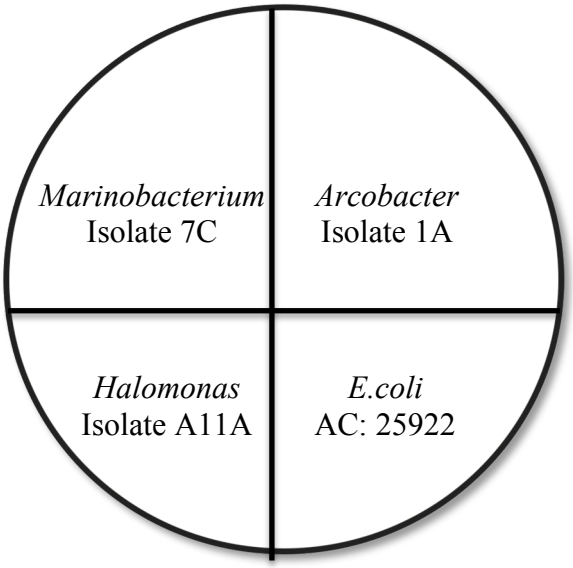


Figure A7. Hydrocarbon degradation screening of isolates obtained from Barnett Shale production water. *Marinobacterim* 7C and *Arcobacter* 1Aobtained as described in Appendix 2 and *Halomonas* A11A obtained from enrichment amended with *n*-alkanes (Chapt. 2) were screened for hydrocarbon degradation potential by streaking onto plates previously spread with a C_5-C_{10} *n*-alkane solution (80 uL 0.1 M) or 80 uL of each individual C_5-C_{10} and C_{16} alkane (100 mM). Plates amended with BTEX were incubated in glass Petri plates with the BTEX solution (25 uL of equimolar 0.1 M) was dropped on to sterile 0.45 um filter paper and placed in the lid of the glass Petri dish and incubated upside down. *Escherichia coli* AC# 25922 was used as a negative control for hydrocarbon degradation. All isolates were streaked on Marine agar as a positive control condition. Plates were incubated in individual sealed plastic bags at 25° C in the dark for 2 weeks.