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Methanogenic biodegradation of crude oil and polycyclic aromatic hydrocarbons

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Methanogenic biodegradation of crude oil and polycyclic aromatic hydrocarbons

by

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Abstract

The methanogenic biodegradation of crude oil is an important process occurring in many subsurface hydrocarbon-associated environments, but little is known about this metabolism in such environments. In this thesis work, the methanogenic biodegradation of crude oil and polycyclic aromatic hydrocarbons (PAH) was investigated. Methanogenic cultures able to metabolize light and heavy crude oil components were enriched from oilfield produced waters. Metabolites (e.g., alkylsuccinates) and genes (e.g. *assA* and *bssA)* associated with a fumarate addition mechanism were detected in the light oil-amended culture. A *Smithella* sp. dominated the community, suggesting this organism was involved in the degradation of the hydrocarbon components. In experiments conducted in sandstone-packed column systems simulating marginal oil fields, the light oil-amended culture was shown to bioconvert alkanes and aromatic hydrocarbons to CH_4. Other oil-associated microbial inocula also enhanced CH_4 production from oil in the column systems. Shifts in the microbial communities were observed after the inocula were incubated in the columns. Methanogenic hydrocarbon metabolism was also investigated using new enrichment cultures that biodegraded 2-ringed PAHs under methanogenic conditions. Metabolite and marker gene analyses were conducted on these cultures to investigate the mechanism(s) involved in PAH metabolism. The PAH-utilizing enrichments were dominated by methanogens closely affiliating with *Methanosaeta* and *Methanoculleus,* and bacterial members most closely related to the *Clostridiaceae* family. Further qPCR analysis with a 2-methylnaphthalene-amended culture suggested that *Clostridium* was the main hydrocarbon degrader in the enrichment. The results of these studies have added new knowledge to the field of methanogenic hydrocarbon biodegradation that may find application in bioremediation or microbial enhanced energy recovery.

Acknowledgements

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For my grandparents, an inspiration of hard work, endurance, and love

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Symbol	Definition
1-MN	1-methylnaphthalene
2, 6-diMN	2,6-dimethylnaphthalene
2-MN	2-methylnaphthalene
°API	American Petroleum Institute gravity
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Ass/*ass*	Alkylsuccinate synthase
Bam/*bam*	Benzoyl-CoA reductase, ATP-independent
Bcr/*bcr*	Benzoyl-COA reductase, ATP dependent
BTEX	Benzene, toluene, ethylbenzene, and xylene
BLAST	Basic local alignment search tool
Bss/*bss*	Bensylsuccinate synthase
BSTFA	*N*, *O*-bis-(trimethylsilyl) trifluoroacetamide
CH_4	Methane
CO_2	Carbon dioxide
CoA	Coenzyme A
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
ENA	European nucleotide archive
Fe (III)	Iron (Ferric)
GC	Gas chromatography/ gas chromatograph
GC-FID	Gas chromatograph with flame ionization detector
GC-MS	Gas chromatography mass spectrometry
H_2	Molecular hydrogen
HC	Hydrocarbon
HCl	Hydrochloric acid
HIGHT	High temperature produced water inoculum
HMN	2, 2,4, 4, 6, 8, 8 -Heptamethylnonane
LB	Luria Bertani
LOWT	Low temperature produced water inoculum
LOWT-EN	Low temperature produced water enrichment
MOF	Most frequently occurring sequence
MS	Mass spectrometry
N_2	Molecular nitrogen
$NaH^{13}CO_3$	^{13}C-labeled bicarbonate
Na_2S	Sodium sulfide
Ncr/*ncr*	Naphthoyl-CoA reductase
Nms*/nms*	Naphthoyl-methylsuccinate synthase
NO_3^-	Nitrate
NRB	Nitrate-reducing bacteria
OTU	Operational taxonomical unit

PAH	Polycyclic aromatic hydrocarbons
PCR	Polymerase chain reaction
PFL	Pyruvate formate lyase
qPCR	Quantitative polymerase chain reaction
RDP	Ribosomal database project
RESOIL	Oil degrading culture from contaminated aquifer
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription quantitative PCR
SIP	Stable isotope probing
SO_4^{2-}	Sulfate
SRB	Sulfate-reducing bacteria
SSU	Small subunit
TMS	Trimethylsilyl
TRFLP	Terminal restriction fragment length polymorphism

Chapter One: Introduction

1.1 Rationale and significance of the project

Microorganisms are found virtually everywhere and they are capable of utilizing a wide variety of available substrates including petroleum hydrocarbons. The ability of microorganisms to metabolize hydrocarbons with O_2 as a terminal electron acceptor has been known for decades (Rosenberg, 2013). Although a few early studies reported hydrocarbon metabolism in the absence of O_2 (e.g., ZoBell, 1946; Muller, 1957), anaerobic biodegradation of hydrocarbons was only recognized and accepted in the last 25 years (e.g., Grbić-Galić and Vogel, 1987; Edwards and Grbić-Galić, 1994; Zengler *et al.*, 1999). It is now known that hydrocarbon biodegradation can also occur with the reduction of other electron acceptors such as NO_3^-, Fe (III), Mn (IV), and SO_4^{2-}. When these electron acceptors are scarce, hydrocarbons can be biodegraded through a low energy yielding metabolic process coupled with CH_4 production (Schink, 1997). This process requires the syntrophic cooperation between at least two groups of organisms, syntrophs and methanogens (McInerney *et al.*, 2009). First, syntrophs/fermenters convert hydrocarbon compounds to intermediate products such as acetate, CO_2, H_2, and formate, and then methanogens consume these metabolic products, and keep them at low concentrations (McInerney *et al.*, 2009), so that reactions are energetically favorable. Under anoxic conditions, the initial activation of hydrocarbons can occur via a fumarate addition mechanism, which has been mainly studied under sulfate- and nitrate-reducing conditions (e.g., Biegert *et al.*, 1996; Beller and Spormann, 1997; Kropp *et al.*, 2000; Grundmann *et al.*, 2008). Other metabolic pathways reported to activate hydrocarbons include carboxylation, hydroxylation, or methylation (reviewed in Foght, 2008; Heider and Schühle, 2013). However, under methanogenic conditions, the metabolic pathways involved in the activation and subsequent degradation of

hydrocarbons are just starting to be elucidated. In addition, the microorganisms in methanogenic consortia and their interactions with each other to metabolize hydrocarbons are not well understood.

During the production and transportation of fossil fuels, petroleum hydrocarbons can be accidentally spilled into the environment. Many of these hydrocarbons are toxic and harmful for humans and other living organisms (Sverdrup *et al.*, 2002). Crude oil components can end up in subsurface environments (e.g., groundwater aquifers or sediments) where O_2 is depleted, and they become available carbon sources for anaerobic microorganisms.

The understanding of hydrocarbon metabolism under anoxic conditions, especially in the absence of electron acceptors not only can help to monitor the natural attenuation of oil contaminants in the subsurface, but can also contribute to improving bioremediation technologies applied to crude oil-contaminated areas. In addition, understanding methanogenic hydrocarbon degradation can help to assess the feasibility of energy-related emerging biotechnologies, such as the bioconversion of residual oil to methane for energy production (Gieg *et al.*, 2008; Jones *et al.*, 2008), or as a means to mobilize heavy oil (Grigoryan and Voordouw, 2008).

1.2 Research objectives

The major goal of this thesis work was to investigate the methanogenic biodegradation of crude oil and polycyclic aromatic hydrocarbons (PAH). Three main objectives were established to direct this work:

1. Identify and characterize metabolic mechanisms involved in crude oil and PAH biodegradation under methanogenic conditions.

2. Characterize microbial communities and key microorganisms involved in methanogenic degradation of crude oil and PAH.
3. Assess the feasibility of microbial communities to bioconvert hydrocarbon components into methane in marginal crude oil reservoir-simulating systems.

1.3 Organization of thesis

This thesis is prepared in a manuscript format that consist of a collection of published manuscripts and unpublished work that describe the methanogenic biodegradation of crude oil with an emphasis on PAH. A brief description of each chapter and the contribution of the co-authors to the work are presented below.

Chapter 2 includes a state of the art literature review describing the main concepts and background information necessary to understand the content of the thesis. Specific background information is also included in each chapter.

Many of the results described in Chapter 3 and 4 on crude oil methanogenesis in cultures and column systems were published in *Frontiers in Microbiology* (Berdugo-Clavijo and Gieg, 2014). For this thesis, the results were split into two chapters because more work was conducted on this topic after the publication of the article. Chapter 3 describes enrichment cultures able to degrade hydrocarbon components from light and heavy crude oils including metabolite analysis, functional gene analysis on light oil (*assA/bssA*), and microbial community characterization. This enrichment culture was used in sandstone-packed column studies (described in Chapter 4).

Chapter 4 also covers part of the work published in *Frontiers in Microbiology* (Berdugo-Clavijo and Gieg, 2014) and focuses on the experiments conducted with sandstone-packed column systems inoculated with a light oil enrichment culture (described in Chapter 3). This

chapter also contains unpublished work conducted with three other different microbial inocula to assess crude oil bioconversion to CH_4 in columns simulating marginal oil fields. Further, hydrocarbon analyses conducted in columns showing the highest levels of CH_4 are also shown in this chapter. In addition, a comparison between the microbial community of the initial inoculum and the community that was enriched after incubation in the sandstone-packed columns is presented.

Chapter 5 consists of a manuscript published in *FEMS Microbiology Ecology* (Berdugo-Clavijo *et al*., 2012). Here, work conducted on methanogenic enrichments capable of degrading polycyclic aromatic hydrocarbons, with an emphasis on 2-methylnaphthalene and 2, 6-dimethylnaphthalene, is described. Metabolite analysis and microbial community characterization of the enrichment cultures are presented in this chapter. Dr. Lisa Gieg established the initial enrichment used in this study while being a research associate at the University of Oklahoma. Xiaoli Dong, Dr. Jung Soh, and Dr. Christoph Sensen provided bioinformatic support for pyrotag and phylogenetic analyses.

Chapter 6 describes qPCR work conducted to identify and quantify microorganisms able to degrade methylnaphthalene, as well as initial work to detect metabolic genes involved in PAH biodegradation under methanogenic conditions. This work has not been published.

Chapter 7 describes the activity, metabolite analysis and microbial communities characterized in enrichment cultures amended with naphthalene and 1-methylnaphthalene (as a continuation of published work, Chapter 5). This work will be submitted for publication in the following months.

Chapter 8 summarizes the main findings and contributions of each chapter to the field of methanogenic hydrocarbon biodegradation, and outlines future work suggested for the continuation of this research.

Appendices A-E include supplementary information related to the work presented in Chapters 3 to 7. Appendix F provides a copy of a review paper on syntrophic hydrocarbon metabolism that I co-authored with Dr. Lisa Gieg and Dr. Jane Fowler.

Please note that as this thesis is prepared as a series of manuscripts, the introductory information for each chapter may be repetitive in some cases.

Chapter Two: Literature Review

2.1 Microorganisms in hydrocarbon-laden environments

Microorganisms are a ubiquitous form of life found virtually everywhere on Earth, including in extreme environments such as those at high or low temperatures, pressures, salinities and pHs (e.g., hot springs, deep sea trenches, and permafrost). In these surroundings microorganisms perform diverse metabolic pathways, and contribute to the biogeochemical cycling of nutrients (e.g., carbon, nitrogen, sulfur, and iron) on the planet.

Since the early 20th-century, microorganisms have been known to inhabit subsurface petroleum reservoirs (Bastin, 1926), and potentially utilize hydrocarbon components, or their metabolites, as an energy source (Sohngen, 1905, 1913). Microorganisms commonly found in petroleum reservoirs can include sulfate-reducing bacteria (SRB), fermentative bacteria, iron-reducers, syntrophs, and methanogens (Magot *et al.*, 2000). SRB were the first microorganisms reported to be found in oil reservoirs, and they are known to be responsible for the accumulation of hydrogen sulfide, which contributes to reservoir "souring", and leads to corrosion of oil pipelines (Bastin, 1926). Fermentative bacteria are able to utilize organic compounds like carbohydrates and peptides, and ferment to organic acids (Birkeland, 2004). Iron (III)-reducing bacteria may also contribute to the cycling of organic matter in the reservoirs. Finally, syntrophs and methanogens also are considered important key players in oil fields and subsurface environments. Recent studies suggest that biodegradation of crude oil to methane is involved in the formation of heavy oil, and it is the primary process that contributes to the biogenic accumulation of methane over geological time in reservoir rocks and fluids (Head *et al.*, 2003; Jones *et al.*, 2008).

The transportation, storage, and usage of crude oil can result in the contamination of natural environments. Here, hydrocarbon components disperse into the soil, sediments and sometimes deep into ground water. These environments can also be the habitat for a variety of microorganisms that can utilize hydrocarbons as a carbon and energy source. Within contaminated ground water systems, aerobic hydrocarbon degraders have been observed at the edges of contaminated plumes where small amounts of pollutants, and relatively high oxygen levels are present. Meanwhile, deep within the plume, groups of anaerobic microbes such as nitrate reducers, metal reducers, sulfate reducers, syntrophs, and methanogens are found depending on the availability of these electron acceptors in the contaminated area (Winderl *et al.*, 2008; Jobelius *et al*., 2010). Other oil containing environments include natural oil and gas seeps. Petroleum seeps are marine or land sites where hydrocarbons leak out of the ground from oil or gas deposits. Aerobic and anaerobic microbes able to degrade methane, and higher molecular weight hydrocarbons have been found in these environments (Michaelis *et al.*, 2002; LaMontagne *et al.*, 2004).

2.2 Anaerobic biodegradation of hydrocarbons

A variety of hydrocarbon components present in oil-associated environments can serve as a source of carbon and energy for microorganisms. Crude oil components may include two groups of hydrocarbons known as aliphatics and aromatics. Saturates are the most common type of aliphatic hydrocarbons, and they comprise *n*-alkanes, branched alkanes, and cycloalkanes. Saturated aliphatic hydrocarbons are highly unreactive due to their stable H-C and C-C bonds (Wilkes and Schwarzbauer, 2010). Unsaturated aliphatic hydrocarbons such as alkenes and alkynes are less common in crude oil. However, the presence of a double bond makes them more

reactive than alkanes. The aromatics group is comprised of mono- and polycyclic hydrocarbons such as benzene or naphthalene, respectively. Aromatic hydrocarbons are also stable compounds that are chemically difficult to attack due to their delocalized bonds (Wilkes and Schwarzbauer, 2010).

The microbial utilization of hydrocarbons in the presence of O_2 is a highly energetic reaction (Widdel and Musat, 2010). In contrast, lower energy is gained from hydrocarbon utilization with other electron acceptors. For example, in the biodegradation of hexadecane with oxygen as a terminal electron acceptor, the free energy change (calculated at standard conditions) is -10,392 kJ.mol^{-1} (Table 2-1a). Lower free energy is released under nitrate- and sulfate-reducing conditions (b and c), and the lowest amount of energy gained during hydrocarbon metabolism occurs under methanogenic conditions (d) (Table 2-1) (Widdel and Musat, 2010).

Table 2-1. Free energy changes for the biodegradation of hexadecane as a model hydrocarbon with oxygen, nitrate, sulfate, or under methanogenic conditions at standard conditions (adapted from Widdel and Musat, 2010).

	ΔG°' (kJ/mol)
a) $2C_{16}H_{34} + 49O_2 \rightarrow 32CO_2 + 34H_2O$	-10,392
b) $5C_{16}H_{34} + 98NO_3^- + 98H^+ \rightarrow 80CO_2 + 49N_2 + 134H_2O$	-9,757
c) $4C_{16}H_{34} + 49SO_4^{2-} + 98H^+ \rightarrow 64CO_2 + 49H_2S + 68H_2O$	-632
d) $4C_{16}H_{34} + 30H_2O \rightarrow 49CH_4 + 15CO_2$	-372

Initially, the ability of microorganisms to metabolize hydrocarbons was believed to occur only with oxygen as the terminal electron acceptor. Aerobic pathways for hydrocarbon biodegradation have been widely investigated (Rosenberg, 2013). Enzymes such as mono- and

di-oxygenases are responsible for catalyzing the degradation of hydrocarbons under aerobic conditions. Alkanes are commonly oxidized to primary alcohols, which are further oxidized to aldehydes, and subsequently metabolized into fatty acids. Finally a conjugated fatty acid is then further degraded by β-oxidation. A key metabolite in aromatic hydrocarbon degradation is catechol which is then metabolized to easily degradable products that will enter the TCA cycle (Whited and Gibson, 1991). Anaerobic hydrocarbon biodegradation was reported in early studies (ZoBell, 1946; Jack *et al.*, 1985). However, only about 25 years ago the biodegradation of hydrocarbons in the absence of oxygen gained more attention. Nowadays, it is known that microorganisms can metabolize different crude oil components with other terminal electron acceptors such as nitrate, iron, sulfate, and in the absence of electron acceptors (Heider and Schühle, 2013).

2.2.1 Metabolic mechanisms involved in anaerobic crude oil biodegradation

The metabolic pathways of anaerobic hydrocarbon metabolism have been elucidated mainly under sulfate- and nitrate- reducing conditions. Studies of hydrocarbon metabolism under methanogenic conditions are scarce. At least four mechanisms may occur for the initial activation of hydrocarbons under anoxic conditions, and they will be described below with an emphasis on naphthalene and methylnaphthalene degradation:

2.2.1.1 Fumarate addition

This process was first described for the biodegradation of toluene under nitrate-reducing conditions (Biegert *et al.*, 1996; Beller and Spormann, 1997). In this reaction, fumarate is added to the methyl carbon of the toluene to form *R*-benzylsuccinate (Figure 2-1A). The oxygen-

sensitive glycyl radical enzyme, known as benzyl succinate synthase (BSS), catalyzes the addition of fumarate to activate the hydrocarbon. This enzyme has been well characterized and purified from the denitrifying bacteria *Azoarcus* sp. and *Thauera aromatica* (Beller and Spormann, 1997; Leuthner and Heider, 1998; Krieger *et al.*, 2001). The BSS enzyme contains three main subunits α, β and γ encoded by the gene *bssABC* (Li *et al.*, 2009). In addition, the BSS operon contains *bssD* gene encoding a glycyl radical activating protein for enzyme function, and other genes encoding proteins of unknown functions (*bssEFGH*) (Hermuth *et al.*, 2002). After benzylsuccinate forms, it is metabolized to benzylsuccinyl-CoA, and it is further degraded by a sequence of β-oxidation reactions to form succinyl-CoA, and benzoyl-CoA. The enzymes catalyzing these reactions are all encoded by the genes *bbsA-H*. (Leutwein and Heider, 2002). Finally, the aromatic ring of benzoyl-CoA is degraded by benzoyl-CoA reductase (BCR). Two types of ring-dearomatizing enzymes are known. The first is the ATP-dependent BCR class that is found in facultative anaerobes (e.g., *Thauera and Azoarcus*), and is encoded by *bcrABCD* genes (Boll and Fuchs, 1995; Song and Ward, 2005). The second type is the ATP independent BCR class, which is typically found in obligate anaerobes (e.g. *Geobacter metallireducens*) and is encoded by the *bamBCDFGHI* genes (Wischgoll *et al.*, 2005; Kuntze *et al.*, 2008). The reduced products of benzoyl-CoA are further degraded most likely by other hydrolytic-like and β-oxidation reactions (Boll *et al*., 2002).

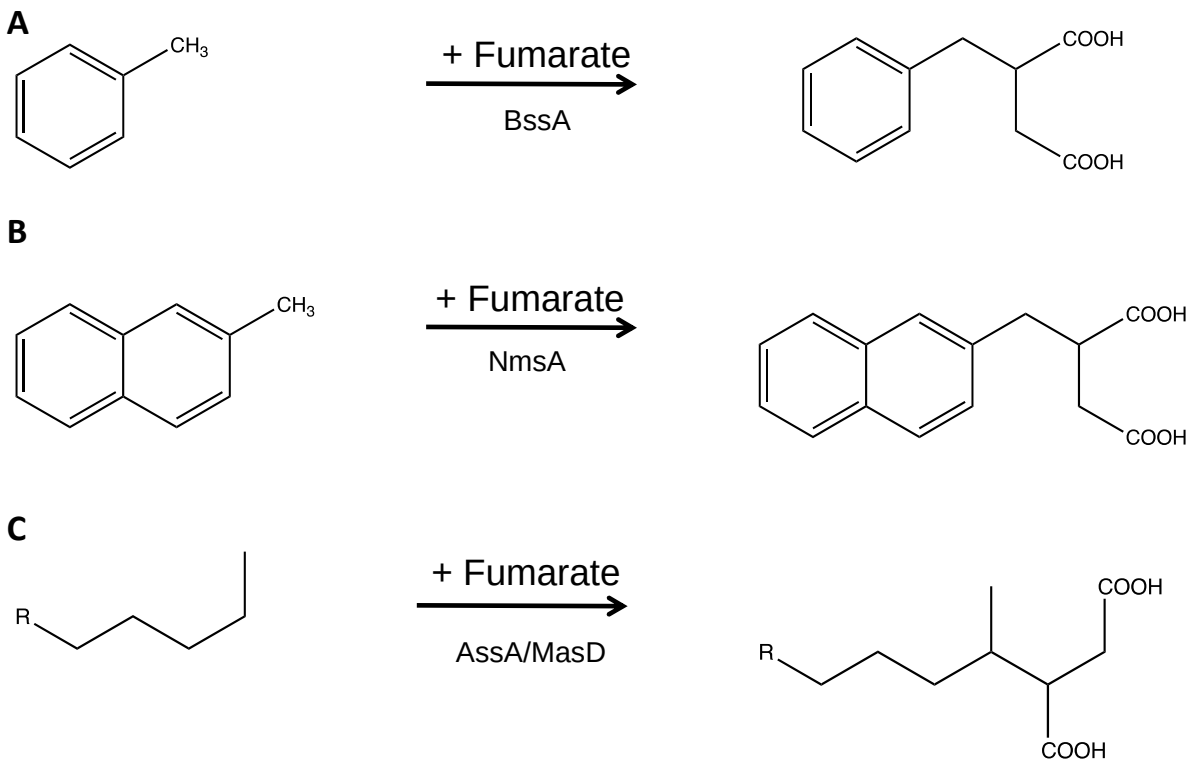

Figure 2-1. Fumarate addition reactions for the initial biodegradation of toluene (A), methylnaphthalene (B), and an *n*-alkane (C). Reactions are catalyzed by the glycyl radical enzymes benzylsucinate synthase (BssA), naphthyl-2-methyl-succinate (NmsA) and alkylsuccinate synthase (AssA/MasD).

Other aromatic hydrocarbons such as *m*-xylene and 2-methylnaphthalene (2-MN) are also known to be biodegraded via fumarate addition reactions under sulfate-reducing conditions (e.g. Harms *et al.*, 1999; Musat *et al.*, 2009). By analogy to the fumarate addition pathway for toluene, a naphthyl succinate metabolite is formed from the initial degradation of 2-MN (Figure 2-1B), and this reaction is catalyzed by a naphthyl-methyl succinate synthase enzyme (NmsA) (Annweiler *et al.*, 2000; Musat *et al.*, 2009). Proteomic, genomic, and metabolomic studies with sulfate-reducing bacterial cultures (Annweiler *et al.*, 2002; DiDonato *et al.*, 2010; Selesi *et al.*, 2010) have contributed to an understanding of the key enzymes and metabolites involved in PAH biodegradation via a fumarate addition pathway, and subsequent reactions (Figure 2-2). A study with the N47 sulfate-reducing culture (Selesi *et al.*, 2010) revealed genes presumably involved in the degradation of the metabolite naphthyl-2-methylsuccinate. These genes were related to the ones involved in the β-oxidation of benzylsuccinate during toluene degradation (*Bbs*ABCDFGH), and were named as *bns* (β-oxidation of naphthylsuccinate) genes comprising the *Bns*ABCDFGH operon coding for different functions (Figure 2-2). Other genes (*Ncr*ABCD) detected in the culture were believed to code for the naphthoyl-CoA reductase enzyme responsible for catalyzying the dearomatization of the ring structure of the 2-naphthoyl-CoA metabolite (Figure 2-2). This is analogous to the *bcr* genes from *Azoarcus* sp. strain CIB, known to be involved in the benzoyl-CoA reductase pathway (López Barragán *et al.*, 2004). Additional genes found in the *ncr* cluster are thought to be involved in subsequent degradation reactions such as β-oxidation and hydrolytic cleavage of the ring structure. Furthermore, corresponding metabolites for the initial β-oxidation reaction of the naphthyl-2-methyl-succinate and the dearomatization of the 2-naphthoyl-CoA have been detected (Annweiler *et al.*, 2002). Central metabolites such as naphthoic acids were found in the degradation of 2-MN and 2, 6-diMN under

methanogenic conditions (Berdugo-Clavijo *et al.*, 2012), but no evidence of fumarate addition reactions exist to date for the methanogenic degradation of PAH.

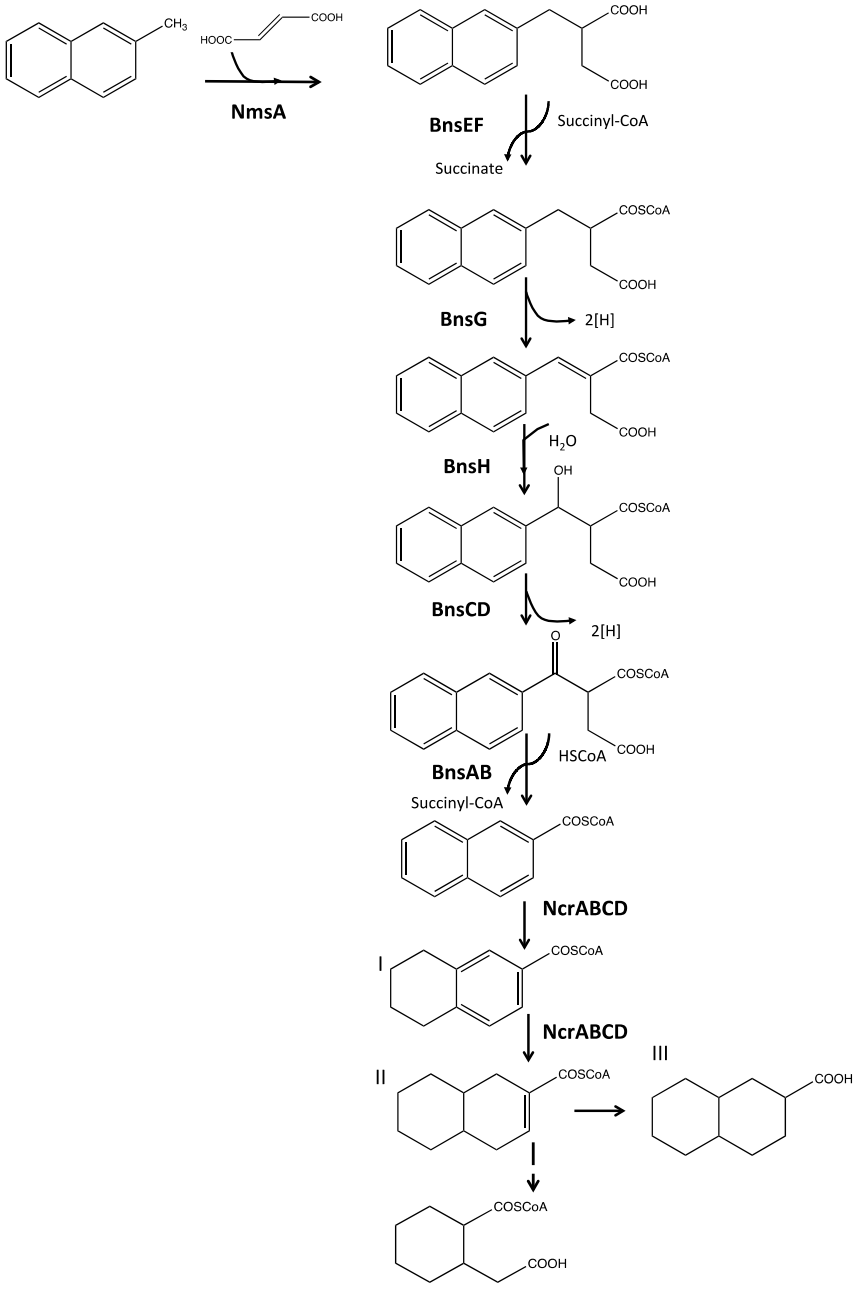

Figure 2-2. Proposed pathway for anaerobic biodegradation of 2-MN via fumarate addition reaction (under sulfate-reducing conditions). I) 5,6,7,8 tetrahydro-2-naphthoic acid, II) octahydro-2-naphthoic acid, and III) decahydro-2-naphthoic acid. Enzyme names are described in text. (Modified from Selesi *et al.*, 2010; Meckenstock and Mouttaki, 2011).

Finally, fumarate addition has also been reported in the anaerobic biodegradation of alkanes. Kropp *et al.* (2000) first observed the formation of dodecylsuccinate during the degradation of dodecane under sulfate-reducing conditions. During the anaerobic metabolism of *n*-alkanes, fumarate is added to the subterminal methylene group of the specific alkane and a methyl-alkyl succinate intermediate is formed (Figure 2-1C) (Rabus *et al.*, 1999; Kropp *et al.*, 2000). The enzyme responsible for catalyzing this reaction is known as alkylsuccinate synthase. This class of glycyl radical enzyme has been well characterized in pure cultures such as the *n*-alkane degrading sulfate-reducing bacterium *Desulfatibacillum alkenivoras*, where the two genes (*assA1* and *assA2*) were detected (Callaghan *et al.*, 2012), and the *n*-hexane degrading nitrate-reducing bacterium strain HxN, where an *assA*-like gene named *masD* was found (Grundmann *et al.*, 2008). The *assA/masD* genes have also been detected in methanogenic *n*-alkane-degrading cultures (Callaghan *et al.*, 2010; Zhou *et al.*, 2012; Aitken *et al.*, 2013). In addition, alkylsuccinate metabolites have only recently been detected under methanogenic conditions (Tan *et al.*, 2013; Berdugo-Clavijo and Gieg, 2014).

2.2.1.2 Carboxylation

This mechanism of activation has been proposed to occur for unsubstituted aromatic hydrocarbons such as benzene, naphthalene, and phenanthrene under nitrate-, iron- and sulfate-reducing conditions (Zhang and Young, 1997; Meckenstock *et al.*, 2000; Rockne *et al.*, 2000; Kunapuli *et al.*, 2008). Typically, carboxylation is investigated with the addition of ^{13}C-bicarbonate to the culture medium. The labelled carbon (from $^{13}C\text{-}HCO_3^-$) is incorporated into the corresponding hydrocarbon molecule, and leads to the formation of ^{13}C-labelled metabolites.

In sulfate-reducing cultures amended with ^{13}C-bicarbonate, labelled metabolites such as ^{13}C-naphthoic acid and ^{13}C-phenanthroic acid, were detected during the degradation of naphthalene and phenanthrene, respectively (Zhang and Young, 1997; Davidova *et al.*, 2007).

During the degradation of naphthalene via carboxylation, a carboxyl group is added into the naphthalene molecule, and 2-naphthoic acid is formed (Figure 2-3) (Meckenstock *et al.*, 2000). In a microarray study with the sulfate-reducing NaphS2 strain, genes coding for aromatic carboxylases were upregulated in the presence of naphthalene (DiDonato *et al.*, 2010). Similarly, Bergmann *et al.* (2011) detected and characterized a gene cluster ORF N47_K27540 coding for the alpha subunit of a naphthalene carboxylase in the sulfate reducer strain N47. Moreover, further ring reduction reactions for naphthoic acid degradation have been investigated. Reduced products formed during further naphthalene degradation include, decahydro-2-naphthoic (Meckenstock *et al.*, 2000), 5,6,7,8-tetrahydro-2-naphthoic acid, and octahydro-2-naphthoic acid (Zhang *et al.*, 2000; Annweiler *et al.*, 2002) (Figure 2-2). Although, naphthalene-degrading cultures have been established under methanogenic conditions (Berdugo-Clavijo *et al.*, 2012), there is no evidence of carboxylation occurring in the absence of electron acceptors.

Initially, benzene degradation by carboxylation was shown to occur in an iron-reducing culture (Kuntze *et al.*, 2008). Proteins showing high similarity to a phenol carboxylase subunit were expressed in the culture, and carboxylase-related genes designated *abcA* and *abcD* were detected (Abu Laban *et al.*, 2010). Furthermore, benzene carboxylase-like genes, as well as genes involved in benzoate degradation were transcribed in a nitrate-reducing enrichment culture amended with benzene or benzoate (Luo *et al.*, 2014).

Fumarate addition is the most common pathway found to be involved in the activation of alkanes, though carboxylation has also been suggested as a metabolic mechanism involved in the

biodegradation of alkanes by SRB (So *et al.*, 2003). By using labelled compounds like ^{13}C-bicarbonate and [1,2 $^{13}C_2$] hexadecane, the authors detected ^{13}C-labelled carboxylic acid metabolites with one carbon number shorter than the parent alkane (C_{15}). Thus, it was suggested that alkane degradation in the culture occurs initially by incorporating a carboxyl group at the C-3 position of the chain, and subsequent removal of the two terminal carbon atoms from the alkyl chain.

2.2.1.3 Methylation

A less common pathway of hydrocarbon activation is the addition of a methyl group to the hydrocarbon parent molecule (Coates *et al.*, 2002). This reaction has been observed during the degradation of benzene and naphthalene (Ulrich *et al.*, 2005; Safinowski and Meckenstock, 2006). ^{13}C-labelled benzene was converted to ^{13}C-toluene in methanogenic and nitrate-reducing cultures (Ulrich *et al.*, 2005). It has been proposed that toluene is then biodegraded via fumarate addition, leading to the formation of benzylsuccinate that subsequently enters the benzoyl-CoA pathway. However, the corresponding metabolites were not identified in the cultures. In a similar way, 2-MN was detected in a naphthalene-degrading sulfate-reducing enrichment culture (Figure 2-3), suggesting methylation as an initial activation mechanism followed by fumarate addition (Safinowski and Meckenstock, 2006). This notion was supported by the detection of metabolites and enzymes potentially associated with methylation and fumarate addition reactions (Annweiler *et al.*, 2002; Safinowski and Meckenstock, 2004).

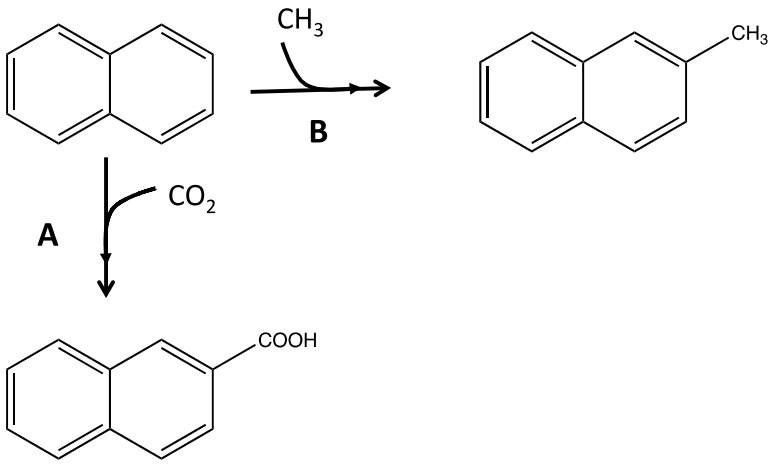

Figure 2-3. Initial anaerobic biodegradation of naphthalene by carboxylation and methylation. Reactions are catalyzed by naphthalene carboxylase (A) and naphthalene-methyl-transferase (hypothetical enzyme) (B) to form naphthoic acid and methyl-naphthalene metabolites, respectively.

2.2.1.4 Hydroxylation

Anaerobic hydroxylation has been observed for the degradation of ethylbenzene under nitrate-reducing conditions (Ball *et al.*, 1996). Here, a hydroxyl group (from water) is added to the carbon-1 of the hydrocarbon chain to produce phenylethanol. This reaction is catalyzed by ethylbenzene dehydrogenase (EBDH), which contains α, β, and γ-subunits (Johnson *et al.*, 2001). Phenylethanol is then converted to acetophenone, and subsequently to benzoylacetate by a number of enzymes previously characterized (Jobst *et al.*, 2010). Furthermore, benzoylacetyl-CoA is formed by a CoA ligase, which is then metabolized to acetyl-coA and benzoyl-CoA (Rabus *et al.*, 2002). Hydroxylation was also reported in the biodegradation of benzene with the formation of phenol (Chakraborty and Coates, 2005; Ulrich *et al.*, 2005). However, Kunapuli *et al.* (2008) showed that phenol is likely formed from abiotic reactions during the sample preparation procedures.

2.3 Syntrophy and methanogenesis

2.3.1 Principles of syntrophic metabolism

The complete mineralization of an organic molecule to methane and CO_2 is possible by a partner-dependent metabolic activity called syntrophy (McInerney *et al.*, 2009). Syntrophic processes typically take place in methanogenic environments including freshwater sediments, rice paddies, sewage treatment plants, landfills, and the intestinal tracts of ruminants. However, there is also evidence that syntrophic processes can happen at higher redox potential, and in the presence of electron acceptors such as nitrate, Fe (III), or sulfate (Kunapuli *et al.*, 2007; Herrmann *et al.*, 2010; van der Zaan *et al.*, 2012). Initially, during syntrophic processes, a group of microorganisms known as fermenters utilize substrates like lipids, proteins, fatty acids, alcohols, amino acids, sugars and aromatic compounds, and produce metabolites such as acetate, long-chain fatty acids, CO_2, H_2 and formate. Then, syntrophic organisms utilize these products and convert them to acetate, CO_2, H_2, and formate, which become substrates for methanogenesis (Figure 2-4). Either hydrogenotrophic (a) or acetotrophic (b) methanogens finalize the reaction to produce CH_4 (and CO_2 or H_2O) (Sieber *et al.*, 2012). The former reactions are only thermodynamically favorable when microbial partners like methanogens consume the metabolic products from syntrophs, and keep them at low concentrations (McInerney *et al.*, 2009). Because of the low energy that is shared between syntrophs and methanogens in these reactions, syntrophy has been considered as an extreme lifestyle (Table 2-2).

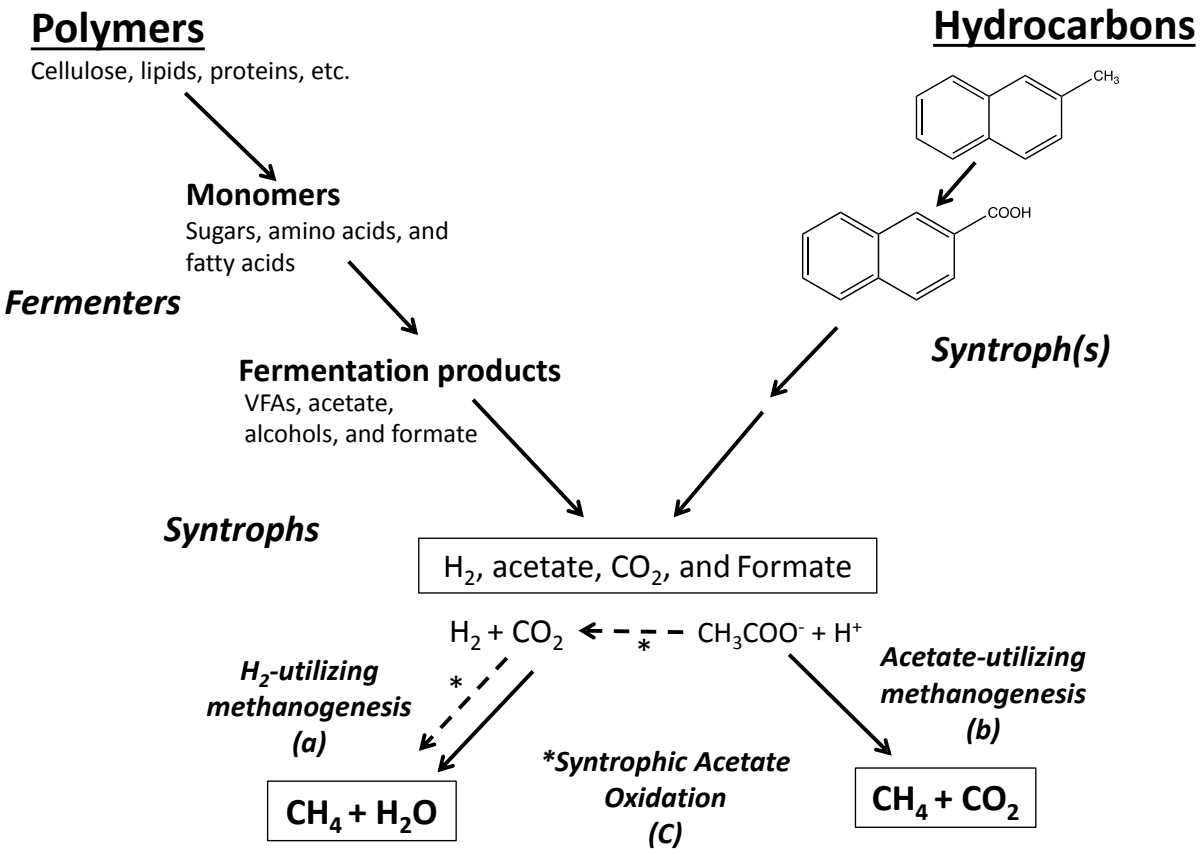

Figure 2-4. Possible routes for syntrophic decomposition of polymers and hydrocarbons, coupled to (a) hydrogen-, (b) acetate-utilizing methanogenesis, and (c) syntrophic acetate oxidation coupled to methane production (dotted lines) (Modified from McInerney *et al.*, 2009).

Syntrophic processes usually occur via interspecies transfer of formate or H_2 (Stams, 2006). Key enzymes and membrane-bound complexes have been well investigated in syntrophic microorganisms exchanging H_2/formate molecules (Sieber *et al.*, 2012). Cysteine has also been shown to be exchanged between cometabolic cultures growing in syntrophic associations (Kaden *et al.*, 2002). Another mechanism of electron transfer in syntrophic processes includes direct electron transfer via nanowires or electronically conductive pili (Reguera, 2005). Syntrophic processes are also known to uptake energy via reverse electron transfer to carry out important redox reactions that are otherwise unfavourable (Sieber *et al.*, 2012).

2.3.2 Syntrophy in methanogenic hydrocarbon degradation

Crude oil hydrocarbons can be completely mineralized to methane via syntrophic reactions. This process can occur through different metabolic routes that are driven by thermodynamics, microbial community composition, and environmental conditions such as acetate/H_2 concentrations, temperature, and pH (Dolfing *et al.*, 2008). For instance, hydrocarbons can be initially degraded to a combination of products such as H_2 and CO_2, acetate and H_2, or acetate alone, and then be coupled to methanogenesis from either H_2 or acetate (Dolfing *et al.*, 2008) (Table 2-2). Laboratory and field studies suggest that hydrogen-utilizing methanogenesis is the dominant process in oil reservoir environments (Nazina *et al.*, 1995; Rozanova *et al.*, 2001; Bonch-Osmolovskaya *et al.*, 2003; Jones *et al.*, 2008). Other studies have shown acetotrophic methanogenesis as a major process occurring in contaminated aquifers (Dojka *et al.*, 1998; Struchtemeyer *et al.*, 2005). The oxidation of hydrocarbons to acetate can also be linked to a thermodynamically feasible process known as syntrophic acetate oxidation (Figure 2-4c; Table 2-2), which is then coupled to hydrogenotrophic methanogenesis (Dolfing *et*

al., 2008). This metabolic route seems to dominate in oil systems (Jones *et al.*, 2008), especially in high temperature (50-70 ℃) oil reservoirs (Nazina *et al.*, 1995; Mayumi *et al.*, 2011).

Table 2-2. Stoichiometry and Gibbs free energy for reactions involved in syntrophic hydrocarbon degradation coupled to methane production (Adapted from Gieg *et al.*, 2014 with permission)

		ΔG°' (kJ/mol)	ΔG'[a] (kJ/mol)
Methanogenic reactions			
Acetotrophic	$CH_3COO^- + H_2O \rightarrow HCO_3^- + CH_4$	-31.0	-15.7
Hydrogenotrophic	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-135.6	-38.7
Syntrophic hydrocarbon oxidation to acetate and H_2			
Hexadecane	$C_{16}H_{34} + 16H_2O \rightarrow 8CH_3COO^- + 8H^+ + 17H_2$	+470.8	-91.3
Naphthalene	$C_{10}H_8 + 10H_2O \rightarrow 5CH_3COO^- + 5H^+ + 4H_2$	+101.1	-90.2
Overall conversion of hydrocarbon to methane[b]			
Hexadecane	$C_{16}H_{34} + 16H_2O \rightarrow 12.25CH_4 + 3.75HCO_3 + 3.75H^+$	-353.5	-380
Naphthalene	$C_{10}H_8 + 12H_2O \rightarrow 6CH_4 + 4HCO_3 + 4H^+$	-189.5	-206.9

[a]Calculated from the equation $\Delta G' = \Delta G°' + RT\ln[(C)^c(D)^d]/(A)^a/(B)^b]$, where R= 0.00831kJ/K.mol, and T= 298 K based on the following concentrations of intermediates: 10 Pa H_2, 50 mM bicarbonate, 50 µM acetate, 50 KPa CH_4, and 100 uM of substrate (values suggested in McInerney *et al.*, 2009)

[b]Assumes product removal via both acetotrophic and hydrogenotrophic methanogenesis

Key microorganisms that play a role in the conversion of hydrocarbons to methane have been investigated. Important members of methanogenic crude oil biodegradation include hydrocarbon degraders, H_2 and acetate utilizing methanogens, and other secondary fermenters or biomass scavengers (Kleinsteuber *et al.*, 2012). Microorganisms reported as hydrocarbon-degraders are usually fermenters such as *Pelotomaculum, Peptococcaceae, Clostridium,* members of the *Deltaproteobacteria, Syntrophus* and *Sporotomaculum.* A recent survey from 26 studies of anoxic hydrocarbon-laden environments revealed that the most frequently found 16S rRNA sequences belonged to the *Firmicutes* phylum, followed by members of the class *Gammaproteobacteria* (Gray *et al.*, 2010). Other important groups identified in the survey were *Deltaproteobacteria* including sulfate- and iron- reducers, and members of the family *Syntrophaceae*. Other genomic approaches have been useful to identify hydrocarbon degraders in microbial cultures. In a methanogenic enrichment amended with crude oil, 16S rRNA genes from a microorganism closely affiliated to the genus *Smithella* were quantified by qPCR analysis (Gray *et al.*, 2011). The growth of *Smithella* was coupled to the removal of alkane hydrocarbons and methanogenesis in the culture, so it was suggested that this organism is the main hydrocarbon-degrader. In a similar way, Sakai *et al.* (2009) detected (by qPCR) the abundance of an organism related to *Syntrophus gentianae* during the methanogenic degradation of benzene. In addition, the authors conducted a DNA-SIP analysis with $^{13}C_6$-benzene showing that this bacterial member had the highest assimilation of ^{13}C, indicating that *Syntrophus* species is the main hydrocarbon-degrader in the culture. More recently, using RNA-SIP and RT-qPCR analyses, Fowler *et al.* (2014) identified *Desulfosporosinus sp.* as the most likely toluene-degrader in a methanogenic enrichment culture. Likewise, Sun *et al.* (2014) concluded by DNA-

SIP and TRFLP that *Desulfosporosinus* was degrading toluene in a methanogenic culture. These studies show how different genera of microorganisms involved in syntrophic processes are able to degrade diverse hydrocarbons. Microbial players that are believed to utilize hydrocarbon intermediates and dead biomass include members of *Chloroflexi, Anaerolineae, and Bacteroidetes* (Kleinsteuber *et al.*, 2012).

Overall, syntrophic partnerships involved in hydrocarbon biodegradation are still not completely understood. New approaches (qPCR, DNA-SIP), transcriptomics, and proteomics are currently helping elucidate important key microorganisms, pathways and interactions involved in syntrophic processes occurring in hydrocarbon-associated environments (Gieg *et al.*, 2014).

2.4 Tools for assessing anaerobic hydrocarbon biodegradation pathways

A number of molecular and chemical approaches are now available to assess microbial activities and metabolic pathways in laboratory cultures, environmental samples or *in situ*. Many of these tools are commonly utilized in the study of anaerobic hydrocarbon biodegradation. The methods that were utilized in this thesis project are reviewed below.

2.4.1 Hydrocarbon metabolomics

This approach is based on the identification of biological markers or intermediate products released during the degradation of a specific parent substrate (Callaghan, 2013). Typically a microbial culture or sub-sample is acidified for sample preservation and protonation of potential metabolites. Then, the sample is liquid-liquid extracted with an organic solvent (e.g. ethylacetate, methylene chloride) or by solid-phase extraction. Extracted sample is then analyzed

by gas chromatography-mass spectrometry (GC-MS) or high performance liquid chromatography coupled with tandem mass spectrometry (LC-MS-MS). Analysis with GC-MS requires derivatization of the sample by methylation or silylation. Derivatized compounds are then identified based on their specific MS fragment ion patterns. Known standards or established libraries are used to confirm the identity of the compounds. LC-MS-MS analysis requires little or no sample preparation and a small volume of initial sample (Agrawal and Gieg, 2013).

Detection of metabolites in microbial cultures or in the environment has helped in defining biodegradation pathways for hydrocarbon compounds. For example, metabolite profiling allowed the detection of benzylsuccinate metabolites associated with the fumarate addition pathway in toluene degradation (Biegert *et al.*, 1996; Beller and Spormann, 1997). Later on, fumarate addition metabolites related to alkane degradation (e.g., alkylsuccinates) were also identified in anaerobic cultures (Rabus *et al.*, 1999; Kropp *et al.*, 2000). Metabolomics tools also have contributed to the monitoring of natural attenuation or bioremediation in contaminated areas such as groundwater aquifers (Beller *et al.*, 1995; Gieg and Suflita, 2002; Rios-Hernandez *et al.*, 2003; Gieg *et al.*, 2009). Beller *et al.* (1995) assessed the presence of benzylsuccinates *in situ* as an indicator of BTEX bioremediation in a groundwater aquifer. Gieg and Suflita (2002) found alkyl- and benzylsuccinate metabolites in contaminated aquifers, indicating *in situ* microbial utilization of alkanes and aromatic hydrocarbons, respectively.

A limitation of hydrocarbon metabolomics is that the identification of metabolites requires knowledge of the compounds that are likely present in the sample, and a detailed understanding of the metabolic mechanisms that may be occurring. Moreover, this approach is limited by the analytical capabilities to resolve certain compounds, especially complex metabolites or high molecular weight metabolites (Callaghan, 2013). Recently, 2-dimensional

GC (GC X GC) analysis coupled to MS was used to identify hydrocarbon-related mixtures such as naphthenic acids (Rowland *et al.*, 2011). The same chromatography approach was later applied to characterize oil fractions, and allowed the identification of various aromatic acids known as intermediates in anaerobic naphthalene biodegradation (West *et al.*, 2014). Thus, these high resolutions techniques could potentially help to characterize complex hydrocarbon metabolites that remain unknown. Furthermore, developing an open-source database that includes anaerobic hydrocarbon degradation metabolites, may also help to make hydrocarbon metabolomics a high-throughput tool (Spiegelman *et al.*, 2005).

2.4.2 PCR -based approaches

2.4.2.1 Functional gene-based analysis

Ribosomal RNA genes, especially 16S rRNA, are commonly used as marker genes to assess microbial diversity in a community. Also, catabolic genes involved in metabolic and functional processes are useful to assess the occurrence of functional abilities in certain microorganisms or communities (Spiegelman *et al.*, 2005). The presence or absence of a targeted gene is assessed by polymerase chain reaction (PCR) amplification of an extracted DNA template. DNA isolation protocols are currently available for the extraction of nucleic acids in soil and water. Purification of extracted DNA is also an important step to remove any inhibitory compound that can interfere with PCR and decrease the amplification efficiency (Liesack and Dunfield, 2003). Oligonucleotide primers that are complementary to the desired genes are designed for PCR, based on a database that is built from reference sequences of common domains or gene functions (e.g. *mcrA, dsrA,* and *pmoA*). Bioinformatic tools can be applied to align the selected sequences and find regions of high similarities. Thus, primers are designed

based on conserved regions of the reference sequences (Junca and Pieper, 2010). For the separation of PCR products of the gene(s) of interest different molecular biology methods can be used including clone library construction, denaturant gradient gel electrophoresis (DGGE), or pyrotag sequencing. Once individual sequences are obtained, they can be identified by available screening tools (e.g. BLAST), used in comparative analysis methods (e.g. phylogenetic tree construction), or further analyzed by other approaches (e.g. qPCR, and DNA-SIP) (Liesack and Dunfield, 2003).

Functional gene-based approaches are commonly applied to assess the potential for anaerobic hydrocarbon biodegradation in anaerobic cultures and environments. Fumarate addition genes such as *assA* and *bssA*, for alkane and aromatic hydrocarbon metabolism, are functional marker genes that can be used to assess natural attenuation or bioremediation in contaminated environments. Beller *et al.* (2002) developed primers for RT-PCR analysis to monitor the presence of benzylsuccinate synthase genes (*bssA*) in nitrate-reducing cultures inoculated with aquifer sediments and amended with BTEX compounds. Toluene degradation was coupled to an increase in the number of *bssA* copy genes. Also, Callaghan *et al.* (2010) designed primer sets to screen for the presence of *assA* and *bssA* genes in a variety of samples from hydrocarbon contaminated environments and enrichment cultures. A wide diversity of the *assA* genes within crude oil-impacted environments was shown with this approach. Recently, von Netzer *et al.* (2013) developed a gene detection assay for screening fumarate addition genes (*assA*, *bssA*, and *nmsA*) in cultures and environmental samples, and for further identification of major lineages in these genes using TRFLP analysis. Functional gene analysis has also helped to monitor genes associated with further degradation of hydrocarbon components such as *bcr*, *bam*,

and *ncr* genes involved in the dearomatization of benzoyl- or naphthoyl-CoA (Song and Ward, 2005; Kuntze *et al.*, 2008; Morris *et al.*, 2014).

2.4.2.2 Pyrotag sequencing

Next-generation sequencing tools have contributed to the genomic exploration of many unexplored surroundings including hydrocarbon-laden environments. With novel sequencing technologies, large-scale sequencing can be performed in a relatively short time, and at a low price (Caffrey, 2011). These new sequencing approaches aim to amplify single strands of a fragment library, and perform sequencing reactions on the strand that was amplified. Specific linkers (adapters) are annealed to blunt-ended fragments from the sample DNA in order to obtain library fragments (Mardis, 2008). In 454 pyrotag sequencing, the fragments are added to agarose beads that contain nucleotides that are complementary to the adapter sequences of the fragments. An individual mixture of "fragment and bead" is isolated into single micelles of oil and water, and then amplified up to about 1 million copies per fragment. Each amplified bead is isolated in extremely small wells (e.g., PicoTiterPlates) where nucleotides and chemiluminescent enzymes are sequentially added across the wells. If a nucleotide is complementary to the template strand, it is incorporated by polymerases. Binding of template and added nucleotides releases pyrophosphate that reacts with APS and ATP cofactors. This reaction is catalyzed by the added enzymes (sulfurylase and luciferase), and generates a flash of light recorded and analyzed by a computer, generating reads of about 400 nucleotides long.

Pyrotag sequencing has been used to characterize the microbial communities within hydrocarbon cultures or enrichments (e.g. Callbeck *et al.*, 2011; Abbai *et al.*, 2012; An *et al.*, 2013). A combination of stable isotope probing (SIP) experiments coupled to pyrotag sequencing

analysis helped to assess microbial community in oil-tar contaminated aquifer, showing a high abundance of toluene-degraders (Pilloni *et al.*, 2011).

2.4.2.3 qPCR

Quantitative polymerase chain reaction (qPCR) is used for the amplification, detection and quantification of PCR products (Spiegelman *et al.*, 2005). qPCR monitors the accumulation of PCR products over time using fluorescent dyes that excite when they are attached to double-stranded DNA (e.g. Syber green I) or specific oligonucleotide probes that release a fluorescent signal when the target DNA is amplified. (Junca and Pieper, 2010). An amplification plot is generated during qPCR analysis, which is used to quantify the target DNA. Four phases comprise a qPCR plot; baseline region, exponential phase, linear phase and plateau phase. qPCR assays also have a quantification cycle (Cq) which is used to determine the initial copy numbers of a specific target. High reproducibility between replicate samples and efficiency close to 100% is optimal for a qPCR assay. The efficiency is based on a calibration curve that is generated from serially diluted standards of the template DNA (Spiegelman *et al.*, 2005).

qPCR assays have been used to quantify 16S rRNA genes to determine key microorganisms growing in response to hydrocarbon substrates (Sakai *et al.*, 2009; Gray *et al.*, 2011). Other assays target functional genes that code for enzymes known to catalyze anaerobic hydrocarbon degrading reactions such as fumarate addition. For instance, the first qPCR assay to detect *bssA* genes was designed by Beller *et al.* (2002) from consensus sequences of toluene degrading nitrate-reducing cultures. Later on, *bssA* sequences were also detected and quantified in sulfate-reducing bacteria and methanogens (Beller *et al.*, 2008). qPCR has also been applied to monitor anaerobic aromatic degradation in contaminated sites (Winderl *et al.*, 2008; Oka *et al.*,

2011), and novel primers targeting fumarate addition genes (*nmsA*) have been designed to potentially be used in qPCR approaches (von Netzer *et al.*, 2013).

2.5 Biotechnology applications of petroleum microbiology

Microbial activities related to the petroleum industry can lead to detrimental or beneficial consequences for human and the environment. For example, the production of hydrogen sulfide by SRB in oil reservoirs can lead to souring and induce microbial corrosion in oil facilities (Voordouw, 2011). Methanogenesis has also been associated with pipeline corrosion in oil reservoir facilities (Suflita *et al*., 2008; Uchyama *et al.*, 2010). In contrast, many microorganisms possess the ability to utilize hydrocarbons or produce compounds (e.g. biosurfactants or biofuels) offering potential industrial applicability. Hence, microbial activities can help minimize the environmental impact of using fossil fuels or improve oil recovery in a more sustainable manner. Hence, knowledge on microbial communities and metabolic processes that are associated with hydrocarbon biodegradation can contribute to overcome the negative effects of microbes in oil facilities, help to improve bioremediation strategies, and investigate the feasibility of energy-emerging biotechnologies. Here we describe those applications that make use of the microbial degradation of hydrocarbons under methanogenic conditions.

2.5.1 Crude oil bioremediation

The production, storage, and transportation of petroleum hydrocarbons can result in accidental oil spills, and lead to the contamination of soil and ground water environments. Naturally the environment can attenuate contaminants by sorption, volatilization, dilution, or biodegradation (Banwart and Thornton, 2010). Initial biodegradation occurs in the presence of

oxygen, but aerobic microorganisms living at the surface of a contaminated site rapidly consume this electron acceptor. Thus, in a natural setting, biodegradation must then proceed through other electron-accepting processes such as with nitrate, iron, sulfate, and/or in the absence of electron acceptors.

Novel tools can be applied in contaminated sites to monitor microbial pathways and microorganisms involved in anaerobic hydrocarbon metabolism. One method to assess microbial activity in polluted environments is detecting and quantifying *in situ* key metabolites involved in anaerobic degradation of contaminants (Beller, 2002; Gieg and Suflita, 2002; Gieg *et al.*, 2009; Jobelius *et al.*, 2010). Moreover, with the use of genomic or proteomic analysis, and stable isotope probing tools, key microorganisms and genes involved in hydrocarbon biodegradation pathways can also be measured in the environment (Griebler *et al.*, 2003; Kunapuli *et al.*, 2007; Beller *et al.*, 2008; Kuntze *et al.*, 2008; Kazy *et al.*, 2010; Winderl *et al.*, 2010). Because of the slow biodegradation rates of anaerobic metabolism, microbial degradation of hydrocarbon contaminants can be enhanced by chemical or physical methods, and bioaugmentation (Hunkeler *et al.*, 2002; Da Silva and Alvarez, 2004; Bauer *et al.*, 2009). A combination of genomic, metabolomic and geochemical tools were used to assess how hydrocarbon biotransformation occurs in a tar-oil contaminated aquifer (Winderl *et al.*, 2008). Increases in the sulfate-reducing activity and number of toluene-degraders carrying *bssA* genes were observed in the depth near the contaminated plume named as "hot spot" zone. These findings support the "plume fringe concept", observed in other studies on contaminated aquifers, which states that the formation of biogeochemical gradients in a contaminated plume allow the formation of areas where microbial degradation occurs at the highest rates (Tuxen *et al*., 2006; Anneser *et al*., 2010, Jobelius *et al*., 2010).

2.5.2 Improved energy production

Current primary and secondary oil extraction technologies can only recover up to about 50% of the crude oil from existing deposits, leaving large amounts of residual oil trapped in reservoir rock pores (Ollivier and Magot, 2005). In addition, the extraction of a great majority of crude oil on the planet is limited by physical properties of the oil such as high viscosity that characterizes heavy oil. Enhanced oil recovery methods based on thermal, chemical, and gas miscible technologies have been developed to mobilize the remaining oil from mature oil fields. However, they can be highly expensive, and some require the use of large amounts of energy and water (Suflita *et al.*, 2004). Microbial products including CO_2, biosurfactants, alcohols, and fatty acids can potentially be used to alter physical properties of crude oils in the reservoirs including viscosity, interfacial tension, permeability, and porosity (Suflita and McInerney, 2008). The alteration of these properties may contribute to enhanced oil recovery. The latter strategy can be considered a tertiary oil recovery technology and is known as microbially enhanced oil recovery (MEOR). Laboratory and field studies have shown possibilities for utilizing microbial products such as gases, fatty acids, alcohols, and biosurfactants as a mean to reduce viscosity and recover oil (Youssef *et al.*, 2007; Youssef *et al.*, 2009; Town *et al.*, 2010), but the technology is not yet widely applied.

Recent work with isotopic fractionation analysis showed that crude oil reservoirs have been biodegraded to methane over geological time (Head *et al.*, 2003). Thus, the activity of microorganisms able to convert crude oil components to methane can potentially contribute to recover crude oil, particularly in reservoirs where primary and secondary extraction technologies are no longer cost-effective (marginal oil fields) (Gray *et al.*, 2010). Gieg *et al.* (2008) obtained a bacterial consortium capable of degrading a variety of hydrocarbon components to methane.

When the culture was amended with a crushed sandstone core from a marginal oil field containing residual oil, methane production was enhanced. Methanogenic rates ranging from 0.15 to 0.40 mol/day /g of core were observed with yields up to 3 mmol CH_4/g core. Gray *et al.* (2009) tested different mechanisms to stimulate methane production in microbial cultures enriched from formation waters of a high temperature oil/gas field. The addition of inorganic nutrients enhanced methane production in incubations amended with H_2/CO_2, and additional methane was seen when the incubations were amended with traces of yeast extract. Microbial conversion of residual oil to methane was also investigated in high-pressure and high temperature column systems that were filled with reservoir brine samples (Maeda *et al.*, 2009). Enhanced levels of methane and carbon dioxide were observed in the columns, and members of the family *Clostridiaceae*, as well as *Desulfotomaculum* and *Thermotoga* members dominated the columns. Also, Sugai *et al.* (2010) examined the microbial community and hydrogen production of brine samples collected from an oilfield with high CO_2 concentrations, to assess the feasibility of converting H_2 and CO_2 to CH_4. Hydrogen producing bacteria including *Thermotoga* sp and *Thermoanaerobacter* sp. and hydrogenotrophic methanogens *Methermicoccus* and *Methanobacterium* were identified in the samples. Recently, Berdugo-Clavijo and Gieg (2014) examined the conversion of crude oil to methane in sandstone-packed columns simulating residual-oil fields. An oil-degrading enrichment from oil production waters was used to inoculate the columns. Methane production was observed in inoculated columns as alkane compounds were shown to be depleted.

One of the limitations of bioconversion of crude oil to methane as potential energy source is the slow methane production rates and the availability of nutrients and water for microbial processes. *In situ* conversion of hydrocarbon to methane will most likely require the stimulation

of native communities, and/or the establishment of external microbial consortia designed to efficiently convert oil to methane in a marginal reservoir (Gray *et al.*, 2010). Investigations on microbial processes participating in the biodegradation of hydrocarbons *in situ* would help to realistically assess the feasibility of this technology (along with engineering studies). Moreover, the high natural abundance of gas in oil shale environments, and the current low price of gas in relation to conventional oil make this technology not very realistic nowadays.

2.6 Conclusions

The study of anaerobic hydrocarbon biodegradation mainly under nitrate- and sulfate-reducing conditions has contributed to the understanding of metabolic mechanisms and microbial communities involved in hydrocarbon metabolism. In more recent years, the anaerobic biodegradation of hydrocarbons in the absence of electron acceptors has gained more interest. The methanogenic biodegradation of hydrocarbons is now known to have an important role in the formation of heavy crude oil, and in the origin of methane accumulations in coal seams and petroleum reservoirs. More studies have emerged on the investigation of hydrocarbon methanogenesis to understand initial hydrocarbon activation mechanisms and subsequent downstream degradation processes. For instance, fumarate addition has been shown to be an important mechanism in the anaerobic hydrocarbon degradation, including under methanogenic conditions. Studies are also focused on understanding syntrophic processes and microbial partners, such as members of the *Firmicutes* and *Deltaproteobacteria* that have been shown to participate in the degradation of hydrocarbon components under methanogenic conditions.

This thesis work aims to investigate the methanogenic biodegradation of whole crude oil and PAH by investigating the metabolic mechanisms and microbial communities involved in this metabolic process.

Preface

Chapter 3 includes the work conducted to develop enrichment cultures able to degrade hydrocarbon components from light and heavy crude oils. In addition, this chapter includes work on metabolite analysis, functional gene analysis on light oil (*assA/bssA*), and microbial community characterization. This enrichment culture was used in sandstone-packed column studies, described in Chapter 4. Part of the work described in Chapter 3 was published in *Frontiers in Microbiology* (Berdugo-Clavijo and Gieg, 2014). The other part of the work published in this article is included in Chapter 4 of this thesis.

Chapter Three: Methanogenic biodegradation of hydrocarbon compounds from light and heavy crude oil

3.1 Introduction

In hydrocarbon-impacted subsurface environments, fuel components can be anaerobically biodegraded via a number of electron accepting processes including nitrate, iron, and sulfate reduction (Widdel *et al.*, 2010). However, when available electron acceptors are depleted in such environments, hydrocarbon biodegradation has to proceed via methanogenesis. Methanogenic hydrocarbon metabolism involves the interaction between syntrophic bacteria and methanogens. Although methanogenic oil biodegradation is a low energy yielding process (Schink, 1997), it is thermodynamically feasible when intermediate products generated by syntrophic bacteria are kept at low concentrations by methanogens (Dolfing *et al.*, 2008). The biodegradation of hydrocarbons under methanogenic conditions has been widely investigated for a variety of crude oil components such as *n*-alkanes (Zengler *et al.*, 1999; Anderson and Lovley, 2000), benzene (Grbić-Galić and Vogel, 1987; Ulrich and Edwards, 2003), toluene (Grbić-Galić and Vogel, 1987; Godsy *et al.*, 1992; Edwards and Grbić-Galić, 1994), and polycyclic aromatic hydrocarbons (Chang *et al.*, 2006; Zhang *et al.*, 2011; Berdugo-Clavijo *et al.*, 2012). Only more recently have reports emerged demonstrating the susceptibility of whole crude oil to methanogenic biodegradation (Townsend *et al.*, 2003; Jones *et al.*, 2008; Gieg *et al.*, 2010) and work is ongoing to elucidate the mechanism(s) involved in the initial activation of hydrocarbons under these conditions. Addition to fumarate as an initial hydrocarbon activation mechanism under anoxic conditions was initially demonstrated with toluene under nitrate-reducing conditions (Biegert *et al.*, 1996) and subsequently for other alkyl-substituted monoaromatic

compounds (e.g., reviewed in Foght, 2008; Widdel *et al.*, 2010). Likewise, *n*-alkanes were shown to be activated via addition to fumarate by nitrate- and sulfate-reducing bacteria (e.g., Kropp *et al.*, 2000; Rabus *et al.*, 2001; Callaghan *et al.*, 2006). Fumarate addition pathway has been shown to occur in methanogenic toluene-degrading enrichments by quantification, expression, or detection of benzylsuccinate synthase genes (Beller *et al.*, 2002; Washer and Edwards, 2007), as well as by metabolite analysis (Fowler *et al.*, 2012). Although fumarate addition genes (e.g., *ass/mas* for alkanes or *bss* for toluene) have been identified in methanogenic oil-degrading enrichments (Zhou *et al.*, 2012; Aitken *et al.*, 2013) and samples from oil-contaminated environments (Callaghan *et al.*, 2010), it is still uncertain whether this metabolic pathway occurs during methanogenic oil biodegradation as the fumarate addition metabolites have been difficult to detect (Aitken *et al.*, 2013). Other putative activation mechanisms may include carboxylation, hydroxylation, or methylation, all of which have been reported to occur under other electron-accepting conditions (e.g., reviewed in Foght, 2008; Widdel *et al.*, 2010).

The understanding of methanogenic crude oil biodegradation can contribute to a number of biotechnological applications related to bioremediation (Kazy *et al.*, 2010; Callaghan, 2013), or to assess the feasibility of energy recovery in the form of CH_4 from marginal oil reservoirs (Parkes, 1999; Gieg *et al.*, 2008; Jones *et al.*, 2008).

In this study, we describe a methanogenic crude oil-degrading culture able to grow with light and heavy oil, and detected putative hydrocarbon metabolites and genes known to be involved in hydrocarbon biodegradation via fumarate addition. In addition, the microbial community of the enrichment culture growing with light oil was characterized using pyrotag sequencing analysis.

3.2 Materials and Methods

3.2.1 Development of a crude oil-degrading enrichment culture

A methanogenic enrichment culture was initially developed from a mixture of production waters of a low temperature reservoir that has been subject to nitrate injection for souring treatment (Agrawal *et al.*, 2012). The production waters were initially enriched with 0.5 -1 mM of phosphate and 5% (by volume) of light crude oil. Following the detection of methane, a secondary enrichment culture was developed by transferring 20 mL of the original production waters into 20 mL of bicarbonate-buffered minimal salt medium (Figure A-1) prepared under strictly anoxic conditions, and containing rezasurin as a redox indicator and cysteine-HCl-sodium sulfide (2.5% solution) as a reducing agent. Triplicate incubations were prepared and amended with 0.5 mL of light crude oil. This culture was incubated for 500 days at 30℃. Finally, a tertiary transfer was prepared by adding 5 mL of the oil-amended enrichment culture into 50 mL of sterile anoxic medium with an atmosphere of N_2/CO_2 (90/10). Incubations were amended with an over layer of 0.5 mL of light crude oil (°API = 37) or with ~0.2 mL of heavy crude oil (°API = 16). In addition, inoculated controls without crude oil were prepared in parallel to account for any background production of methane. Incubations were prepared in triplicate for each tested condition. All enrichments were incubated in the dark at 30°C for approximately 48 weeks.

3.2.2 Methane measurements

Methane production from the oil-amended enrichments was monitored over time by injecting 0.2 mL of an incubation head space into a HP model 5890 gas chromatograph (GC) equipped with a flame ionization detector held at 200°C. Injections were done at 150°C onto a

packed stainless steel column (6 ft. x 1/8 in., Poropak R, 80/100, Supelco) held isothermally at 100°C. Methane amounts were determined with calibration curves prepared from five standards containing known methane concentrations that were analyzed every time methane was measured in the samples.

3.2.3 Metabolite analysis

Supernatants (40 mL) from the crude-oil degrading enrichments and controls were subsampled when substantial amounts of methane were produced. Samples were acidified with HCl (pH = 2), extracted with 3 volumes of ethylacetate that were dried over anhydrous sodium sulfate, and were initially concentrated by rotary evaporation at 60°C. Samples were further concentrated under a stream of N_2 to a volume of 100 µL then silylated with 100 µL of *N, O*-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) (Thermo Scientific, Waltham, MA) for 20 minutes at 60°C. All samples were prepared to the exact same volume (200 µL). Sample components were separated and identified using an Agilent 7890A gas chromatograph equipped with an HP-5MS column (50 m × 0.25 mm × 0.25 µm; Agilent) and an Agilent 5975C mass selective detector. The oven temperature was held at 45°C for 5 min, then increased at a rate of 4°C/min to 270°C, then held at this final temperature for 5 min. The injector, operated in split mode (50:1) was held at 270°C. Putative hydrocarbon metabolites were identified through mass spectral analysis and comparisons to literature reports, or confirmed by matching GC retention times and MS profiles with commercially available authentic standards.

3.2.4 DNA isolation from light oil-degrading culture

A liquid sample (5 mL) was removed from the crude oil degrading culture and was pelleted by centrifugation at 17 000 × *g* for 5 min. Genomic DNA was extracted from the cell pellet using a commercially available kit (FastDNA Spin Kit for Soil; MP Biomedicals). DNA was quantified using a Qubit Fluorometer (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions.

3.2.5 Identification of putative bssA and assA sequences

Genomic DNA from the light oil degrading enrichment was used to test for the presence of fumarate addition genes involved in the initial degradation of alkanes (*assA/masD*) and monoaromatic (*bssA*) hydrocarbons. PCR amplification for the *assA/bssA* genes was performed using nine published primers sets from Callaghan *et al.* (2010) (Table 3-1). The PCR reactions (50 µL) were conducted with the following temperature program: 95℃ for 3 min, followed by 40 cycles of denaturing step at 95℃ for 45 sec, primer annealing at 55℃ for 1 min, 72 ℃ for 2 min and a final extension at 72℃ for 10 min. Amplified products were cloned and transformed into *E.coli* plasmid using the TOPO TA cloning kit (Invitrogen) following the manufacturer's protocol. Positive transformants were incubated overnight in LB medium (5 mL). Plasmids were isolated using a QIAprep Miniprep kit (Qiagen), and sent for sequencing (Eurofins).

The *ass/bssA* sequences from the light oil degrading culture were screened and confirmed against the NCBI database using BLASTN. Selected sequences from this study were aligned with other relevant sequences (retrieved from NCBI) from other hydrocarbon degrading cultures using MuscleV3.3 (Edgar, 2004). Aligned sequences (~750 bp) were used to construct a maximum likelihood phylogenetic tree using MEGA6 (Tamura *et al.*, 2013). The tree was

conducted with a bootstrap phylogeny test (100 replications), and Kimura2-parameter model with a uniform substitution rate.

Table 3-1. Primer sets (from Callaghan *et al.*, 2010) that were used to target *assA* and *bssA* genes in the light crude oil degrading culture.

Primer set	Forward (F) and Reverse (R) primer name	Sequence (5'-3')	Expected size (bp)
1	*assA/bssA* F *assA/bss* R	TTTGAGTGCATCCGCCAYGGICT TCGTCRTTGCCCCATTTIGGIGC	assA:661 bssA: 682
2	*bssA* 1213F bssA1987R	GACATGACCGAYGCCATYCT TCRTCGTCRTTGCCCCAYTT	793
3	*bssA* 1294F *bssA* 1936R	TTSGARTGCATCCGNCACGGN TCRTCATTNCCCCAYTTNGG	661
4	*assA* 1294F *assA* 2457R	TTSGARTGCATCCGNCACGGN TTGTCCTGNCTYTTGCGG	1180
5	*assA* 1294F *assA* 1936R	TTYGAGTGYATNCGCCASGGC TCRTCATTNCCCCAYTTNGG	661
6	*assA* 1294F *assA* 2457R	TTYGAGTGYATNCGCCASGGC TTGTCCTGNGTYTTGCGG	1180
7	*assA* 1432F *assA* 1936R	CCNACCACNAAGCAYGG TCRTCATTNCCCCAYTTNGG	523
8	*assA* 1432F *assA* 2457R	CCNACCACNAAGCAYGG TTGTCCTGNGTYTTGCGG	1042
9	*assA1432F* *assA/bss* R	CCNACCACNAAGCAYGG TCGTCRTTGCCCCATTTIGGIGC	523

3.2.6 Microbial community analysis

Genomic DNA from the light oil degrading culture was amplified using a two round PCR method with universal microbial primers 926F and 1392R (Ramos-Padrón *et al.*, 2010), and barcoded FLX titanium amplicon primers 454T-RA and 454T-FB (Berdugo-Clavijo *et al.*, 2012). PCR products were purified, quantified with a Qubit Fluorometer (Invitrogen, Carlsbad, USA), and sent to Genome Quebec and McGill University Innovation Centre for pyrotag sequencing analysis with Roche GS-FLX Titanium technology. 16S rRNA sequencing results were analyzed with a locally installed Phoenix 2 pipeline (Soh *et al.*, 2013). For this study, taxonomic

annotation was done with RDP classifier on the Silva small subunit rRNA database using a 5% cutoff clustering distance.

3.3 Results

3.3.1 Methanogenic microbial activity and hydrocarbon metabolites

Two microbial cultures able to utilize hydrocarbon components from light or heavy crude oil were established from production waters of a low temperature heavy oil-containing reservoir. After 48 weeks of incubation, enhanced levels of methane were observed in the methanogenic enrichments amended with light and heavy crude oil relative to oil-free controls (Figure 3-1). Enrichments amended with light oil produced higher amounts of methane (up to 850 μmol) than enrichments amended with heavy oil (up to 310 μmol) and relative to the oil-free controls. The average methane production rate for the light oil amended incubations was 6.2 μmol CH_4/g of oil/day, while that for the heavy oil amended incubations was 4.6 μmol CH_4/g of oil/day. The loss of crude oil was not measured in the oil-amended incubations because the oil over-layer was too thin for sampling over time or it would have required sacrificing the cultures.

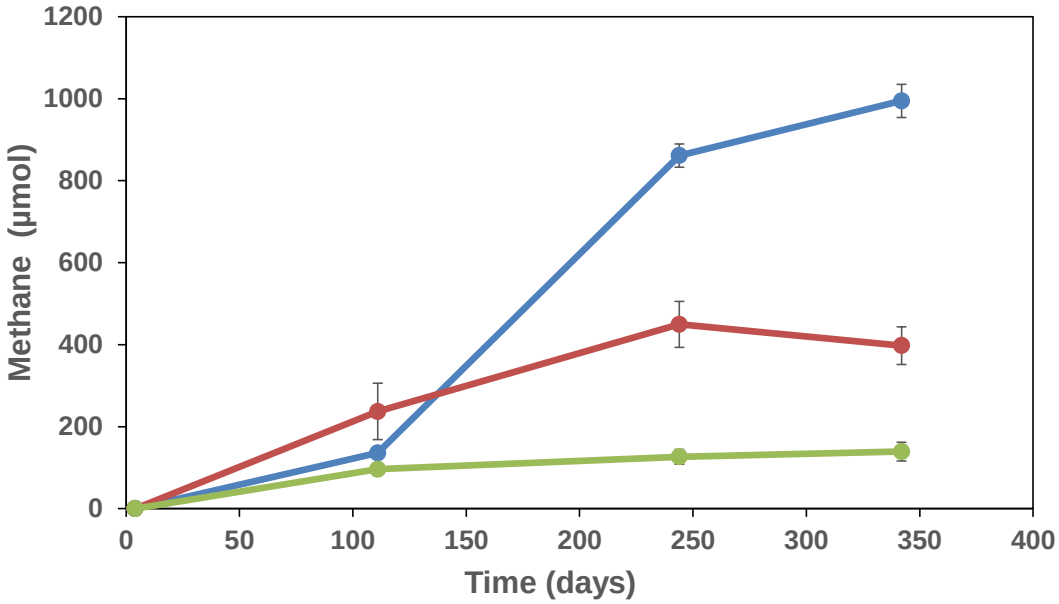

Figure 3-1. Methane production in incubations with light (blue) or heavy (red) crude oil, enriched from production waters, relative to oil-free controls (green). Error bars represent a standard error of the mean of triplicate incubations.

Putative metabolites known to be formed during the anaerobic biodegradation of hydrocarbon compounds were searched in the enrichment cultures amended with light and heavy oil, and in the oil-free controls after incubation for 342 days. Various silylated compounds with characteristic mass spectra of hydrocarbon metabolites were identified in the enrichments by GC-MS analysis (Table 3-2). These compounds were either not present or were in higher abundances than in the oil-free control incubations. Hydrocarbon metabolites detected in the light and heavy oil enrichments included benzoate (m/z 194, M^+-15) and cyclohexane carboxylate (m/z 185, M^+-15), known metabolites of anaerobic aromatic compound degradation. Other metabolites detected in both enrichments had mass profiles corresponding to toluic acids (m/z 208, M^+-15) and carboxybenzylaldehyde (m/z 222, M^+-15), suggesting the degradation of monoaromatic hydrocarbons. Benzylsuccinate, a fumarate addition metabolite formed from the degradation of toluene was not detected in any of the extracted samples. Metabolites associated with the degradation of PAH such as carboxy-PAHs, (e.g., Berdugo-Clavijo *et al.*, 2012) were also not found in the cultures. In contrast, peaks with MS fragment ions diagnostic of silylated fumarate addition metabolites of *n*-alkanes (e.g., alkylsuccinates) were detected in the extracts amended with light and heavy oil (Table 3-2). Metabolites detected in the light oil-amended enrichment culture included methylsuccinate (m/z 276, M^+-15), pentylsuccinate (m/z 317, M^+-15) (Figure 3-2B), and hexylsuccinate (m/z 331, M^+-15) (Figure 3-2C). Other tentatively identified metabolites included heptyl (m/z 345, M^+-15) and octyl (m/z 359, M^+-15) succinates (based on published MS profiles; Gieg and Suflita, 2002). A MS profile of a hexylsuccinate was also identified in the enrichment culture amended with heavy oil. Although these alkylsuccinate peaks were present at low abundance, they were not detected in sterile controls or were found above the levels of the oil-free controls (Figure 3-2A). It is possible that low levels of some of

the metabolites were detected in the oil-free controls due to the presence of small amounts of crude oil that were transferred from the oil-amended enrichment during the inoculation of the oil-free controls.

Table 3-2. Metabolites detected in methanogenic enrichment cultures amended with light and heavy crude oil. The characteristic mass spectra (m/z) for the identified compounds is also shown. M represents the molecular mass of the compound and bold numbers indicate the M^+-15 fragment ion and distinctive TMS fragment.

Tentatively-identified metabolite	Characteristic m/z	Enrichment cultures	
		Light oil	Heavy oil
Benzoate	194 (M), **179**, 135, 105, 77, 51, 45	X	X
Cyclohexanecarboxylate	200 (M), **185**, 145, 117, 73	X	X
Glutarate	276 (M), **261**, 233, 204, 158, 147, 73	X	
Dimethylbenzoate	222 (M), **207**, 163, 133, 105	X	X
Toluic acid	208 (M), **193**, 149, 119, 91, 65	X	X
Carboxybenzaldehyde	222 (M), **207**, 193, 179, 163, 133, 105, 77		
C1-alkylsuccinate	276 (M), **261**, 217, 172, 147, 73	X	
C5-alkylsuccinate	332 (M), **317, 262**, 217, 172, 147, 73	X	
C6-alkylsuccinate	346 (M), **331, 262**, 217, 172, 147, 73	X	X
C7-alkylsuccinate	360 (M), **345, 262**, 217, 172, 147, 73	X	
C8-alkylsuccinate	374 (M), **359, 262**, 217, 172, 147, 73	X	

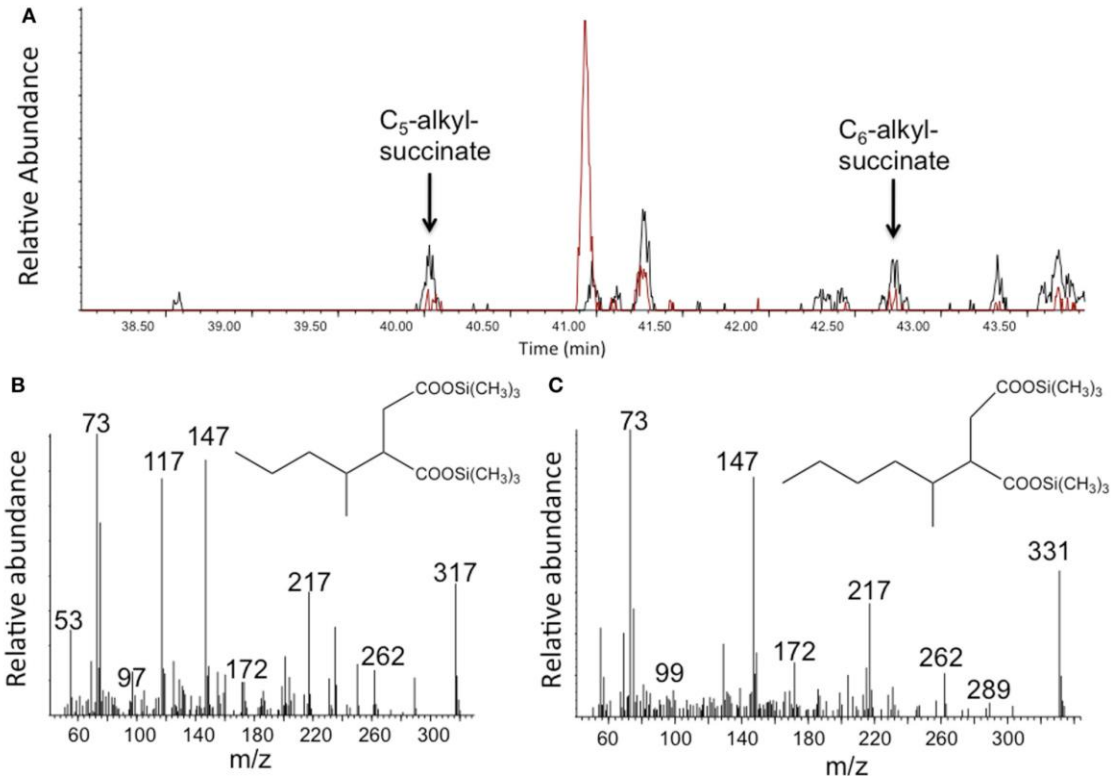

Figure 3-2. Detection of putative alkylsuccinates in methanogenic light crude oil-amended incubations. (A) A portion of a GC total ion chromatogram showing larger peaks detected in oil-amended culture extracts (black) relative to oil-free controls (red) whose mass spectral profiles are indicative of (B) pentylsuccinate and (C) hexylsuccinate. Metabolites were detected as their trimethylsilylated derivatives.

In accordance with the metabolite results, genes encoding for the enzymes involved in anaerobic degradation of alkanes via fumarate addition, alkylsuccinate synthase (*assA/masD*) were found in the enrichment culture amended with light oil. Although the compound benzylsuccinate was not detected in the culture, a benzylsuccinate synthase coding gene (*bssA*) was identified. Using primer sets 2 and 7 from Callaghan *et al.* (2010) (Table 3-1), we obtained a single fragment (771 bp) that showed homology (86%) to known benzyl succinate synthase subunit A sequences, and another fragment (501 bp) that also showed homology (92%) to known alkylsuccinate synthase gene sequences (Figure A-2). Amplified PCR products were cloned and confirmed by sequencing. The *assA* clones recovered from this study (designated as assA-OILEN) aligned closely with *assA* sequences from other crude oil degrading cultures (Figure 3-3). For example, assA-OILEN1 was clustered with an *assA* gene from a different methanogenic alkane degrading consortium (Zhou *et al.*, 2012). The assA-OILEN2 clone was closely related to an *assA* gene from a crude oil degrading SRB enrichment (Sherry *et al.*, 2013). Also, the latter clone obtained from our enrichment culture was closely aligned with an *assA* sequence from *Smithella* sp. (Tan *et al.*, 2014) isolated from produced waters of the same oil reservoir from where our culture was enriched. Interestingly, *Smithella* was the most dominant genus in our light oil degrading culture (Figure 3-4), suggesting this organism may play an important role in the degradation of alkanes in the culture. In addition, the *bssA* clones retrieved from the enrichment culture in this study were related to benzylsuccinate clones previously published from methanogenic cultures able to degrade toluene (Washer and Edwards, 2007) and crude oil components (Tan *et al.*, 2013) (Figure 3-3). Other *bss* sequences from microbial cultures growing under other electron accepting conditions (e.g., with NO_3^-, SO_4^{2-}, or Fe (III)) were

clustered together and were more distantly related to the sequences obtained from the methanogenic oil enrichment cultures in this study (Figure 3-3).

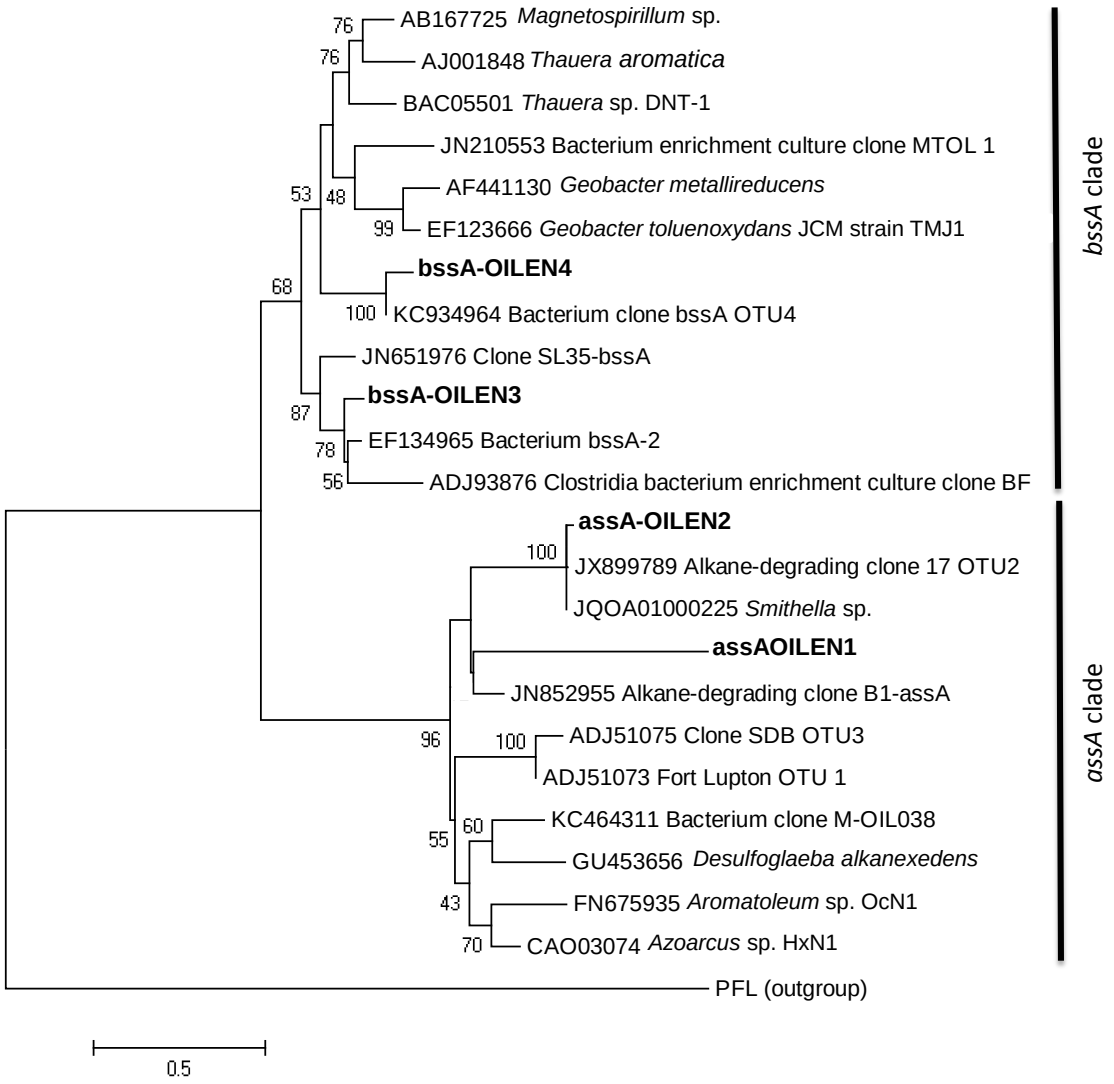

Figure 3-3. Phylogenetic tree of *ass/bssA* sequences recovered from a light oil degrading enrichment culture (bold), and other published *ass/bssA* sequences. A maximum likelihood phylogenetic tree was constructed with aligned sequences (~750 bp) using 100 bootstrap replicates. Only bootstraps values greater than 40% are shown at the nodes. Pyruvate formate lyase (PFL) from *Clostridium beijerinckii* (CP000721) was used to root the tree. The scale bar represents 0.5 change per nucleotide position.

3.3.2 Microbial community of the light oil-degrading culture

The microbial community composition of the light oil-degrading enrichment was assessed by pyrotag sequencing analysis of 16S rRNA genes. At the phylum level, the community of the enrichment culture was dominated by members of the phyla *Euryarchaeota, Spirochaetes, Firmicutes,* and *Proteobacteria* (Table A-1). Other phyla detected at lower relative abundances included *Bacteroidetes, Thermotogae, Chloroflexi* and *Synergistetes*. At the genus level, the microbial community of the enrichment culture was dominated by bacterial members affiliated with *Smithella*, while most dominant archaeal members were affiliated with *Methanosaeta* and *Methanoculleus* (Figure 3-4). Bacterial genera detected in lower proportion in the enrichment included *Desulfotobacterium, Kosmotoga, Thermanaerovibrio,* and *Sedimentibacter* (Figure 3-4A). *Methanocalculus* and *Methanolinea* were also found in lower relative abundances in the enrichment culture (Figure 3-4).

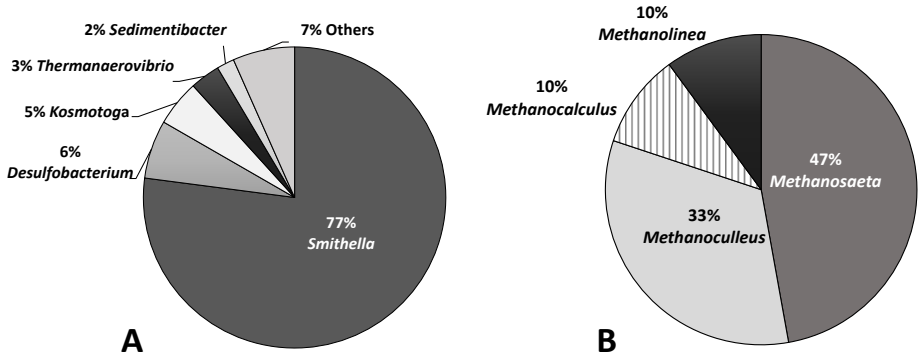

Figure 3-4. Distribution of bacterial (A) and archaeal (B) sequences obtained from pyrotag sequencing analysis of 16S rRNA genes in the light oil-degrading enrichment. Taxa identified at the genus level are shown.

3.4 Discussion

In this work, methanogenic communities enriched from production waters of a low-temperature oil reservoir were found to be capable of utilizing hydrocarbon components present in light and heavy crude oil. Although the demonstration of hydrocarbon-degrading oilfield-derived consortia remained elusive for many years, this finding adds to the handful of reports that now exist showing that microbes enriched from oilfield fluids can utilize hydrocarbons under methanogenic conditions (Gieg *et al.*, 2010; Mbadinga *et al.*, 2011; Zhou *et al.*, 2012; Cheng *et al.*, 2013).

The detection of putative anaerobic hydrocarbon metabolites at higher levels in the oil-amended than in the oil-free controls provides further evidence that the consortium metabolizes crude oil components. The detection of alkylsuccinates (Figure 3-2), along with alkyl- and benzylsuccinate synthase genes (Figure 3-3), suggests that addition to fumarate is at least one mechanism being used by a member(s) of the enrichment culture to activate hydrocarbons.

Fumarate addition to *n*-alkanes has been proposed to occur in methanogenic microbial enrichments (Callaghan *et al.*, 2010; Zhou *et al.*, 2012) and in oil-contaminated environments (Callaghan *et al.*, 2010) where fumarate addition genes (*assA*) have been detected. However, detecting the products in laboratory cultures has proven difficult (Gieg *et al.*, 2010; Zhou *et al.*, 2012). Using a combined approach of metabolite analysis and qPCR analysis of the *assA* gene in a different methanogenic crude oil-degrading enrichment culture, Aitken *et al.* (2013) did not detect alkylsuccinates nor increases in copy numbers of the *assA* gene at significant levels above controls even though *n*-alkanes were metabolized over time. These results suggested that an alternate mechanism for activation of alkanes was occurring by members of this methanogenic consortium. In contrast, Tan *et al.* (2013) were able to detect fumarate addition metabolites from

branched alkanes (but not from *n*-alkanes) in a short chain alkane-degrading microbial culture enriched from oil sands tailings ponds, which corresponded to the identification of *assA* in the metagenome of the consortium. These differing results demonstrate that the activation of hydrocarbons under methanogenic conditions remains uncertain, and that multiple (unidentified) mechanisms may occur, likely depending on the microbial/genetic composition of the consortium under study. In the present culture, alkylsuccinates were only assessed at one time point, thus additional work to determine whether these form transiently during methanogenic crude oil degradation is a goal of future work.

The microbial characterization of the oil degrading enrichment culture described here revealed that bacterial members of the genus *Smithella* dominated (Figure 3-4). The genome sequence for *Smithella* was recently obtained (Tan *et al*., 2014) from produced waters of an oil field from Southern Alberta (Voordouw *et al.*, 2009). The *Smithella* genome revealed the presence of alkylsuccinate synthase genes involved in alkane metabolism (Tan *et al.*, 2014). Interestingly, an *assA* sequence recovered from our light oil-degrading culture that was enriched from produced waters of the same oil field showed high sequence identity to one *assA* sequence from this *Smithella* sp. sequenced in Tan *et al.* (2014). This observation suggests that in our crude oil-degrading enrichment, an organism affiliated with *Smithella* may be involved in the degradation of alkanes. *Smithella* is a syntrophic member of the *Deltaproteobacteria* shown to be prevalent in several methanogenic oil-degrading cultures or environments (Gray *et al.*, 2010). Gray *et al.* (2011) observed increased numbers of 16S rRNA genes from members of the genera *Smithella* and *Syntrophus* during methanogenic degradation of alkanes. In a separate culture, a member of the *Syntrophaceae* closely related to *Smithella propionicus* was identified by DNA-SIP analysis as a key player in the methanogenic degradation of hexadecane (Cheng *et al.*, 2013).

Other organisms detected in lower abundance in our enrichment culture were *Sedimentibacter* and *Desulfotomaculum* species. Members of these genera have been detected in other hydrocarbon-degrading enrichments, and have been proposed to act as either primary or secondary syntrophs in such consortia (Kleinsteuber *et al.*, 2012). In addition, sequencing analysis revealed that acetate- and H_2-utilizing methanogens in the enrichment culture are found in similar proportions. To date, there is no clear consensus regarding the predominant route (via acetotrophic or hydrogenotrophic methanogenesis) involved in methanogenic hydrocarbon biodegradation since both mechanisms have been observed in hydrocarbon studies (Gieg *et al.*, 2008; Jones *et al.*, 2008). However, further experiments are required to assess the specific roles that these abundant organisms play in the anaerobic degradation of crude oil components.

Overall, the results of the present study demonstrate that the methanogenic microbial consortium enriched from oilfield production waters can convert hydrocarbons from light or heavy oil to methane. Also, metabolite and marker gene analysis indicate that the activation of *n*-alkanes and monoaromatic hydrocarbons in the enrichment culture amended with light oil can occur via a fumarate addition mechanism. Further, the community of the oil-degrading enrichment culture was dominated by bacterial members of the genus *Smithella,* previously identified as alkane-degrading bacterium under methanogenic conditions. Pyrotag analysis also revealed that both hydrogen and acetate-utilizing methanogens were present in the enrichment culture.

Preface

Chapter 4 presents experiments conducted with sandstone-packed column systems including a set of columns inoculated with a light oil enrichment culture which is introduced in Chapter 3. This chapter also covers part of the work published in *Frontiers in Microbiology* (Berdugo-Clavijo and Gieg, 2014), and contains unpublished work conducted with three other different microbial inocula to assess crude oil bioconversion to CH_4 in columns simulating marginal oil fields, as a continuation of the work showed in Chapter 3. This work includes hydrocarbon analyses conducted in columns showing the highest levels of CH_4, and a comparison between the microbial community of the initial inoculum and the community that was enriched after incubation in the sandstone-packed columns.

Chapter Four: Methanogenic biodegradation of hydrocarbons in marginal reservoir-simulating columns

4.1 Introduction

The ability of microorganisms to metabolize crude oil hydrocarbons to methane has been reported for several microbial enrichments and in oil-associated samples (Townsend *et al.*, 2003; Jones *et al.*, 2008; Gieg *et al.*, 2010; Mayumi *et al.*, 2011; Aitken *et al.*, 2013). Further, isotopic analysis also showed that hydrocarbon methanogenesis takes place *in situ* in biodegrading oil reservoirs (Larter and di Primio, 2005; Jones *et al.*, 2008). Thus, it has been suggested that in marginal oil fields, the residual oil that remains after conventional recovery technologies (up to 50%) can be microbiologically converted to methane gas, and be recovered as an energy source (Head *et al.*, 2003; Gieg *et al.*, 2008; Jones *et al.*, 2008; Gray *et al.*, 2009). In addition, the production of CH_4 from residual oil degradation can potentially be used to mobilize heavy oil by pressurizing the reservoir and decreasing its viscosity (Grigoryan and Voordouw, 2008; Gray *et al.*, 2009). However, before this technology becomes a reality, there are challenges concerning the engineering of the oil reservoirs, and it is necessary to understand the methanogenic microbial activities and communities that can survive and actively metabolize oil in the subsurface (Gray *et al.*, 2010). Microbial communities present in oil reservoirs likely need to be stimulated with nutrients to accelerate the slow methanogenesis process, or new microbial amendments able to survive reservoir conditions need to be established and added to reservoirs (Gray *et al.*, 2010). Gieg *et al.* (2008) enriched a methanogenic crude oil degrading culture from sediments of a gas condensate contaminated aquifer. This culture was able to enhance methane production from sandstone core containing residual oil sampled from a marginal oil field relative to controls without the inoculum. Also, Gray *et al.* (2009) showed the stimulation of a

methanogenic community from oil reservoir formation waters by the addition of inorganic nutrients and yeast extract. Methane production was also investigated in high temperature and high pressure bioreactors amended with liquid petroleum brine (Maeda *et al.*, 2009; Sugai *et al.*, 2010). However, the microbial conversion of oil to methane, and the methanogenic communities able to utilize these hydrocarbons have not been well studied in sediment environments mimicking actual oil reservoir rocks that are typically comprised of porous, permeable sediments such as limestone and sandstone (Larter and di Primio, 2005).

This study aimed to test the feasibility of various microbial inocula to grow and produce methane from residual oil trapped in sandstone-packed columns, simulating marginal oil reservoirs. Four different microbial inocula were incubated in the column systems for up to 400 days and they included; 1) a crude oil enrichment culture originally established by Gieg *et al.* (2008) able to degrade crude oil components to methane, 2) produced waters from a low temperature reservoir, 3) an enrichment culture that was originated from produced waters of a low temperature oil field (Chapter 3), and 4) produced waters from a high temperature oil field. Here we report methane production, and the loss of saturate and aromatic hydrocarbons in the columns after 400 days of incubation. In addition, we characterize the microbial community for all three inocula before inoculation into the column and after the microbial inocula were incubated in the sandstone-packed columns.

4.2 Material and methods

4.2.1 Enrichment cultures and microbial inocula

Four different microbial inocula were used for the column experiments in this study. The first was a methanogenic oil-degrading culture that was able to metabolize alkanes from crude oil

into methane (Gieg *et al.*, 2008). This culture previously produced up to 500 µmol of CH_4 when incubated with 0.2 mg of a light oil, and it is referred to in this chapter as RESOIL. The second source of microbial inoculum was a mixture of unenriched produced water samples (PW4 and PW10), obtained from a shallow low temperature (30℃) reservoir near Medicine Hat, Alberta (Voordouw *et al.*, 2009). The microbial communities from these waters were previously found to contain methanogens (Dr. Akhil Agrawal, unpublished). This inoculum is described in this study as LOWT. The third inoculum, referred to as LOWT-EN, was a methanogenic enrichment culture established from produced waters also from the Medicine Hat oil reservoir that was able to degrade light and heavy oil components (described in Chapter 3). The last source of microbial inoculum was obtained from a mixture of produced waters sampled from a high-temperature (80℃) reservoir in Argentina (Agrawal *et al.*, 2014) and its methanogenic potential was unknown at the time of the experiment. This inoculum is described here as HIGHT.

4.2.2 Column set-up

Sandstone-packed columns systems simulating mature oil fields were established to test the ability of microbial inocula to biodegrade crude oil to methane. Sterile glass syringes (30 mL) were used as columns, fitted with a layer of polymeric mesh at the bottom, and packed with 40 g of crushed Berea sandstone core (that was sieved to < 250 µm grain size). The tops of the columns were sealed with rubber stoppers that were modified to incorporate an effluent port (Figure 4-1). Residual oil conditions were simulated by first injecting anoxic mineral salts medium (McInerney *et al.*, 1979) through the columns, then flooding the columns with light crude oil (°API 37) until saturation was obtained. The columns amended with HIGHT received crude oil (°API 25) from the same reservoir of the inoculum (Agrawal *et al.*, 2014). Columns sat

for 5 days to allow the crude oil to be attached to the sandstone, and then anoxic sterile medium was injected again into the columns to displace the non-attached crude oil. Residual oil-simulating columns were considered ready when the water and oil volume ratio reached equilibrium (e.g., measured from the volume of oil and water). A mineral salts medium was used (McInerney *et al.*, 1979) to provide N, P, and other essential nutrients (Figure A-1), so that they were not limiting the hydrocarbon biodegradation processes; such biostimulation would likely be required for a field application to enhance the recovery of gas from oil in marginal reservoirs (Gray *et al.*, 2010). All injections were done with Tygon tubing connected to the bottom of the column and using a multichannel peristaltic pump (Minipuls 3 Gilson) at a flow rate of 27 mL/h. Between 40 to 50% of the crude oil initially injected remained trapped in the columns. The average porosity of the columns was 32% ± 5 (n=6) and the estimated permeability was 1100 mD. Columns were inoculated with 4 mL of either RESOIL, LOWT, LOW-EN, or HIGHT by first injecting 2 mL of the inoculum followed by 5 mL of sterile anoxic mineral medium (injected at 5.3 mL/h) to push the microbial culture through the column, and then repeating the injection again with 2 mL inoculum and 3 mL of medium. These volumes were added to help ensure that the inoculum was distributed throughout the pore volumes of the columns. Column conditioning and inoculation were conducted inside an anaerobic glove bag (90:10, N_2/CO_2). Triplicate columns were made for each tested inoculum. Also, two controls were prepared in parallel, one without crude oil, and another one without inoculum. A serum bottle flushed with argon gas was attached at the top of the column to collect methane gas over time (Figure 4-1). All columns were shut-in and incubated at 22℃ inside an anaerobic glove bag filled with a N_2/CO_2 (90/10) atmosphere for up to 400 days. Cumulative methane in the columns was

monitored over time by sampling 0.2 mL of headspace from the attached serum bottle, and injecting it into a GC-FID as described in Chapter 3.

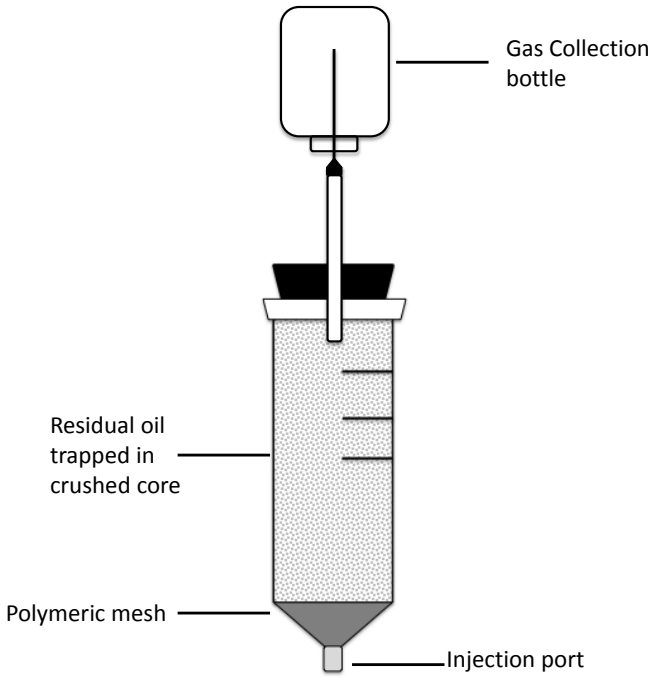

Figure 4-1. Diagram of a sandstone-packed column used in this study to simulate a marginal oil reservoir.

4.2.3 Oil analysis

After the incubation period (~400 days), sandstone-packed columns that were actively producing methane over time relative to the oil-unamended controls were then taken out of the anaerobic hood and aseptically opened for DNA and oil analysis. Subsamples were removed for hydrocarbon analysis. Crude oil hydrocarbons entrained in the sandstone were recovered with a Soxhlet extractor method by running methylene chloride continuously through 10 g of sand for 8 hours. About 0.8 g of crude oil were extracted, and a portion of the oil (0.2 g) was used for saturate and aromatic component separation. Extracted oil was passed through a silica column

developed with 10 mL of 100% pentane, 20 mL of 20% methylene chloride in pentane, and 25 mL of 50% methylene chloride in pentane, to allow for the separation of saturate and aromatic fractions (Fedorak and Westlake, 1981). Squalene and *p*-terphenyl (0.48 μmol) were added as internal standards during the extraction of the saturate and aromatic fractions, respectively. Extracted fractions were collected in separate tubes, completely dried under nitrogen, and diluted in 1 mL of methylene chloride. The extracted samples (1 μL) were analyzed using an Agilent 7890A GC-FID equipped with an HP-5 capillary column (30 m × 0.32 mm × 0.25 μm; Agilent) and a FID detector held at 200℃. The oven was held at 90˚C for 2 min, increased at a rate of 4˚C/min to 250˚C, and then held at this temperature for 18 min. The injector was operated in split mode (50:1) and held at 250°C. For the hydrocarbon analysis, ratios between the peak areas of selected saturate and aromatic hydrocarbons and the peak area of a squalene or *p*-terphenyl standard were calculated. Hydrocarbon loss was determined by comparing the area ratios of the oils from the inoculated columns relative to those from the uninoculated oil-containing columns. In addition, mass balance calculations for the bioconversion of hydrocarbons to methane were determined by estimating the amount (μmol) of saturate and select aromatic hydrocarbons in each replicate and control samples based on calibration curves prepared with hexadecane (saturates) and *p*-terphenyl (aromatics), assuming a similar response factor for all saturates and aromatics. The amount of hydrocarbons consumed by the cultures was determined by subtracting the amounts in the inoculated, oil-amended column from those in the oil-amended, uninoculated control. Then, the expected amount of methane for that amount of hydrocarbon consumed was estimated for each hydrocarbon, based on stoichiometric reactions determined using the Symons and Buswell (1933) equation (a).

$$C_nH_aO_b + \left(n - \frac{a}{4} - \frac{b}{2}\right) H_2O \rightarrow \left(\frac{n}{2} - \frac{a}{8} + \frac{b}{4}\right) CO_2 + \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4}\right) CH_4 \quad \text{(a)}$$

4.2.4 Protein content assay

To determine biomass, the protein content in the initial inocula (LOWT-EN, RESOIL, LOWT, and HIGHT) was determined with a colorimetric test using the DC protein assay (BioRad). From each inoculum 1 mL of liquid culture was taken and centrifuged at 17 000 × *g* for 8 minutes. The cell pellet was then resuspended with 1mL of NaOH (0.5 N), and a subsample (100 µL) was mixed with the assay reagents according to the BioRad manual instructions. Standards were prepared with dilutions of albumin (1.55 mg/mL) in NaOH. Protein content was obtained by measuring the absorbance of each reaction with a spectrophotometer at 750 nm.

4.2.5 Microbial community analysis

Genomic DNA was extracted from the initial microbial inocula used for the experiments and from the sacrified columns using a commercially available kit (FastDNA Spin Kit for Soil; MP Biomedicals). For the initial inocula (RESOIL, LOWT, LOWTEN and HIGHT) 5 mL of liquid sample were collected and pelleted by centrifugation at 17 000 × *g* for 5 min. For the columns, 500 mg of sandstone material were aseptically collected from the columns after they were cut open. DNA was amplified using a two round PCR method described elsewhere (Ramos-Padrón *et al.*, 2010) with universal microbial primers 926F and 1392R, and barcoded FLX titanium amplicon primers 454T-RA and 454T-FB (Berdugo-Clavijo and Gieg, 2014). PCR products were purified and sent to Genome Quebec and McGill University Innovation Centre for pyrotag sequencing analysis as descried in Chapter 3. 16S rRNA sequencing results were analyzed with a locally installed Phoenix 2 pipeline (Soh *et al.*, 2013). For this study, taxonomic

annotation was done with RDP classifier on the Silva small subunit rRNA database using a 5% cutoff clustering distance.

4.3 Results

4.3.1 Methanogenic activity

Enhanced levels of methane were observed in all of the inoculated and oil-amended columns relative to controls. However, due to the variability in methane production within the replicate columns that received the same inoculum (except HIGHT), mean values were not calculated. The highest methane production was measured in one column amended with LOW-EN (Table 4-1). In this column, up to 160 μmol of CH_4 were produced relative to controls after 48 days of incubation. The methane production rate for this column was 1.43 μmol CH_4/g of oil/day. Columns amended with the other inocula (RESOIL, LOWT, or HIGHT) showed lower methane production than the column inoculated with LOWT-EN (Table 4-1). Within these latter columns, the highest methane production was measured in a column amended with RESOIL (up to 14 μmol) after 99 days of incubation, and similar amounts of CH_4 (up to 13 μmol) were measured for a column amended with LOWT after a lag phase of about 207 days (Table 4-1). The columns amended with HIGHT produced only 1.24 μmol (± 0.6, n=3) of CH_4 after 400 days, suggesting the poor growth of this community (Table 4-1). The amount of biomass (e.g., protein content) in each inocula used to inoculate the columns was determined. The highest biomass was measured for the enrichment cultures RESOIL and LOWT-EN, while the samples obtained from unenriched produced waters, HIGHT and LOWT, had far lower biomass (Table 4-1).

Table 4-1. CH_4 production of columns amended with LOWT-EN, RESOIL, LOWT, and HIGHT and incubated at 22°C with residual oil (light oil) for up to 400 days. Only results from the best CH_4 producer of three replicate columns are shown. Protein content (e.g., biomass) is also shown for each initial inocula.

Inoculum	Maximum CH_4 production (µmol)[a]	CH_4 production rate (µmol/g of oil/day)	Duration of lag phase (days)[b]	Biomass of initial inoculum (µg/ml)[c]
LOWT-EN	160.0	1.430	48	30.6 ± 1.8
RESOIL	14.4	0.018	99	58.0 ± 5.2
LOWT	12.7	0.022	207	2.2 ± 0
HIGHT[d]	1.2	0.0003	400	7.6 ± 2.6

[a] Calculated relative to oil-unamended and uninoculated controls

[b] Time before onset of CH_4 production

[c] Standard deviations were determined from duplicates

[d] Columns amended with heavy oil. Calculations from three replicate samples

4.3.2 Hydrocarbon analysis

The loss of saturate and aromatic components was determined in the columns that showed the highest amount of methane relative to an uninoculated (only oil) control for the LOW-EN, RESOIL, and LOWT inocula. Hydrocarbon analysis was not conducted for the HIGHT columns as relatively low CH_4 was produced (Table 4-1). In the column inoculated with LOWT-EN, between 40 to 50 % of *n*-alkanes ranging from C_8 to C_{19} were depleted, except for C_{17} that showed only 20 % of depletion (Figure 4-2). The rest of the *n*-alkanes quantified in the column (C_{19}, C_{20}, C_{24}, C_{30}, and C_{36}) were less depleted (< 40%) (Figure 4-2). Also, oil analysis showed that PAH such as methylnaphthalene, dimethylnaphthalene, phenanthrene, and methylanthracene were also partially depleted in the inoculated replicate columns relative to the inoculum-free control (Figure 4-2). The two-ringed PAH were consumed to a greater extent than the three-ringed PAH analyzed. According to mass balance calculations of the hydrocarbons that were quantified (e.g., the *n*-alkanes, the four known PAH compounds, and other 2- or 3-ringed aromatics) approx. 11 μmol of hydrocarbons were consumed, and approximately 113 μmol of CH_4 were predicted to form in the column (Table B-1 and Table B-2). Experimentally, the column amended with LOWT-EN produced 160 μmol CH_4. The difference in methane concentrations from predicted to actual measured amounts (~ 47 μmol) is likely due to the consumption of other hydrocarbons (such as branched alkanes or PAHs with > 3 rings) that were not quantified in this study and were thus not taken into account in the mass balance calculations.

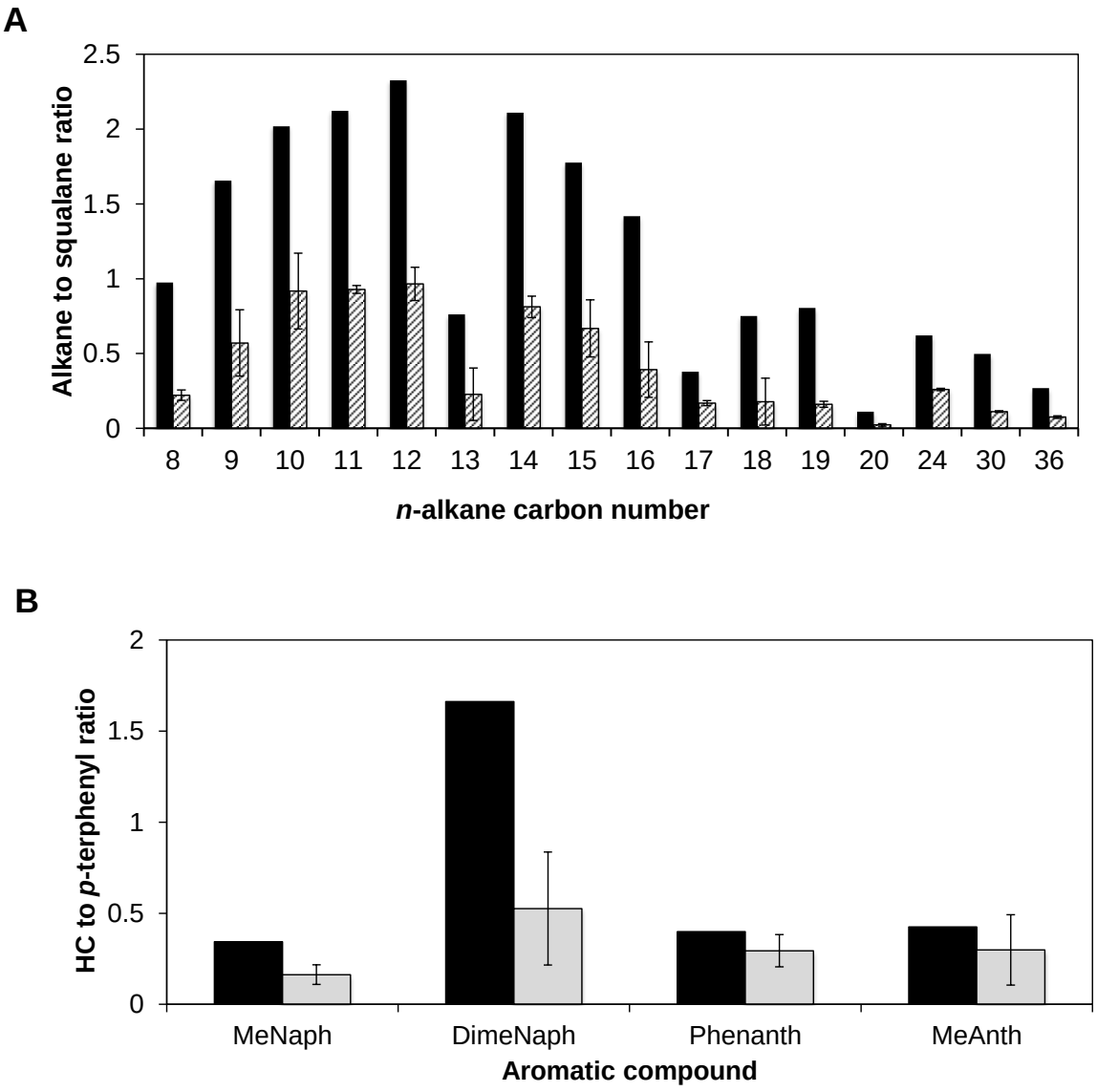

Figure 4-2. Hydrocarbon loss measured as hydrocarbon (HC) to squalane/terphenyl peak area ratios for (A) *n*-alkanes and (B) select PAH. Data shown are for columns inoculated with LOWT-EN. Striped-bars represent the results from inoculated columns, while the black bars represent the results from a control with no inoculum. Error bars represent standard deviations of duplicates. Columns were amended with light oil. MeNaph, methylnaphthalene; DimeNaph, dimethylnaphthalene; Phenanth, phenanthrene; and MeAnth, methylanthracene.

Oil analysis was also performed for a column amended with RESOIL showing the highest CH_4 production. In this column, between 6 to 40% of *n*-alkanes ranging from C_9 to C_{18} were depleted (Figure 4-3A). The highest depletion (~90%) was observed for C_8, C_{20}, C_{24}, and C_{30} alkanes (Figure 4-3A). Mass stoichiometric calculations showed that from the amount of *n*-alkanes that were depleted (~1µmol), 12 µmol of CH_4 were expected to be produced (Table B-3). For the identified aromatic components, only dimethylnaphthalene was found to be degraded, and its degradation only accounts for 0.2 µmol of CH_4. Experimentally 14 µmol of CH_4 were measured in the column. It is possible that the extra 1.8 µmol of CH_4 detected comes from other aromatic compounds that were depleted, but not identified in the analysis, or from the degradation of branched alkanes, which were not quantified.

The column amended with LOWT that exhibited the highest CH_4 production also showed depletion of *n*-alkanes relative to an oil-only control (Figure 4-3B). Up to 33% of *n*-alkanes from C_8 to C_{30} were depleted after incubation. Compared to the RESOIL column, LOWT showed a lower percentage of biodegraded compounds, but overall more *n*-alkanes were consumed (1.5 µmol) in the LOWT-inoculated column. Stoichiometric calculations, based on the *n*-alkanes that were utilized, predicted that 17 µmol of CH_4 should have been produced (Table B-4). Identified aromatic compounds such as methyl and dimethyl naphthalene were also depleted, but in much lower proportions (<13% loss). Experimentally, up to 13 µmol of CH_4 were measured in the column.

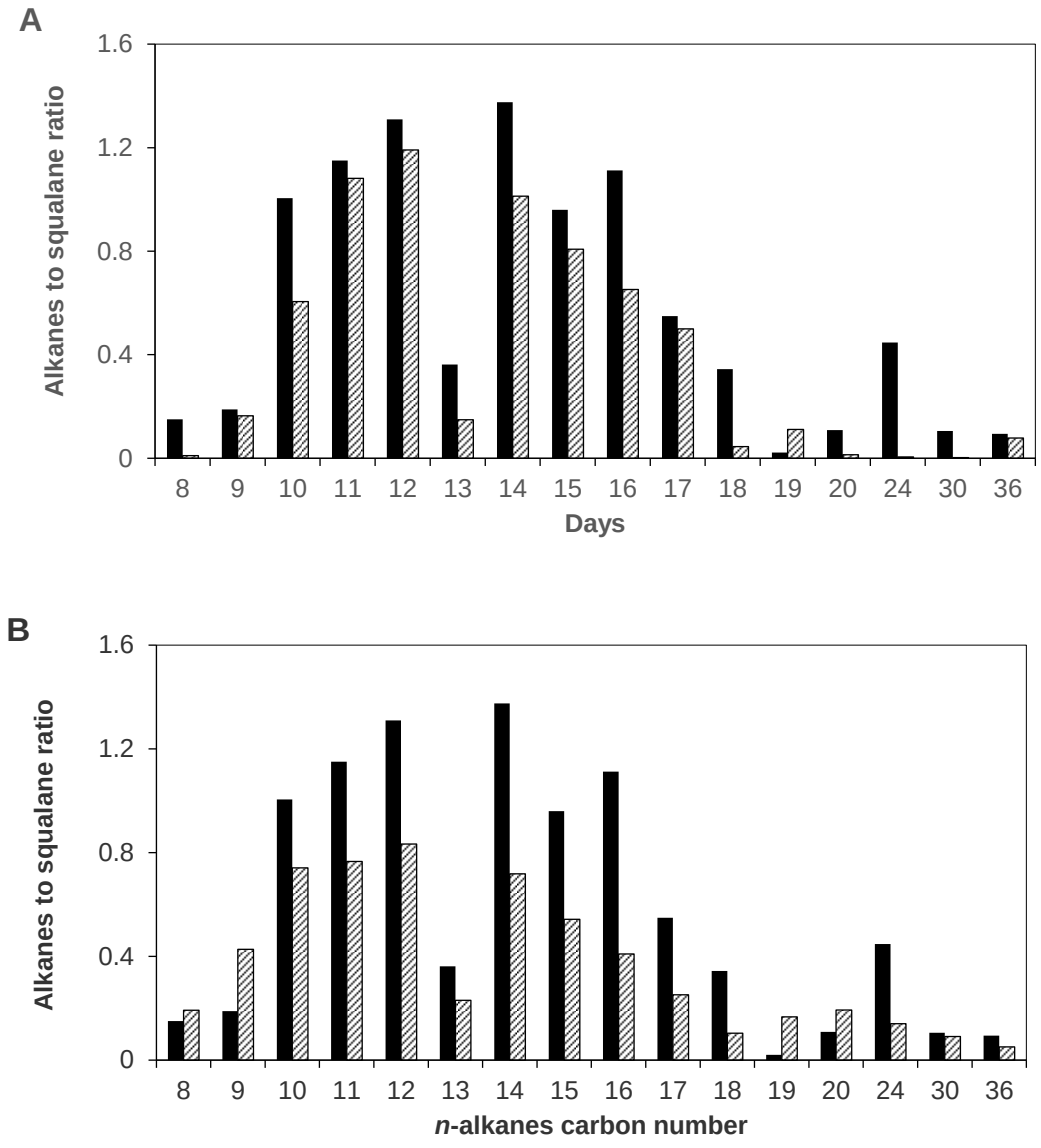

Figure 4-3. *n*-Alkane loss measured as hydrocarbon (HC) to squalane peak area ratios for columns inoculated with (A) RESOIL and (B) LOWT. Results for one technical replicate from each inoculum are shown. Columns were amended with light oil.

4.3.3 Microbial community characterization

The microbial communities of all inocula tested for their ability to produce CH_4 were assessed before and after the column experiments. The microbial communities enriched after the incubation in sand-packed columns were determined only for those columns that showed the highest CH_4 production within each inocula tested.

There was a dramatic shift in the dominant microbial community members of the LOWT-EN culture after the column experiments compared to the initial inoculum. At the phylum level, the percentage of microbial reads from the *Euryarchaeota*, mainly methanogens, increased from 25% in the enrichment culture to 61% in the column system (Figure 4-4A). Other microbial members that were enriched in the column community belonged to the *Proteobacteria* and *Actinobacteria* phyla (Figure 4-4A). In contrast, the relative abundance of *Firmicutes* decreased (~17%) after the incubation in the sand-packed columns (Figure 4-4A). At the genus level, the microbial community of the enrichment culture was dominated by members of *Smithella* (27% total reads), followed by *Methanosaeta* (25% total reads) and *Methanoculleus* (23% total reads) (Figure 4-4B). In contrast, the composition of the community following incubation in the sandstone column was dominated by the hydrogen-utilizing methanogen, *Methanobacterium* (56% of total reads), and bacterial members affiliated with *Pseudomonas* (16%) (Figure 4-4B). Other microbial members in the column community that increased in their relative abundance belonged to the genera *Eggerthella*, *Halomonas, Clostridium,* and *Desulfovibrio* (Figure 4-4B).

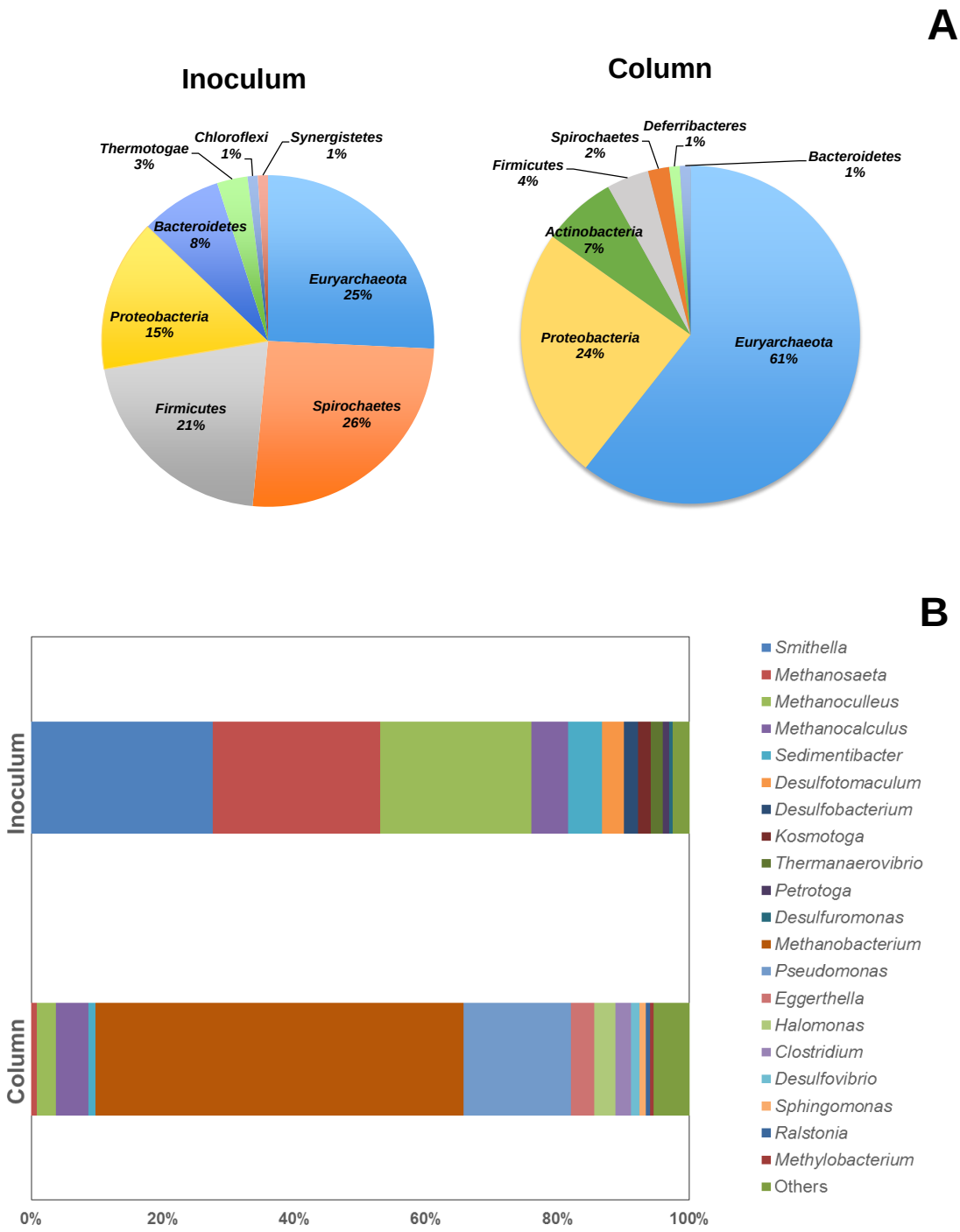

Figure 4-4. Distribution of microbial sequence reads identified at the (A) phylum and (B) genus level for LOWT-EN before ("inoculum") and after ("column") incubation in the sandstone-packed column. Only taxa with relative abundance higher than 1% are shown.

The microbial community composition of the RESOIL culture also showed a substantial change after the column experiment. Prior to the inoculation, RESOIL was dominated by members of the *Euryarcheaota*, specifically the methanogens *Methanosaeta* and *Methanolinea* (Table 4-2). The majority of bacterial members in the enrichment culture belonged to the phylum *Firmicutes,* followed by members of the *Proteobacteria* and *Chloroflexi* groups (Figure B-1). *Clostridium* species had the highest relative abundance within the *Firmicutes,* and *Smithella,* found within the *Proteobacteria* group, was the next most dominant genus. In contrast, the dominant bacterial phylum in the column community was *Proteobacteria*, with microbial members affiliated with *Halomonas* and *Pseudomonas*. *Actinobacteria* and *Bacteriodetes* also showed an increase in their relative abundance after incubation in the column (Figure B-1). Further, the relative abundance of archaeal members decreased in the culture, but the methanogens *Methanosaeta* and *Methanolinea* were still the dominant groups within the *Euryarcheaota*. Other groups that were decreased in the column community were affiliated with the *Firmicutes, Chloroflexi,* and *Spirochaetes* phyla (Figure B-1).

The microbial community of the produced water LOWT inoculum was also assessed before and after the column experiments. The initial community was dominated by members of the *Euryarchaeota,* including *Methanosarcina* and *Methanofollis* (Table 4-2). The most dominant bacterial phylum was *Proteobacteria* (Figure B-1). Within the *Proteobacteria,* members of the genus *Geobacter* had the highest relative abundance (Table 4-2). In the column community, members of the phylum *Proteobacteria* were also predominant. Within the *Proteobacteria,* an identified phylotype within the family *Rhodocyclaceae* (within the *Betaproteobacteria*) was the most abundant followed by other bacteria affiliating with *Thauera, Pseudomonas, Smithella, Brachymonas, and* members of the *Proteobacteria* phylum (Table 4-2).

The relative abundance of the archaeal members was decreased, but methanogens such as *Methanosaeta* and *Methanocalculus* were enriched after incubation in the sandstone-packed column (Table 4-2).

At the phylum level the community of the high temperature production water (HIGHT) was apparently not very different after it was incubated in the column (Figure B-1). Before and after the incubation in the column, *Proteobacteria* (94% of relative abundance) was the most dominant phylum. In the original inoculum, the majority of *Proteobacteria* members were closely related to *Desulfovibrio* and *Desulfuromonadales* (Table 4-2). Meanwhile, the column system enriched for unidentified members of *Proteobacteria,* as well as *Marinobacter, Rhodospirallaceae,* and *Rhodobacteraceae* (Table 4-2). In the community obtained after the incubation in the sandstone-packed columns, *Firmicutes* and *Spirochaetes* phyla were decreased (Figure B-1). In contrast, bacterial members affiliated to the *Bacteriodetes, Thermotogae* and *Deferribacter* increased after incubation in the column (Table 4-2). Moreover, the relative abundance of the phyla *Euryarchaeota,* specifically *Methanosarcina* that comprise all the methanogens in the community, decreased after the incubation in the sandstone-packed column.

Table 4-2. Ten most abundant OTUs determined by pyrotag sequences of the 16S rRNA genes before ("inoculum") and after ("column") incubation in sandstone-packed columns for RESOIL, LOWT, and HIGHT inocula. Percentages represent the proportion of reads associated with each taxon relative to the domain.

RESOIL

Inoculum		Column	
Microbial OTUs	**% of reads**	**Microbial OTUs**	**% of reads**
Methanosaeta	40.0	*Halomonas*	25.0
Methanolinea	26.7	*Coriobacteriaceae*	14.1
Clostridium	18.4	*Pseudomonas*	10.8
Smithella	4.4	*Proteiniphilum*	7.5
Euryarchaeota	2.3	*Methanosaeta*	6.5
Methanoregula	2.2	*Sphingomonas*	3.4
Anaerolineaceae	0.9	*Sediminibacterium*	3.1
Firmicutes	0.9	*Enterobacter*	3.0
Enterobacter	0.5	*Clostridium*	2.9
Bacteroidetes	0.4	*Proteobacteria*	2.7

LOWT

Inoculum		Column	
Microbial OTUs	**% of reads**	**Microbial OTUs**	**% of reads**
Methanosarcina	23.9	*Rhodocyclaceae*	37.8
Methanofollis	16.8	*Methanosaeta*	7.6
Geobacter	7.3	*Methanocalculus*	6.9
Spirochaetes	6.6	*Thauera*	6.5
Methanolinea	4.4	*Proteobacteria*	6.0
Acholeplasma	4.3	*Brachymonas*	4.0
Thermanaerovibrio	3.8	*Pseudomonas*	3.0
Chloroflexi	3.5	*Smithella*	2.9
Deferribacteraceae	2.9	*Deferribacteraceae*	2.8
Halomonas	2.3	*Rhizobium*	2.1

HIGHT

Inoculum		Column	
Microbial OTUs	**% of reads**	**Microbial OTUs**	**% of reads**
Desulfovibrio	40.8	*Proteobacteria*	33.2
Desulfuromonadales	40.7	*Proteobacteria*	13.5
Marinobacter	4.2	*Marinobacter*	13.4
Marinobacterium	3.8	*Rhodospirillaceae*	9.6
Methanosarcina	1.7	*Rhodobacteraceae*	7.7
Rhodobacteraceae	1.0	*Parvibaculum*	6.3
Thiomicrospira	0.9	*Shewanella*	2.2
Proteobacteria	0.8	*Thalassospira*	1.9
Bacteroidetes	0.7	*Pusillimonas*	1.8
Acetobacterium	0.5	*Geoalkalibacter*	1.8

Microbial OTUs: Lowest identified taxon (phylum, family or genus) is shown

According to the Shannon biodiversity index, which measures the evenness of a community with a focus on rare species, LOWT-EN was less biodiverse after it was incubated in the sandstone-packed column system (Table 4-3). An increase in the Simpson index, which focuses on the number of abundant species, also indicated lower biodiversity in the community after column incubation. These results suggest that the LOWT-EN community was enriched in more specialized organisms able to survive the column conditions. In accordance with these results, the biodiversity of the LOWT community, originating from the same oil field, was also decreased after the inoculum was incubated in a sandstone-pack column.

In contrast, based on the Shannon and Simpsons indexes, RESOIL and HIGHT communities were more biodiverse after incubation in the sandstone-packed column (Table 4-3). This can also be observed with the Chao estimator that indicates the richness of a community (based on OTUs), showing that the microbial richness for the RESOIL and HIGHT increased after the column experiments (Table 4-3). The increase in the number of OTUs in these samples may be explained by the introduction of new OTUs coming from the oil amended in the columns. Although the light oil that was amended in the sandstone-packed columns had its own microbial community (Table B-6) and it may explain the present of "new" microbial taxa in the columns, pyrosequencing results showed that specific microorganisms were enriched in the column experiments, and the new community was not just a mix of two microbial communities.

Table 4-3. Alpha-diversity indexes obtained from 16S rRNA gene pyrotag sequencing of RESOIL, HIGHT, LOWT, and LOWT-EN before and after incubation in the column systems.

	RESOIL		HIGHT		LOWT		LOWT-EN	
	Inoculum	Column	Inoculum	Column	Inoculum	Column	Inoculum	Column
Shannon	1.76	3.08	1.70	2.38	3.26	2.85	3.57	1.99
Simpson	0.27	0.08	0.32	0.16	0.10	0.17	0.08	0.33
Chao	117	207	107	157	282	201	1798	157

4.4 Discussion

In an effort to determine how hydrocarbon methanogenesis would proceed in a system more closely resembling a marginal oilfield, different microbial inocula were incubated in sandstone packed columns containing residual oil. Although the columns were not prepared under pressurized conditions (e.g., that would truly simulate an actual oilfield), they were used as a 'proof-of-concept' assessment of whether hydrocarbon methanogenesis would occur in a system characterized by residual oil-laden porous rock. From the column experiments, we were able to measure the evolution of methane at rates of up to ~ 1.4 μmol CH_4/g of oil/day (Table 4-1), and after a 'shut-in' period, more than 40% aliphatic hydrocarbons were biodegraded, presumably to methane, relative to uninoculated controls. The highest CH_4 production was observed in a column amended with LOWT-EN. This methanogenic consortium was able to utilize crude oil components both as a planktonic culture (Chapter 3), and enriched for sessile organisms in the sandstone-packed columns (Figure 4-2). Notably, the microbial community composition of the LOWT-EN enrichment shifted substantially when grown planktonically in liquid medium versus growth on a solid support (Figure 4-4). First, the relative abundance of methanogens increased, especially *Methanobacterium* (a H_2-user), in the sessile community

relative to the original planktonic enrichment culture (Figure 4-4). *Methanocalculus* remained at a similar abundance (~ 5% of total reads at the genus level), while the relative abundances of *Methanoculleus* and *Methanosaeta* decreased substantially. Second, bacterial members of the genus *Pseudomonas* dominated the community of the sand-packed column, whereas the most predominant bacterial members in the original enrichment were related to *Smithella.*

Lower CH_4 production was observed in columns amended with RESOIL and LOWT. However, *n*-alkanes were also depleted in these columns relative to uninoculated controls (Figure 4-3). Archaeal members from the sandstone-packed columns inoculated with RESOIL and LOWT were not enriched, and in contrast to the LOWT-EN community, these columns were dominated mainly by methanogens that strictly utilize acetate (e.g., *Methanosaeta*) (Table 4-2). Members of the phylum *Proteobacteria* were increased in the RESOIL and LOWT communities after the column experiments. For example, a betaproteobacterium member of the *Rhodocyclaceae* family was detected in high relative abundance in the column amended with LOWT (Table 4-2). Members of the *Rhodocyclaceae* family were previously shown to grow under anoxic conditions, and to degrade polycyclic aromatic hydrocarbons (Rotaru *et al.*, 2010; Singleton *et al.*, 2012). Other members of *Proteobacteria* that were enriched in the RESOIL and LOWT columns were affiliated with *Pseudomonas* and *Halomonas* (Table 4-2). These two organisms were also enriched in the column inoculated with LOWT-EN. This was a surprising finding as *Pseudomonas* species are often described as hydrocarbon-degrading aerobes, while the column incubations were strictly anoxic. However, these organisms are facultative and have been associated with oil biodegradation under some anoxic conditions (Grossi *et al.*, 2008). Further, *Pseudomonas* spp. have been detected in samples collected from anoxic oil reservoirs (Li *et al.*, 2012; Zhang *et al.*, 2012; Meslé *et al.*, 2013). It is possible that the proliferation of

Pseudomonas sp. in the sandstone column environment may be related to the known ability of these organisms to form biofilms (e.g., Klausen *et al.,* 2006; Li *et al.,* 2009), thus giving sessile organisms a competitive advantage in the sandstone column. *Halomonas* sp., also enriched in the sandstone columns with LOWT-EN, RESOIL, and LOWT (Figure 4-4 and Table 4-2), are known biofilm and exopolysaccharide-forming organisms that may allow for the solubilization of hydrocarbons attached to rock (Llamas *et al.,* 2006; Qurashi and Sabri, 2012; Gutierrez *et al.,* 2013). Members of this genus have also been found to be associated with oil reservoirs and the deep biosphere (Mnif *et al.*, 2009; Dong *et al.*, 2014).

Given the relatively high abundance of *Pseudomonas* in the sandstone-packed column experiment amended with LOWT-EN, we hypothesize that these bacteria were at least partially responsible for the anaerobic biodegradation of crude oil components, potentially acting as syntrophic organisms in conjunction with methanogens. While such a hypothesis remains to be tested, it should be noted that pyrotag sequencing of several produced water samples from the oilfield from which the enrichment was derived revealed the presence of *Pseudomonas* sp. and other nitrate-reducers along with methanogens (Agrawal *et al.*, 2012), thus interactions between these kinds of microbes may be occurring in some oilfield environments. Interestingly, the coexistence between these two groups of microorganisms in bioreactor systems has been previously noted (Percheron *et al.*, 1999). Other bacteria that were enhanced in the column amended with LOW-EN were members of the genera *Clostridium* and *Desulfovibrio* (Figure 4-4). These bacteria and other members from the *Firmicutes* and *Deltaproteobacteria* groups have been associated with anaerobic oil biodegradation (Gray *et al.*, 2010). The enrichment of *Eggerthella* in the sandstone incubations is unclear, given that this organism is a known gut-

associated organism, although clones related to this genus (e.g., in the *Actinobacteria*) were previously identified in RESOIL (Gieg *et al.*, 2008).

All the columns amended with HIGHT had very low CH_4 production after a 400-day incubation period. The microbial community of HIGHT was dominated by sulfate-reducing bacteria such as *Desulfovibrio* and *Desulfuromonadales* (Table 4-2), while the other inocula tested in this study were dominated by methanogens (Table 4-2 and Figure 4-4). Methanogens affiliating with *Methanosarcina* were present in the HIGHT inoculum at low abundance (1.7%), and were decreased after incubation in the column. Thus, the microbial composition of HIGHT may explain why the column amended with this inoculum had relatively low CH_4 production. Although the amount of biomass in produced waters from HIGHT was higher than in LOWT, the microbial composition of the inoculum seemed to play a more important role in the ability to bioconvert hydrocarbons to CH_4.

It was hypothesized that the oil degrading culture RESOIL would produce the highest amount of methane when inoculated in the column systems, as this was an established culture with high biomass (Table 4-1) and high methanogenic activity (Gieg *et al.*, 2008). It was surprising to see that the columns amended with RESOIL produced relatively low CH_4, especially compared to the column amended with LOWT-EN. A possible explanation for these findings is that the cells were not well distributed in the columns (e.g., cells were stuck in the injection ports), thus oil and nutrients were not readily available to cells. The RESOIL culture inoculum contained sediments from the crushed core used to enhance methane production in the enrichment culture, which could have affected the mobilization of cells through the columns when it was injected. In addition, the inocula from production waters were expected to take a longer time than the enrichments to start producing CH_4. In accordance with this prediction, the

column inoculated with LOWT with the highest CH_4 production had a longer lag period than the ones amended with LOWT-EN and RESOIL prior to the onset of CH_4 production. Regarding the differences in CH_4 production within the columns amended with the same inocula, it is possible that this variation was due to the slightly different amounts of residual oil remained in each column (Figure B-4) or slight variations in physical properties (e.g., differences in permeability or biomass distribution) that were hard to replicate in the column systems. Thus, more attention should be given in the future on the designing and preparation of anaerobic bioreactors simulating marginal oil fields for better reproducibility.

Overall, this study showed that at least three tested microbial communities previously exposed to hydrocarbons were able to convert residual oil to methane according to stoichiometric calculations, and that they survived in columns comprising Berea sandstone rock. This work also showed that in sandstone-packed columns, more similar to actual sediment rock reservoirs, enriched for different microbial communities than the planktonic organisms found in the enrichments and produced waters. The information gained in this study would help to understand the syntrophic communities inhabiting the subsurface, and may lead to the development of specialized microbial consortia able to grow on oil reservoir sediment rocks while biodegrading crude oil to methane.

Preface

Chapter 5 consists of a manuscript published in *FEMS Microbiology Ecology* (Berdugo-Clavijo et al., 2012). The work described in this chapter shows the study of methanogenic enrichments capable of degrading polycyclic aromatic hydrocarbons, with an emphasis on 2-methylnaphthalene and 2, 6-dimethylnaphthalene. This work is directly related to the study of hydrocarbon metabolism described in Chapter 3 and 4. Metabolite analysis and microbial community characterization of the enrichment cultures are also presented in this chapter.

Chapter Five: Methanogenic Metabolism of Two-Ringed Polycyclic Aromatic Hydrocarbons

5.1 Abstract

Polycyclic aromatic hydrocarbons (PAH) are widespread in subsurface environments, such as oil reservoirs and fuel-contaminated aquifers, however little is known about the biodegradation of these compounds under methanogenic conditions. To assess the metabolism of PAH in the absence of electron acceptors, a crude oil-degrading methanogenic enrichment culture was tested for the ability to biodegrade naphthalene, 1-methylnaphthalene (1-MN), 2-methylnapthalene (2-MN) and 2, 6-dimethylnaphthalene (2, 6-diMN). When methane was measured as an indicator of metabolism, nearly 400 µmol of methane was produced in the 2-MN- and 2, 6-diMN-amended cultures relative to substrate-unamended controls, which is close to the amount of methane stoichiometrically predicted based on amount of substrate added (51-56 µmol). In contrast, no substantial methane was produced in the naphthalene- and 1-MN-amended enrichments. In time course experiments, metabolite analysis of enrichments containing 2-MN and 2, 6-diMN revealed the formation of 2-naphthoic acid and 6-methyl-2-naphthoic acid, respectively. Microbial community analysis by 454 pyrotag sequencing revealed that these PAH-utilizing enrichments were dominated by the archaeal members most closely affiliated with the *Methanosaeta* and *Methanoculleus* species and bacterial members most closely related to the *Clostridiaceae,* suggesting that these organisms play an important role in the methanogenic metabolism of the substituted naphthalenes in these cultures.

5.2 Introduction

Polycyclic aromatic hydrocarbons (PAH) comprise a substantial fraction of petroleum mixtures, including crude oils, coal tar creosote, and refined fuel mixtures such as diesel. When such fuel mixtures are accidentally spilled or seep into the natural environment, some of the more water-soluble PAH, such as the 2-ringed naphthalenes and methyl-substituted naphthalenes, can migrate away from the source, while the larger ringed, less water-soluble PAH can adhere to sediments and soils (Hughes *et al.*, 1997). Many PAH can be toxic and/or carcinogenic (Sverdrup *et al.*, 2002), thus PAH contamination in the natural environment is of ecological and human health concern. The microbial degradation of hydrocarbons has been widely investigated to understand how these compounds can be remediated in petroleum-contaminated ecosystems. The aerobic biodegradation of PAH has been known for decades (Haritash and Kaushik, 2009) and it is now evident that PAH can also be biodegraded under anaerobic conditions. As recently reviewed, PAH biodegradation has been most frequently shown under nitrate- and sulfate-reducing conditions (e.g. Foght, 2008; Widdel *et al.*, 2010; Meckenstock and Mouttaki, 2011). Reported mechanisms for the initial activation of unsubstituted PAH include carboxylation (Zhang and Young, 1997; Davidova *et al.*, 2007), methylation (Safinowski and Meckenstock, 2006), or hydroxylation (Bedessem *et al.*, 1997). Fumarate addition occurs for the metabolism of 2-methylnaphthalene under sulfate reducing conditions (Annweiler *et al.*, 2000; Selesi *et al.*, 2010). Naphthyl-2-methylsuccinate (-succinyl-CoA) forms and is further metabolized to 2-naphthoate (2-naphthoyl-CoA) in a manner similar to that elucidated for anaerobic toluene metabolism (Beller and Spormann, 1997). 2-Naphthoate is then metabolized by ring reduction to form 5, 6, 7, 8-tetrahydro-2-naphthoate, followed by further stepwise ring reduction (Annweiler *et al.*, 2000; Zhang *et al.*, 2000; Annweiler *et al.*,

2002), and cleavage of the reduced ring (Annweiler *et al.*, 2002). Key genes and enzymes responsible for the biotransformation of 2-methylnaphthalene under sulfate-reducing conditions have been recently identified (Selesi *et al.*, 2010).

As PAH are widely present in methanogenic environments such as crude oil reservoirs and fuel-contaminated sediments, they can potentially serve as carbon sources for deep and near-surface methanogenic communities. Although syntrophic PAH metabolism coupled with methane production is considered a low energy yielding process (Schink, 1997), it is thermodynamically feasible (Dolfing *et al.*, 2009) and has been reported in a handful of laboratory-based studies. Methanogenic enrichments from sewage sludge were shown to utilize PAH of more than 3 aromatic rings (Trably *et al.*, 2003; Christensen *et al.*, 2004). Chang *et al.* (2006) observed up to 50% loss of naphthalene and phenanthrene, relative to controls when contaminated harbor sediments were incubated in the absence of electron acceptors. Similarly, Yuan and Chang (2007) reported the anaerobic degradation of five PAH by river sediment enrichments incubated under methanogenic conditions. The addition of acetate, lactate, or pyruvate to these incubations enhanced the methane production and PAH degradation rates. However, H_2 was included in the headspace (5-10%), so it is not clear what portion of the CH_4 was formed from H_2 versus the added PAH. Recently, Siegert *et al.* (2011) reported low amounts of methane production from naphthalene-amended enrichments, Zhang *et al.* (2012) described methanogenic anthracene-amended cultures, and Gieg *et al.* (2010) showed that a thermophilic anaerobic consortium consumed specific PAH and alkanes in crude oil-amended incubations relative to controls, with overall hydrocarbon loss correlating with methane production.

Recent biogeochemical evidence also suggests that PAH can be methanogenically biodegraded *in situ*. Aitken *et al.* (2004) detected several anaerobic PAH metabolites in biodegraded crude oils sampled from reservoirs across the globe, offering evidence that PAH can serve as substrates in deep anaerobic subsurface environments. Such a finding, coupled with isotopic analyses indicating that a substantial portion of the CH_4 and CO_2 present in oil-bearing formations is biogenic, also suggests that biodegradation of oil components in reservoirs, including PAHs, occurs under methanogenic conditions and can lead to the formation of heavy oil (Gray *et al.*, 2010). Many near-surface fuel-contaminated aquifers also contain PAH and can be methanogenic (e.g. Godsy *et al.*, 1992; Gieg *et al.*, 1999; Kleikemper *et al.*, 2005; Essaid *et al.*, 2011). Bianchin *et al.* (2006) showed the *in situ* mineralization of ^{14}C-naphthalene to $^{14}CO_2$ in a creosote-contaminated aquifer characterized by iron-reducing and methanogenic conditions, offering evidence that *in situ* methanogenic PAH degradation can potentially occur. Some studies have revealed the presence of anaerobic PAH metabolites in fuel-contaminated aquifers (Gieg and Suflita, 2002; Griebler *et al.*, 2003) although these sites were characterized as primarily sulfate- or nitrate-reducing rather than methanogenic. Little is known about the metabolic pathways and microorganisms involved in the methanogenic biodegradation of PAH, thus an understanding of their fate in the absence of electron acceptors is important for optimizing bioremediation efforts and *in situ* energy production applications.

In a previous study examining the methanogenic bioconversion of crude oil to methane, the alkane fraction was consumed with concomitant methane production (Gieg *et al.*, 2008). Periodic analyses of this culture for the presence of known anaerobic hydrocarbon metabolites did not reveal the presence of key anaerobic alkane metabolites, such as alkylsuccinic acids (unpublished data). However, several putative anaerobic naphthalene metabolites were detected

relative to controls. We thus hypothesized that the microbial community could also metabolize PAH under methanogenic conditions. Here, we report the activity of enrichment cultures capable of metabolizing 2-ringed PAH to methane, describe the microbial community members found in these enrichments, and identify metabolites that were produced during the degradation of the tested naphthalenes.

5.3 Materials and methods

5.3.1 Enrichments

The inoculum for all experiments originated from a crude oil-degrading enrichment previously shown to consume alkanes under methanogenic conditions (Townsend *et al.*, 2003; Gieg *et al.*, 2008). PAH-utilizing enrichments were developed by adding 2 mL of the inoculum previously incubated with crude oil to16 mL of anoxically prepared mineral salts medium (Figure A-1) reduced with 2.5% cysteine-HCl−sodium sulfide solution. These initial anaerobic incubations were prepared in 26-mL glass tubes capped with butyl rubber stoppers and aluminum seals. Substrate stock solutions containing 20 mg of 2-methylnaphthalene (2-MN), 2, 6-dimethylnaphthalene (2, 6-diMN), 1-methylnaphthalene (1-MN) or naphthalene (Naph) were anoxically prepared in 20 mL of the inert carrier 2, 2, 4, 4, 6, 8, 8-heptamethylnonane (HMN) by bubbling the solution with N_2 for 20 min prior to autoclaving. Two milliliters of each stock solution (2 mg of the PAH) were added to triplicate incubations. Substrate-free controls containing HMN only were also established. Methanogenic activity was periodically monitored by methane formation for over a year. After this incubation time all cultures containing the different PAH substrates showed enhanced methane production relative to the substrate-free controls. The cultures were then prepared for transfer. Replicate cultures containing the same

PAH substrate were combined, centrifuged, and the resulting cell pellet was resuspended in a small volume of sterile anaerobic medium (12 mL). An equal volume of this 'concentrated' inoculum (3 mL) was then added to each of four 120-mL serum bottles containing 60 mL sterile anaerobic medium. Three of the bottles received the PAH with which the cells were initially incubated (2-MN, 2, 6-diMN, 1-MN, or Naph) and one bottle contained the medium but no substrate. Thus, the substrate-free control for each PAH was derived from a separate inoculum source. For these transfers, the naphthalene substrates (6 to 8 mg) were pre-adsorbed to Amberlite XAD-7 (0.3 g), as previously described by Morasch *et al.* (2001) to allow for the addition of higher amounts of substrate while minimizing toxicity. Abiotic experiments examining the sorption of 2-MN to XAD-7 (carried out as described by Morasch *et al.* (2001)) showed that the aqueous phase concentrations of 2-MN was close to 40 µM with 6 mg of substrate in 60 mL of medium (Figure C-1). All cultures were incubated in the dark at room temperature (21-23°C) for 16 to 20 weeks.

5.3.2 Analytical Methods

Methane formation was monitored in the enrichment cultures by injecting 0.2 mL of an incubation headspace, using a sterile syringe preflushed with N_2/CO_2 (90/10), into a HP model 5890 gas chromatograph (GC) equipped with a flame ionization detector maintained at 200°C. Injections were carried out at 150°C onto a packed stainless steel column (6 ft. x 1/8 in., Poropak R, 80/100, Supelco) held isothermally at 100°C. Methane amounts were determined based on calibration curves prepared from standards containing known methane concentrations.

To determine the putative PAH metabolites produced during the methanogenic degradation of 2-MN or 2, 6-diMN, time course experiments were carried out. For each

substrate, newly transferred incubations were prepared as described above (e.g. using a washed cell concentrate as the inoculum and adsorbing the PAH to Amberlite). Substrate-containing, substrate-free, and sterile control incubations were prepared in triplicate for each PAH. The incubations were monitored for methane production and culture fluids were subsampled (20 mL) as increasing amounts of methane were produced. The samples were acidified with HCl to a pH of 2 and extracted with 3 volumes of ethyl acetate. For some incubations, the whole culture was sacrificed by acidifying and extracting in the same manner. The organic layers were dried over sodium sulfate and concentrated by rotary evaporation and under a stream of N_2 to a volume of approximately 100 µL. The samples were then silylated with 100 µL of BSTFA (Thermo Scientific, Waltham, MA) for 20 minutes at 60˚C. Putative metabolites were separated and identified using an Agilent 7890A gas chromatograph equipped with an HP-5MS column (30 m × 0.25 mm × 0.25 µm; Agilent Technologies) and an Agilent 5975C mass selective detector. The oven was held at 45˚C for 5 min, increased at a rate of 4˚C/min to 270˚C, and then held at this temperature for 5 minutes. The injector was operated in split mode (50:1) and was held at 270°C. Metabolite identifications were confirmed by matching GC retention times and MS profiles with those of authentic standards or tentatively identified through mass spectral analysis. Metabolite concentrations were calculated based on calibration curves prepared with authentic 2-naphthoic acid.

5.3.3 Microbial community analysis

The 2-MN and 2, 6-diMN enrichment cultures were subject to DNA extraction, 16S rRNA gene amplification, and subsequent microbial community analysis by 454 pyrotag sequencing in a manner similar to that described by Ramos-Padrón *et al.*, 2011. DNA was

extracted from 5 mL of the culture fluids using a commercial kit according to the provided instructions (FastDNA Spin Kit for Soil; MP Biomedicals). The 16S rRNA genes were amplified by PCR (95 °C, 3 min; 25 cycles of 95 °C 30 s, 55 °C 45 s, 72 °C 90 s; 72 °C 10 min; final hold at 4 °C) using the FLX Titanium amplicon primers 454T-RA and 454T-FB (20 pmol μL^{-1}) that have the sequences for 16S primers 926f (aaa ctY aaa Kga att gac gg) and 1392r (acg ggc ggt gtg tRc) as their 3'-ends. Primer 454T-RA had a 25 nucleotide A-adaptor sequence of CGTATCGCCTCCCTCGCGCCATCAG, whereas primer 454T-FB had a 25 nucleotide B-adaptor sequence of CTATGCGCCTTGCCAGCCCGCTCAG. The resulting PCR products were purified using a commercial kit (QIAquick PCR Purification Kit, Qiagen) and concentrations were determined by fluorometry (on a Qubit Fluorometer using a Quant-iT™ dsDNA HS Assay Kit, Invitrogen). PCR products were sent to the McGill University and Genome Quebec Innovation Centre for pyrotag sequencing where they underwent 10 PCR cycles with primers 454T-RA-X and 454T-FB, where X is a 10 nucleotide multiplex identifier barcode (to enable the simultaneous analysis of multiple samples in one run). The barcoded PCR products were analyzed by pyrotag sequencing using a GS FLX Titanium Series Kit XLR70 (Roche Diagnostics Corporation). All the pyrotag sequencing reads were deposited at the Sequence Read Archive (SRA) in NCBI. The accession numbers of the reads in the 2-MN and 2, 6-diMN enrichment samples are SRS151517 and SRS151516, respectively.

5.3.4 Phylogenetic analysis

Phoenix 2, an in-house developed SSU rRNA data analysis pipeline, was used to conduct the Pyrotag analysis (Ramos-Padrón *et al.*, 2011). There were 3,540 and 13,178 raw reads in the 2-MN and 2, 6-diMN enrichment samples, respectively. After quality control by Phoenix 2, there

were 2,024 reads with an average read length of 371 (median 380.5) from the 2-MN enrichment sample and 7,183 reads with an average read length of 375 (median 391) from the 2, 6-diMN enrichment sample. From these reads, 321 operational taxonomic units (OTUs) were generated using average neighbor clustering at a 3% distance cutoff. The most frequently occurring sequence in an OTU was chosen as its representative sequence. The taxonomic annotation of each OTU was based on the blast search results of the representative sequences against SSU Ref NR 102 database (http://www.arb-silva.de/no_cache/download/archive/release_102/Exports). The representative sequences of those OTUs of bacterial origin, whose frequencies were > 25 reads, were selected as the bacterial sequences, and the representative sequences of those OTUs of archaeal origin, whose frequencies were > 40 reads, were selected as the archaeal sequences for this study to build phylogenetic trees. Sequences from known aromatic hydrocarbon degrading species or environments relevant to our study were retrieved from the ENA database (European Nucleotide Archive). All sequences were aligned with SINA (the SILVA web aligner, Pruesse *et al.*, 2007). SINA aligned sequences were imported and inserted into the 'All-Species Living Tree' alignment version 106 (Yarza *et al.*, 2008) using the ARB parsimony approach with ARB (http://www.arb-silva.de/projects/living-tree). For each inserted sequence, we searched and marked the closest relatives in the tree. The phylogenetic trees were calculated using the distance matrix neighbor-joining method with a Jukes-Cantor correction model and FMX filter in ARB. The representative OTUs in the 2-MN and 2, 6-diMN cultures were designated MethylNaph1, MethylNaph2, MethlyNaph3, etc. (Figure 5-3, Figure C-2, Table C-1).

5.4 Results

5.4.1 Methanogenic microbial activity

To evaluate whether the 2-ringed PAH substrates were metabolized under methanogenic conditions, methane production was monitored over time. Enhanced levels of methane were observed in the 2-MN- and 2, 6-diMN-amended incubations relative to the corresponding substrate-unamended controls, demonstrating the methanogenic bioconversion of these 2-ringed PAH. For the cultures amended with 2-MN, an average of 422 µmol methane was detected, while the 2, 6-diMN-amended cultures produced an average of 388 µmol methane after 110 days of incubation (Figures 5-1A and 5-1B). These methane values were close to those expected based on theoretical stoichiometric predictions (Table 5-1) calculated from the amounts of the substrates added. In contrast, methanogenesis was inhibited by the presence of 1-MN (Figure 5-1C), as the unamended controls produced substantially more methane than the 1-MN-amended incubations. Although methanogenesis was not inhibited in the Naph-amended cultures, methane was produced at similar levels as the unamended controls after 140 days of incubation (Figure 5-1D), suggesting that naphthalene did not serve as a substrate for the enrichment culture. Due to the increased methanogenic activity observed with 2-MN and 2, 6-diMN-amended enrichments, further studies focused on these two cultures.

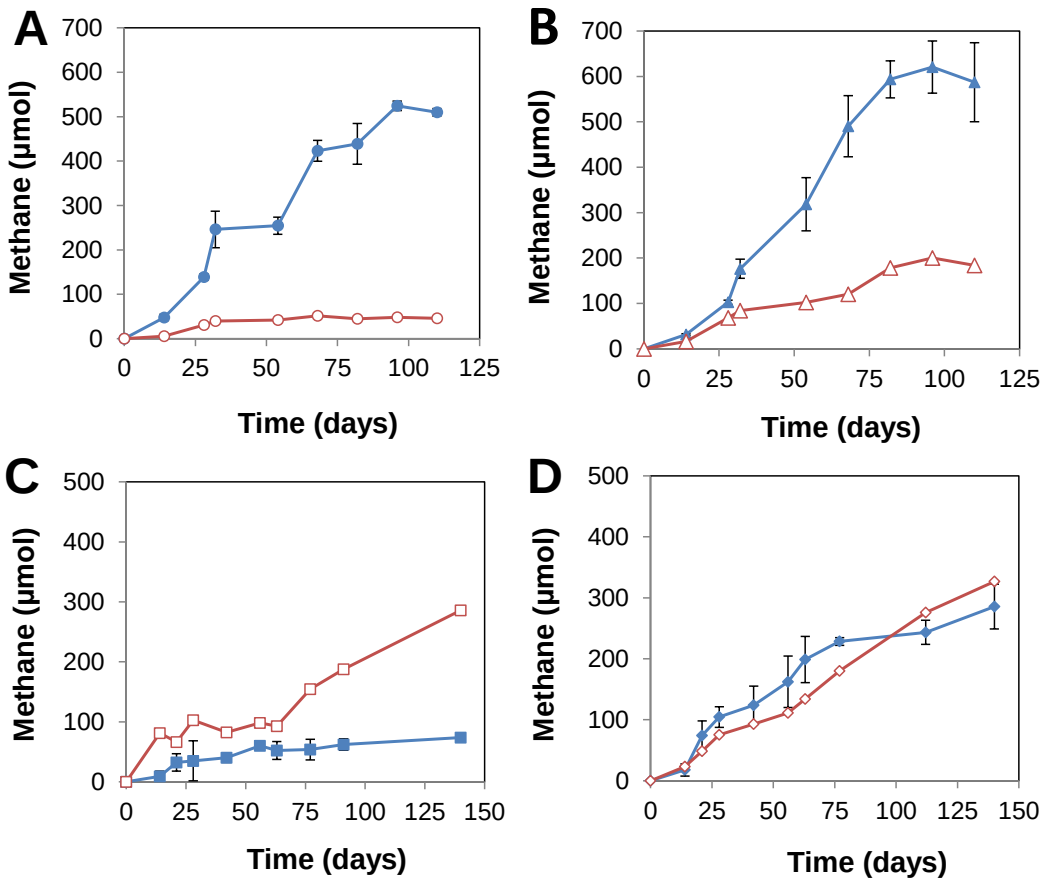

Figure 5-1. Methane production in incubations amended with (A) 2-MN: (B) 2, 6-diMN; (C) 1-MN; or (D) Naph. Closed symbols show the mean of replicate incubations amended with the requisite PAH, and open symbols represent the corresponding substrate-unamended controls. Error bars indicate the standard deviation for triplicate PAH-amended incubations.

Table 5-1. Stoichiometric calculations for the biodegradation of 2-MN and 2, 6-diMN under methanogenic conditions relative to unamended controls.

Substrate	Stoichiometric reaction	Predicted CH_4 (µmol)*	Measured CH_4 (µmol)	Percentage of predicted CH_4 recovered
2-MN	$C_{11}H_{10} + 8.5H_2O \rightarrow 4.25CO_2 + 6.75CH_4$	379	422 ±6	111 ±1.6
2, 6-diMN	$C_{12}H_{12} + 9H_2O \rightarrow 4.5CO_2 + 7.5CH_4$	384	388 ±62	101 ±16

*Based on the amount of substrate added (8 mg; 56.3 µmol 2-MN; 51.2 µmol 2, 6-diMN)

5.4.2 Identification of metabolites

Subsequent transfers of the methanogenic cultures amended with 2-MN or 2, 6-diMN were used to determine the formation of metabolites in time course experiments. Substrate-unamended and sterile controls were incubated and analyzed in parallel. Mass spectral analyses of the incubation extracts revealed the presence of 2-naphthoic acid in the 2-MN-amended enrichments (Figure 5-2a) and a presumed methylnaphthoic acid in the 2, 6-diMN-amended cultures (Figure 5-2b). The mass spectral profile of the latter metabolite revealed fragment ions that were 14 mass units higher than those of an authentic 2-naphthoic acid standard. Using 2-naphthoic acid as the calibration standard, the maximum concentrations of the naphthoic acids averaged 32 nM (n=6). These products were not detected in the corresponding substrate-unamended or sterile controls. In addition to these compounds, other putative methyl-substituted naphthalene metabolites were sought (based on literature reports) including naphthyl-2-methylsuccinate, 1, 2, 3, 4-tetrahydro- and 5, 6, 7, 8-tetrahydro-2-naphthoic acids, octahydronaphthoic acid, and decahydronaphthoic acid (and their methylated analogs in the case of the 2, 6-diMN cultures). However, none of these compounds was detected during the time course experiments.

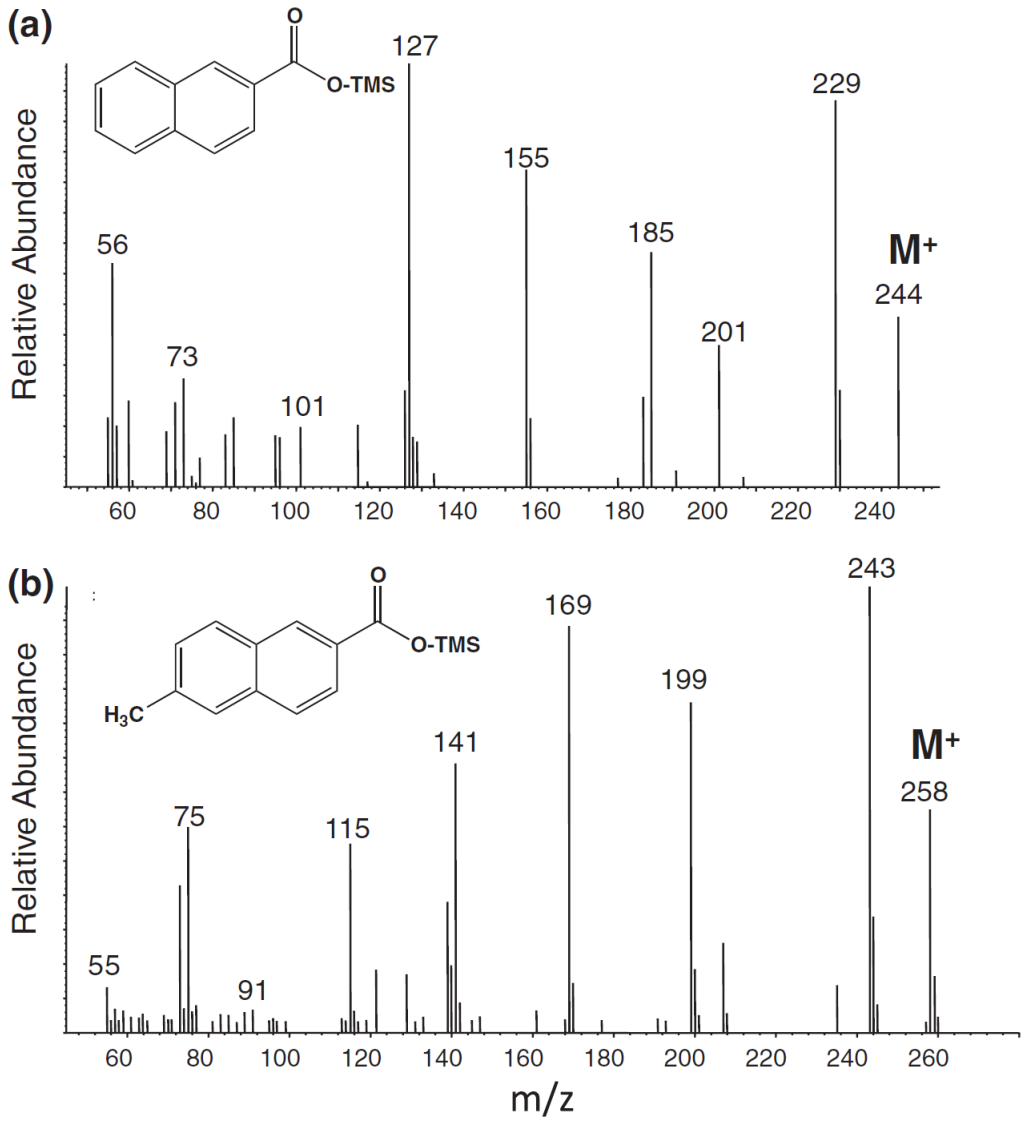

Figure 5-2. Mass spectral profiles of (a) 2-naphthoic acid (silylated) detected in the 2-MN-amended culture and (b) the tentatively identified 6-methyl-2-naphthoic acid (silylated) detected in the 2, 6-diMN-amended culture. The major fragment ions in (B) are shifted 14 mass units higher than in (a), denoting the presence of an extra methyl group.

5.4.3 Microbial community analysis of enrichments

Pyrotag sequencing analysis of the 16S rRNA genes amplified from the 2-MN- and 2, 6-diMN-amended cultures revealed the predominance of organisms belonging primarily to the phyla *Euryarchaeota*, *Firmicutes*, and *Proteobacteria* (Table 5-2). Sequences falling within the *Chloroflexi*, *Spirochaetes*, *Bacteroidetes*, and *Actinobacteria* were also present in low abundance. Both methanogenic enrichment cultures were dominated by archaeal members affiliating with the genera *Methanoculleus* and *Methanosaeta*, representing approximately 50 to 65% of the sequences identified at the genus level (Table 5-2). Members of the *Methanolinea* and *Candidatus Methanoregula* comprised the next most abundant archaeal genera. The dominant representative archaeal OTU in the cultures (MethylNaph3, Figure C-2, Table C-1) matched most closely with other uncultured *Methanosaeta* clones derived from the original aquifer sediments (Struchtemeyer *et al.*, 2005). In addition, both enrichment cultures were dominated by bacterial members belonging to the genus *Clostridium* (Table 5-2), representing 24% and 32% of the sequences identified at the genus level in the 2-MN and 2, 6-diMN cultures, respectively. The dominant representative bacterial OTU in the cultures (MethylNaph1, representing 27% of the sequences identified at the genus level) also aligned most closely with a *Clostridia* clone retrieved from the aquifer sediments from which the cultures were initially derived (Callaghan *et al.*, 2010) (Figure 5-3). Another minor representative OTU (MethylNaph5 0.7% abundance) also fell within this class (Figure 5-3). The OTUs present in our PAH-degrading enrichment were only distantly related to clones retrieved from other PAH-degrading methanogenic enrichments (clones RFLP U10 and RFLP U27; (Chang *et al.*, 2005). The other seven representative OTUs aligned within the *Desulfuromonadales*, *Desulfovibrionales*, *Betaproteobacteria, and Chloroflexi* (Figure 5-3, Table C-1) but were not closely related to other

sulfate-reducing, naphthalene-degrading strains or clones in PAH-degrading cultures (e.g. NaphS2, NaphS3, NaphS6, clone N47-1, clone N47-2) (Galushko *et al.*, 1999; Musat *et al.*, 2009; Selesi *et al.*, 2010).

Table 5-2. Phylogenetic affiliations of the microbial reads identified at the phylum and genus levels by pyrotag sequencing of the 16S rRNA genes in the 2-MN and 2, 6-diMN-amended methanogenic enrichment cultures.

	Percentage of sequence reads*	
Taxon (*Phylum*, *Genus*)	2-MN	2, 6-diMN
Euryarchaeota	68.56	60.61
Methanoculleus	37.97	21.79
Methanosaeta	29.91	33.53
Methanolinea	4.03	3.34
Candidatus Methanoregula	0.59	3.18
Methanomethylovorans	0.27	0.34
Methanobacterium	ND	1.32
Methanosarcina	ND	0.16
Firmicutes	23.21	32.03
Clostridium	24.17	32.24
Cryptanaerobacter	0.32	0.13
Sedimentibacter	0.21	0.33
Streptococcus	ND	0.10
Proteobacteria	6.70	5.09
Desulfobulbus	1.24	0.10
Desulfovibrio	0.59	1.11
Smithella	0.11	0.12
Methylibium	ND	0.30
Geobacter	ND	0.70
Diaphorobacter	ND	0.22
Chloroflexi	0.64	0.51
Spirochetes	0.59	1.08
Spirochaeta	0.21	0.28
Bacteroidetes	0.15	0.32
Proteiniphilum	ND	0.10
Actinobacteria	0.10	0.28

ND, not detected at the given level.

*Only sequences with $\geq$ 0.1% abundance are shown. Percentages are based on the number of reads identified at the phylum or genus levels. For the 2-MN culture at the phylum level = 2029 reads; at the genus level = 862 reads. For the 2, 6-diMN culture at the phylum level = 7230 reads; at the genus level = 6728 reads.

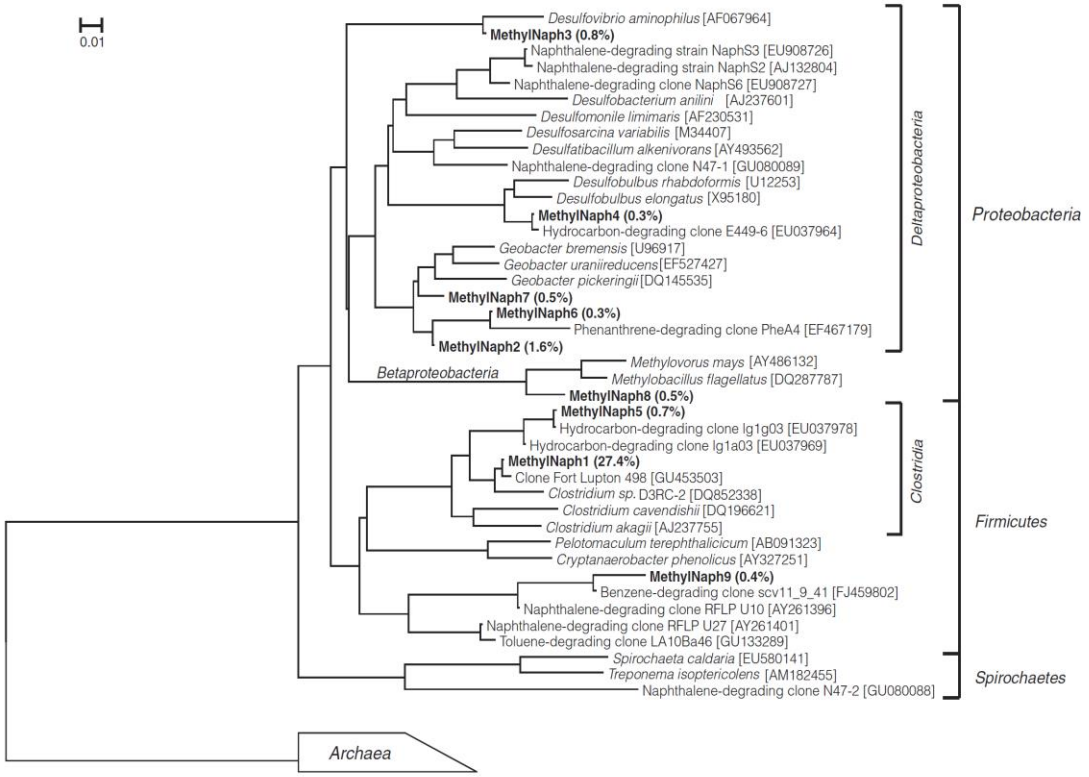

Figure 5-3. Phylogenetic relationships of bacterial 16S rRNA gene representative sequences (bold) identified in the 2-MN and 2, 6-diMN-amended enrichments with respect to other bacterial sequences including those in other anaerobic hydrocarbon degrading cultures or environments. The phylogenetic tree was constructed using the distance matrix neighbour-joining method with Jukes-Cantor model and FMX filter in ARB. The number in parenthesis for the MethylNaph sequences indicates the percentage of abundance compared to all OTUs. The scale bar indicates 1% of sequence divergence.

5.5 Discussion

We have developed enrichment cultures capable of metabolizing 2-ringed PAH in order to assess the microbial community composition and metabolites associated with methanogenic PAH degradation. Incubations containing Naph or 1-MN did not produce substantial methane over 140 days relative to their requisite substrate-unamended controls (Figure 5-1C and 5-1D). However, cultures amended with 2-MN or 2, 6-diMN produced near stoichiometrically predicted amounts of methane relative to their substrate-free controls (Figure 5-1A and 5-1B, Table 5-1) within 110 days and were further transferred and studied for their community composition and pathways of metabolism. The compounds 2-naphthoic acid and 6-methyl-2-naphthoic acid were the only metabolites detected during time course experiments of cultures amended with 2-MN and 2, 6 di-MN, respectively. 2-Naphthoic acid was previously identified as a central intermediate in sulfate-reducing enrichment cultures able to degrade naphthalene or 2-methylnaphthalene (e.g. Zhang *et al.*, 2000; Morasch *et al.*, 2001; Davidova *et al.*, 2007). Our work shows that (methyl) naphthoic acid is also an important metabolite produced during the methanogenic metabolism of methyl-substituted 2-ringed PAH. The amounts of the naphthoic acids detected were approximately three orders of magnitude lower than that of the starting substrate, consistent with other studies reporting anaerobic hydrocarbon metabolite concentrations (Ulrich *et al.*, 2005; Fowler *et al.*, 2012). Previous studies under sulfate-reducing conditions showed that 2-MN is activated via addition to fumarate prior to conversion to 2-naphthoic acid (Annweiler *et al.*, 2000; Annweiler *et al.*, 2002), but we were not able to detect the predicted naphthylmethylsuccinates in our cultures. Thus, the metabolic mechanism responsible for the attack of the 2-MN or 2, 6-diMN in the absence of electron acceptors leading to the production of naphthoic acid is still unknown. Based on the fact that we added a similar

amount of PAH to our cultures (e.g. 8 mg per 60 mL culture) as was added to sulfate-reducing cultures wherein the fumarate addition product was detected (4-6 mg PAH per 50 mL culture) (Annweiler *et al.*, 2000), and our own experience in detecting fumarate addition metabolites in methanogenic cultures metabolizing low concentrations of toluene (Fowler *et al.*, 2012), we would expect to detect such compounds if formed. Ongoing efforts to scale up our enrichments will help determine whether the lack of detection of the fumarate addition metabolites is a scale issue or whether another mechanism of activation is occurring.

Microbial community analysis of the 2-MN- and 2, 6-diMN-amended enrichments revealed that the cultures were dominated by methanogens (Table 5-2, Figure C-2) and bacterial members affiliating within the phylum *Firmicutes* and class *Clostridia* (Table 5-2, Figure 5-3). In the crude oil-degrading methanogenic culture from which the PAH-degrading enrichments were derived, the most dominant bacterial OTU (clone lg1a03, EU037969) was also a member of the *Firmicutes* aligning within the *Clostridiaceae* (Gieg *et al.*, 2008). In the PAH-degrading enrichments described here, only a small portion of the identified sequences (MethylNaph4, 0.3%) was most closely related to this OTU (Figure 5-3). Instead, the most dominant OTU within the *Firmicutes* in the PAH-degrading enrichments (MethylNaph1, 27%, Figure 5-3) aligned most closely with a clone retrieved directly from the aquifer sediments from which the enrichments were initially derived (Clone Fort Lupton 498, Callaghan *et al.*, 2010). Although these results suggest that a shift occurred in the enrichment cultures as the community was transferred from whole crude oil to specific PAH substrates, further experiments (such as monitoring the growth of specific groups of organisms in the cultures or nucleic acid-based stable isotope probing) are still necessary to help pinpoint the roles played by these abundant organisms in PAH-amended cultures relative to substrate-free controls. Interestingly, Chang *et*

al. (2005), using RFLP analyses of a 16S rRNA gene library created from naphthalene and phenanthrene-amended methanogenic enrichments, also found that the most abundant RFLP types in the cultures most closely affiliated with members of the phylum *Firmicutes* and class *Clostridia*. In fact, an increasing number of studies are revealing the importance of Gram-positive organisms within the *Firmicutes* as key players in anaerobic hydrocarbon degradation in contaminated sites and enrichment cultures. For example, Winderl *et al.* (2008) examined the microbial community distribution with depth of a tar oil-contaminated aquifer and found that a specialized aromatic hydrocarbon-degrading microbial community of iron- and sulfate-reducing bacteria, as well as organisms belonging to the *Firmicutes* (uncultured *Peptococcaceae,* and *Desulfosporosinus, Desulfotomaculum,* and *Sedimentibacter* spp.), dominated hydrocarbon-sulfate transition zone. Winderl *et al.* (2010) further analyzed a microbial population from the same contaminated site by DNA-SIP and found that the most active population corresponded to the toluene-degrading Gram-positive sulfate-reducing *Desulfosporosinus* spp. within the class *Clostridia*. Kunapuli *et al.* (2010) isolated two iron-reducing bacteria capable of degrading monoaromatic hydrocarbons such as toluene, phenol and *m*-cresol. Phylogenetic analyses revealed that one of the strains was closely related to *Desulfitobacterium* also within the *Clostridia*. Fowler *et al.* (2012) also found that members of the *Clostridiales* dominated a methanogenic toluene-degrading community. The potential importance of the *Firmicutes* in hydrocarbon-associated environments in general was recently noted in a survey conducted by Gray *et al*. (2010). These authors compared and clustered the microbial community information from 26 different studies related to hydrocarbon-associated environments, and found that bacterial sequences affiliating with the *Firmicutes* were the most frequently detected in the diverse habitats, representing 31% of the surveyed bacterial sequences (amassed from over 3000

16S rRNA gene sequences). Based on the abundance of *Clostridia* in our 2-MN- and 2, 6-diMN-degrading cultures, we speculate that these organisms play a key role in PAH activation but as stated earlier, further studies are needed to support this hypothesis. The dominant methanogenic OTUs in the PAH-degrading enrichments, MethylNaph3 (22.5%) and MethyNaph2 (19%), affiliated most closely with uncultured *Methanosaeta* clones and *Methanoculleus* spp., respectively (Figure C-2). These findings suggest that both acetate- and hydrogen-utilizing methanogens also function in the syntrophic metabolism of the 2-ringed PAH.

Thus, in this work, we demonstrated the stoichiometric conversion of 2-ringed methyl substituted PAH to methane and identified naphthoic acids to be key PAH metabolites under methanogenic conditions. Our pyrotag sequencing data suggest the importance of *Clostridia*, *Methanosaeta*, and *Methanoculleus* species in methanogenic PAH metabolism although further study will help reveal the exact roles played by these organisms. Interestingly, Zhang *et al.* (2012) recently used SIP to identify the microbes involved in methanogenic anthracene degradation, and found that members of the deltaproteobacterial genera *Methylibium* and *Legionella* and an unclassified *Rhizobiales*, were the dominant phylotypes. Such results demonstrate not only the diversity of microbes that can be involved in methanogenic PAH biodegradation, but also that our understanding of such metabolism is far from complete.

Preface

Chapter 6 describes additional work that was conducted on the 2-MN-degrading enrichment described in Chapter 5. These work includes, qPCR work conducted to identify and quantify microorganisms able to degrade methylnaphthalene. In addition, initial work to detect metabolic genes involved in PAH biodegradation under methanogenic conditions conducted with the same enrichment cultures is included, as part of the study of hydrocarbon metabolism.

Chapter Six: Detection of key microorganisms and functional genes involved in the degradation of two-ringed polycyclic aromatic hydrocarbons

6.1 Introduction

During the production and usage of fossil fuels, hydrocarbons can be spilled into the environment, leading to the contamination of soil and water ecosystems. Hydrocarbons can be initially degraded in the environment under aerobic conditions (Rosenberg, 2013), but when oxygen is depleted biodegradation proceeds via anoxic mechanisms with other electron acceptors (Heider and Schühle, 2013). Aromatic hydrocarbons are major components of crude oil that can be metabolized by microorganisms. Various aromatic hydrocarbons including *m*-xylene, naphthalene, or toluene can be biodegraded via a fumarate addition mechanism (e.g. Harms *et al.*, 1999; Beller and Edwards, 2000; Musat *et al.*, 2009). In the degradation of toluene, the double bond of fumarate is added to the methyl carbon of the hydrocarbon to form benzylsuccinate, and this reaction is catalyzed by an oxygen-sensitive glycyl radical enzyme known as benzylsuccinate synthase (BSS) (Beller and Spormann, 1997; Beller and Edwards, 2000). In a similar way, a fumarate addition enzyme named naphyl-2-methylsuccinate synthase is known to activate the degradation of 2-methylnaphthalene with the formation of naphthylsuccinate (DiDonato *et al.*, 2010; Selesi *et al.*, 2010). The fumarate addition mechanism for hydrocarbon activation has been most frequently studied under denitrifying or sulfate-reducing conditions. Under methanogenic conditions, fumarate addition was found to initiate the degradation of toluene (Washer and Edwards, 2007; Fowler *et al.*, 2012). Thus, it is expected that methanogenic degradation of PAH would occur via the addition of fumarate, but there is no direct evidence that this mechanism is taking place under methanogenic conditions. Hence, the metabolic mechanism (s) for activation of PAH under methanogenic conditions is still unknown.

Many microorganisms thought to degrade aromatic hydrocarbons under anoxic conditions have also been identified. Sulfate- and nitrate-reducing bacteria known to degrade aromatic hydrocarbons (e.g., xylene, toluene, or naphthalene) include *Azoarcus, Thauera, Georfuchsia, Desulfotomaculum, Desulfobacula,* and *Desulfosarcina* (Heider and Schühle, 2013). *Desulfosporosinus* was recently reported as the initial toluene-degrader in methanogenic enrichment cultures (Fowler *et al.*, 2014; Sun *et al.*, 2014). Other aromatic hydrocarbon degraders involved in syntrophic metabolism include *Pelotomaculum, Peptococcaceae, Thermincola,* and *Syntrophus* (Kleinsteuber *et al.*, 2012). In a survey of over 3000 16S rRNA sequences from hydrocarbon-associated cultures and environments (Gray *et al.*, 2010), members of the *Firmicutes* phylum were the most frequently detected, and within this phylum, *Clostridia* were dominant. In another study involving a methanogenic consortium able to grow with PAHs (Chang *et al.*, 2005), members of the *Clostridia* were also enriched, suggesting these organisms were involved in the initial PAH degradation. However, there has been no direct evidence to demonstrate that specific members of the class *Clostridia* can activate PAHs.

As described in Chapter 5, 2-methylnaphthalene (2-MN) degrading enrichments were developed from sediments of a contaminated aquifer. Members of the *Firmicutes*, specifically *Clostridium* species, dominated in these cultures. In this study, quantitative PCR (qPCR) was used to measure the abundance of 16S rRNA genes (targeting most dominant bacterial taxa based on pyrotag analysis). We hypothesized that the taxa that increased in abundance during the course of incubation with 2-MN play an important role in its biodegradation. Further, cultures were exposed to pasteurization to study the ability of spore-forming bacteria to degrade the hydrocarbon. In addition, 2-MN and 2, 6-diMN degrading cultures were screened for the presence of genes known to be involved in the initial activation of the hydrocarbon (*e.g. bssA,*

nmsA) (Washer and Edwards, 2007; von Netzer *et al.*, 2013) and in the subsequent degradation of the aromatic ring structure (*e.g. ncr*) using primers available in the literature (2013; Morris *et al.*, 2014).

6.2 Materials and methods

6.2.1 Quantification of 16S rRNA genes

The abundance of bacterial 16S rRNA genes of *Clostridium, Desulfovibrio, Geobacter*, and *Bacteria* was quantified over time in 2-MN degrading cultures by qPCR. These organisms were selected based on previous pyrotag sequencing results using the most frequently occurring sequence analysis (MOF) (Table D-1). Enrichment cultures were prepared with 10 mL of anaerobic mineral medium (Figure A-1) and reduced with 0.05 mL of a sodium sulfide solution (Widdel *et al.*, 2006). Each bottle was inoculated with 10% of a resuspended cell pellet collected from previous cultures growing with 2-MN. Incubations were amended with 2 mL of HMN containing 2.5 mg of 2-MN. In addition, substrate-free (unamended) and autoclaved (sterile) control incubations were prepared in parallel. All cultures were incubated at 22℃ in the dark for 41 weeks, and regularly monitored for CH_4 production as previously described (Berdugo-Clavijo *et al.*, 2012). Numerous replicates (44 bottles) of 2-MN-amended and -unamended incubations were prepared in order to sacrifice individual incubations in triplicate at different time points (0, 13, 35, 119, and 206 days). For each time point, 10 mL incubations were opened in the anaerobic glove bag and centrifuged at 12 000 × *g* for 10 minutes. Cell pellets from sacrificed incubations were used for DNA extraction using a commercially available kit (FastDNA Spin Kit for Soil; MP Biomedicals). DNA concentration from all samples was normalized for qPCR analysis. Each qPCR reaction (10 µL) was prepared with 5 µL of 2X Evagreen supermix (Bio-Rad), 0.5 µM of

each forward/reverse primer, and 1 µL of DNA template (previously normalized) from sacrified incubations and run on a C1000 thermocycler (Bio-Rad). qPCR primers used in this study to quantify the selected 16S rRNA genes in the culture were obtained from the literature and optimized by temperature gradient PCR (Table 6-1).

Table 6-1. 16S rRNA gene primers used for qPCR analysis.

16s rRNA target gene	ID name (Forward/Reverse)	Sequence (5'-3')	Template size (bp)	Annealing temperature (℃)	Reference
Clostridium	Clos2-16S F Clos2-16S R	AGCGTTGTCCGGATTTACTG TTCGCCACTGGTGTTCCTCC	182	52	Wang *et al*., 2008 (F); Fowler *et al*., 2014 (R)
Desulfovibrio	DSV 691 F DSV 826 R	CCGTAGATATCTGGAGGAACATCAG ACATCTAGCATCCATCGTTTACAGC	135	62	Fite *et al*., 2004
Geobacter	Geo546F Geo840R	AAGCGTTGTTCGGAWTTAT GGCACTGCAGGGGTCAATA	294	63	Cumming *et al.*, 2003
Bacteria	Bac338F Bac530R	ACTCCTACGGGAGGCAGC GTATTACCGCGGCTGCTG	~130-200[a]	55	Whiteley and Bailey, 2000 (F); Lane, 1991 (R)

[a]Amplicon size was between 130 to 200 bp due to variations in 16S RNA gene sequences of various organisms

The temperature program for *Geobacter* primers was: 94°C, 4 min; 40 cycles of 94°C 30 s, 63°C 30 s (Cummings *et al.*, 2003). The following qPCR program was used for *Clostridium*, *Desulfovibrio* and *Bacteria* primers: 95°C, 3 min; 40 cycles of 95°C 20 s, 25 s at the optimized annealing temperature (Table 6-1). All programs were followed by a melt curve analysis to detect possible primer dimer formation, with a temperature gradient from 65℃ to 95℃ at an increment of 0.5℃/min. 16S rRNA genes were quantified using prepared standards (10^2 to 10^9 copies/µL) from diluted plasmids. For the preparation of the standards, amplified products for each bacterial gene were cloned from culture DNA and transformed into *E.coli* plasmid using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's protocol. Successful transformants were grown in 5 mL of LB liquid medium, plasmids were isolated with a Miniprep kit (Qiagen), and confirmed by sequencing (Eurofins). The slopes of the calibration curves

ranged from -3.0 to -3.4 and their efficiencies were higher than 97% (Figure D-1). Changes in 16S rRNA gene abundance were calculated from mean differences of abundances between the lag phase data points (0, 13 and 35 days) and the exponential phase points (119 and 206 days). Raw data was log-transformed for statistical analysis. A one-tail one sample t-test analysis was used to determine if there were significant changes in the gene abundances over time using IBM SPSS Statistics Program, version 22.0 (Armonk, NY).

6.2.2 Pasteurization

Based on the 16S rRNA gene sequencing results from pyrotag analysis described in Chapter 6, 2-MN and 2, 6-diMN degrading cultures were dominated by *Firmicutes*, specifically members of the genus *Clostridium.* In order to test whether a spore former like *Clostridium* was degrading the hydrocarbon, a 2-MN degrading culture was pasteurized. Incubations were established with 25 mL of anoxic mineral medium (McInerney *et al.*, 1979) amended with 0.125 g of amberlite resin pre-adsorbed with 2.5 mg of 2-MN. Culture medium was initially conditioned by placing the bottles in a water bath at 80℃ for about 20 minutes. Then, medium was inoculated with 30% of an actively growing culture able to degrade 2-MN. Immediately after, the bottles were moved back to the water bath and pasteurized at 80℃ for 10 minutes (Sanford *et al.*, 1996). The next morning, pasteurized cultures were inoculated with 6% of a pure H_2/CO_2-using methanogenic culture *Methanospirillum hungatei* strain JF1 to allow methanogenesis to proceed. Thus, if the heat-resistant bacterium survived and degraded the hydrocarbon, its metabolic products would be converted to methane by strain JF1. Unpasteurized incubations were also prepared and incubated in parallel. All bottles were incubated at room temperature for 295 days. During that time methane was monitored in the incubations by

injecting 0.2 mL of the headspace into a GC-FID system (Chapter 5; Berdugo-Clavijo *et al.*, 2012). Acetate was measured in the pasteurized and non-pasteurized incubations at the end of the incubation period by ion chromatography as previously described (Grigoryan *et al.*, 2008).

6.2.3 Functional gene analysis

Functional genes associated with aromatic hydrocarbon biodegradation were sought in genomic DNA from the 2-MN and 2, 6-diMN degrading cultures described in Chapter 5. Primers from published literature targeting genes coding for glycyl radical enzymes known to catalyze fumarate addition reactions were used, including three primer sets for the *bssA* gene (Washer and Edwards, 2007) and three primer sets for the *nmsA* gene (von Netzer *et al.*, 2013) (Table 6-2). In addition, genes coding for 2-naphthoyl-CoA reductase (*ncrA*) shown to be involved in the dearomatization of the unsubstituted aromatic ring (Morris *et al.*, 2014) were screened (Table 6-2). A PCR reaction with *bssA* gene primers was conducted with the following PCR program: initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, primer annealing for 1 min at an specific temperature (Table 6-2), 72°C for 1.5 min, and a final extension step at 72°C for 10 min. The temperature program for the PCR with *nmsA* primers was initially 94℃ for 3 min, followed by 35 cycles starting at 94℃ for 30 sec, primer annealing for 30 sec at 58℃, 72°C for 1 min, and a final extension step at 72°C for 5 min. The PCR reaction with the *ncr* primers was conducted with the following program: 95℃ for 3 min, followed by 30 cycles at 95℃ for 30 sec, primer annealing for 30 sec at 55℃, 72°C for 2 min, and a final extension step at 72°C for 10 min. PCR products of all reactions were observed on a 1% agarose gel and detected using SYBR safe gel stain to visualize amplified bands. DNA from *Thauera aromatica* (Biegert *et al.*, 1996) extracted from a culture grown in our lab was used as a positive

control for *bssA* genes. For *nmsA* and *ncr* genes, positive controls were not available at the time of the experiment. Molecular grade water used in the PCR reactions was used as the negative control for all the tested genes.

Table 6-2. PCR primers used to amplify genes potentially involved in anaerobic aromatic hydrocarbon degradation (*bssA*, *nmsA* and *ncr*). S1=primer set 1, S2=primer set 2, S3=primer set 3.

Target gene	Primer set	ID name (Forward/Reverse)	Sequence (5'-3')	Template size (bp)	Annealing temperature (℃)	Reference
bssA	S1	BssA327F BssA2004R	CGAATTCATCNTCGGCTACC GTCGTCRTTGCCCCAYTTNGG	1677[a]	54	Washer and Edwards., 2007
	S2	MBssA1516F BssA2524R	AGACCCAGAAGACCAGGTC ATGATSGTGTTYTGSCCRTAGGT	1008[a]		
	S3	BssA 1985F BssA 347R	CNAARTGGGGCAAYGACGA TGYTCNGGNCGRTTGATCTCYTC	1638[a]	49	
nmsA		8543R	TCGTCRTTGCCCCAYTTNGG	NA	58	von Netzer *et al* ., 2013
	S1	7363F	TCGCCGACAATTTCGAYTTG	1180		
	S2	7374F	TTCGAYTTGAGCGACAGCGT	1169		
	S3	7768F	CAAYGATTTAACCRACGCCAT	775		
ncr	S1	Ncr1F Ncr1R	CGTTATWCKCCYTGCCGTG CGATAAGCCATRCADATRGG	~320	55	Morris *et al* ., 2014
	S2	Ncr2F Ncr2R	TGGACAAAYAAAMGYACVGAT GATTCCGGCTTTTTTCCAAVT			

[a]Degenerate primers designed to amplified putative *bssA* genes. Expected region based on positive control *T. aromatica* was 300 bp. NA = not applicable

6.3 Results and discussion

6.3.1 qPCR analysis

The methanogenic activity of the microbial enrichment culture amended with 2-MN was enhanced compared to unamended and uninoculated control incubations (Figure 6-1). After 289 days, the culture produced up to 32 µmol of CH_4 (relative to unamended controls) that corresponds to almost 30% of the expected CH_4. In order to minimize disturbing the cultures, 2-MN was not measured over time. From the amount of CH_4 produced, it can be expected that almost 5 µmol of 2-MN were consumed by the culture (based on the stoichiometric reaction shown in Chapter 5).

16S rRNA genes of *Bacteria*, *Clostridium*, *Desulfovibrio*, and *Geobacter* were quantified in the culture over time (0, 13, 35,119 and 206 days) to determine their importance in the degradation of the PAH. The changes in abundance of these 16S rRNA genes were calculated from the mean differences of abundances (log copy genes/mL) measured between the lag phase (0-35 days) and the time points showing the highest methane production (119 and 206 days) (Figure 6-1). The average increase in abundance of *Clostridium* in 2-MN-amended triplicates was 2.01 ± 0.61 log units ($p < 0.05$), while the average increase in abundance of *Clostridium* for the substrate-unamended controls was 0.76 ± 0.45 log units ($p > 0.05$) (Figure 6-2). *Desulfovibrio* had an average change in abundance of 1.13 ± 0.09 log units in the 2-MN-amended replicates, but a similar increase in abundance was observed in the substrate-unamended controls (1.25 ± 0.41 log units) (Figure 6-2). The highest increase in abundance of all the quantified 16S rRNA genes corresponded to *Geobacter*, however the changes of abundance in 2-MN-amended and unamended samples were not significantly different from each other (Figure 6-2). After 206 days of incubation, the total *Bacteria* in the culture showed a significant increase in abundance (1.8 ± 0.7 log units) in the triplicate cultures amended with 2-MN, while the increase in the abundance of total *Bacteria* in the unamended samples was not significant ($p > 0.05$) (Figure 6-2). These findings suggest that the bacterial community in the culture was enhanced by the presence of 2-MN.

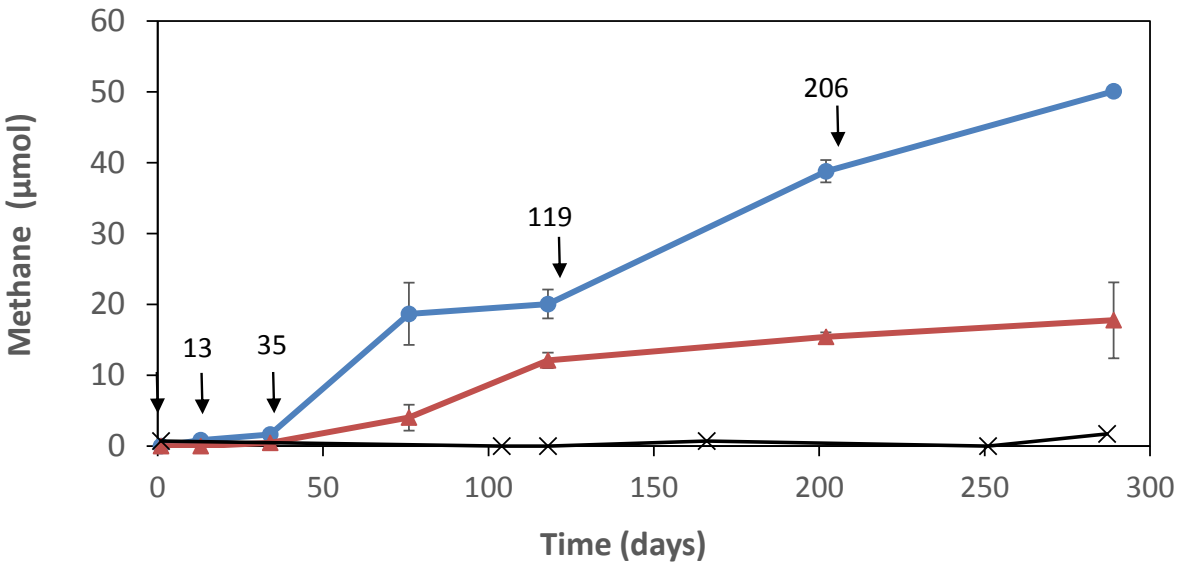

Figure 6-1. Methane production from enrichment cultures amended with 2-MN (blue), substrate-unamended incubations (red) and sterile controls (black). Arrows indicate the time points wherein cultures were sacrificed for qPCR analysis. Error bars indicate the standard error of the mean of triplicates.

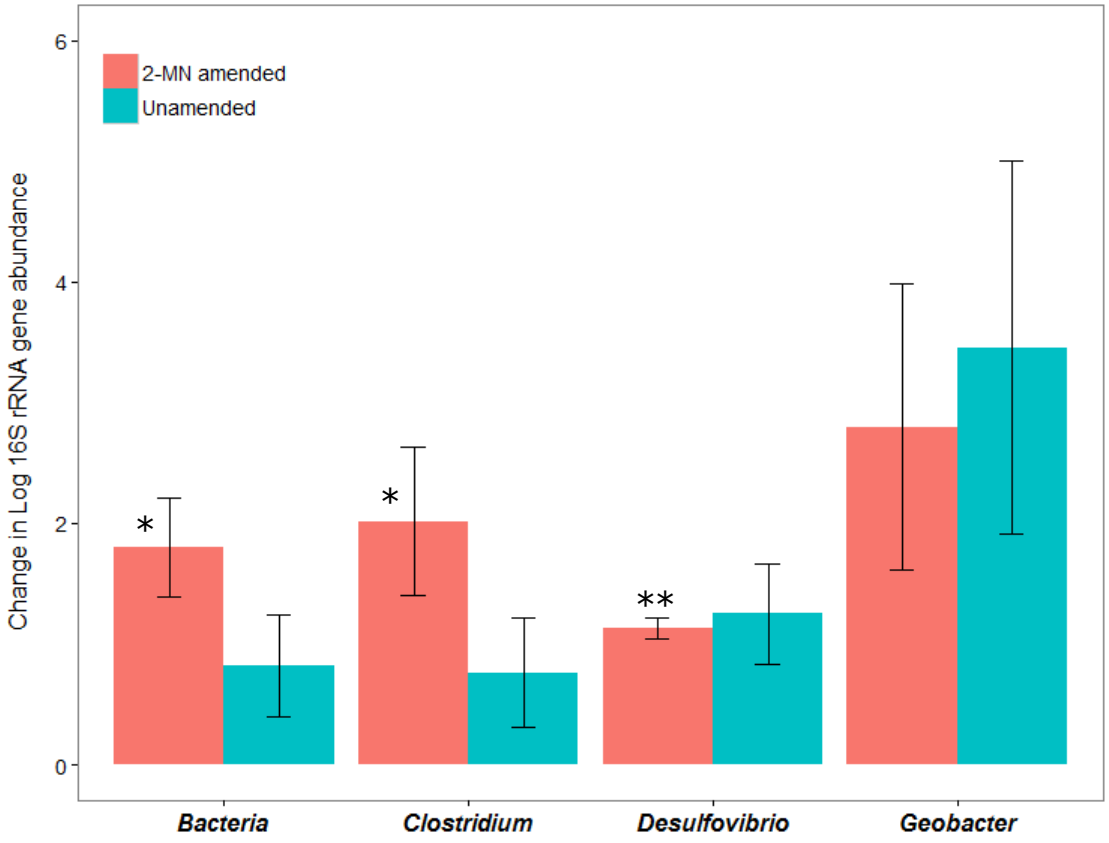

Figure 6-2. Average increase of 16S rRNA gene abundance (log copy gene/mL) for taxonomic groups selected for qPCR analysis in response to 2-MN amendment. The data show changes in abundances between data collected during lag phase (0-35 days) and the exponential phase (119 and 206 days). A value greater than zero indicates increase in abundance from the lag phase to the exponential phase. Unamended: from incubations without 2-MN. Error bars represent standard error of the mean of three replicates. T-test: * p<0.05; **p<0.01 (log-transformed data was used).

The qPCR data show that of the taxa monitored, the increase in *Clostridium* abundance was coupled to the production of CH_4 over time, suggesting the importance of this bacterium in the initial degradation of 2-MN. These results were in agreement with previous pyrotag sequencing analysis of the microbial enrichment that revealed the predominance of bacterial members affiliated with the genus *Clostridium* (e.g. comprised 24% of microbial sequences, Chapter 5). Microbial members closely related to *Clostridium* genus or within the *Clostridia* class have been found to be dominant in other enrichments amended with naphthalene and phenanthrene (Chang *et al.*, 2005). Other genera found in lower relative abundance in our 2-MN degrading culture included *Desulfovibrio, Geobacter* and *Desulfobulbus.* According to qPCR data, *Geobacter* and *Desulfovibrio* abundances were not significantly different between the 2-MN-amended incubations and unamended controls. However, it is possible that *Desulfovibrio* may be acting as a syntrophic H_2-utilizing partner in the 2-MN culture. This organism was previously shown able to switch to a syntrophic metabolism in the absence of sulfate (Meyer *et al.*, 2013) and has been detected in other microbial consortia degrading hydrocarbons (Kleinsteuber *et al.*, 2008; Herrmann *et al.*, 2010; Fowler *et al.*, 2012; Fowler *et al.*, 2014). *Geobacter* have also been implicated in utilizing hydrocarbon intermediate products that form during hydrocarbon degradation (Botton *et al.*, 2007). Although *Desulfobulbus* was also found in a low relative abundance in our culture, this organism was not targeted for qPCR. However, it may also play a role in downstream degradation as this bacterium has been previously associated with syntrophic propionate utilization (Widdel and Pfennig, 1982).

6.3.2 Pasteurization of 2-MN-degrading cultures

Prior to qPCR analysis, the 2-MN-degrading culture was used to test whether a spore-forming bacterium (e.g., *Clostridium*) would survive pasteurization and degrade the added PAH substrate. It was hypothesized that the 2-MN would be converted to CH_4 if the right syntrophic partner(s) such as a hydrogen-utilizing methanogen (strain JF1) were present. However, the pasteurization results indicated that CH_4 production in the enrichment cultures that were pasteurized and then amended with JF1 was inhibited, as they produced only 8% of the expected CH_4 (9.5 µmol) after 295 days (Figure 6-3). In contrast, incubations that were not pasteurized, but received the same inoculum as the pasteurized cultures, produced 80% of the expected CH_4 (95 µmol, relative to unamended), which indicates that the 2-MN was almost completely degraded. Thus, it was not possible to confirm with this experiment whether *Clostridium* was responsible for the initial degradation of 2-MN as these results suggest that the bacterium degrading the substrate did not survive pasteurization. This is unexpected because *Clostridium* is a known spore-forming bacterium that would be expected to survive pasteurization (Yang *et al.*, 2009). However, it is also possible that the enrichment culture that was pasteurized was not re-amended with the requisite syntrophic partner(s) following pasteurization. According to pyrotag results from the 2-MN degrading culture (Chapter 5), H_2-utilizing methanogens comprised up to 38% of all methanogens in the culture, while acetate-utilizing methanogens had a relative abundance of 26%. Based on these findings, it was expected that at least one third of the predicted methane in the culture would be produced in the pasteurized cultures amended with JF1.

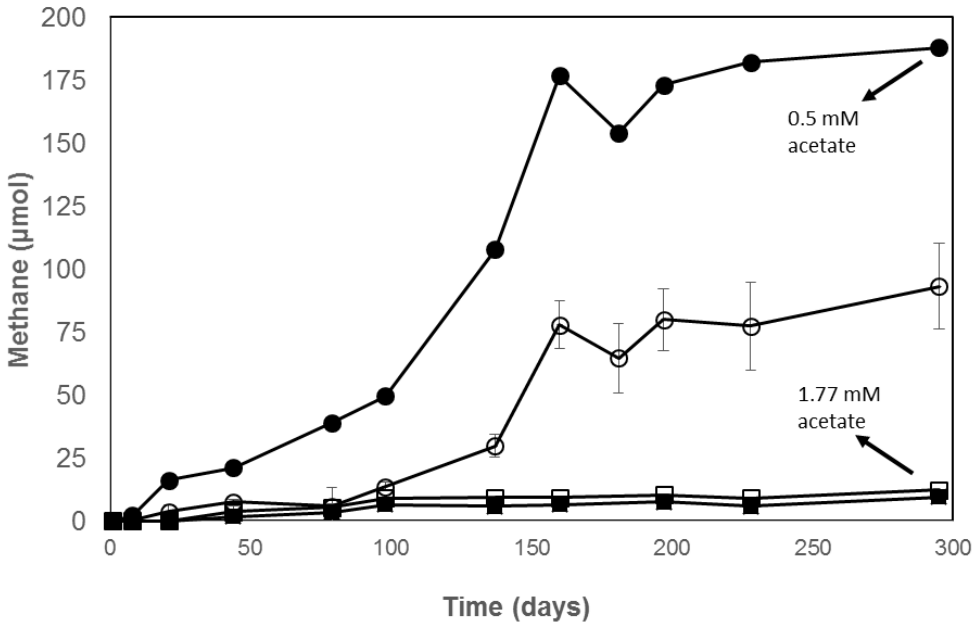

Figure 6-3. Methane production of unpasteurized (●) and pasteurized (■) methanogenic cultures amended with 2-MN. Unamended cultures are shown with open symbols. Error bars represent standard deviation of duplicate samples.

Acetate measurements conducted at the end of the incubation showed that the unpasteurized cultures contained 0.5 mM of acetate, while the pasteurized cultures amended with the H_2-utilizing methanogen JF1 accumulated up to 1.8 mM of acetate, showing that acetate-utilizing methanogens were likely also needed for syntrophic conversion to methane. Thus, a major reason for the "failure" of the pasteurization experiment to help demonstrate the importance of *Clostridium* in the degradation of 2-MN is likely because a methanogen able to utilize acetate was not present. It is also possible that pasteurization killed other important syntrophic bacteria that may be involved in syntrophic 2-MN degradation. Ficker *et al.* (1999) suggested that in the methanogenic degradation of toluene, an initial fermenter (*Chloroflexi* sp.) activates the hydrocarbon, and then a secondary fermenter (*Desulfotomaculum* sp.) converts the fatty acids to acetate and hydrogen for methanogens. Thus, it is probable that in our enrichment culture a necessary secondary fermenter did not survive pasteurization, and methanogenesis could not be completed.

6.3.3 Gene screening analysis

Evidence of fumarate addition in the 2-MN and 2, 6-diMN degrading cultures was initially assayed by searching for benzylsuccinate synthase coding genes (*bssA*). BSS is known to catalyze the addition to fumarate in the anaerobic activation of toluene. It is believed that anaerobic methylated PAH degradation occurs via an analogous reaction catalyzed by a naphthylsuccinate synthase enzyme (NMS). Since there were no *nms* primers available initially when this experiment was conducted, primer sets targeting *bssA* genes previously used to obtain amplicons in a methanogenic toluene degrading culture (Washer and Edwards, 2007) were used in this study (Table 6-2).

PCR conducted with *bssA* primer sets S1 and S3 did not result in the amplification of any bands (Figure 6-4), while PCR done with *bssA* primer set S2 resulted in the amplification of two bands using the genomic DNA of 2-MN and 2, 6 diMN degrading cultures (Figure 6-4). The first band had a similar size (~300 bp) to the positive control (*T. aromatica*), but it could not be confirmed by sequencing due to low DNA concentration (following gel purification or cloning). The second amplified band (smaller size) had a higher intensity (Figure 6-4), but as expected it did not show similarity to any benzylsuccinate synthase coding genes.

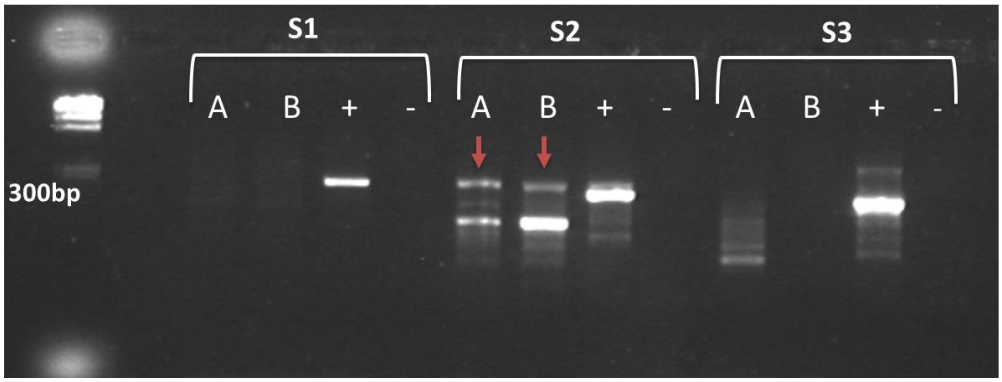

Figure 6-4. Amplification of *bssA* genes in methanogenic cultures degrading 2-MN (A) and 2, 6-diMN (B). The positive control shows amplification with *T. aromatica* (+), while the negative control was prepared with PCR water (-). S1, S2 and S3 correspond to the three primer sets that were used to target *bssA* genes (Table 6-2).

Later on, primer sets targeting genes coding naphthylmethylsuccinate synthase (NMS) were reported (von Netzer *et al.*, 2013), thus they were used in PCR analysis with our PAH degrading cultures (Table 6-2). Amplification was not observed with primer sets S1 and S2 (data not shown). These primer sets were designed to target a sequence motif region present in *nmsA* genes of the cultures N47 and NaphS strains that are able to degrade PAHs under sulfate-reducing conditions (von Netzer *et al.*, 2013). It is possible that the *nmsA* genes in our methanogenic cultures do not share high identity to these *nmsA* sequences retrieved from SRB cultures. However, an amplified band of the expected size (775 bp) was obtained with *nmsA* primer set S3 from the DNA of the 2-MN degrading culture (Figure 6-5A). Further, PCR done with DNA of the 2, 6-diMN degrading culture resulted in the amplification of a band of smaller expected size (~300 bp) (Figure 6-5B). The primers S3 were designed to target *bssA*/*nmsA* homolog regions (von Netzer *et al.*, 2013). Thus, it is possible that the band amplified with the *nmsA* primer set 3 in the 2,6-dMN degrading culture (Figure 6-5B) was the same as the band observed with the *bssA* primer S2 for the same culture (Figure 6-4). Amplified *nmsA* band obtained from 2-MN-degrading culture were gel excised, but the DNA concentrations were too low for sequencing analysis (despite repeated attempts). Thus, PCR protocols still need to be optimized to conclusively indicate the identity of these *nmsA* genes in the PAH-degrading cultures.

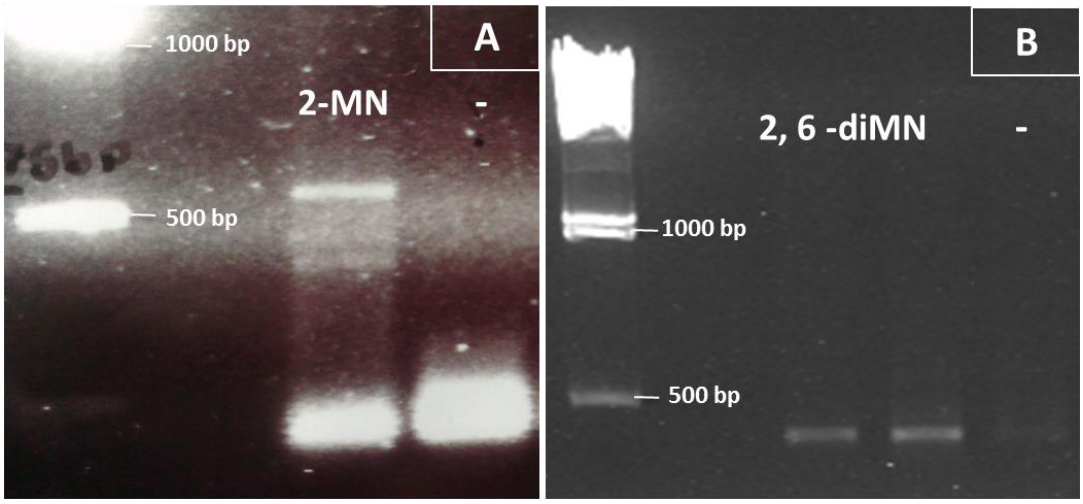

Figure 6-5. DNA amplification obtained with *nmsA* primer set 3 (S3) using A) 2-MN and B) 2, 6-di-MN degrading cultures. Negative control (-) prepared with PCR water. No positive control was available.

In downstream conversion of 2-MN under sulfate-reducing conditions, the reduction and ring cleavage of 2-naphthoic acid was shown to be catalyzed via a 2-naphthoyl-CoA reductase pathway (Eberlein *et al.*, 2013). 2-Methylnaphthoic and dimethylnaphthoic acids were detected in the cultures amended with 2-MN and 2, 6-diMN, respectively (Chapter 5). Thus, in order to assess the further degradation of the PAH, 2-naphthoyl-CoA reductase genes (*ncr*) were sought in our cultures using two *ncr* primers sets (Table 6-2). PCR reactions did not result in the amplification of NCR from genomic DNA from any of the PAH-degrading cultures. Although the first *ncr* primer set was specifically designed to target SRB cultures (Morris *et al.*, 2014), the second set was designed to target a broader range of bacteria (Morris *et al.*, 2014). It is possible that microbes carrying the *ncr* genes were too low in abundance in the culture to amplify the targeted genes. However, in our PAH-degrading cultures other reduced metabolites such as decahydro-naphthoic acid or tetrahydro-naphthoic acids, known to be formed from the reduction of 2-naphthoic acid, were not detected. Thus, these findings suggest that other mechanisms may be involved in the reductive dearomatization of naphthoic acid under methanogenic conditions. Future cultivation of the PAH-degrading cultures with 2-naphthoic acid or dimethyl-naphthoic acid as a sole carbon source may help to identify metabolites and enzymes involved in an aromatic compound reduction pathway under methanogenic conditions.

In summary, 16S rRNA genes of the dominant bacterial taxa found in 2-MN degrading cultures were quantified. qPCR analysis showed an increase in *Clostridium* abundance over time in 2-MN amended cultures compared to unamended controls, suggesting that *Clostridium* plays an important role as a hydrocarbon degrader in the culture. Other taxa (*Geobacter* and *Desulfovibrio*) did not show higher abundance compared to substrate-unamended controls. The possibility that other microorganisms detected at lower relative abundances in the culture

(Chapter 5) and not targeted by this qPCR assay should not be ruled out. Also, the significant increase in *Bacteria* abundance in the 2-MN-amended samples compared to the unamended controls, indicates higher bacterial growth in the cultures amended with 2-MN. It should be noted that qPCR results in this study showed gene abundances of the 2-MN culture when only 30% of the expected CH_4 production was reached. It is likely that if the cultures were incubated for a longer period of time, the differences in the *Bacteria* and *Clostridium* abundances would be even higher. DNA-SIP analysis can help to confirm if this organism is the initial degrader in the culture, however this approach was not conducted in this study due to the lack of availability of ^{13}C-labeled 2-MN (and the high cost of its synthesis). Methanogenic culture studies with hydrocarbon substrates are limited by the long doubling times of these microbial consortia (e.g., Gray *et al.,* 2011; Berdugo-Clavijo *et al.*, 2012; Fowler *et al.,* 2012). Thus, different strategies to enhance methanogenic activity in established enrichment cultures are currently under investigation in our lab. Although the pasteurization experiment did not help to confirm the role of *Clostridium* (spore-forming bacterium) in the initial degradation of 2-MN, it revealed the importance of acetate-utilizing methanogens in the syntrophic conversion of PAH to methane. The quantification of gene copy numbers of *Methanosaeta (*acetate-utilizing methanogen with the highest relative abundance in PAH degrading enrichments) may help to determine the role of this methanogen in the culture. The screening of functional genes in the culture involved in the initial degradation of PAH, suggested that fumarate addition is the mechanism utilized in the enrichment to degrade 2-MN and 2, 6-diMN as shown by the DNA amplification with *nmsA* targeting primers although we were not able to find the corresponding metabolites (Chapter 5). Further experiments to scale up the cultures and to develop an optimized PCR approach may help to obtain amplicons of higher intensities to confirm the identity of these *nmsA* sequences.

Preface

Chapter 7 describes the activity, metabolite analysis and microbial communities characterized in enrichment cultures amended with naphthalene and 1-methylnaphthalene, as a continuation of published work described in Chapter 5, where these PAH-degrading cultures are initially introduced.

Chapter Seven: Methanogenic biodegradation of naphthalene and 1-methylnaphthalene

7.1 Introduction

Polycyclic aromatic hydrocarbons (PAH) can be found naturally or released into the environment during the production and transportation of crude oil or coal (Preuss *et al.*, 2003; Krauss *et al.*, 2005). Many PAHs are considered to be environmental and human health concern as they can be toxic and carcinogenic compounds (Sverdrup *et al.*, 2002). A number of PAHs are currently known to be susceptible to microbial mineralization. Naphthalene is the simplest PAH that can be found in nature, but it can be more difficult to enzymatically attack due to the absence of a methyl or other functional group (Wilkes and Schwarzbauer, 2010). Aerobic biodegradation of naphthalene and other PAHs has been well studied for decades (Seo *et al.*, 2009), while studies on naphthalene biodegradation under anoxic conditions are more recent and fewer in number. In the absence of oxygen, naphthalene mineralization can take place under nitrate- (Mihelcic and Luthy, 1988; Rockne *et al.*, 2000) and sulfate-reducing conditions (Bedessem *et al.*, 1997; Zhang and Young, 1997; Galushko *et al.*, 1999; Meckenstock *et al.*, 2000). Iron- and manganese-reducing cultures have also been found capable of degrading naphthalene (Coates *et al.*, 1996; Langenhoff *et al.*, 1996). The degradation of naphthalene and methyl substituted naphthalenes has been most often studied under sulfate-reducing conditions, especially in strain NaphS2 (Galushko *et al.*, 1999) and the N47 culture (Meckenstock *et al.*, 2000). This thesis work has shown that 2-methylnaphthalenes can also be degraded under methanogenic conditions (Chapter 5).

Naphthalene activation was proposed to occur via initial carboxylation (Zhang and Young 1997; Meckenstock *et al*., 2000; Musat *et al*., 2009) or by methylation, followed by a fumarate addition reaction (Safinowski *et al*., 2006). The hydroxylation of naphthalene to naphthol was also suggested as a possible mechanism to activate the naphthalene molecule (Bedessem *et al.*, 1997), however, little evidence was shown to support this pathway. Based on the formation of putative metabolites (e.g., naphthyl-2-methylsuccinate), and the activity of enzymes typical of the fumarate addition pathway, it was suggested that methylation was the mechanism used to activate naphthalene in the SRB N47 culture (Safinowski and Meckenstock, 2006). However, in recent years the carboxylation mechanism for naphthalene degradation has gained more attention. Initially, ^{13}C-naphthoic acid and other labeled metabolites were detected in a sulfate-reducing enrichment amended with ^{13}C-bicarbonate, indicating that an initial carboxylation reaction was occurring during the degradation of naphthalene (Zhang and Young, 1997). In support of this mechanism, carboxylase encoding genes were found to be upregulated in anaerobic cultures when exposed to benzene or naphthalene (Abu Laban *et al.*, 2010; Bergmann *et al.*, 2011). Moreover, microarray and proteomic analyses with the NaphS2 culture (DiDonato *et al.*, 2010), showed that although fumarate addition genes were detected in the culture, naphthyl-2-methylsuccinate synthase (NMS) and most of the ß-oxidation reactions (BNS) subunits and genes were not upregulated when grown with naphthalene. Thus, these findings contradicted the occurrence of the methylation pathway in naphthalene degradation. Later on, Mouttaki *et al.* (2012) identified a naphthalene carboxylase in the SRB N47 culture, suggesting carboxylation was in fact the more likely mode of activation by this culture. Genomic and proteomic studies with the N47 and NaphS2 cultures have also revealed degradation mechanisms involved in the dearomatization and ring cleavage of 2-naphthoic acid

(naphthoyl-CoA). Naphthoyl-CoA reductase (NCR) encoding genes were expressed during the growth in naphthalene (DiDonato *et al.*, 2010) and were also detected in the N47 enrichment (Selesi *et al.*, 2010). Moreover, genes coding for fumarate addition enzymes such as naphthyl-2-methylsuccinate synthase (NMS), and other genes like *bns* involved in the ß-oxidation of naphthyl -2-methylsuccinate to 2-naphthoyl-CoA were characterized in the N47 culture amended with 2-methylnaphthalene (Selesi *et al.*, 2010). Under methanogenic conditions, the biodegradation of naphthalene and other PAHs has been less investigated. Thus, it is not clear whether the same metabolic mechanisms occurring for the degradation of PAH under sulfate reducing conditions, can be used to metabolize PAH in the absence of electron acceptors. Although methanogenic aromatic hydrocarbon degradation is a low energy yielding process, naphthalene biodegradation was shown to be thermodynamically possible under methanogenic conditions (Dolfing *et al.*, 2009). There are a number of studies indirectly showing the biodegradation of PAH under methanogenic conditions (Yuan and Chang, 2007; Larsen *et al.*, 2009). Naphthalene loss was also observed in microbial communities from contaminated harbor sediments in the absence of electron acceptors (Chang *et al.*, 2006). However, in this latter study incubations were amended with hydrogen gas, so it is unclear what portion of naphthalene actually contributed to the formation of methane. Hence, knowledge on the metabolic pathways and microorganisms involved in the mineralization of PAHs under methanogenic conditions is scarce.

In order to test the ability of microbial communities to biodegrade PAHs under methanogenic conditions, microbial cultures previously shown to degrade crude oil components under methanogenic conditions (Townsend *et al.*, 2003; Gieg *et al.*, 2008), were amended with various two ringed PAHs. In Chapter 5 enrichment cultures able to metabolize 2-

methylnaphthalene and 2, 6-dimethylnaphthalene were described. Initially, cultures amended with naphthalene and 1-methylnaphthalene (1-MN) did not produce substantial amounts of CH_4 relative to controls (Chapter 5). However, in this chapter, we show that after long incubation periods and transfers, these more chemically stable PAHs (Naph and 1-MN) were also metabolized under methanogenic conditions. Further, in this study we show the methanogenic activity of a naphthalene-amended culture, and the formation of several metabolites present in 1-MN- and naphthalene-amended cultures relative to controls. In addition, the microbial community characterization of the two PAH degrading cultures is described.

7.2 Methods

7.2.1 Enrichment cultures

The inoculum used in these experiments was originally obtained from an anaerobic microbial culture able to degrade *n*-alkanes in crude oil (Townsend *et al.*, 2003; Gieg *et al.*, 2008). The initial incubations amended with naphthalene and 1-MN were prepared as previously described in chapter five, briefly by adding 3 mL of resuspended cells from the original culture to 60 mL of sterile anoxic mineral medium (Figure A-1) flushed with N_2 / CO_2 (90:10). Incubations were amended with 8 mg of naphthalene or 10 mg of 1-MN pre-adsorbed in 0.3 g of resin (Amberlite XAD-7) as described by Morasch *et al.* (2001). The medium was reduced with 2.5% cysteine-HCl−2.5% Na_2S solution (2 mL/100 mL). After 140 days of incubation, these enrichments produce lower or equal amounts of CH_4 relative to controls that were not amended with the PAH (Chapter 5). In order to test whether the cultures were able to utilize naphthalene, new transfers (10%) were prepared and amended with 3 mL of HMN containing 7.5 mg of

naphthalene in 70 mL of anoxic mineral medium described above. Transfers were incubated at 30℃ to enhance microbial activity.

7.2.2 Naphthalene loss

Naphthalene loss in the cultures was determined by sampling ~ 0.1 mL of the HMN layer and injecting 1 µL onto a GC-FID system (Agilent 7890A). The GC was equipped with a HP-5 capillary column (30m × 320µm × 0.25µm; Agilent). The oven was held at 50℃ for 2 min, increased at a rate of 7℃ / min to 140℃, and held at this temperature for 3 min. The injector was held at 275℃ in split mode (50:1), and the detector was held at 300℃. Naphthalene concentrations were determined based on calibration curves prepared from naphthalene standards of known concentrations, which were run every time naphthalene was measured.

7.2.3 Metabolite analysis

Putative metabolites formed during the degradation of naphthalene or 1-MN were examined in the enrichment cultures by subsampling 50 mL of culture supernatant at different time points, or by using the whole volume of a sacrified culture. Liquid samples from the microbial cultures were acidified with HCl (pH 2) and extracted with 3 volumes of ethyl acetate. The extracted organic layers were initially concentrated by rotary evaporation at 60℃, and under a stream of N_2 to a volume of approximately 50 or 100 µL. Concentrated samples were then reacted with an equal volume of BSTFA (Thermo Scientific, Waltham, MA) to form trimethylsilyl (TMS) esters. Extracted samples were injected and analyzed using a GC (Agilent 7890A) with a HP-5MS column (50 m × 0.25 mm × 0.25 µm; Agilent) and equipped with a mass selective detector (Agilent 5975C). The oven temperature was held at 45℃ for 5 min, increased

to 270℃ at a rate of 4℃/min, and then held for 5 min. The injector was held at 270℃ and operated in split mode (50:1). In some cases, injections were made in splitless mode to obtain maximum sensitivity for metabolite detection.

7.2.4 ^{13}C-bicarbonate incorporation

To test whether carboxylation was the mechanism involved in the initial microbial activation of naphthalene, new incubations were prepared with ^{13}C-bicarbonate, as a co-substrate. A microbial pellet from an established naphthalene-degrading culture was collected by centrifugation at 12 000 × *g* for 10 min, and resuspended in fresh anoxic mineral salts medium. Collected biomass (5 mL) was distributed into 70 mL of sterile anaerobic medium that was buffered with 25 mM of HEPES instead of bicarbonate, and flushed under N_2. Incubations were amended with 3 mL of a HMN layer containing 7.5 mg of naphthalene, and 23 mM of $NaH^{13}CO_3$ (Cambridge Isotope Laboratories Inc., Andover, MA, USA). Cultures were incubated at 22℃ for over a year and metabolites were extracted and analyzed by GC-MS in the same way as described above.

7.2.5 DNA extraction and sequencing analysis

Liquid samples (4 mL) from the naphthalene and 1-MN degrading cultures were collected, and their cell pellets centrifuged at 17 000 × *g* for 5 minutes. Genomic DNA was isolated using a commercially available kit (FastDNA Spin Kit for Soil; MP Biomedicals) and quantified with a Qubit Fluorometer using a dsDNA HS assay kit (Invitrogen, Carlsbad, USA). The 16S rRNA genes from isolated DNA were PCR amplified using a two round PCR method of 25 and 10 cycles. For the initial PCR method, universal primers 926F and 1392F (as described

in Chapter 5) were used at the following conditions: 95 °C, 3 min; 25 cycles of 95°C 30 s, 55°C 45 s, 72°C 90 s; 72°C 10 min; final hold at 4°C. For the 10-cycle PCR method FLX Titanium amplicon primers 454T-RA and 454T-FB (20 pmol μL^{-1}) were used. PCR products were purified with a MP purification kit (MP Biomedicals, Santa Ana, USA) and then quantified with a Qubit flourometer (Invitrogen, Carlsbad, USA). Prepared DNA was sent to McGill University and Genome Quebec Innovation Centre for pyrotag sequencing analysis with a GS FLX Titanium Series kit (Roche Diagnostics Corporation). Sequencing data was analyzed using an in-house SSU rRNA pipeline data system (Phoenix 2) as described by Soh *et al.* (2013). For this study taxonomic annotation results done with RDP classifier on the Silva SSU rRNA database were used at 5% cutoff clustering distance.

7.2.6 Screening for functional genes

Functional genes involved in the degradation of PAHs by fumarate addition (*nmsA*) or carboxylation were assayed for by PCR in isolated DNA from enrichment cultures amended with naphthalene. PCR amplification was conducted using three available primer sets (von Netzer *et al.*, 2013) targeting fumarate addition genes coding naphthylmethylsuccinate synthase (NMS), *nmsA*1F (TCGCCGACAATTTCGAYTTG), *nmsA*2F (TTCGAYTTGAGCGACAGCGT) and *nmsA*3F (CAAYGATTTAACCRACGCCAT) with the reverse primer *nmsA*R (TCGTCRTTGCCCCAYTTNGG). The PCR reaction (50 µL) with *nmsA* primer was conducted with the following program: initial denaturation at 95°C, 3 min; 35 cycles of 94°C 30 s, annealing at 58°C 30 s, 72°C 1 min; final extension at 72 °C 5 min, and final hold at 4 °C. DNA was also screened with a primer set designed in our lab (Dr. Jane Fowler, unpublished) to target

benzylcarboxylase coding genes, benCarbF (GTGGTCTTCGCACCGTTAAT) and benCarbR (TCTCCGGTAACTGGGTGTTC). The PCR reaction (25 µL) with the carboxylase primers was run with the following program: denaturation at 95°C, 5 min; 25 cycles of 95°C 30 s, annealing at 54°C 45 s, 72°C 40 min; final extension at 72°C 7 min; final hold at 4°C.

7.3 Results

7.3.1 Methanogenic activity of the PAH-degrading cultures

Methane production was enhanced in the naphthalene-amended incubations relative to unamended controls (Figure 7-1) and after incubation at 30℃. After 320 days of incubation, methane production in the cultures amended with naphthalene produced up to 120 µmol of CH_4 relative to controls without naphthalene. According to stoichiometric calculations (Table 7-1), the cultures produced 37% of the expected methane for the complete degradation of the substrate (56 µmol). Even though CH_4 production was also observed in the "substrate unamended controls", naphthalene was shown to be depleted in those incubations amended with the PAH. Stoichiometric calculations indicated (Table 7-1) that the amount of naphthalene that was consumed was in agreement with the amount of methane produced in the naphthalene-amended cultures (relative to unamended control). It was hypothesized that the CH_4 produced in the "substrate-free" controls was formed from the degradation of the cysteine that was added to the medium as a reducing agent. From the amount of cysteine added to these incubations (286 µmol), it was calculated that 357 µmol of CH_4 could be produced (Table 7-2). In the "substrate-free" controls up to 236 µmol of CH_4 were observed corresponding to 66% of the CH_4 predicted from the degradation of the added cysteine. However, cysteine loss was not monitored to conclusively confirm that CH_4 produced in the controls was formed from cysteine utilization.

Other possible carbon sources in the medium could be small amount of naphthalene likely introduced into the "substrate-free" controls from the inoculum during the transfer of the culture. GC-MS analysis revealed that about a third of the naphthalene amended in the cultures was present in the incubations labelled as "substrate-free" controls. Thus, from this amount of naphthalene up to 117 µmol of CH_4 should be produced. It is also possible that the microbial culture might be utilizing dead biomass accumulated in the culture after various transfers. Further studies are necessary to conclusively confirm the reason for the methane production in the substrate-unamended controls. Nevertheless, for the following transfers the cysteine used as a reducing agent in the medium was replaced with Na_2S in all the PAH-degrading cultures.

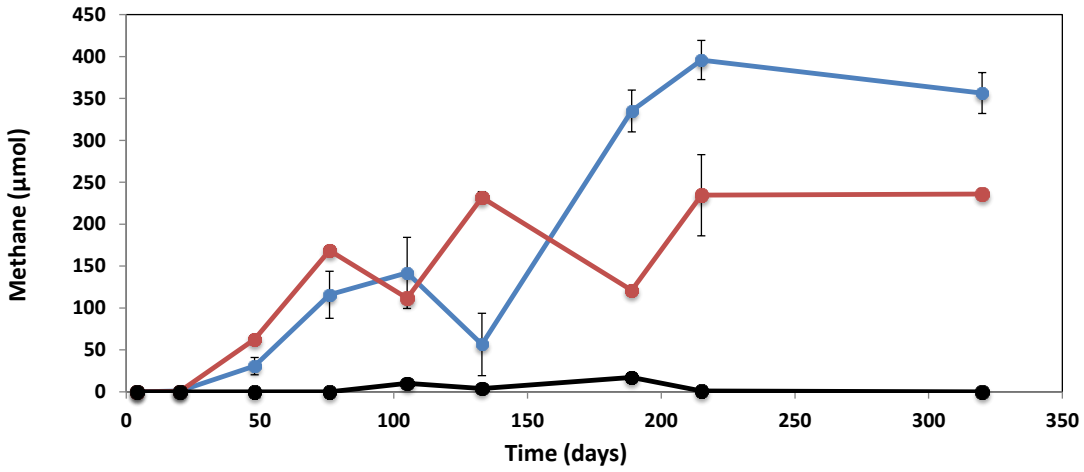

Figure 7-1. Methane production from incubations amended with naphthalene (blue), "substrate-unamended" controls (red) and sterile controls (black). Error bars were calculated from standard error of duplicate incubations.

Table 7-1. Stoichiometry of naphthalene degradation coupled to methane production. Calculations based on the results from duplicate samples.

$C_{10}H_8 + 8H_2O \rightarrow 6CH_4 + 4HCO_3^- + 4H^+$					
Incubation time	**Naphthalene loss (µmol)[a]**	**Expected CH_4 (µmol)**	**Total CH_4 in amended (µmol)**	**Calculated CH_4 in amended (µmol)[b]**	**Predicted CH_4 recovered (%)[c]**
320 days	20.5 ±6	334 ±26	356 ±30	120 ±22	36.8 ±9

[a] Based on the amount of substrate amended (7.1 ±0.5 mg; 56 ±4 µmol naphthalene)

[b] Calculated by substracting the amount of CH_4 observed in unamended from the total CH_4 in amended

[c] From the amount of naphthalene loss

Table 7-2. Stoichiometry of cysteine degradation coupled to methane production. Calculations based on the results from duplicate samples.

$4C_3H_8O_2SN^+ + 6H_2O \rightarrow 5CH_4 + 7CO_2 + 4NH_4^+ + 4\ H_2S$			
Incubation time	**Expected CH_4 (µmol)[a]**	**Observed CH_4 in controls (µmol)**	**Predicted CH_4 recovered (%)**
320 days	357	236 ±8	66

[a] Based on the amount of cysteine amended (35 mg; 286 µmol)

The microbial enrichment amended with 1-MN was also stimulated by incubation at 30℃ (Figure E-1), but the CH_4 production rate of the enrichment was still low (0.08 µmol CH_4 /day) compared to the CH_4 production rate of the naphthalene-degrading culture (0.96 µmol CH_4/day). This was in accordance to previous studies with methanogenic enrichments that reported an increased PAH removal when incubations were grown at higher temperatures (Trably *et al.*, 2003; Christensen *et al.*, 2004).

7.3.2 Metabolites in PAH-degrading cultures

Despite the slow growth of 1-MN degrading incubations, those bottles with the highest CH_4 production were subject to metabolite extraction. GC-MS analysis of the extracts obtained from this culture revealed the presence of a metabolite (Figure 7-2) that matches the MS profile and retention time of a 1-naphthoic acid standard. This compound was not found in extracts from controls. 2-Methylnaphthoic acid was detected in sulfate-reducing cultures able to degrade naphthalene and 2-methylnaphthalene (Zhang and Young, 1997; Meckenstock *et al*., 2000), and we previously detected 2-naphthoic acid in methanogenic cultures able to degrade 2-MN (Berdugo-Clavijo *et al*., 2012). Thus, our result shows that 1-methylnaphthoic acid is formed from 1-MN, indicating that despite the long doubling time of the culture (based on low CH_4 production rates), the enrichment was able to biotransform 1-MN.

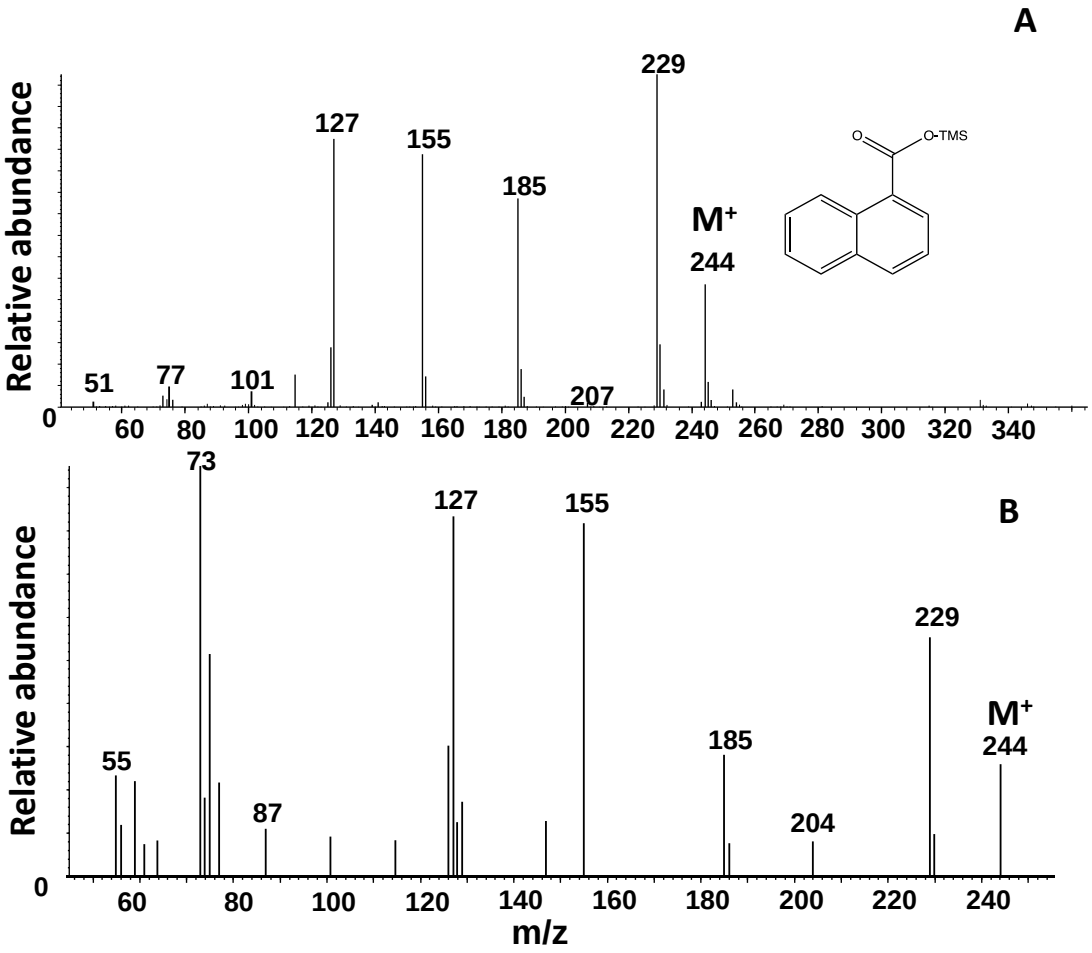

Figure 7-2. Mass spectral profiles (analyzed as TMS esters) of 1-naphthoic acid authentic standard (A) and a tentatively detected naphthoic acid in the 1-MN-degrading culture (B).

Repeated extractions of supernatants from cultures amended with naphthalene were conducted to identify putative metabolites that may be formed during the degradation of naphthalene by carboxylation or methylation reactions (Figure 7-3). Naphthoic acid (IV) or methylated naphthalene (II) were not detected in samples extracted from the naphthalene-degrading culture. Also, metabolites associated with the fumarate addition pathway such as naphthyl-2-methylsuccinate (III) were not detected in the extracted samples from the naphthalene-amended culture. Naphthol (I) was detected in various extractions, but it was also found in sterile controls containing naphthalene, indicating the abiotic formation of this compound. However, GC-MS analysis revealed the presence of various compounds that were not found in sterile or unamended controls, and thus may be metabolites formed during the anaerobic degradation of naphthalene under methanogenic conditions (Table 7-2). For example, a cyclohexane-diacid compound ($C_{11}H_{14}O_4$-diacid) was tentatively detected in the culture. This metabolite was 2 mass units lower than a $C_{11}H_{16}O_4$-diacid detected in a sulfate-reducing culture able to degrade naphthalene (Annweiler *et al*., 2002) (Figure 7-3, compound X). It is hypothesized that $C_{11}H_{14}O_4$-diacid (in our culture) formed from the reduction of 5,6,7,8-tetrahydro-2-naphthoic acid (V). However, the latter metabolite or the further degradation product, octahydro-naphthoic acid (VI) were not detected in the extracts from the naphthalene-degrading culture. A metabolite with the mass spectral profile of decahydro-2-naphthoic acid (m/z 254, 239, Figure 7-3, compound VII) was tentatively detected, but the retention time of this compound was found a minute higher than that of the authentic standard, suggesting that it is a possible stereoisomer. In addition, a monoaromatic compound with MS fragments similar to hydroxyl-methylbenzoic acid (Table 7-2), was also detected in some extracted samples.

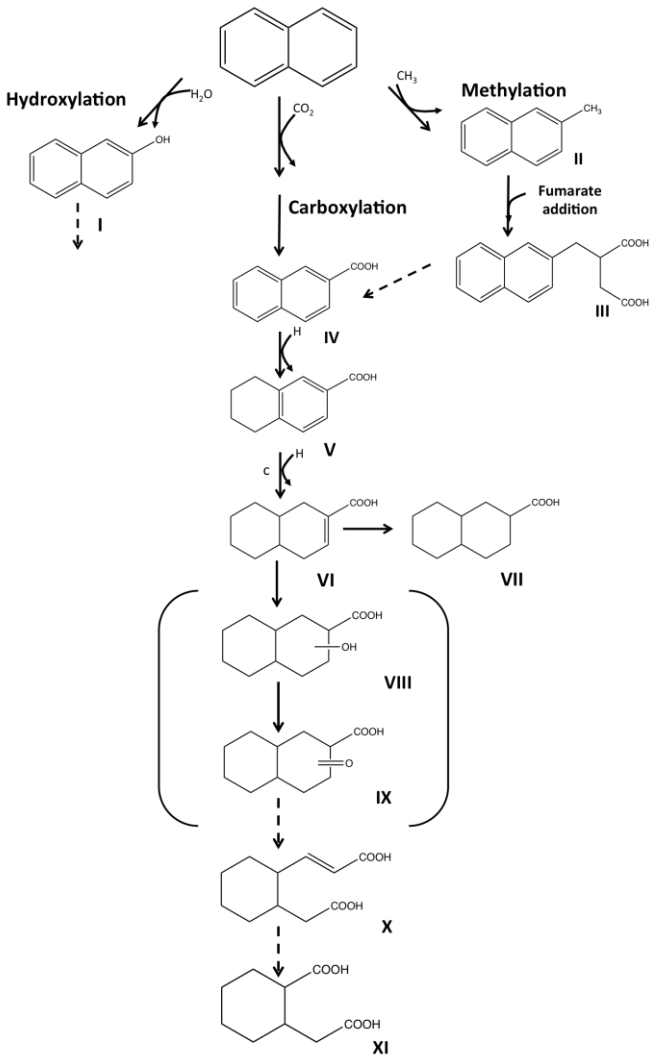

Figure 7-3. Proposed metabolic pathways for the initial biodegradation of naphthalene based on prior studies with sulfate-reducing cultures (Bedessem *et al.*, 1997; Annweiler *et al.*, 2002; Safinowski and Meckenstock, 2006). I) naphthol, II) methylnaphthalene, III) naphthyl-2-methylsuccinate, IV) 2-naphthoic acid V) 5,6,7,8-tetrahydro-naphthoic acid, VI) octahydro-naphthoic acid, VII) decahydro-naphthoic acid, VIII) hydroxy-decahydro-naphthoic-acid, IX) oxo-decahydro-naphthoic acid, X) $C_{11}H_{16}O_4$-diacid, XI) carboxycyclohexyl acetic acid. Metabolites VIII and IX have not been detected in cultures (theoretical).

Table 7-3. Metabolites detected in extracts from microbial cultures amended with naphthalene and 1-MN

Metabolite detected in enrichments			
Characteristic m/z	**Naph**	**1-MN**	**Tentatively identified metabolite**
73, 127, 155, 185, **229, 244**		X	1-naphthoic acid
73, 129, 145, 161, 176, 189, **239, 254**	X		Decahydronaphthoic acid
73, 103, 129, 178, 251, **339, 354**	X		$C_{11}H_{12}O_4$-diacid
73, 147, 195, 207, 237, **281, 296**	X		Hydroxy-methylbenzoic acid
73, 111, 147, 173, 201, **213, 228**	X		unknown
73, 91, 129, 147, 192, **279, 294**	X		unknown

These findings suggests the occurrence of two possible metabolic mechanisms in the reduction of the aromatic ring of naphthalene via formation of a cyclohexane ring structure with two carboxylic acids (Figure 7-4A) or via the benzoyl-CoA pathway (Figure 7-4B) possibly from the reduction of 1,2,3,4 tetrahydro-2-naphthoic acid. Annweiler *et al*. (2002) suggested that the ring cleavage of the naphthoic acid structure from naphthalene degradation proceed via the formation of 5,6,7,8-tetrahydro-2-naphthoic acid that was further reduced to a cyclohexane ring with two carboxylic acid side chains. In fact, a naphthoyl-CoA-reductase enzyme (NCR) has been identified (Selesi *et al*., 2010) and characterized (Eberlein *et al*., 2013) in the N47 culture. As genes involved in the anoxic benzoate degradation (*bamB* to *bamI*) were not detected in the culture, it was concluded that a monocyclic reduction (e.g. benzoyl-CoA pathway) does not occur in N47 (Selesi *et al*., 2010). In contrast, DiDonato *et al*. (2010) identified reductases in the NaphS2 culture that showed similarity to some *Bam* subunits, suggesting a benzoate metabolism. Other compounds with fragment ions indicative of silylated metabolites (e.g., m/z M^+-15 and other key fragment ions (e.g.,73)) were detected in the extracts of naphthalene-degrading enrichments (not in controls), but their identity remains unknown (Table 7-2).

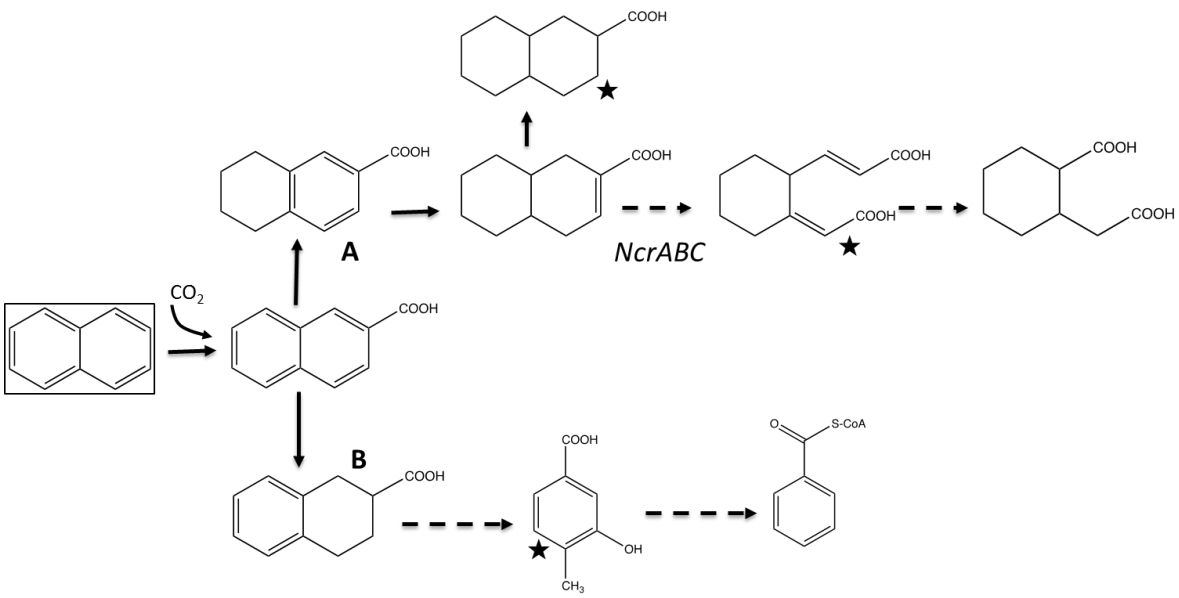

Figure 7-4. Proposed metabolic routes involved in the degradation of naphthalene and reduction of naphthoic acid (naphthoyl-CoA) based on detected metabolites via cyclohexanoic acid formation (A) or benzoyl-CoA pathway (B). Stars indicate the metabolites that have been tentatively identified in the methanogenic naphthalene-degrading culture.

Experiments with ^{13}C-bicarbonate were conducted with actively growing enrichments to test for the incorporation of COO^- to naphthalene as an indication of the carboxylation pathway (Figure 7-3). ^{13}C-labeled metabolites were not found in enrichment cultures amended with ^{13}C-bicarbonate after repeated incubations and extractions, suggesting that carboxylation is not involved in the initial activation of the naphthalene in the enrichment culture described here. Again, it is possible that the products were formed, but they were too low (< 2 nmol) to be detected by GC-MS analysis. Metabolite detection in methanogenic enrichment cultures can be limited by the low biomass and slow degradation rate of the enrichments. Enhancing the CH_4 production in the naphthalene culture may help to accumulate microbial products and contribute to identifying putative metabolites.

7.3.3 Functional gene analysis

In accordance with the metabolite analysis, DNA amplification from enrichment culture amended with naphthalene was not observed with primer sets targeting fumarate addition genes (*nmsA*), suggesting that methylnaphthalene, expected to be formed from the methylation of naphthalene (Safinowski and Meckenstock, 2006), was not activated via fumarate addition. More specifically, these findings show that methylation was probably not the mechanism used in the initial activation of naphthalene. A carboxylation reaction was previously demonstrated for the degradation of naphthalene under sulfate-reducing conditions (Zhang and Young, 1997), thus in this study the presence of carboxylase genes in the culture amended with naphthalene was assayed. No DNA amplification was observed using carboxylase primers. Although primers used in this study were designed to target benzyl carboxylase coding genes found in a toluene degrading methanogenic enrichment, they were expected to amplify DNA with our naphthalene

culture. Bergmann *et al.* (2011) found that the protein sequence of a naphthalene carboxylase alpha-subunit detected in the N47 culture was 48% identical to a benzene carboxylase sequence, and 45% similar to a phenylcarboxylase sequence. It is possible that the primers used in this study were not specific enough to amplify the naphthalene carboxylase coding genes in the culture. However, our gene analysis results were in agreement with metabolite analysis conducted with the ^{13}C-bicarbonate-amended incubations that shows no evidence of carboxylation metabolites formed in the naphthalene-amended culture. Thus, these findings suggest that carboxylation was not the initial activation mechanism for the degradation of naphthalene in our culture. However, it is still important to test whether specific naphthalene carboxylase coding genes are present or not in the PAH-degrading culture to conclusively rule out carboxylation reaction occurring in the culture. Specific primers can be designed based on previously identified sequences (if available) of naphthalene carboxylases obtained from a combination of proteomic and genomic analyses (Bergmann *et al*., 2011) on a sulfate-reducing culture N47 able to degrade naphthalene.

7.3.4 Microbial community analysis

The enrichments amended with naphthalene or 1-MN had a similar microbial community composition. At the phylum level (Figure 7-5), the communities were dominated by members of the *Firmicute*s, followed by *Euryarchaeota* and *Proteobacteria*. Other phyla detected at a lower abundance included *Synergistetes, Chloroflexi, Spirochaetes,* and *Bacteriodetes. Firmicutes,* specifically members of the *Clostridia* class have previously been found to dominate in methanogenic enrichments able to degrade aromatic hydrocarbons such as toluene, naphthalene, and phenanthrene (Chang *et al.,* 2005; Yuan and Chang, 2007; Fowler *et al.*, 2012). The

importance of the *Firmicutes* has also been noted in a survey conducted with over 3000 16S rRNA sequences from 26 different hydrocarbon-related communities (Gray *et al.*, 2010), here sequences belonging to the *Firmicutes* phylum were the most frequently detected.

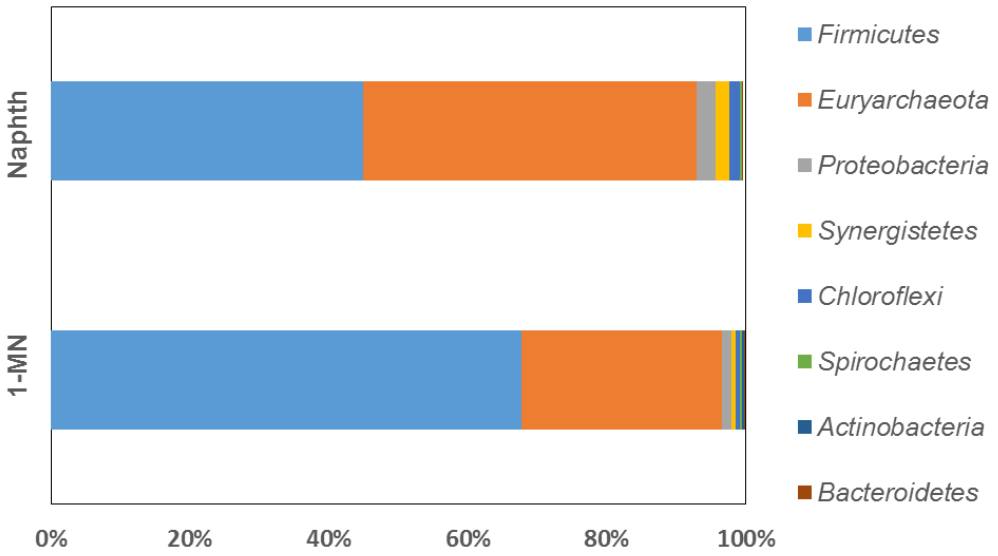

Figure 7-5. Microbial community of methanogenic cultures amended with naphthalene (Naphth) and 1-MN assayed by pyrotag sequencing. Percentages are based on the number of reads (≥ 0.1%) identified at the phylum level.

At the genus level, microbial members most dominant in both enrichment cultures were mainly organisms affiliated with the genus *Clostridium* (Table 7-3). A number of studies have directly identified hydrocarbon-degraders within the *Firmicutes* phylum. Winderl *et al.* (2010) observed using DNA-SIP analysis that *Desulfosporosinus* (within *Clostridiales* order) was the main toluene-degrader in a community of iron- and sulfate-reducing bacteria from a contaminated aquifer. RNA-SIP along with RT-qPCR analyses revealed that *Desulfosporosinus* was the toluene-degrader in a methanogenic toluene-degrading consortium (Fowler *et al.*, 2014). The same phylotype was also detected by DNA-SIP in another toluene-degrading enrichment growing under methanogenic conditions (Sun *et al.*, 2014). Other *Firmicutes* members that were found in the enrichments in lower relative abundances include *Anaerobacter* and an unidentified member of the order *Ruminococcaceae* (Table 7-3). However, these organisms more likely play a role as secondary fermenters or scavengers (Kleinsteuber *et al.*, 2012). Furthermore, *Desulfobulbus* and *Smithella* genera were found in higher relative abundance in the 1-MN amended culture (Table 7-3). Organisms within *Desulfobulbaceae* have been detected in benzene-degrading cultures under sulfate- and iron- reducing conditions (Kunapuli *et al.*, 2007; Herrmann *et al.*, 2010). Also, a strain of the *Desulfobulbaceae* family was shown to degrade toluene within a syntrophic coculture (Meckenstock, 1999). However, members of this taxon are also known to utilize H_2 and grow in syntrophy with hydrocarbon degraders (Kleinsteuber *et al.*, 2012). Members of the family *Syntrophaceae* including *Smithella* and the close relative *Syntrophus* have been identified by DNA-SIP or qPCR analyses as alkane (Gray *et al.*, 2011; Cheng *et al.*, 2013) or benzene (Sakai *et al.*, 2009) degrading organisms. It is possible that *Smithella* was the organism activating 1-MN in the enrichment culture, however this has to be further evaluated as *Clostridium* had a much higher relative abundance in the culture. Other

bacterial members found in lower abundance in the two microbial enrichments were *Geobacter* and *Desulfovibrio* (Table 7-3), which may be playing a role as synthrophs within the microbial consortia. Furthermore, *Proteiniphilum* was found in low relative abundance (~0.1%) in both enrichment cultures. Although members of this genus are commonly known as a protein-utilizing bacterium, *Proteiniphilum* has also been associated with PAH biodegradation. *Proteiniphilum acetatigenes* was isolated from activated sludge from a wastewater treatment plant, and was used to remediate PAHs (Larsen *et al.*, 2009). This may suggest that *Proteiniphilum* may play an important role in naphthalene-degradation, however other approaches are necessary to arrive to this conclusion as no other studies have reported the ability of this bacterium to degrade PAHs. Methanogens were also an important part of the community in the enrichment cultures. The most predominant methanogen member in both cultures was affiliated to the genus *Methanosaeta*, an acetate-utilizing methanogen (Table 7-3). In the naphthalene-degrading culture the genus with the next most relative abundance (14%) was *Methanoculleus*, a hydrogen-utilizing methanogen. The methanogen *Methanomethylovorans* was found in lower abundance in the 1-MN-degrading culture. These results suggest the importance of acetate metabolism in the enrichment cultures amended with naphthalene or 1-MN.

Table 7-4. Phylogenetic affiliations of microbial reads assessed by pyrotag sequencing analysis of 16S rRNA genes in the 1-MN and naphthalene (Naph) degrading cultures. Percentages are based on the number of reads (≥ 1%) identified at the genus level.

Lineage	% of microbial reads	
Sample	**1-MN**	**Naph**
Clostridium	66.71	43.58
Methanosaeta	26.45	30.17
Methanomethylovorans	0.93	<0.1
Methanoculleus	0.87	14.76
Geobacter	0.45	0.66
Desulfobulbus	0.28	<0.1
Methanobacterium	0.26	1.94
Desulfovibrio	0.24	1.38
Smithella	0.18	<0.1
Proteiniphilum	0.16	0.13
Anaerobacter	0.12	<0.1
Spirochaeta	<0.1	0.26
Syntrophorhabdus	<0.1	0.13
Desulfuromonas	<0.1	0.10

7.4 Discussion

In this work, enrichment cultures able to degrade naphthalene and 1-MN were established. The enrichments amended with 1-MN showed a lower methane production rate than the cultures growing with naphthalene. However 1-naphthoic acid was formed from the degradation of 1-MN, as an indication that this PAH was biotransformed. Reports on anaerobic degradation of 1-MN are very scarce. Kleemann and Meckenstock (2011) showed an iron-reducing culture (N49) enriched from sediments of a tar-oil contaminated site that was able to utilize 1-MN. However, the N47 SRB culture enriched from the same site, and able to degrade 2-MN, was not able to utilize 1-MN (Meckenstock *et al.*, 2000). To our knowledge, the biodegradability of 1-MN compared to the isomer 2-MN has not been investigated. According to our results, the low CH_4 production rate observed in the culture amended with 1-MN compared to the culture amended with 2-MN (Chapter 5), suggests that 1-MN is harder to activate than 2-MN under methanogenic conditions.

In this study, the loss of naphthalene coupled to methane production was also demonstrated. Initially, this enrichment did not produce higher amounts of CH_4 relative to the controls (Chapter 5). However, after transferring and incubating the culture at a slightly higher temperature (30℃), the methanogenic activity of this enrichment was enhanced (Figure 7-1). Although putative metabolites characteristic of proposed metabolic pathways (e.g., carboxylation or methylation) were not detected in the cultures, compounds formed from the reduction of the naphthoic acid ring via benzoyl-CoA or cyclohexanoic acid pathways were tentatively identified. *Firmicutes*, specifically members of the *Clostridium* genus, dominated the microbial community in both cultures, suggesting the role of this organism in the degradation of naphthalene and 1-MN. Also, the high relative abundance of acetate-utilizing methanogens (*Methanosaeta*)

suggests the importance of acetate metabolism in the enrichments. These microbial community results align with those found for 2-MN and 2, 6-diMN methanogenic degradation (Chapter 5). While pyrotag sequencing showed a general characterization of the communities in the enrichments, other approaches such as qPCR and SIP would help to specifically identify the key organisms in the naphthalene amended cultures. In addition, it is expected that further analysis with larger incubation samples (at least 500 mL) will help to identify key metabolites in the degradation of naphthalene and elucidate main metabolic pathways.

Chapter Eight: Conclusions and future directions

The main goal of this dissertation work was to investigate the methanogenic biodegradation of crude oil components with an emphasis on PAH. Three main objectives were established to guide this work: 1) Identify and characterize metabolic mechanisms involved in crude oil and PAH biodegradation under methanogenic conditions, 2) Characterize microbial communities and key microorganisms involved in methanogenic degradation of crude oil and PAH, and 3) assess the feasibility of microbial communities to bioconvert hydrocarbon components into methane in marginal oil reservoir-simulating systems. These objectives were executed using different approaches including establishing hydrocarbon degrading cultures, hydrocarbon metabolomics, 16S rRNA pyrotag gene sequencing, and molecular biology methods (e.g., cloning, PCR, and qPCR analysis).

8.1 Key findings and conclusions

The study of microbial communities exposed to hydrocarbon-laden environments such as crude oil contaminated areas or petroleum reservoirs, allows for the investigation of hydrocarbon biodegradation processes occurring in the environment. Methanogenic consortia enriched from produced waters of a low temperature oil reservoir were found to be capable of utilizing light and heavy crude oil components (Chapter 3). Metabolites known to be formed under anoxic conditions during the activation and further degradation of *n*-alkanes and monoaromatic hydrocarbons were identified in the enrichments. In addition, the results showed that the enrichment culture amended with light oil can activate alkanes and aromatic hydrocarbons via the addition of fumarate (indicated by the detection of *bssA* and *assA genes* and alkylsuccinic

acids). In the latter enrichment the most dominant bacterial phylotype was closely related to *Smithella,* which has been associated with alkane degradation in other methanogenic cultures. The *assA* gene identified in our enrichment culture showed high sequence identity to published *assA* gene identified in the genome of *Smithella,* which is able to degrade branched alkanes (Tan *et al.*, 2013; Tan *et al.*, 2014). This suggests that *Smithella* is likely able to degrade the alkane compounds in our enrichment culture amended with light oil. Crude oil bioconversion to CH_4 was further investigated in sandstone-packed column systems simulating sediment rock reservoirs (Chapter 4). Three tested microbial inocula incubated in sandstone-packed columns showed enhanced levels of CH_4 compared to controls and corresponding to the loss of several hydrocarbon components in crude oil. The enrichment culture developed from produced waters (Chapter 3), stimulated the highest CH_4 production in the columns. Also, this work showed that the composition and abundance of microbial communities growing in planktonic systems (cultures) were different from the microbes enriched in communities growing in sessile environments (sandstone-packed columns). Surprisingly, microorganisms commonly found in aerobic environments (e.g., *Pseudomonas* and *Halomonas*) were enriched in the columns despite incubation under strict anoxic conditions. Further, the high relative abundances of *Pseudomonas* and *Methanoculleus* (H_2-using methanogen) in the columns amended with the produced water-derived oil-degrading culture suggests the ability of these organisms to grow syntrophically in a consortium.

In addition to studying methanogenic biodegradation with whole crude oils, this work explored the metabolism of PAH under methanogenic conditions. From a crude oil-degrading culture established from the sediments of a contaminated aquifer (Gieg *et al*., 2008), microbial cultures able to degrade 2-ringed aromatic compounds including methylnaphthalene isomers (2-

MN, 1-MN and 2, 6-diMN) and naphthalene (Chapter 5 and Chapter 7) were established. Methanogenic activity along with mass balance calculations allowed us to characterize these cultures. Naphthoic acids, considered central metabolites in PAH degradation, were detected in the cultures amended with methylnaphthalenes. Although fumarate addition metabolites were not found in these cultures, *nmsA* and *bssA* genes coding for enzymes catalyzing the addition of fumarate were amplified in the 2-MN and 2, 6-diMN-amended cultures (Chapter 6), suggesting that this mechanism is possible during methanogenic PAH metabolism. According to the microbial characterization of the cultures, key organisms in the enrichments belonged primarily to the phyla *Firmicutes, Proteobacteria,* and *Euryarchaeota* (based on their high relative abundances). In the 2-MN and 2, 6-diMN amended enrichments, methanogens related to *Methanosaeta* and *Methanoculleus* were dominant and present in similar abundances, thus both acetate- and hydrogen-utilizing methanogens may be playing an important role in the syntrophic metabolism of the 2-ringed PAH. In addition, all the PAH-degrading enrichments from this study were dominated by organisms closely related to *Clostridium* (Chapter 5 & Chapter 7). Further qPCR analysis with the 2-MN amended culture showed that an increase in the abundance of *Clostridium* over time was coupled to the production of CH_4 (Chapter 6), suggesting that this bacterium is a key hydrocarbon degrader in this enrichment.

In summary, the work presented in this thesis has contributed with new knowledge to the field of anaerobic hydrocarbon biodegradation. Notably, the work conducted here has: 1) established new methanogenic enrichment cultures able to degrade a variety of PAHs and crude oil components allowing for the study of hydrocarbon metabolism, 2) directly demonstrated the biodegradation of two-ringed PAHs in the absence of electron acceptors, using the model hydrocarbons naphthalene, 2-MN, 2,6-diMN and 1-MN, 3) provided proof of principle for the

microbial conversion of crude oil to methane in sandstone column systems simulating oil reservoirs, giving insight of the microbial communities that are able to grow in this system while metabolizing crude oil, 4) identified microorganisms involved in PAH and crude oil degradation under methanogenic conditions, and 5) identified metabolites and genes potentially involved in the methanogenic biodegradation of alkanes, monoaromatic hydrocarbons, and PAH.

8.2 Future directions

The methanogenic biodegradation of hydrocarbon compounds, especially the metabolism of alkanes and PAH, is only starting to be explored. Thus, there is still a wide research potential to improve our understanding of how microbial consortia are able to metabolize different hydrocarbon components when electron acceptors are scarce or absent. From the work conducted in this dissertation, the following future research directions are proposed:

8.2.1 Further investigation of the fumarate addition reaction

Although fumarate addition genes (e.g., *assA* and *bssA*) were identified in a culture amended with light oil, the demonstration that these genes are transiently increasing over time when growing with hydrocarbons will add a stronger evidence of the role of the glycyl radical enzymes in catalyzing hydrocarbon metabolism under methanogenic conditions. Thus, the specific quantification and expression of the *assA* and *bssA* genes overtime by RT-qPCR analysis specifically amended with a model *n*-alkane (e.g., hexane) and/or a mono-aromatic hydrocarbon (e.g., toluene) is suggested as a future approach to continue the investigation of this enrichment.

8.2.2 Investigation of microbial sessile communities

Facultative anaerobic bacteria, especially *Pseudomonas* and *Halomonas,* were enriched in strictly anoxic communities growing in sandstone-packed columns. Thus, it is suggested that the proliferation of these organisms in porous rock environments (e.g. sandstone) is related to the ability of these microbes to form biofilms. The sessile growth of *Pseudomonas* or *Halomonas,* and their ability to form biofilms can be further evaluated on the same culture used to inoculate the sandstone-column, by using fluorescence microscopy. Testing the activity of the oil-degrading culture in an oil reservoir-like environment will also help to investigate whether a syntrophic relationship between *Pseudomonas* and methanogens is possible in a strictly anoxic system.

8.2.3 Expanding our knowledge on methanogenic PAH activation

Identifying the main mechanisms of hydrocarbon activation that are occurring in the PAH-degrading cultures would be of great importance to advance in the area of anaerobic hydrocarbon degradation. The information gained in this study (e.g. via gene analysis) suggests that the methanogenic activation of 2-ringed PAHs (e.g., 2-MN and 2, 6-diMN) can possibly occur via fumarate addition. If the presence of *nmsA* encoding gene(s) is conclusively confirmed (by PCR optimization, sequencing, and BLAST), it is proposed that reverse transcriptome-qPCR should be used to determine whether the *nmsA* gene is expressed and if it increases over time in the 2MN-degrading cultures. Also, it is believed that obtaining a metagenome of the 2-MN-degrading culture will provide a great advantage to understanding the genetic capabilities of the culture able to degrade PAH under methanogenic conditions.

In the culture amended with naphthalene, the metabolite analysis conducted with the ^{13}C-bicarbonate experiment did not show evidence that the initial activation mechanism for naphthalene was via carboxylation. However, no evidence of other activation pathway was found. Developing carboxylase primers, based on the carboxylase-protein sequences recently obtained in the SRB N47 culture (Mouttaki *et al*., 2012) for PCR analysis will help to confirm if the carboxylation pathway can occur in the naphthalene-amended culture. Also, as ^{13}C-naphthalene is more commercially available that the other 2-ring PAH (e.g., 2-MN), it is proposed that a DNA-SIP approach can be conducted with this substrate to confirm if *Clostridium* (bacterial OTU with the highest abundance in the culture) is the main degrader in the culture amended with naphthalene or other PAHs.

In addition to identifying the organism(s) that activate PAH in our enrichment cultures, it is important to investigate the role of specific methanogens (acetate or H_2-utilizers) in syntrophic microbial associations degrading PAHs. This can be achieved by quantification of 16S rRNA genes of methanogens such as *Methanosaeta* and *Methanoculleus* (by qPCR), which dominated in the 2-ringed PAH degrading enrichments.

8.2.4 Improving cultivation of methanogenic hydrocarbon-degrading cultures

Methanogenic communities degrading hydrocarbons usually grow with a very low energy yield, so their metabolic processes can be very slow, and their cultivation can require long incubation times. This issue slows the pace of research in this area. The study of methanogenic communities can be facilitated by improving their metabolic activities via optimizing cultivation procedures. Experiments are currently being conducted in our lab to enhance methanogenic degradation with the addition of nutrients such as yeast extract (by Courtney Toth). It is proposed

that improving the substrate and nutrient uptake of the microbes will also help to stimulate the activity of the methanogenic communities in the cultures. The activity of microorganisms can be enhanced by adding small beads/resins that offer small surface-areas for the microbes to attach and obtain the hydrocarbons. Initial cultivation of the PAH-degrading cultures in this study was conducted by absorbing the substrates in amberlite-resin (beads). This allowed for the development of methanogenic activity in the cultures. Continuous cultivation with a solid support may help to speed up the rates of hydrocarbon degradation. Another challenge in studying methanogenic hydrocarbon-degrading cultures is to maintain the hydrocarbon degrading capabilities over time. The future (on-going) scaling-up of the cultures will not only avoid constant transferring, but also may facilitate the detection of metabolites. In addition, the information gain from the metagenome of a PAH-degrading culture (e.g., 2-MN enrichment described in this thesis) will potentially help to develop better cultivation strategies in the PAH-degrading cultures, allowing for more efficient ways to study of methanogenic hydrocarbon degradation.

References

Abbai, N., Govender, A., Shaik, R., and Pillay, B. (2012). Pyrosequence analysis of unamplified and whole genome amplified DNA from hydrocarbon-contaminated groundwater. *Mol. Biotechnol.* 50, 39-48.

Abu Laban, N., Selesi, D., Rattei, T., Tischler, P., and Meckenstock, R.U. (2010). Identification of enzymes involved in anaerobic benzene degradation by a strictly anaerobic iron-reducing enrichment culture. *Environ. Microbiol.* 12**,** 2783-2796.

Agrawal, A., Park, H.S., Nathoo, S., Gieg, L.M., Jack, T.R., Miner, K., Ertmoed, R., Benko, A., and Voordouw, G. (2012). Toluene depletion in produced oil contributes to souring control in a field subjected to nitrate injection. *Environ. Sci. Technol.* 46**,** 1285-1292.

Agrawal, A., and Gieg, L.M. (2013). *In situ* detection of anaerobic alkane metabolites in subsurface environments. *Front. Microbiol.* 140**,** 1-11.

Agrawal, A., An, D., Cavallaro, A., and Voordouw, G. (2014). Souring in low-temperature surface facilities of two high-temperature Argentinian oil fields. *Appl. Microbiol. Biotechnol.* 98**,** 8017-8029.

Aitken, C.M., Jones, D.M., and Larter, S.R. (2004). Anaerobic hydrocarbon biodegradation in deep subsurface oil reservoirs. *Nature* 431**,** 291-294.

Aitken, C.M., Jones, D.M., Maguire, M.J., Gray, N.D., Sherry, A., Bowler, B.F.J., Ditchfield, A.K., Larter, S.R., and Head, I.M. (2013). Evidence that crude oil alkane activation proceeds by different mechanisms under sulfate-reducing and methanogenic conditions. *Geochim. Cosmochim. Acta* 109**,** 162-174.

An, D., Caffrey, S.M., Soh, J., Agrawal, A., Brown, D., Budwill, K., Dong, X., Dunfield, P.F., Foght, J., Gieg, L.M., Hallam, S.J., Hanson, N.W., He, Z., Jack, T.R., Klassen, J., Konwar, K.M., Kuatsjah, E., Li, C., Larter, S., Leopatra, V., Nesbø, C.L., Oldenburg, T., Pagé, A.P., Ramos-Padrón, E., Rochman, F.F., Saidi-Mehrabad, A., Sensen, C.W., Sipahimalani, P., Song, Y.C., Wilson, S., Wolbring, G., Wong, M.-L., and Voordouw, G. (2013). Metagenomics of hydrocarbon resource environments indicates aerobic taxa and genes to be unexpectedly common. *Environ. Sci. Technol.* 47, 10708-10717.

Anderson, R.T., and Lovley, D.R. (2000). Biogeochemistry: Hexadecane decay by methanogenesis. *Nature* 404**,** 722-723.

Anneser, B., Pilloni, G., Bayer, A., Lueders, T., Griebler, C., Einsiedl, F., and Richters, L. (2010). High resolution analysis of contaminated aquifer sediments and groundwater - What can be learned in terms of natural attenuation? *Geomicrobiol. J.* 27, 130-142.

Annweiler, E., Materna, A., Safinowski, M., Kappler, A., Richnow, H.H., Michaelis, W., and Meckenstock, R.U. (2000). Anaerobic degradation of 2-methylnaphthalene by a sulfate-reducing enrichment culture. *Appl. Environ. Microbiol.* 66**,** 5329-5333.

Annweiler, E., Michaelis, W., and Meckenstock, R.U. (2002). Identical ring cleavage products during anaerobic degradation of naphthalene, 2-methylnaphthalene, and tetralin indicate a new metabolic pathway. *Appl. Environ. Microbiol.* 68**,** 852-858.

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

Banwart, S.A., and Thornton, S.F. (2010). Natural attenuation of hydrocarbon compounds in groundwater. *Handbook of hydrocarbon and lipid microbiology*, (K.N Timmis, ed.), pp 2473-2486. Springer, Berlin.

Bastin, E.S. (1926). The presence of sulphate reducing bacteria in oil field waters. *Science* 63, 21-24.

Bauer, R.D., Rolle, M., Bauer, S., Eberhardt, C., Grathwohl, P., Kolditz, O., Meckenstock, R.U., and Griebler, C. (2009). Enhanced biodegradation by hydraulic heterogeneities in petroleum hydrocarbon plumes. *J. Contam. Hydrol.* 105, 56-68.

Bedessem, M.E., Swoboda-Colberg, N.G., and Colberg, P.J.S. (1997). Naphthalene mineralization coupled to sulfate reduction in aquifer-derived enrichments. *FEMS Microbiol. Lett.* 152**,** 213-218.

Beller, H.R., Ding, W.-H., and Reinhard, M. (1995). Byproducts of anaerobic alkylbenzene metabolism useful as indicators of in situ bioremediation. *Environ. Sci. Technol.* 29, 2864-2870.

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

Beller, H.R., and Edwards, E.A. (2000). Anaerobic toluene activation by benzylsuccinate synthase in a highly enriched methanogenic culture. *Appl. Environ. Microbiol.* 66**,** 5503-5505.

Beller, H.R. (2002). Analysis of benzylsuccinates in groundwater by liquid chromatography/tandem mass spectrometry and its use for monitoring in situ BTEX biodegradation. *Environ. Sci. Technol.* 36, 2724-2728.

Beller, H.R., Kane, S.R., Legler, T.C., and Alvarez, P.J.J. (2002). A real-time polymerase chain reaction method for monitoring anaerobic, hydrocarbon-degrading bacteria based on a catabolic gene. *Environ. Sci. Technol.* 36**,** 3977-3984.

Beller, H.R., Kane, S.R., Legler, T.C., Mckelvie, J.R., Sherwood Lollar, B., Pearson, F., Balser, L., and Mackay, D.M. (2008). Comparative assessments of benzene, toluene, and xylene natural attenuation by quantitative polymerase chain reaction analysis of a catabolic gene, signature metabolites, and compound-specific isotope analysis. *Environ. Sci. Technol.* 42, 6065-6072.

Berdugo-Clavijo, C., and Gieg, L. (2014). Conversion of crude oil to methane by a microbial consortium enriched from oil reservoir production waters. *Front. Microbiol.* 5**,** 197.

Berdugo-Clavijo, C., Dong, X., Soh, J., Sensen, C.W., and Gieg, L.M. (2012). Methanogenic biodegradation of two-ringed polycyclic aromatic hydrocarbons. *FEMS Microbiol. Ecol.* 81**,** 124-133.

Bergmann, F., Selesi, D., and Meckenstock, R.U (2011). Identification of new enzymes potentially involved in anaerobic naphthalene degradation by the sulfate-reducing enrichment culture N47. *Arch. Microbiol.***,** 1-10.

Bianchin, M., Smith, L., Barker, J.F., and Beckie, R. (2006). Anaerobic degradation of naphthalene in a fluvial aquifer: A radiotracer study. *J. Contam. Hydrol.* 84**,** 178-196.

Biegert, T., Fuchs, G., and Heider, J. (1996). Evidence that anaerobic oxidation of toluene in the denitrifying bacterium *Thauera aromatica* is initiated by formation of benzylsuccinate from toluene and fumarate. *Eur. J. Biochem.* 238**,** 661-668.

Birkeland, N.K. (2004). The microbial diversity of deep subsurface oil reservoirs, *Studies in surface science and catalysis,* (R. Vazques-Duhalt and R. Quintero-Ramirez, eds.), pp 385-403. Elsevier, Amsterdam.

Boll, M., and Fuchs, G. (1995). Benzoyl-coenzyme A reductase (dearomatizing), a key enzyme of anaerobic aromatic metabolism. *Eur. J. Biochem.* 234**,** 921-933.

Boll M, Fuchs G, Heider J (2002). Anaerobic oxidation of aromatic compounds and hydrocarbons. *Curr. Opin. Chem. Biol*. 6, 604–611.

Bonch-Osmolovskaya, E.A., Miroshnichenko, M.L., Lebedinsky, A.V., Chernyh, N.A., Nazina, T.N., Ivoilov, V.S., Belyaev, S.S., Boulygina, E.S., Lysov, Y.P., Perov, A.N., Mirzabekov, A.D., Hippe, H., Stackebrandt, E., L'haridon, S., and Jeanthon, C. (2003). Radioisotopic, culture-based, and oligonucleotide microchip analyses of thermophilic microbial communities in a continental high-temperature petroleum reservoir. *Appl. Environ. Microbiol.* 69**,** 6143-6151.

Botton, S., Van Harmelen, M., Braster, M., Parsons, J.R., and Röling, W.F.M. (2007). Caffrey, S.M. (2011). Which microbial communities are present? *Applied microbiology and molecular biology in oilfield systems.* (C. Whitby and T.L. Skovhus, eds.), pp 63-76 Springer, Dordrecht.

Callaghan, A.V. (2013). Metabolomic investigations of anaerobic hydrocarbon-impacted environments. *Curr. Opin. Biotech.* 24**,** 506-515.

Callaghan, A.V., Davidova, I.A., Savage-Ashlock, K., Parisi, V.A., Gieg, L.M., Suflita, J.M., Kukor, J.J., and Wawrik, B. (2010). Diversity of benzyl- and alkylsuccinate synthase genes in hydrocarbon-impacted environments and enrichment cultures. *Environ. Sci. Technol.* 44**,** 7287-7294.

Callaghan, A.V., Morris, B.E.L., Pereira, I.A.C., McInerney, M.J., Austin, R.N., Groves, J.T., Kukor, J.J., Suflita, J.M., Young, L.Y., Zylstra, G.J., and Wawrik, B. (2012). The genome sequence of *Desulfatibacillum alkenivorans* AK-01: A blueprint for anaerobic alkane oxidation. *Environ. Microbiol.* 14**,** 101-113.

Callbeck, C., Dong, X., Chatterjee, I., Agrawal, A., Caffrey, S., Sensen, C., and Voordouw, G. (2011). Microbial community succession in a bioreactor modeling a souring low-temperature oil reservoir subjected to nitrate injection. *Appl. Microbiol. Biotechnol.* 91**,** 799-810.

Chakraborty, R., and Coates, J.D. (2005). Hydroxylation and carboxylation—two crucial steps of anaerobic benzene degradation by *Dechloromonas* strain Rcb. *Appl. Environ. Microbiol.* 71**,** 5427-5432.

Chang, W., Um, Y., Hoffman, B., and Holoman, T.R.P. (2005). Molecular characterization of polycyclic aromatic hydrocarbon (PAH)-degrading methanogenic communities. *Biotechnol. Prog.* 21**,** 682-688.

Chang, W., Um, Y., and Holoman, T. (2006). Polycyclic aromatic hydrocarbon (PAH) degradation coupled to methanogenesis. *Biotechnol. Lett.* 28**,** 425-430.

Cheng, L., Ding, C., Li, Q., He, Q., Dai, L.-R., and Zhang, H. (2013). DNA-SIP reveals that *Syntrophaceae* play an important role in methanogenic hexadecane degradation. *PLoS ONE* 8**,** e66784.

Christensen, N., Batstone, D.J., He, Z., Angelidaki, I., and Schmidt, J.E. (2004). Removal of polycyclic aromatic hydrocarbons (PAHs) from sewage sludge by anaerobic degradation. *Water Sci. Technol.* 50**,** 237-244.

Coates, J.D., Anderson, R.T., Woodward, J.C., Phillips, E.J.P., and Lovley, D.R. (1996). Anaerobic hydrocarbon degradation in petroleum-contaminated harbor sediments under sulfate-reducing and artificially imposed iron-reducing conditions. *Environ. Sci. Technol.* 30**,** 2784-2789.

Coates, J.D., Chakraborty, R., and Mcinerney, M.J. (2002). Anaerobic benzene biodegradation-a new era. *Res. Microbiol.* 153**,** 621-628.

Cummings, D.E., Snoeyenbos-West, O.L., Newby, D.T., Niggemyer, A.M., Lovley, D.R., Achenbach, L.A., and Rosenzweig, R.F. (2003). Diversity of *Geobacteraceae*

species inhabiting metal-polluted freshwater lake sediments ascertained by 16S rDNA analyses. *Microb. Ecol.* 46**,** 257-269.

Da Silva, M.L.B., and Alvarez, P.J.J. (2004). Enhanced anaerobic biodegradation of benzene-toluene-ethylbenzene-xylene-ethanol mixtures in bioaugmented aquifer columns. *Appl. Environ. Microbiol.* 70**,** 4720-4726.

Davidova, I.A., Gieg, L.M., Duncan, K.E., and Suflita, J.M. (2007). Anaerobic phenanthrene mineralization by a carboxylating sulfate-reducing bacterial enrichment. *ISME J.* 1**,** 436-442.

DiDonato, R.J., Jr., Young, N.D., Butler, J.E., Chin, K.-J., Hixson, K.K., Mouser, P., Lipton, M.S., Deboy, R., and Methé, B.A. (2010). Genome sequence of the deltaproteobacterial strain Naphs2 and analysis of differential gene expression during anaerobic growth on naphthalene. *PLoS ONE* 5**,** e14072.

Dojka, M.A., Hugenholtz, P., Haack, S.K., and Pace, N.R. (1998). Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. *Appl. Environ. Microbiol.* 64**,** 3869-3877.

Dolfing, J., R. Larter, S., and Ian, M.H. (2008). Thermodynamic constraints on methanogenic crude oil biodegradation. *ISME J.* 2**,** 442-452.

Dolfing, J., Xu, A., Gray, N.D., Larter, S.R., and Head, I.M. (2009). The thermodynamic landscape of methanogenic PAH degradation. *Microb. Biotechnol.* 2**,** 566-574.

Dong, Y., Kumar, C.G., Chia, N., Kim, P.-J., Miller, P.A., Price, N.D., Cann, I.K.O., Flynn, T.M., Sanford, R.A., Krapac, I.G., Locke, R.A., Hong, P.-Y., Tamaki, H., Liu, W.-T., Mackie, R.I., Hernandez, A.G., Wright, C.L., Mikel, M.A., Walker, J.L., Sivaguru, M., Fried, G., Yannarell, A.C., and Fouke, B.W. (2014). *Halomonas sulfidaeris*-dominated microbial community inhabits a 1.8 km-deep subsurface cambrian sandstone reservoir. *Environ. Microbiol.* 16**,** 1695-1708.

Eberlein, C., Estelmann, S., Seifert, J., Von Bergen, M., Müller, M., Meckenstock, R.U., Edwards, E.A., and Grbić-Galić, D. (1994). Anaerobic degradation of toluene and *o*-xylene by a methanogenic consortium. *Appl. Environ. Microbiol.* 60**,** 313-322.

Eberlein, C., Estelmann, S., Seifert, J., Von Bergen, M., Müller, M., Meckenstock, R.U., and Boll, M. (2013). Identification and characterization of 2-naphthoyl-coenzyme A reductase, the prototype of a novel class of dearomatizing reductases. *Mol. Microbiol.* 88**,** 1032-1039.

Essaid, H.I., Bekins, B.A., Herkelrath, W.N., and Delin, G.N. (2011). Crude oil at the Bemidji site: 25 years of monitoring, modeling, and understanding. *Ground Water* 49**,** 706-726.

Fedorak, P.M., and Westlake, D.W.S. (1981). Microbial degradation of aromatics and saturates in Prudhoe Bay crude oil as determined by glass capillary gas chromatography. *Can. J. Microbiol.* 27**,** 432-443.

Ficker, M., Krastel, K., Orlicky, S., and Edwards, E. (1999). Molecular characterization of a toluene-degrading methanogenic consortium. *Appl. Environ. Microbiol.* 65**,** 5576-5585.

Fite, A., Macfarlane, G.T., Cummings, J.H., Hopkins, M.J., Kong, S.C., Furrie, E., and Macfarlane, S. (2004). Identification and quantitation of mucosal and faecal *Desulfovibrios* using real time polymerase chain reaction. *Gut* 53**,** 523-529.

Foght, J. (2008). Anaerobic biodegradation of aromatic hydrocarbons: Pathways and prospects. *J. Mol. Microbiol. Biotechnol.* 15**,** 93-120.

Fowler, S.J., Dong, X., Sensen, C.W., Suflita, J.M., and Gieg, L.M. (2012). Methanogenic toluene metabolism: Community structure and intermediates. *Environ. Microbiol.* 14**,** 754-764.

Fowler, S.J., Gutierrez-Zamora, M.-L., Manefield, M., and Gieg, L.M. (2014). Identification of toluene degraders in a methanogenic enrichment culture. *FEMS Microbiol. Ecol.***,** 89, 625-636.

Galushko, A., Minz, D., Schink, B., and Widdel, F. (1999). Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulfate-reducing bacterium. *Environ. Microbiol.* 1**,** 415-420.

Gieg, L.M., Kolhatkar, R.V., Mcinerney, M.J., Tanner, R.S., Harris, S.H., Sublette, K.L., and Suflita, J.M. (1999). Intrinsic bioremediation of petroleum hydrocarbons in a gas condensate-contaminated aquifer. *Environ. Sci. Technol.* 33**,** 2550-2560.

Gieg, L.M., and Suflita, J.M. (2002). Detection of anaerobic metabolites of saturated and aromatic hydrocarbons in petroleum-contaminated aquifers. *Environ. Sci. Technol.* 36**,** 3755-3762.

Gieg, L.M., Duncan, K.E., and Suflita, J.M. (2008). Bioenergy production via microbial conversion of residual oil to natural gas. *Appl. Environ. Microbiol.* 74**,** 3022-3029.

Gieg, L.M., Alumbaugh, R.E., Field, J., Jones, J., Istok, J.D., and Suflita, J.M. (2009). Assessing *in situ* rates of anaerobic hydrocarbon bioremediation. *Microb. Biotechnol.* 2**,** 222-233.

Gieg, L.M., Davidova, I.A., Duncan, K.E., and Suflita, J.M. (2010). Methanogenesis, sulfate reduction and crude oil biodegradation in hot alaskan oilfields. *Environ. Microbiol.* 12**,** 3074-3086.

Gieg, L.M., Fowler, S.J., and Berdugo-Clavijo, C. (2014). Syntrophic biodegradation of hydrocarbon contaminants. *Curr. Opin. Biotechnol.* 27**,** 21-29.

Godsy, E.M., Goerlitz, D.F., and Grbić-Galić, D. (1992). Methanogenic biodégradation of creosote contaminants in natural and simulated ground-water ecosystems. *Ground Water* 30**,** 232-242.

Gray, N., Sherry, A., Larter, S., Erdmann, M., Leyris, J., Liengen, T., Beeder, J., and Head, I. (2009). Biogenic methane production in formation waters from a large gas field in the north sea. *Extremophiles* 13**,** 511-519.

Gray, N.D., Sherry, A., Hubert, C., Dolfing, J., and Head, I.M. (2010). *Methanogenic degradation of petroleum hydrocarbons in subsurface environments: Remediation, heavy oil formation, and energy recovery, Adv. Appl. Microbiol.* (A. I. Laskin and M.G. Geoffrey, eds.), pp 137-161. Academic Press, San Diego, CA.

Gray, N.D., Sherry, A., Grant, R.J., Rowan, A.K., Hubert, C.R.J., Callbeck, C.M., Aitken, C.M., Jones, D.M., Adams, J.J., Larter, S.R., and Head, I.M. (2011). The quantitative significance of *Syntrophaceae* and syntrophic partnerships in methanogenic degradation of crude oil alkanes. *Environ. Microbiol.* 13**,** 2957-2975.

Grbić-Galić, D., and Vogel, T.M. (1987). Transformation of toluene and benzene by mixed methanogenic cultures. *Appl. Environ. Microbiol.* 53**,** 254-260.

Griebler, C., Safinowski, M., Vieth, A., Richnow, H.H., and Meckenstock, R.U. (2003). Combined application of stable carbon isotope analysis and specific metabolites determination for assessing *in situ* degradation of aromatic hydrocarbons in a tar oil-contaminated aquifer. *Environ. Sci. Technol.* 38**,** 617-631.

Grigoryan, A.A., Cornish, S.L., Buziak, B., Lin, S., Cavallaro, A., Arensdorf, J.J., and Voordouw, G. (2008). Competitive oxidation of volatile fatty acids by sulfate- and nitrate-reducing bacteria from an oil field in Argentina. *Appl. Environ. Microbiol.* 74**,** 4324-4335.

Grigoryan, A., and Voordouw, G. (2008). Microbiology to help solve our energy needs. *Ann. NY. Acad. Sci.* 1125**,** 345-352.

Grossi, V., Cravo-Laureau, C., Guyoneaud, R., Ranchou-Peyruse, A., and Hirschler-Réa, A. (2008). Metabolism of *n*-alkanes and *n*-alkenes by anaerobic bacteria: A summary. *Org. Geochem.* 39**,** 1197-1203.

Grundmann, O., Behrends, A., Rabus, R., Amann, J., Halder, T., Heider, J., and Widdel, F. (2008). Genes encoding the candidate enzyme for anaerobic activation of *n*-alkanes in the denitrifying bacterium, strain Hxn1. *Environ. Microbiol.* 10**,** 376-385.

Gutierrez, T., Berry, D., Yang, T., Mishamandani, S., Mckay, L., Teske, A., and Aitken, M.D. (2013). Role of bacterial exopolysaccharides (eps) in the fate of the oil released during the deepwater horizon oil spill. *PLoS ONE* 8**,** e67717.

Haritash, A.K., and Kaushik, C.P. (2009). Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): A review. *J. Haz. Mat.* 169**,** 1-15.

Harms, G., Zengler, K., Rabus, R., Aeckersberg, F., Minz, D., Rossello-Mora, R., and Widdel, F. (1999). Anaerobic oxidation of *o*-xylene, *m*-xylene, and homologous alkylbenzenes by new types of sulfate-reducing bacteria. *Appl. Environ. Microbiol.* 65**,** 999-1004.

Head, I.M., Jones, D.M., and Larter, S.R. (2003). Biological activity in the deep subsurface and the origin of heavy oil. *Nature* 426**,** 344-352.

Heider, J., and Schühle, K. (2013). Anaerobic biodegradation of hydrocarbons including methane. *The prokaryotes,* (E. Rosenberg, E. Delong, S. Lory, E. Stackebrandt and F. Thompson, eds.), pp 605-634. Springer, Berlin.

Hermuth, K., Leuthner, B., and Heider, J. (2002). Operon structure and expression of the genes for benzylsuccinate synthase in *Thauera aromatica* strain K172. *Arch. Microbiol.* 177**,** 132-138.

Herrmann, S., Kleinsteuber, S., Chatzinotas, A., Kuppardt, S., Lueders, T., Richnow, H.-H., and Vogt, C. (2010). Functional characterization of an anaerobic benzene-degrading enrichment culture by DNA stable isotope probing. *Environ. Microbiol.* 12**,** 401-411.

Hughes, J.B., Beckles, D.M., Chandra, S.D., and Ward, C.H. (1997). Utilization of bioremediation processes for the treatment of PAH-contaminated sediments. *J. Ind. Microbiol. Biotechnol.* 18**,** 152-160.

Hunkeler, D., Höhener, P., and Zeyer, J. (2002). Engineered and subsequent intrinsic in situ bioremediation of a diesel fuel contaminated aquifer. *J. Contam. Hydrol.* 59**,** 231-245.

Jack, T.R., Lee, E., and Mueller, J. (1985). Anaerobic gas production from crude oil: Microbes and oil recovery. *Int. Bioresour. J.* 1**,** 167–180.

Jobelius, C., Ruth, B., Griebler, C., Meckenstock, R.U., Hollender, J., Reineke, A., Frimmel, F.H., and Zwiener, C. (2010). Metabolites indicate hot spots of biodegradation and biogeochemical gradients in a high-resolution monitoring well. *Environ. Sci. Technol.* 45**,** 474-481.

Jobst, B., Schühle, K., Linne, U., and Heider, J. (2010). ATP-dependent carboxylation of acetophenone by a novel type of carboxylase. *J. Bacteriol.* 192**,** 1387-1394.

Johnson, H.A., Pelletier, D.A., and Spormann, A.M. (2001). Isolation and characterization of anaerobic ethylbenzene dehydrogenase, a novel Mo-Fe-S enzyme. *J. Bacteriol*. 183**,** 4536-4542.

Jones, D.M., Head, I.M., Gray, N.D., Adams, J.J., Rowan, A.K., Aitken, C.M., Bennett, B., Huang, H., Brown, A., Bowler, B.F.J., Oldenburg, T., Erdmann, M., and Larter, S.R. (2008).

Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs. *Nature* 451**,** 176-180.

Junca, H., and Pieper, D.H. (2010). Functional marker gene assays for hydrocarbon degrading microbial communities: Aerobic. *Handbook of hydrocarbon and lipid microbiology,* (K.N. Timmis, ed.), pp 4289-4312. Springer, Berlin.

Kaden, J., S. Galushko, A., and Schink, B. (2002). Cysteine-mediated electron transfer in syntrophic acetate oxidation by cocultures of *Geobacter sulfurreducens* and *Wolinella succinogenes*. *Arch. Microbiol.* 178**,** 53-58.

Kazy, S.K., Monier, A.L., and Alvarez, P.J. (2010). Assessing the correlation between anaerobic toluene degradation activity and *bss*A concentrations in hydrocarbon-contaminated aquifer material. *Biodegradation* 21**,** 793-800.

Kleemann, R., and Meckenstock, R.U. (2011). Anaerobic naphthalene degradation by gram-positive, iron-reducing bacteria. *FEMS Microbiol. Ecol.* 78**,** 488-496.

Kleikemper, J., Pombo, S.A., Schroth, M.H., Sigler, W.V., Pesaro, M., and Zeyer, J. (2005). Activity and diversity of methanogens in a petroleum hydrocarbon-contaminated aquifer. *Appl. Environ. Microbiol.* 71**,** 149-158.

Kleinsteuber, S., Schleinitz, K.M., Breitfeld, J., Harms, H., Richnow, H.H., and Vogt, C. (2008). Molecular characterization of bacterial communities mineralizing benzene under sulfate-reducing conditions. *FEMS Microbiol. Ecol.* 66**,** 143-157.

Kleinsteuber, S., Schleinitz, K., and Vogt, C. (2012). Key players and team play: Anaerobic microbial communities in hydrocarbon-contaminated aquifers. *Appl. Microbiol. Biotechnol.* 94**,** 851-873.

Krauss, M., Wilcke, W., Martius, C., Bandeira, A.G., Garcia, M.V.B., and Amelung, W. (2005). Atmospheric versus biological sources of polycyclic aromatic hydrocarbons (PAHs) in a tropical rain forest environment. *Environ. Pollut.* 135**,** 143-154.

Krieger, C.J., Roseboom, W., Albracht, S.P.J., and Spormann, A.M. (2001). A stable organic free radical in anaerobic benzylsuccinate synthase of *Azoarcus* sp. Strain T. *J. Biol. Chem.* 276**,** 12924-12927.

Kropp, K.G., Davidova, I.A., and Suflita, J.M. (2000). Anaerobic oxidation of *n*-dodecane by an addition reaction in a sulfate-reducing bacterial enrichment culture. *Appl. Environ. Microbiol.* 66**,** 5393-5398.

Kunapuli, U., Lueders, T., and Meckenstock, R.U. (2007). The use of stable isotope probing to identify key iron-reducing microorganisms involved in anaerobic benzene degradation. *ISME J.* 1**,** 643-653.

Kunapuli, U., Griebler, C., Beller, H.R., and Meckenstock, R.U. (2008). Identification of intermediates formed during anaerobic benzene degradation by an iron-reducing enrichment culture. *Environ. Microbiol.* 10**,** 1703-1712.

Kunapuli, U., Jahn, M.K., Lueders, T., Geyer, R., Heipieper, H.J., and Meckenstock, R.U. (2010). *Desulfitobacterium aromaticivorans* sp. Nov. and *Geobacter toluenoxydans* sp. Nov., iron-reducing bacteria capable of anaerobic degradation of monoaromatic hydrocarbons. *Int. J. Syst. Evol. Microbiol.* 60**,** 686-695.

Kuntze, K., Shinoda, Y., Moutakki, H., Mcinerney, M.J., Vogt, C., Richnow, H.-H., and Boll, M. (2008). 6-Oxocyclohex-1-ene-1-carbonyl-coenzyme a hydrolases from obligately anaerobic bacteria: Characterization and identification of its gene as a functional marker for aromatic compounds degrading anaerobes. *Environ. Microbiol.* 10**,** 1547-1556.

Lamontagne, M.G., Leifer, I., Bergmann, S., Van De Werfhorst, L.C., and Holden, P.A. (2004). Bacterial diversity in marine hydrocarbon seep sediments. *Environ. Microbiol.* 6**,** 799-808.

Lane, D. (1991). 16S/23S RNA sequencing. *Nucleic Acid Techniques in Bacterial Systematics,* (E. Stackebrandt and M. Goodfellow, eds.), pp 115-175. John Wiley & Sons, Inc, New York, NY.

Langenhoff, A.M., Zehnder, A.B., and Schraa, G. (1996). Behaviour of toluene, benzene and naphthalene under anaerobic conditions in sediment columns. *Biodegradation* 7**,** 267-274.

Larsen, S.B., Karakashev, D., Angelidaki, I., and Schmidt, J.E. (2009). *Ex-situ* bioremediation of polycyclic aromatic hydrocarbons in sewage sludge. *J. Haz. Mat.* 164**,** 1568-1572.

Larter, S., and Di Primio, R. (2005). Effects of biodegradation on oil and gas field pvt properties and the origin of oil rimmed gas accumulations. *Org. Geochem.* 36**,** 299-310.

Leuthner, B., and Heider, J. (1998). A two-component system involved in regulation of anaerobic toluene metabolism in thauera aromatica. *FEMS Microbiol. Lett.* 166**,** 35-41.

Leutwein, C., and Heider, J. (2002). (R)-Benzylsuccinyl-CoA dehydrogenase of *Thauera aromatica*, an enzyme of the anaerobic toluene catabolic pathway. *Arch. Microbiol.* 178**,** 517-524.

Li, D., Midgley, D., Ross, J., Oytam, Y., Abell, G.J., Volk, H., Daud, W., and Hendry, P. (2012). Microbial biodiversity in a Malaysian oil field and a systematic comparison with oil reservoirs worldwide. *Arch. Microbiol.* 194**,** 513-523.

Li, L., Patterson, D.P., Fox, C.C., Lin, B., Coschigano, P.W., and Marsh, E.N.G. (2009). Subunit structure of benzylsuccinate synthase. *Biochemistry* 48**,** 1284-1292.

Liesack, W., and Dunfield, P.F. (2003). Biodiversity in soils: Use of molecular methods for its characterization. *Encyclopedia of environmental microbiology*. John Wiley & Sons, Inc, New York.

Llamas, I., Moral, A., Martínez-Checa, F., Arco, Y., Arias, S., and Quesada, E. (2006). *Halomonas maura* is a physiologically versatile bacterium of both ecological and biotechnological interest. *Antonie van Leeuwenhoek* 89**,** 395-403.

López Barragán, M.J., Carmona, M., Zamarro, M.T., Thiele, B., Boll, M., Fuchs, G., García, J.L., and Díaz, E. (2004). The *bzd* gene cluster, coding for anaerobic benzoate catabolism, in *Azoarcus* sp. Strain CIB. *J. Bacteriol.* 186**,** 5762-5774.

Luo, F., Gitiafroz, R., Devine, C.E., Gong, Y., Hug, L.A., Raskin, L., and Edwards, E.A. (2014). Metatranscriptome of an anaerobic benzene-degrading nitrate-reducing enrichment culture reveals involvement of carboxylation in benzene ring activation. *Appl. Environ. Microbiol.* 80, 4095-4107.

Maeda, H., Y. Miyagawa, M. Ikarashi, Kobayashi, H., K. Sato, Sakata, S., and Mochimaru, H. (2009). Development of microbial conversion process of residual oil to methane in depleted oil fields. *SPE* paper 122557.

Magot, M., Ollivier, B., and Patel, B.K.C. (2000). Microbiology of petroleum reservoirs. *Antonie van Leeuwenhoek.* 77**,** 103-116.

Mardis, E.R. (2008). Next-generation DNA sequencing methods. *Annu. Rev. Genomics Human Genet.* 9**,** 387-402.

Mayumi, D., Mochimaru, H., Yoshioka, H., Sakata, S., Maeda, H., Miyagawa, Y., Ikarashi, M., Takeuchi, M., and Kamagata, Y. (2011). Evidence for syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis in the high-temperature petroleum reservoir of Yabase oil field (Japan). *Environ. Microbiol.* 13**,** 1995-2006.

McInerney, M.J., Bryant, M.P., and Pfennig, N. (1979). Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. *Arch. Microbiol.* 122**,** 129-135.

McInerney, M.J., Sieber, J.R., and Gunsalus, R.P. (2009). Syntrophy in anaerobic global carbon cycles. *Curr. Opin. Biotechnol.* 20**,** 623-632.

Meckenstock, R.U. (1999). Fermentative toluene degradation in anaerobic defined syntrophic cocultures. *FEMS Microbiol. Lett.* 177**,** 67-73.

Meckenstock, R.U., Annweiler, E., Michaelis, W., Richnow, H.H., and Schink, B. (2000). Anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. *Appl. Environ. Microbiol.* 66**,** 2743-2747.

Meckenstock, R.U., and Mouttaki, H. (2011). Anaerobic degradation of non-substituted aromatic hydrocarbons. *Curr. Opin. Biotechnol.* 22**,** 406-414.

Meslé, M., Dromart, G., and Oger, P. (2013). Microbial methanogenesis in subsurface oil and coal. *Res. Microbiol.* 164**,** 959-972.

Meyer, B., Kuehl, J., Deutschbauer, A.M., Price, M.N., Arkin, A.P., and Stahl, D.A. (2013). Variation among *Desulfovibrio* species in electron transfer systems used for syntrophic growth. *J. Bacteriol.* 195**,** 990-1004.

Michaelis, W., Seifert, R., Nauhaus, K., Treude, T., Thiel, V., Blumenberg, M., Knittel, K., Gieseke, A., Peterknecht, K., Pape, T., Boetius, A., Amann, R., Jørgensen, B.B., Widdel, F., Peckmann, J., Pimenov, N.V., and Gulin, M.B. (2002). Microbial reefs in the black sea fueled by anaerobic oxidation of methane. *Science* 297**,** 1013-1015.

Mihelcic, J.R., and Luthy, R.G. (1988). Degradation of polycyclic aromatic hydrocarbon compounds under various redox conditions in soil-water systems. *Appl. Environ. Microbiol.* 54**,** 1182-1187.

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

Morasch, B., Annweiler, E., Warthmann, R.J., and Meckenstock, R.U. (2001). The use of a solid adsorber resin for enrichment of bacteria with toxic substrates and to identify metabolites: Degradation of naphthalene, *o*-, and *m*-xylene by sulfate-reducing bacteria. *J. Microbiol. Meth.* 44**,** 183-191.

Morris, B.E.L., Gissibl, A., Kümmel, S., Richnow, H.-H., and Boll, M. (2014). A PCR-based assay for the detection of anaerobic naphthalene degradation. *FEMS Microbiol. Lett.* 354**,** 55-59.

Mouttaki, H., Johannes, J., and Meckenstock, R.U. (2012). Identification of naphthalene carboxylase as a prototype for the anaerobic activation of non-substituted aromatic hydrocarbons. *Environ. Microbiol.* 14**,** 2770-2774.

Muller, F. (1957). On methane fermentation of higher alkanes. *Antonie van Leeuwenhoek* 23**,** 369-384.

Musat, F., Galushko, A., Jacob, J., Widdel, F., Kube, M., Reinhardt, R., Wilkes, H., Schink, B., and Rabus, R. (2009). Anaerobic degradation of naphthalene and 2-methylnaphthalene by strains of marine sulfate-reducing bacteria. *Environ. Microbiol.* 11**,** 209-219.

Nazina, T.N., Ivanova, A.E., Borzenkov, I.A., Belyaev, S.S., and Ivanov, M.V. (1995). Occurrence and geochemical activity of microorganisms in high**-**temperature, water**-**flooded oil fields of Kazakhstan and western Siberia. *Geomicrobiol. J.* 13**,** 181-192.

Oka, A.R., Phelps, C.D., Zhu, X., Saber, D.L., and Young, L.Y. (2011). Dual biomarkers of anaerobic hydrocarbon degradation in historically contaminated groundwater. *Environ. Sci. Technol.* 45**,** 3407-3414.

Ollivier , B.A., and Magot, M. (2005). *Petroleum microbiology*, ASM press, Washington, D.C.

Percheron, G., Bernet, N., and Moletta, R. (1999). Interactions between methanogenic and nitrate-reducing bacteria during the anaerobic digestion of an industrial sulfate rich wastewater. *FEMS Microbiol. Ecol.* 29**,** 341-350.

Pilloni, G., Von Netzer, F., Engel, M., and Lueders, T. (2011). Electron acceptor-dependent identification of key anaerobic toluene degraders at a tar-oil-contaminated aquifer by Pyro-SIP. *FEMS Microbiol. Ecol.* 78**,** 165-175.

Preuss, R., Angerer, J., and Drexler, H. (2003). Naphthalene-an environmental and occupational toxicant. *Int. Arch. Occup. Environ. Health* 76**,** 556-576.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., and Glöckner, F.O. (2007). Silva: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* 35**,** 7188-7196.

Qurashi, A.W., and Sabri, A.N. (2012). Biofilm formation in moderately halophilic bacteria is influenced by varying salinity levels. *J. Basic Microbiol.* 52**,** 566-572.

Rabus, R., Wilkes, H., Schramm, A., Harms,H., Behrends, A., Amann, M, and Widdel, F. (1999). Anaerobic utilization of alkylbenzenes and *n*-alkanes from crude oil in an enrichment culture of denitrifying bacteria affiliating with the β-subclass of proteobacteria. *Environ. Microbiol.* 1**,** 145-157.

Rabus, R., Kube, M., Beck, A., Widdel, F., and Reinhardt, R. (2002). Genes involved in the anaerobic degradation of ethylbenzene in a denitrifying bacterium, strain EBN1. *Arch. Microbiol.* 178**,** 506-516.

Ramos-Padrón , E., Bordenave, S., Lin, S., Bhaskar, I.M., Dong, X., Sensen, C.W., Fournier, J., Voordouw, G., and Gieg, L.M. (2011). Carbon and sulfur cycling by microbial communities in a gypsum-treated oil sands tailings pond. *Environ. Sci. Technol.* 45**,** 439-446.

Reguera, G. (2005). Extracellular electron transfer via microbial nanowires. *Nature* 435**,** 1098-1101.

Rios-Hernandez, L.A., Gieg, L.M., and Suflita, J.M. (2003). Biodegradation of an alicyclic hydrocarbon by a sulfate-reducing enrichment from a gas condensate-contaminated aquifer. *Appl. Environ. Microbiol.* 69**,** 434-443.

Rockne, K.J., Chee-Sanford, J.C., Sanford, R.A., Hedlund, B.P., Staley, J.T., and Strand, S.E. (2000). Anaerobic naphthalene degradation by microbial pure cultures under nitrate-reducing conditions. *Appl. Environ. Microbiol.* 66**,** 1595-1601.

Rosenberg, E. (2013). Hydrocarbon-oxidizing bacteria. *The prokaryotes*, (E. Rosenberg, E. Delong, S. Lory, E. Stackebrandt and F. Thompson, eds.), pp 201-214. Springer, Berlin.

Rotaru, A.-E., Probian, C., Wilkes, H., and Harder, J. (2010). Highly enriched betaproteobacteria growing anaerobically with *p*-xylene and nitrate. *FEMS Microbiol. Ecol.* 71**,** 460-468.

Rowland, S.J., West, C.E., Scarlett, A.G., Jones, D., and Frank, R.A. (2011). Identification of individual tetra- and pentacyclic naphthenic acids in oil sands process water by comprehensive two-dimensional gas chromatography/mass spectrometry. *Rap. Comm. Mass Spec.* 25**,** 1198-1204.

Rozanova, E.P., Borzenkov, I.A., Tarasov, A.L., Suntsova, L.A., Dong, C.L., Belyaev, S.S., and Ivanov, M.V. (2001). Microbiological processes in a high-temperature oil field. *Microbiology* 70**,** 102-110.

Safinowski, M., and Meckenstock, R.U. (2004). Enzymatic reactions in anaerobic 2-methylnaphthalene degradation by the sulphate-reducing enrichment culture N47. *FEMS Microbiol. Lett.* 240**,** 99-104.

Safinowski, M., and Meckenstock, R.U. (2006). Methylation is the initial reaction in anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. *Environ. Microbiol.* 8**,** 347-352.

Sakai, N., Kurisu, F., Yagi, O., Nakajima, F., and Yamamoto, K. (2009). Identification of putative benzene-degrading bacteria in methanogenic enrichment cultures. *J. Biosci. Bioeng.* 108**,** 501-507.

Sanford, R.A., Cole, J.R., Löffler, F.E., and Tiedje, J.M. (1996). Characterization of *Desulfitobacterium chlororespirans* sp. Nov., which grows by coupling the oxidation of lactate to the reductive dechlorination of 3-chloro-4-hydroxybenzoate. *Appl. Environ. Microbiol.* 62**,** 3800-3808.

Schink, B. (1997). Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol. Mol. Biol. Rev.* 61**,** 262-280.

Selesi, D., Jehmlich, N., Von Bergen, M., Schmidt, F., Rattei, T., Tischler, P., Lueders, T., and Meckenstock, R.U. (2010). Combined genomic and proteomic approaches identify gene clusters involved in anaerobic 2-methylnaphthalene degradation in the sulfate-reducing enrichment culture N47. *J. Bacteriol.* 192**,** 295-306.

Seo, J.-S., Keum, Y.-S., and Li, Q. (2009). Bacterial degradation of aromatic compounds. *Int. J. Environ. Res. Publ. Health* 6**,** 278-309.

Sieber, J.R., McInerney, M.J., and Gunsalus, R.P. (2012). Genomic insights into syntrophy: The paradigm for anaerobic metabolic cooperation. *Annu. Rev. Microbiol.* 66**,** 429-452.

Siegert, M., Cichocka, D., Herrmann, S., Gründger, F., Feisthauer, S., Richnow, H.-H., Springael, D., and Krüger, M. (2011). Accelerated methanogenesis from aliphatic and aromatic hydrocarbons under iron- and sulfate-reducing conditions. *FEMS Microbiol. Lett.* 315**,** 6-16.

Singleton, D.R., Hu, J., and Aitken, M.D. (2012). Heterologous expression of polycyclic aromatic hydrocarbon ring-hydroxylating dioxygenase genes from a novel pyrene-degrading betaproteobacterium. *Appl. Environ. Microbiol.* 78**,** 3552-3559.

So, C.M., Phelps, C.D., and Young, L.Y. (2003). Anaerobic transformation of alkanes to fatty acids by a sulfate-reducing bacterium, strain Hxd 3. *App. Environ. Microbiol.* 69**,** 3892-3900.

Soh, J., Dong, X., Caffrey, S.M., Voordouw, G., and Sensen, C.W. (2013). Phoenix 2: A locally installable large-scale 16S rRNA gene sequence analysis pipeline with web interface. *J. Biotechnol.* 167**,** 393-403.

Sohngen, N. (1905). Methane as carbon-food and source of energy for bacteria. *Proc. Kon. Akad. Wetensch.* 8**,** 327.

Sohngen, N. (1913). Oxidation of petroleum, paraffin, paraffin-oil and benzene by microbes. *Proc. Kon. Akad. Wetensch.* 15**,** 1145.

Song, B., and Ward, B.B. (2005). Genetic diversity of benzoyl coenzyme A reductase genes detected in denitrifying isolates and estuarine sediment communities. *Appl. Environ. Microbiol.* 71**,** 2036-2045.

Spiegelman, D., Whissell, G., and Greer, C.W. (2005). A survey of the methods for the characterization of microbial consortia and communities. *Can. J. Microbiol.* 51**,** 355-386.

Stams, A.J.M. (2006). Exocellular electron transfer in anaerobic microbial communities. *Environ. Microbiol.* 8**,** 371-382.

Struchtemeyer, C.G., Elshahed, M.S., Duncan, K.E., and Mcinerney, M.J. (2005). Evidence for aceticlastic methanogenesis in the presence of sulfate in a gas condensate-contaminated aquifer. *Appl. Environ. Microbiol.* 71**,** 5348-5353.

Suflita, J.M, and McInerney, M.J. (2008). Microbial approaches for the enhanced recovery of methane and oil from mature reservoirs. *Bioenergy.* (J. Wall, C.S. Harwood and A.L Demain. eds) pp 389-403. ASM press, Washington D.C.

Suflita, J.M., Davidova, I.A., Gieg, L.M., Nanny, M., and Prince, R.C. (2004). Anaerobic hydrocarbon biodegradation and the prospects for microbial enhanced energy production. *Studies in surface science and catalysis,* (R, Vazquez-Duholt and R, Quintero-Ramirez, eds.), pp 283-305. Elsevier. Boston

Suflita, J.M., Phelps, T.J., and Little, B. (2008). Carbon dioxide corrosion and acetate: A hypothesis on the influence of microorganisms. *Corrosion* 64**,** 854-859.

Sugai, Y., Purwasena, I.A., Sasaki, K., And, K.F., and Okatsu, K. (2010). Evaluation of the potential of microbial conversion process of CO_2 into CH_4 by investigating the microorganisms in high-CO_2 content oilfield. *CSUG/SPE 137568.*

Sun, W., Sun, X., and Cupples, A.M. (2014). Identification of *Desulfosporosinus* as toluene-assimilating microorganisms from a methanogenic consortium. *Int. Biodeterior. Biodeg.* 88**,** 13-19.

Sverdrup, L.E., Nielson, T., and Krogh, P.H. (2002). Soil ecotoxicity of polycyclic aromatic hydrocarbons in relation to soil sorption, lipophilicity and water solubility. *Environ. Sci. Technol*. 36**,** 2429-2435.

Symons, G.E., and Buswell, A.M. (1933). The methane fermentation of carbohydrates. *J. Am. Chem. Soc.* 55**,** 2028-2036.

Tan, B., De Araújo E Silva, R., Rozycki, T., Nesbø, C., and Foght, J. (2014). Draft genome sequences of three *Smithella* spp. obtained from a methanogenic alkane-degrading culture and oil field produced water. *Genome Announc.* 2.

Tan, B., Dong, X., Sensen, C.W., and Foght, J. (2013). Metagenomic analysis of an anaerobic alkane-degrading microbial culture: Potential hydrocarbon-activating pathways and inferred roles of community members. *Genome* 56**,** 599-611.

Town, K., Sheehy, A. J., & Govreau, B. R. (2010, October 1). MEOR Success in Southern Saskatchewan. Society of Petroleum Engineers. doi:10.2118/124319-PA

Townsend, G.T., Prince, R.C., and Suflita, J.M. (2003). Anaerobic oxidation of crude oil hydrocarbons by the resident microorganisms of a contaminated anoxic aquifer. *Environ. Sci. Technol.* 37**,** 5213-5218.

Trably, E., Patureau, D., and Delgenes, J.P. (2003). Enhancement of polycyclic aromatic hydrocarbons removal during anaerobic treatment of urban sludge. *Water Sci. Technol.* 48**,** 53-60.

Tuxen, N., Albrechtsen, H.J., and Bjerg, PL. (2006). Identification of a reactive degradation zone at a landfill leachate plume fringe using high resolution sampling and incubation techniques *J. Contam. Hydrol*. 85, 179-194.

Uchiyama, T., Ito, K., Mori, K., Tsurumaru, H., and Harayama, S. (2010). Iron-corroding methanogen isolated from a crude-oil storage tank. *Appl. Environ. Microbiol.* 76**,** 1783-1788.

Ulrich, A.C., Beller, H.R., and Edwards, E.A. (2005). Metabolites detected during biodegradation of $^{13}C_6$-benzene in nitrate-reducing and methanogenic enrichment cultures. *Environ. Sci. Technol.* 39**,** 6681-6691.

Van Der Zaan, B.M., Saia, F.T., Stams, A.J.M., Plugge, C.M., De Vos, W.M., Smidt, H., Langenhoff, A.A.M., and Gerritse, J. (2012). Anaerobic benzene degradation under denitrifying conditions: *Peptococcaceae* as dominant benzene degraders and evidence for a syntrophic process. *Environ. Microbiol.* 14**,** 1171-1181.

von Netzer, F., Pilloni, G., Kleindienst, S., Krüger, M., Knittel, K., Gründger, F., and Lueders, T. (2013). Enhanced gene detection assays for fumarate-adding enzymes allow uncovering of anaerobic hydrocarbon degraders in terrestrial and marine systems. *Appl. Environ. Microbiol.* 79**,** 543-552.

Voordouw, G., Grigoryan, A.A., Lambo, A., Lin, S., Park, H.S., Jack, T.R., Coombe, D., Clay, B., Zhang, F., Ertmoed, R., Miner, K., and Arensdorf, J.J. (2009). Sulfide remediation by pulsed injection of nitrate into a low temperature Canadian heavy oil reservoir. *Environ. Sci. Technol.* 43**,** 9512-9518.

Voordouw, G. (2011). Production-related petroleum microbiology: Progress and prospects. *Curr. Opin. Biotechnol.* 22**,** 401-405.

Wang, M.-Y., Tsai, Y.-L., Olson, B.H., and Chang, J.-S. (2008). Monitoring dark hydrogen fermentation performance of indigenous *Clostridium butyricum* by hydrogenase gene expression using RT-PCR and qPCR. *Int. J. Hydrogen Energ.* 33**,** 4730-4738.

Washer, C.E., and Edwards, E.A. (2007). Identification and expression of benzylsuccinate synthase genes in a toluene-degrading methanogenic consortium. *Appl. Environ. Microbiol.* 73**,** 1367-1369.

West, C.E., Pureveen, J., Scarlett, A.G., Lengger, S.K., Wilde, M.J., Korndorffer, F., Tegelaar, E.W., and Rowland, S.J. (2014). Can two-dimensional gas chromatography/mass spectrometric identification of bicyclic aromatic acids in petroleum fractions help to reveal further details of aromatic hydrocarbon biotransformation pathways? *Rapid Comm. Mass Spec.* 28**,** 1023-1032.

Whited, G.M., and Gibson, D.T. (1991). Toluene-4-monooxygenase, a three-component enzyme system that catalyzes the oxidation of toluene to *p*-cresol in *Pseudomonas mendocina* Kr1. *J. Bacteriol.* 173**,** 3010-3016.

Whiteley, A.S., and Bailey, M.J. (2000). Bacterial community structure and physiological state within an industrial phenol bioremediation system. *Appl. Environ. Microbiol.* 66**,** 2400-2407.

Widdel, F., and Pfennig, N. (1982). Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. Incomplete oxidation of propionate by *Desulfobulbus propionicus* gen. Nov., sp. Nov. *Arch. Microbiol.* 131**,** 360-365.

Widdel, F., Boetius, A., and Rabus, R. (2006). Anaerobic biodegradation of hydrocarbons including methane *The prokaryotes,* (M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer and E. Stackebrandt, eds.), pp 1028-1049. Springer, New York.

Widdel, F., and Musat, F. (2010). Energetic and other quantitative aspects of microbial hydrocarbon utilization. *Handbook of hydrocarbon and lipid microbiology,* (K.N. Timmis, ed.), pp 729-763. Springer, Berlin.

Widdel, F., Knittel, K., and Galushko, A. (2010). Anaerobic hydrocarbon-degrading microorganisms: an overview. *Handbook of hydrocarbon and lipid microbiology*, (K.N. Timmis, ed.), pp 1997-2021. Springer, Berlin.

Wilkes, H., and Schwarzbauer, J. (2010). Hydrocarbons: An introduction to structure, physico-chemical properties and natural occurrence. *Handbook of hydrocarbon and lipid microbiology* (K.N. Timmis, ed.), pp 1-48. Springer, Berlin.

Winderl, C., Anneser, B., Griebler, C., Meckenstock, R.U., and Lueders, T. (2008). Depth-resolved quantification of anaerobic toluene degraders and aquifer microbial community patterns in distinct redox zones of a tar oil contaminant plume. *Appl. Environ. Microbiol.* 74**,** 792-801.

Winderl, C., Penning, H., Netzer, F.V., Meckenstock, R.U., and Lueders, T. (2010). DNA-SIP identifies sulfate-reducing *Clostridia* as important toluene degraders in tar-oil-contaminated aquifer sediment. *ISME J.* 4**,** 1314-1325.

Wischgoll, S., Heintz, D., Peters, F., Erxleben, A., Sarnighausen, E., Reski, R., Van Dorsselaer, A., and Boll, M. (2005). Gene clusters involved in anaerobic benzoate degradation of *Geobacter metallireducens*. *Mol. Microbiol.* 58**,** 1238-1252.

Yang, W.W., Crow-Willard, E.N., and Ponce, A. (2009). Production and characterization of pure *Clostridium* spore suspensions. *J. Appl. Microbiol.* 106**,** 27-33.

Yarza, P., Richter, M., Peplies, J., Euzeby, J., Amann, R., Schleifer, K.-H., Ludwig, W., Glöckner, F.O., and Rosselló-Móra, R. (2008). The all-species living tree project: A 16S rRNA-based phylogenetic tree of all sequenced type strains. *Syst. Appl. Microbiol.* 31**,** 241-250.

Youssef, N., Simpson, D.R., Duncan, K.E., McInerney, M.J., Folmsbee, M., Fincher, T., and Knapp, R.M. (2007). *In situ* biosurfactant production by *Bacillus* strains injected into a limestone petroleum reservoir. *Appl. Environ. Microbiol.* 73**,** 1239-1247.

Youssef, N., Elshahed, M.S., and McInerney, M.J. (2009). Microbial processes in oil fields: Culprits, problems, and opportunities. *Advances in applied microbiology*, (I. A. Laskin, S. Sariaslani, G.M. Gadd, eds, pp 141-251. Academic Press. Boston.

Yuan, S.Y., and Chang, B.V. (2007). Anaerobic degradation of five polycyclic aromatic hydrocarbons from river sediment in Taiwan. *J. Environ. Sci. Health. B.* 42**,** 63 - 69.

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

Zhang, F., She, Y.-H., Chai, L.-J., Banat, I.M., Zhang, X.-T., Shu, F.-C., Wang, Z.-L., Yu, L.-J., and Hou, D.-J. (2012). Microbial diversity in long-term water-flooded oil reservoirs with different *in situ* temperatures in China. *Sci. Rep.* 2, Article 760.

Zhang, S., Wang, Q., and Xie, S. (2012). Stable isotope probing identifies anthracene degraders under methanogenic conditions. *Biodegradation***,** 23, 221-230

Zhang, X., and Young, L. (1997). Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. *Appl. Environ. Microbiol.* 63**,** 4759-4764.

Zhang, X., Sullivan, E.R., and Young, L.Y. (2000). Evidence for aromatic ring reduction in the biodegradation pathway of carboxylated naphthalene by a sulfate reducing consortium. *Biodegradation* 11**,** 117-124.

Zhou, L., Li, K.-P., Mbadinga, S., Yang, S.-Z., Gu, J.-D., and Mu, B.-Z. (2012). Analyses of *n*-alkanes degrading community dynamics of a high-temperature methanogenic consortium enriched from production water of a petroleum reservoir by a combination of molecular techniques. *Ecotoxicology* 21**,** 1680-1691.

Zobell, C.E. (1946). Action of microorganisms on hydrocarbons. *Bacteriol. Rev.* 10**,** 1-49.

APPENDIX A: SUPPLEMENTARY MATERIAL FROM CHAPTER 3. METABOLISM OF A METHANOGENIC CRUDE OIL DEGRADING ENRICHMENT CULTURE

Pfennig general anaerobic medium for fresh water anaerobes

	Per 100 mL
Pfennig I	5 mL
Pfennig II	5 mL
Wolin metals	1 mL
Balch vitamins	1 mL
Rezasurin	0.1 mL
$NaHCO_3$	0.35 g

pH adjusted to 7.1-7.3

Pfennig mineral solutions:

		Per 1 Liter
Pfennig I	K_2HPO_4	10 g
Pfennig II	$MgCL_2$	6.6 g
	NaCL	8 g
	NH_4Cl	8 g
	$CaCl_2$ X $2H_2O$	1 g

Reducing agents:

▪Cysteine-HCl–sodium sulfide:

2 mL of solution added to 100 mL medium (4 mM)

	Per 100 mL
Cysteine-HCl	2.5 g
Sodium sulfide	2.5 g

▪Sodium sulfide[a]:

0.5 mL of solution added to 100 mL

	Per 100 mL
Na_2S X $9H_2O$	48 g

[a] Widdel *et al*., 2006

Figure A-1. Minimal salt medium used for the cultivation of microbial cultures. Adapted from McInerney *et al*., 1979.

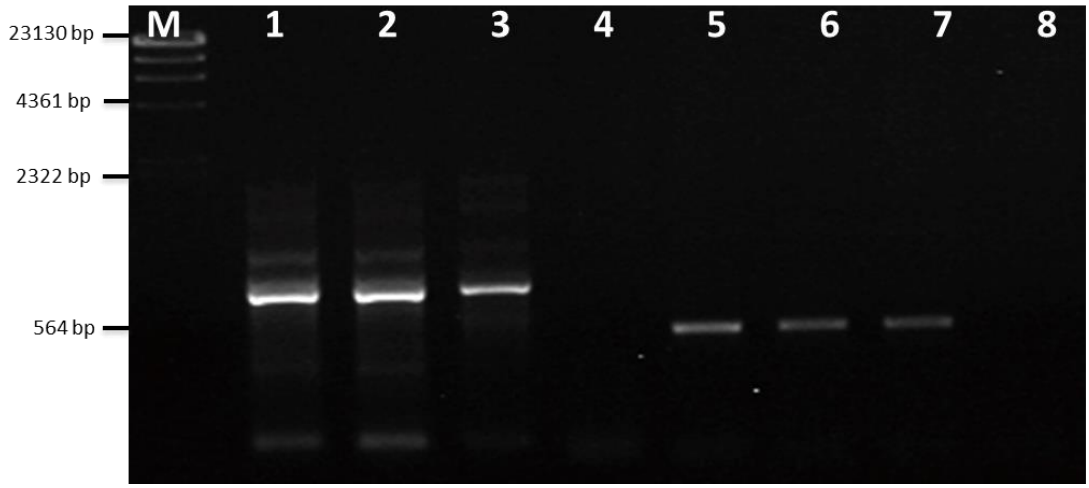

Figure A-2. Agarose gel showing amplified bands of alkyl or benzyl succinate synthases, M: DNA marker, lanes 1-3: replicate samples with primer *bssA* set #2 (793 bp), lane 4: negative control with primer set #2, lanes 5-7: replicate samples with primer *assA* set #7 (523 bp), and lane 8: negative control with primer set #7.

Table A-1. Phylogenetic affiliations of the microbial reads identified at the phylum level by pyrotag sequencing of the 16S rRNA genes in the light oil-degrading enrichment culture.

Taxon (Phylum)	Reads	Percentage of microbial reads
Euryarchaeota	3526	31.373
Spirochaetes	3326	29.596
Firmicutes	2399	21.347
Proteobacteria	2100	18.686
Bacteroidetes	894	7.955
Thermotogae	236	2.1
Chloroflexi	160	1.424
Synergistetes	118	1.05
Actinobacteria	14	0.124
Planctomycetes	5	0.044

Table A-2. Taxonomic distribution of 16S rRNA microbial sequences obtained by pyrotag sequencing analysis in the light oil-degrading culture. Total number of reads: 13269

Taxonomic term						Reads	Percentage of microbial reads
Domain	**Phylum**	**Class**	**Order**	**Family**	**Genus/taxonomic term**		
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosaetaceae*	*Methanosaeta*	1763	13.29
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanomicrobiaceae*	*Methanoculleus*	1588	11.97
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanocalculus*		382	2.88
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanomicrobiaceae*		34	0.26
Archaea	*Euryarchaeota*	*Thermoplasmata*			Kazan-3A-21	31	0.23
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*			24	0.18
Archaea	*Euryarchaeota*	Methanomicrobia	*Methanomicrobiales*	*Methanolinea*		17	0.13
Archaea	*Euryarchaeota*	*Halobacteria*	*Halobacteriales*		DHVEG-6	16	0.12
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*			15	0.11
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*		Candidatus _Methanoregula	4	0.03
Archaea	*Euryarchaeota*	*Methanomicrobia*				3	0.02
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanomicrobiaceae*	*Methanosphaerula*	3	0.02
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*		uncultured	3	0.02
Archaea						1	0.01
Archaea	*Euryarchaeota*	*Methanobacteria*	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobacterium*	1	0.01
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanomicrobiaceae*	*Methanoplanus*	1	0.01
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanospirillaceae*	*Methanospirillum*	1	0.01
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcinaceae*	*Methanomethylovorans*	1	0.01
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Family_XI_Incertae_Sedis*		2173	16.38
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*	*Smithella*	1908	14.38
Bacteria	*Spirochaetes*	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	uncultured	1665	12.55
Bacteria	*Bacteroidetes*					907	6.84
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Family_XI_Incertae_Sedis*	*Sedimentibacter*	362	2.73
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Peptococcaceae*	*Desulfotomaculum*	226	1.70
Bacteria	*Spirochaetes*	*Spirochaetes*				224	1.69
Bacteria	*Firmicutes*					213	1.61

Bacteria						199	1.50
Bacteria	*Chloroflexi*	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	uncultured	165	1.24
Bacteria	*Candidate_division_OP9*					149	1.12
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobacteraceae*	*Desulfobacterium*	148	1.12
Bacteria	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Rikenellaceae*		147	1.11
Bacteria	*Thermotogae*	*Thermotogae*	*Thermotogales*	*Thermotogaceae*	*Kosmotoga*	136	1.03
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*Synergistaceae*	*Thermanaerovibrio*	124	0.94
Bacteria	*Thermotogae*	*Thermotogae*	*Thermotogales*	*Thermotogaceae*	*Petrotoga*	66	0.50
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*			60	0.45
Bacteria	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*		53	0.40
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfuromonadales*	*Desulfuromonadaceae*	*Desulfuromonas*	37	0.28
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophorhabdaceae*	*Syntrophorhabdus*		33	0.25
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*Synergistaceae*		27	0.20
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*	26	0.20
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobacteraceae*		25	0.19
Bacteria	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*			20	0.15
Bacteria	*Proteobacteria*	*Deltaproteobacteria*				17	0.13
Bacteria	*Chloroflexi*	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*		15	0.11
Bacteria	*Spirochaetes*	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	*Spirochaeta*	15	0.11
Bacteria	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*		ML635J-40	14	0.11
Bacteria	*Firmicutes*	*Clostridia*				14	0.11
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Family_XI_Incertae_Sedis*	*Tissierella*	14	0.11
Bacteria	*Spirochaetes*	*Spirochaetes*	*Spirochaetales*			14	0.11
Bacteria	*Firmicutes*	*Bacilli*				13	0.10
Bacteria	*Chloroflexi*	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	*Leptolinea*	12	0.09
Bacteria	*Candidate_division_WS6*					11	0.08
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Fastidiosipila*	11	0.08
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*		11	0.08
Bacteria	*Proteobacteria*					10	0.08
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Family_XIII_Incertae_Sedis*	*Anaerovorax*	8	0.06
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Peptococcaceae*		8	0.06
Bacteria	*Chloroflexi*					7	0.05
Bacteria	*Planctomycetes*	*Phycisphaerae*				7	0.05
Bacteria	*Actinobacteria*	*Subclass: Coriobacteridae*	*Coriobacteriales*	*Coriobacteriaceae*	*Eggerthella*	5	0.04
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfuromonadales*			5	0.04

Bacteria	*Actinobacteria*					4	0.03
Bacteria	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*		env.OPS_17	4	0.03
Bacteria	*Candidate_division_OP8*					4	0.03
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*	*Syntrophus*	4	0.03
Bacteria	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Petrimonas*	3	0.02
Bacteria	*Candidate_division_WS1*					3	0.02
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	3	0.02
Bacteria	*Firmicutes*	*Clostridia*	*Thermoanaerobacteral*	*Family_III_Incertae_Sedis*	*Tepidanaerobacter*	3	0.02
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*			3	0.02
Bacteria	*Proteobacteria*	*Gammaproteobacteria*				3	0.02
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	3	0.02
Bacteria	*Spirochaetes*	*Spirochaetes*				3	0.02
Bacteria	*Chloroflexi*	*Caldilineae*	*Caldilineales*	*Caldilineaceae*		2	0.02
Bacteria	*Chloroflexi*					2	0.02
Bacteria	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Carnobacteriaceae*	*Trichococcus*	2	0.02
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Eubacteriaceae*	*Alkalibacter*	2	0.02
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Syntrophomonadaceae*	*Syntrophomonas*	2	0.02
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfuromonadales*	*Desulfuromonadaceae*		2	0.02
Bacteria	*Spirochaetes*	*Spirochaetes*				2	0.02
Bacteria	*Spirochaetes*	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*		2	0.02
Bacteria	*Actinobacteria*					1	0.01
Bacteria	*Actinobacteria*		*Rubrobacteridae*		AKIW543	1	0.01
Bacteria	*Bacteroidetes*	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*		1	0.01
Bacteria	*Chlorobi*	*Chlorobia*	*Chlorobiales*	*OPB56*		1	0.01
Bacteria	*Firmicutes*	*Bacilli*	*Bacillales*			1	0.01
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Anaerobacter*	1	0.01
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Family_XI_Incertae_Sedis*	*Sporanaerobacter*	1	0.01
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Peptococcaceae*	*Desulfosporosinus*	1	0.01
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Oscillibacter*	1	0.01
Bacteria	*Planctomycetes*	*Planctomycetacia*	*Planctomycetales*	*Planctomycetaceae*	uncultured	1	0.01
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Caulobacterales*	*Caulobacteraceae*	uncultured	1	0.01
Bacteria	*Proteobacteria*	*Betaproteobacteria*				1	0.01
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Ralstonia*	1	0.01
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Brachymonas*	1	0.01
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Variovorax*	1	0.01
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Hydrogenophilales*	*Hydrogenophilaceae*	*Thiobacillus*	1	0.01
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Thauera*	1	0.01
Bacteria	*Proteobacteria*	*Deltaproteobacteria*				1	0.01
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfovibrionales*			1	0.01
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*	uncultured	1	0.01
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*Synergistaceae*	*Anaerobaculum*	1	0.01

APPENDIX B: SUPPLEMENTARY MATERIAL FROM CHAPTER 4. METHANOGENIC BIODEGRADATION OF HYDROCARBONS IN MARGINAL OIL RESERVOIR-SIMULATING COLUMNS

Table B-1. Amount (μmol) of *n*-alkanes detected in uninoculated (Control) and replicate column (Rep) amended with LOWT-EN, and expected amounts (μmol) of methane based on predicted stoichiometric reactions.

n-alkane	μmol of *n*-alkanes			μmol of *n*-alkane consumed		μmol of CH_4 /μmol	Expected CH_4 consumed		% of biodegradation	
	Control	Rep 1	Rep 2	Rep 1	Rep 2	of *n*-alkane	Rep 1	Rep 2	Rep 1	Rep 2
8	1.05	0.64	0.42	0.41	0.63	6.25	2.58	3.93	39.16	59.76
9	1.52	0.90	0.65	0.63	0.87	7	4.38	6.12	41.09	57.37
10	1.78	1.03	1.00	0.75	0.77	7.75	5.80	6.00	42.17	43.62
11	1.85	0.97	1.08	0.88	0.77	8.5	7.48	6.56	47.66	41.77
12	1.99	0.96	1.13	1.03	0.86	9.25	9.50	7.91	51.65	43.04
13	0.91	0.50	0.57	0.40	0.33	10	4.05	3.35	44.68	36.92
14	1.84	0.85	1.03	0.99	0.80	10.75	10.65	8.65	53.90	43.77
15	1.61	0.75	0.93	0.86	0.68	11.5	9.86	7.78	53.34	42.06
16	1.36	0.66	0.64	0.70	0.72	12.25	8.59	8.79	51.56	52.76
17	0.64	0.57	0.42	0.07	0.22	13	0.90	2.89	10.78	34.72
18	0.90	0.49	0.51	0.41	0.39	13.75	5.59	5.32	45.22	43.02
19	0.94	0.49	0.49	0.45	0.44	14.5	6.52	6.40	48.08	47.19
20	0.46	0.39	0.40	0.07	0.06	15.25	0.99	0.88	14.26	12.67
24	0.81	0.56	0.56	0.25	0.25	18.25	4.51	4.61	30.61	31.25
30	0.72	0.46	0.45	0.26	0.27	22.75	5.97	6.17	36.31	37.50
36	0.56	0.43	0.44	0.14	0.13	27.25	3.80	3.47	24.69	22.58
	Total μmol of *n*-alkane consumed			8.29	8.20	**Expected CH_4 (μmol)**	91.17	88.82		

Table B-2. Amount (μmol) of aromatic compounds (2 and 3 ringed PAHs) detected in uninoculated (Control) and replicate column (Rep) amended with LOWT-EN, and expected amounts (μmol) of methane based on predicted stoichiometric reactions.

Sum of aromatic analogs	μmol of aromatic			μmol of aromatic consumed		μmol of CH_4	Expected CH4	
	Control	Replicate 1	Replicate 2	Replicate 1	Replicate 2	per μmol aromatic	Replicate 1	Replicate 2
MeNaphthalene	0.07	0.06	0.07	0.01	0.00	6.75	0.06	0.03
DimethylNaphaphthalene	3.22	2.04	4.43	1.18	0.08	7.5	8.85	0.65
Phenanthrene	1.67	1.13	1.88	0.54	0.06	8.25	4.62	0.86
MethylPhenanthrene	4.55	2.72	3.96	1.83	1.50	9	18.09	12.54
	Total μmol of aromatic consumed			3.56	1.65	**Expected CH4 (μmol)**	31.63	14.07

Table B-3. Amount (μmol) of *n*-alkanes detected in uninoculated (Control) and replicate column (Rep) amended with RESOIL, and expected amounts (μmol) of methane based on predicted stoichiometric reactions.

Carbon number of *n* -alkane	μmol of *n* -alkanes		μmol of *n* -alkane consumed	μmol of CH_4 per μmol of alkane	Expected CH_4 from alkane consumed	% of biodegradation
	Control	Rep				
8	0.44	0.38	0.05	6.25	0.34	93.48
9	0.45	0.44	0.01	7	0.07	13.21
10	0.76	0.61	0.15	7.75	1.18	39.74
11	0.82	0.79	0.03	8.5	0.22	5.99
12	0.88	0.83	0.05	9.25	0.42	9.03
13	0.52	0.48	0.04	10	0.36	25.70
14	0.90	0.77	0.14	10.75	1.49	26.41
15	0.75	0.69	0.06	11.5	0.67	15.86
16	0.80	0.63	0.18	12.25	2.16	41.40
17	0.59	0.57	0.02	13	0.25	8.98
18	0.51	0.46	0.05	13.75	0.75	41.36
20	0.42	0.38	0.04	15.25	0.56	88.01
24	0.55	0.38	0.17	18.25	3.09	98.96
30	0.42	0.38	0.04	22.75	0.89	97.81
36	0.41	0.41	0.01	27.25	0.17	17.81
Total n-alkane consumed (μmol)			1.02	**Expected CH_4**	12.61	

Table B-4. Amount (μmol) of *n*-alkanes detected in uninoculated (Control) and replicate column (Rep) amended with LOWT, and expected amounts (μmol) of methane based on predicted stoichiometric reactions.

n -alkane C#	μmol of *n* -alkanes		μmol of *n* -alkane consumed	μmol of CH_4 per μmol of alkane	Expected CH_4 from alkane consumed	% of biodegradation
	Control	Rep				
7	0.38	0.39	0.00	5.50	0.00	0.00
8	0.44	0.45	0.00	6.25	0.00	0.00
9	0.45	0.54	0.00	7.00	0.00	0.00
10	0.76	0.66	0.10	7.75	0.78	13.21
11	0.82	0.67	0.15	8.50	1.25	17.99
12	0.88	0.70	0.18	9.25	1.68	20.71
13	0.46	0.38	0.05	10.00	0.50	9.67
14	0.90	0.65	0.25	10.75	2.70	27.78
15	0.75	0.59	0.16	11.50	1.83	21.36
16	0.80	0.54	0.27	12.25	3.29	33.43
17	0.59	0.47	0.11	13.00	1.48	19.33
18	0.51	0.42	0.09	13.75	1.26	17.98
20	0.42	0.45	0.00	15.25	0.00	0.00
24	0.55	0.43	0.12	18.25	2.14	21.38
30	0.42	0.41	0.01	22.75	0.13	1.34
Total *n* -alkane consumed (μmol)			1.49	**Expected CH_4 (μmol)**	17.05	

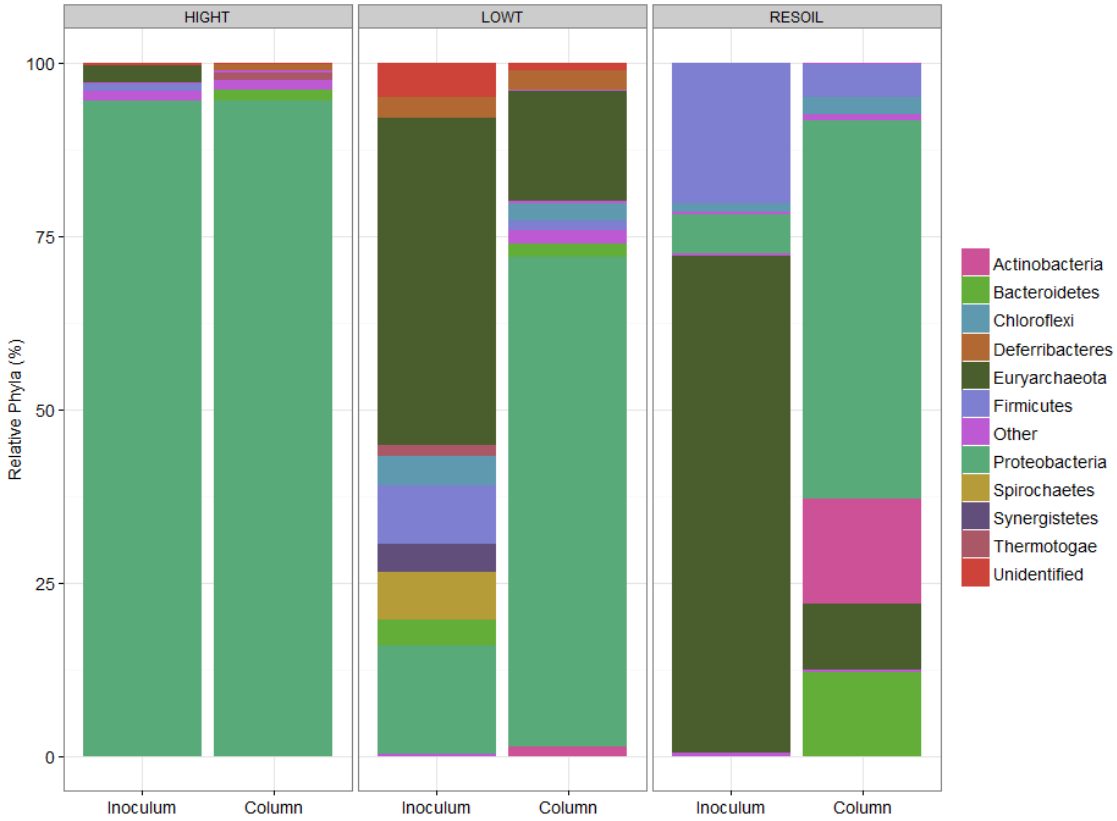

Figure B-1. Relative abundances of microbial reads identified at the phylum level by pyrotag analysis of the 16S rRNA genes in the columns amended with RESOIL, LOWT, and HIGHT before (inoculum) and after (column) incubation in sandstone-packed columns.

Table B-5. Methane production rates measured from columns amended with LOWT-EN, RESOIL, LOWT, and HIGHT after incubation in the sandstone-packed columns. Amount of residual oil and methane production rate calculated for each column replicate are also shown. Values shown for column replicates (R1, R2, and R3).

	LOWT-EN			RESOIL			LOWT		HIGHT		
Column Inocula	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R1**	**R2**	**R3**
Highest CH_4 (µmol)	1.73	8.20	161.78	14.40	1.13	1.13	12.74	0.59	1.99	0.84	0.91
Lag phase (days)	209	127	48	99	44	44	207	400	400	400	400
Residual oil (g)	6.24	1.86	2.36	6.2	1.65	5.26	2.82	3.17	6.69	6.98	6.40
µmol of CH_4/g oil/day	0.0013	0.0347	1.4308	0.0235	0.0155	0.0049	0.0219	0.0005	0.0007	0.0003	0.0004

Table B-6 Taxonomic classification of 16S rRNA microbial genes identified at the genus level in light-oil amended in sandstone-packed columns

Taxonomic term					% of microbial reads
Phylum	**class**	**order**	**family**	**genus**	
Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanoculleus	24.66
Bacteroidetes	Sphingobacteria	Sphingobacteriales	Chitinophagaceae	Sediminibacterium	15.84
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	10.78
Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosaetaceae	Methanosaeta	5.42
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae		5.27
Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcina	3.89
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas	3.22
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	uncultured	3.13
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	2.85
Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Methyloversatilis	2.33
Proteobacteria	Betaproteobacteria	Hydrogenophilales	Hydrogenophilaceae	Thiobacillus	1.99
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Delftia	1.62
Bacteroidetes	Sphingobacteria	Sphingobacteriales	Chitinophagaceae		1.56
Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium	1.35
Euryarchaeota	Thermoplasmata	WCHA1-57			1.29
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax	1.26
Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Herbaspirillum	1.16
Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Halomonas	1.10
Bacteroidetes	Sphingobacteria	Sphingobacteriales	env.OPS_17		0.89
Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	Weissella	0.77
Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Thauera	0.77
Proteobacteria	Gammaproteobacteria				0.64
Proteobacteria	Alphaproteobacteria	Rhizobiales			0.61
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter	0.58
Proteobacteria	Gammaproteobacteria	Chromatiales	Chromatiaceae		0.52
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae		0.49
Actinobacteria	Actinobacteria	Acidobacteriales	Propionibacteriaceae	Propionibacterium	0.49
Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium	0.49

Bacteroidetes	Sphingobacteria	Sphingobacteriales	Cytophagaceae	Spirosoma	0.43
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Incertae_Sedis	0.43
Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	Knoellia	0.37
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Brachymonas	0.37
Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Brevibacillus	0.34
Firmicutes	Bacilli	Bacillales	Bacillaceae	Geobacillus	0.31
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	uncultured	0.31
Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium	0.28
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	0.25
Proteobacteria					0.25
Acidobacteria	Acidobacteria	Acidobacteriales	Acidobacteriaceae	uncultured	0.18
Cyanobacteria	Chloroplast				0.18
Cyanobacteria	SubsectionIII	Leptolyngbya			0.18
Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Pedomicrobium	0.15
Actinobacteria	Actinobacteria	Actinomycetales	Dietziaceae	Dietzia	0.12
Actinobacteria	Actinobacteria	Actinobacteridae	Actinomycetales	Micrococcineae	0.12
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Proteiniphilum	0.09
Proteobacteria	Alphaproteobacteria				0.06
Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	Methylosinus	0.06
Proteobacteria	Alphaproteobacteria	Sphingomonadales			0.06
Proteobacteria	Deltaproteobacteria	Desulfuromonadales	Desulfuromonadaceae	Desulfuromonas	0.06
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	0.06
Euryarchaeota	Methanomicrobia	Methanomicrobiales			0.03
Euryarchaeota	Methanomicrobia	Methanomicrobiales	Candidatus_Methanoregula		0.03
Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanolinea		0.03
Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Rhodococcus	0.03
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Paracoccus	0.03
Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Ralstonia	0.03
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae		0.03
Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Sorangium	0.03
Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae	Legionella	0.03
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	0.03
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	Nevskia	0.03

APPENDIX C: SUPPLEMENTARY MATERIAL FROM CHAPTER 5: METHANOGENIC METABOLISM OF TWO-RINGED POLYCYCLIC AROMATIC HYDROCARBONS

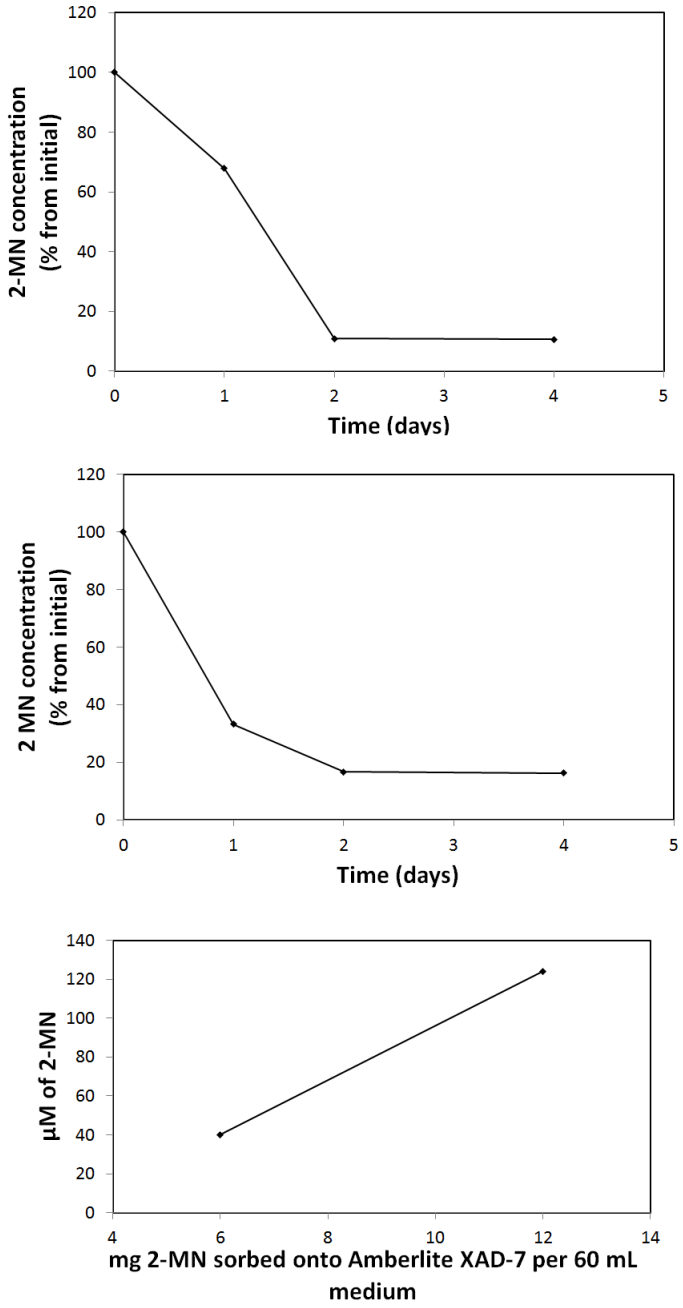

Figure C-1. Percentage of adsorbed 2-MN onto 0.3 g of XAD-7 amberlite with (A) 6 mg or (B) 12 mg of substrate in 60 mL of medium. A plot of the 2-MN equilibrium concentrations in the aqueous phase with 6 mg and 12 mg of substrate after 4 days is shown in (C). The water solubility of 2-MN is 25 mg/L.

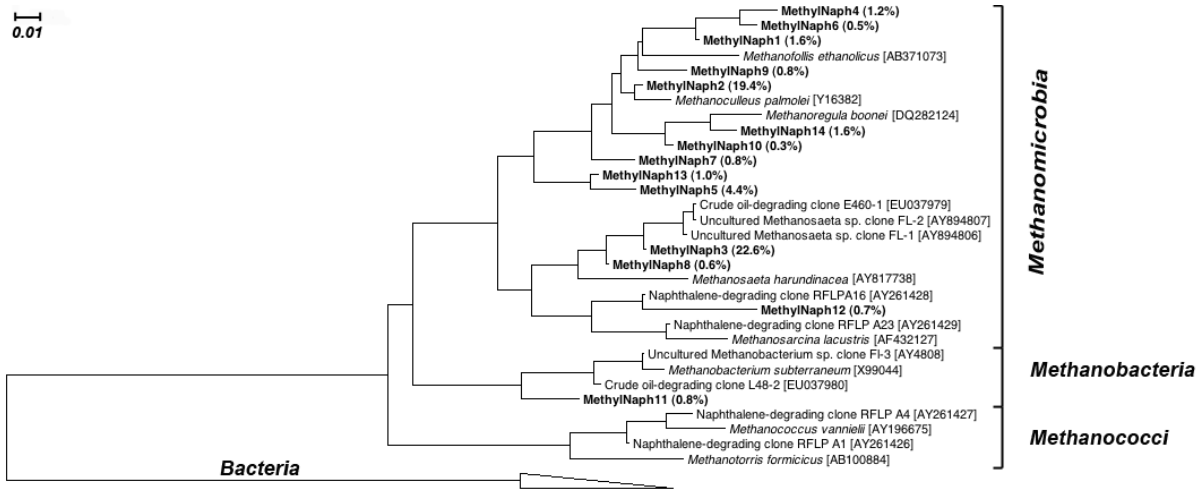

Figure C-2. Phylogenetic relationships of archaea 16S rRNA gene representative sequences (bold) identified in the 2-MN and 2, 6-diMN amended enrichments with respect to other archaeal strains and sequences including those found in anaerobic hydrocarbon-degrading cultures. The phylogenetic tree was calculated using the distance matrix neighbor-joining method with a Jukes-Cantor correction model and FMX filter in ARB. The number in parentheses for the MethylNaph sequences indicates the percentage of abundance compared to all the OTUs. The scale bar indicates 1% of sequence divergence.

Table C-1. Accession numbers in the NCBI Sequence Read Archive and other information about the most abundant archaeal and bacterial OTUs used for phylogenetic tree construction.

Bacteria						
Name	**OTU size**	**%**	**Taxon**	**Accesion No.**	**Alias**	**Culture**
MethylNaph1	2518	27.4	Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Clostridium	SRR090437	GJ45CIU02HKDZF	2-MN
MethylNaph2	147	1.6	Bacteria;Proteobacteria;Deltaproteobacteria;Desulfuromonadales;JN18-A94-J	SRR090436	GJ45CIU02GD4Z1	2,6-diMN
MethylNaph3	75	0.8	Bacteria;Proteobacteria;Deltaproteobacteria;Desulfovibrionales;Desulfovibrionaceae;Desulfovibrio	SRR090436	GJ45CIU02GKCSY	2,6-diMN
MethylNaph5	64	0.7	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Incertae Sedis	SRR090436	GJ45CIU02H6VCN	2,6di-MN
MethylNaph7	43	0.5	Bacteria;Proteobacteria;Deltaproteobacteria;Desulfuromonadales;Geobacteraceae;Geobacter	SRR090436	GJ45CIU02JQ0J0	2,6-diMN
MethylNaph8	43	0.5	Bacteria;Proteobacteria;Betaproteobacteria;Methylophilales;Methylophilaceae	SRR090437	GJ45CIU02IBOF7	2-MN
MethylNaph4	25	0.3	Bacteria;Proteobacteria;Deltaproteobacteria;Desulfobacterales;Desulfobulbaceae;Desulfobulbus	SRR090437	GJ45CIU02GZZHZ	2-MN
MethylNaph9	36	0.4	Bacteria;Chloroflexi;Anaerolineae;Anaerolineales;Anaerolineaceae;uncultured	SRR090436	GJ45CIU02HEHH9	2,6-diMN
MethylNaph6	25	0.3	Bacteria;Proteobacteria;Deltaproteobacteria;Desulfuromonadales;Geobacteraceae;Geobacter	SRR090436	GJ45CIU02HDF0Z	2,6-diMN
Archaea						
Name	**OTU size**	**%**	**Taxon**	**Accesion No.**	**Alias**	**Culture**
MethylNaph3	2076	22.6	Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosaetaceae;Methanosaeta	SRR090437	GJ45CIU02HB2RU	2-MN
MethylNaph2	1786	19.4	Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Methanomicrobiaceae;Methanoculleus	SRR090437	GJ45CIU02IWUWB	2-MN
MethylNaph5	401	4.4	Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Methanolinea	SRR090437	GJ45CIU02HELD2	2-MN
MethylNaph14	147	1.6	Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Candidatus Methanoregula	SRR090436	GJ45CIU02HZ6H2	2,6-diMN
MethylNaph1	149	1.6	Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Methanomicrobiaceae;Methanoculleus	SRR090437	GJ45CIU02GV9WZ	2-MN
MethylNaph4	106	1.2	Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Methanolinea	SRR090437	GJ45CIU02J3N84	2-MN
MethylNaph13	92	1.0	Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Methanolinea	SRR090437	GJ45CIU02F8L8K	2-MN
MethylNaph11	75	0.8	Archaea;Euryarchaeota;Methanobacteria;Methanobacteriales;Methanobacteriaceae;Methanobacterium	SRR090436	GJ45CIU02FPGGI	2,6-di-MN
MethylNaph7	72	0.8	Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Methanomicrobiaceae;Methanoculleus	SRR090437	GJ45CIU02H523K	2-MN
MethylNaph9	70	0.8	Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Methanomicrobiaceae;Methanoculleus	SRR090437	GJ45CIU02J7BDA	2-MN
MethylNaph12	62	0.7	Archaea;Euryarchaeota;Thermoplasmata;WCHA1-57	SRR090436	GJ45CIU02IXMXW	2,6-di-MN
MethylNaph8	55	0.6	Archaea;Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosaetaceae;Methanosaeta	SRR090437	GJ45CIU02H5Q18	2-MN
MethylNaph6	42	0.5	Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Methanolinea	SRR090437	GJ45CIU02GDKIK	2-MN
MethylNaph10	29	0.3	Archaea;Euryarchaeota;Methanomicrobia;Methanomicrobiales;Candidatus Methanoregula	SRR090437	GJ45CIU02HPZ5U	2-MN

Table C-2. Taxonomic distribution of 16S rRNA microbial sequences obtained by pyrotag sequencing analysis in the 2-MN degrading culture. Total number of reads: 2023

Taxonomic term						Reads	Percentage of microbial reads
Domain	**Phylum**	**Class**	**Order**	**Family**	**Genus**		
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanomicrobiaceae*	*Methanoculleus*	707	34.95
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosaetaceae*	*Methanosaeta*	557	27.53
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanolinea*		75	3.71
Archaea	*Euryarchaeota*	*Thermoplasmata*	*WCHA1-57*			24	1.19
Archaea	Euryarchaeota	*Methanomicrobia*	*Methanomicrobiales*	*Candidatus Methanoregula*		11	0.54
Archaea	*Euryarchaeota*	*Thermoplasmata*	*014H09-A-SD-P15*			9	0.45
Archaea	Euryarchaeota	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcinaceae*	*Methanomethylovorans*	5	0.25
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcinaceae*	uncultured	2	0.10
Archaea	*Euryarchaeota*	*Thermoplasmata*			059A02-A-SD-P93	1	0.05
Archaea	*Euryarchaeota*	*Methanobacteria*	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobacterium*	0	0.00
Archaea	*Crenarchaeota*			*Miscellaneous Crenarchaeotic Group*		0	0.00
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcinaceae*	*Methanohalophilus*	0	0.00
Archaea	*Euryarchaeota*	*Thermoplasmata*	*Thermoplasmatales*	*Terrestrial Miscellaneous Gp(TMEG)*		0	0.00
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcinaceae*	*Methanosarcina*	0	0.00
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	450	22.24
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfuromonadales*		JN18-A94-J	82	4.05
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobulbaceae*	*Desulfobulbus*	23	1.14
Bacteria	*Chloroflexi*	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	uncultured	12	0.59
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*	11	0.54
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Methylophilales*	*Methylophilaceae*		9	0.45
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Peptococcaceae*	*Cryptanaerobacter*	6	0.30

Bacteria	*Spirochaetes*	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	*Spirochaeta*	4	0.20
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Family XI Incertae Sedis*	*Sedimentibacter*	4	0.20
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Peptococcaceae*	uncultured	3	0.15
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	uncultured	3	0.15
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfuromonadales*	*BVA59*		3	0.15
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Syntrophomonadaceae*	uncultured	2	0.10
Bacteria	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*SB-1*		2	0.10
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*	*Smithella*	2	0.10
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Incertae Sedis*	2	0.10
Bacteria	*Spirochaetes*	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	uncultured	2	0.10
Bacteria	*Acidobacteria*	*Holophagae*	*Holophagales*	*Holophagaceae*		1	0.05
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Rhodoferax*	1	0.05
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Cupriavidus*	1	0.05
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*GR-WP33-30*	*uncultured*		1	0.05
Bacteria	*Actinobacteria*	*Actinobacteria*	*Coriobacteriales*	*Coriobacteriaceae*		1	0.05
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Methylibium*	1	0.05
Bacteria	*Chloroflexi*	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	*Anaerolinea*	1	0.05
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Eubacteriaceae*	*Anaerofustis*	1	0.05
Bacteria	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*vadinHA17*		1	0.05
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Naxibacter*	1	0.05
Bacteria	*Candidate division WS1*					1	0.05
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Methylobacteriaceae*	*Methylobacterium*	1	0.05

Table C-2. Taxonomic distribution of 16S rRNA microbial sequences obtained by pyrotag sequencing analysis in the 2, 6-diMN degrading culture. Total number of reads: 7180

Taxonomic term						Reads	Percentage of microbial reads
Domain	**Phylum**	**Class**	**Order**	**Family**	**Genus**		
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosaetaceae*	*Methanosaeta*	2256	31.42
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanomicrobiaceae*	*Methanoculleus*	1466	20.42
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanolinea*		225	3.13
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*		*Candidatus Methanoregula*	214	2.98
Archaea	*Euryarchaeota*	*Methanobacteria*	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobacterium*	89	1.24
Archaea	*Euryarchaeota*	*Thermoplasmata*	WCHA1-57			63	0.88
Archaea	*Euryarchaeota*	*Thermoplasmata*	014H09-A-SD-P15			28	0.39
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcinaceae*	*Methanomethylovorans*	23	0.32
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcinaceae*	*Methanosarcina*	11	0.15
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcinaceae*	uncultured	4	0.06
Archaea	*Euryarchaeota*	*Thermoplasmata*	059A02-A-SD-P93			2	0.03
Archaea	*Crenarchaeota*			*Miscellaneous Crenarchaeotic Group*		2	0.03
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcinaceae*	*Methanohalophilus*	1	0.01
Archaea	*Euryarchaeota*	*Thermoplasmata*	*Thermoplasmatales*	*Terrestrial Miscellaneous Gp(TMEG)*		0	0.00
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	2169	30.21
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfuromonadales*	*JN18-A94-J*		110	1.53
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*	75	1.05
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Incertae Sedis*	71	0.99
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfuromonadales*	*Geobacteraceae*	*Geobacter*	47	0.66
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Methylophilales*	*Methylophilaceae*		40	0.56
Bacteria	*Chloroflexi*	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	uncultured	35	0.49
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Family XI Incertae Sedis*	*Sedimentibacter*	22	0.31
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Methylibium*	20	0.28
Bacteria	*Spirochaetes*	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	uncultured	20	0.28
Bacteria	*Spirochaetes*	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	*Spirochaeta*	19	0.27
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	uncultured	15	0.21
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Diaphorobacter*	15	0.21
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*GR-WP33-30*	*uncultured*		12	0.17
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Incertae Sedis*	11	0.15
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Peptococcaceae*	*Cryptanaerobacter*	9	0.13
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*	*Smithella*	8	0.11
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfuromonadales*	*BVA59*		8	0.11
Bacteria	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Proteiniphilum*	7	0.10

Bacteria	*Actinobacteria*	*Actinobacteria*	*Coriobacteridae*	*Coriobacteriales*	*Coriobacterineae*	7	0.10
Bacteria	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	*Streptococcus*	7	0.10
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobulbaceae*	*Desulfobulbus*	7	0.10
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Rhodoferax*	5	0.07
Bacteria	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*SB-1*		5	0.07
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Peptococcaceae*	uncultured	4	0.06
Bacteria	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*vadinHA17*		4	0.06
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Lachnospira*	3	0.04
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Hydrogenophilales*	*Hydrogenophilaceae*	*Thiobacillus*	3	0.04
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Hydrogenophilales*	*Hydrogenophilaceae*	*Tepidiphilus*	2	0.03
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	2	0.03
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	2	0.03
Bacteria	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Paludibacter*	2	0.03
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Veillonellaceae*	*Thermosinus*	2	0.03
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Acidovorax*	2	0.03
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Ralstonia*	2	0.03
Bacteria	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Rikenellaceae*	*vadinBC27 wastewater-sludg*	2	0.03
Bacteria	*Bacteroidetes*	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Cloacibacterium*	2	0.03
Bacteria	*Bacteroidetes*	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Chryseobacterium*	1	0.01
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Cupriavidus*	1	0.01
Bacteria	*Actinobacteria*	*Actinobacteria*	*Rubrobacteridae*	*AKIW543*		1	0.01
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfomicrobiaceae*	*Desulfomicrobium*	1	0.01
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Ruminococcus*	1	0.01
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfuromonadales*	*Desulfuromonadaceae*	*Desulfuromonas*	1	0.01
Bacteria	*Chloroflexi*	*GIF9*				1	0.01
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingomonas*	1	0.01
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*Synergistaceae*	uncultured	1	0.01
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophorhabdaceae*	*Syntrophorhabdus*		1	0.01
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Enteric Bacteria cluster*	1	0.01
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Propionivibrio*	1	0.01
Bacteria	*Actinobacteria*	*Actinobacteria*	*Coriobacteridae*	*Coriobacteriales*	*Coriobacterineae*	1	0.01
Bacteria	*Actinobacteria*	*Actinobacteria*	*OPB41*			1	0.01
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Eubacteriaceae*	*Anaerofustis*	1	0.01
Bacteria	*Chloroflexi*	*vadinBA26*				1	0.01
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	uncultured	1	0.01
Bacteria	*Acidobacteria*	*Acidobacteria*	*Acidobacteriales*	*Acidobacteriaceae*	uncultured	1	0.01
Bacteria	*Planctomycetes*	*Phycisphaerae*		vadinBA30 marine sediment group		1	0.01
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Fastidiosipila*	1	0.01

APPENDIX D: SUPPLEMENTARY MATERIAL FROM CHAPTER 6. DETECTION OF KEY MICROORGANISMS AND FUNCTIONAL GENES INVOLVED IN THE DEGRADATION OF TWO-RINGED POLYCYCLIC AROMATIC HYDROCARBONS

Table D-1. Taxonomic affiliations of 16S rRNA microbial reads identified at the genus level (>1 reads) from 2-MN-degrading culture by pyrotag sequencing analysis using most frequently occurring sequence.

Taxon (genus)	Microbial Reads[a]	% of microbial reads
Methanoculleus	770	38.04
Methanosaeta	543	26.83
Clostridium	442	21.84
Geobacter	79	3.90
Desulfobulbus	21	1.04
Desulfovibrio	10	0.49
Anaerobacter	9	0.45
Methanomethylovorans	8	0.40
Cryptanaerobacter	5	0.25
Spirochaeta	4	0.20
Sedimentibacter	3	0.15
Thermosinus	2	0.10
Smithella	2	0.10
Ralstonia	1	0.05
Alkalibacter	1	0.05
Methylobacterium	1	0.05

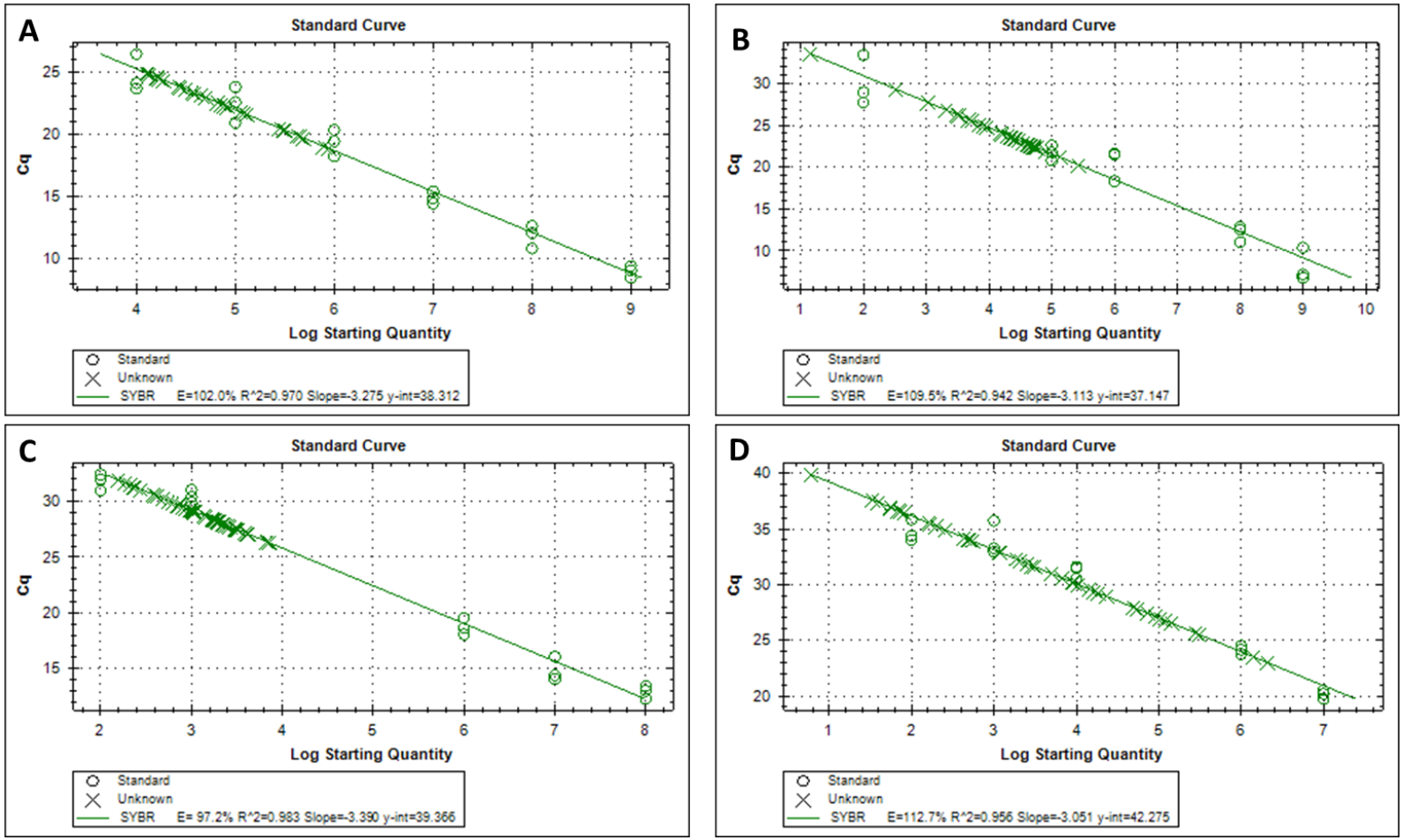

Figure D-1. Calibration curves for the 16s rRNA genes of (A) *Bacteria*, (B) *Clostridium*, (C) *Desulfovibrio*, (E) *Geobacter* obtained for qPCR analysis.

APPENDIX E: SUPPLEMENTARY MATERIAL FROM CHAPTER 7. METHANOGENIC BIODEGRADATION OF NAPHTHALENE AND 1-METHYLNAPHTHALENE

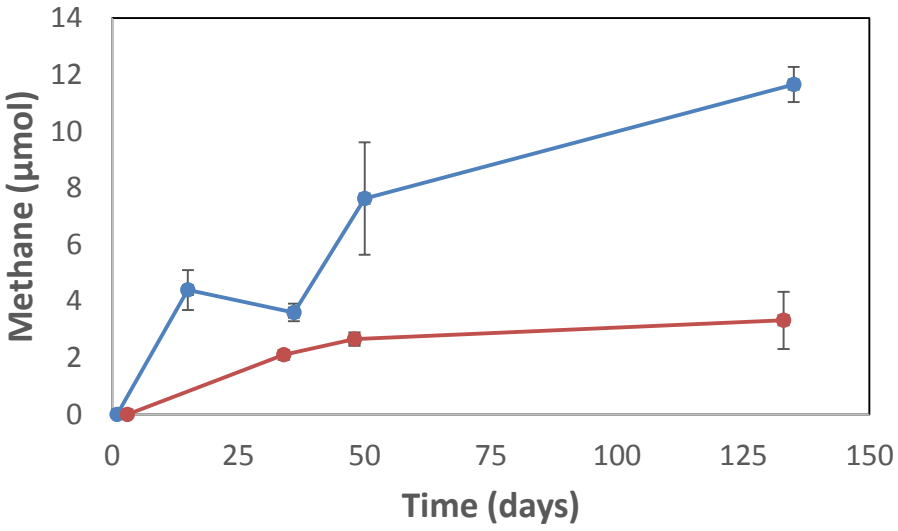

Figure E-1. Methane production of methanogenic cultures amended with 1-methyl naphthalene (blue) after 130 days of incubation at 30℃. Substrate-unamended control is shown in red. Error bars represent standard error of two replicates.

Table E-1. Taxonomic distribution of 16S rRNA microbial sequences obtained by pyrotag sequencing analysis in the naphthalene-degrading culture. Total number of reads: 3915

Taxonomic term						Number of reads	% of microbial reads
Domain	**Phylum**	**Class**	**Order**	**Family**	**Genus**		
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosaetaceae*	*Methanosaeta*	1181	30.17
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanomicrobiaceae*	*Methanoculleus*	578	14.76
Archaea	*Euryarchaeota*	*Methanobacteria*	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobacterium*	76	1.94
Archaea	*Euryarchaeota*	*Thermoplasmata*			*WCHA1-57*	28	0.72
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*		*Candidatus _Methanoregula*	14	0.36
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcinaceae*	*Methanomethylovorans*	2	0.05
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanomicrobiaceae*		1	0.03
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanospirillaceae*	*Methanospirillum*	1	0.03
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	1706	43.58
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*Synergistaceae*		74	1.89
Bacteria	*Chloroflexi*	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	*uncultured*	58	1.48
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*	54	1.38
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*		31	0.79
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfuromonadales*	*Geobacteraceae*	*Geobacter*	26	0.66
Bacteria						14	0.36
Bacteria	*Spirochaetes*	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	*Spirochaeta*	10	0.26
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Clostridiaceae*		6	0.15
Bacteria	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Proteiniphilum*	5	0.13
Bacteria	*Firmicutes*					5	0.13
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophorhabdaceae*	*Syntrophorhabdus*		5	0.13

Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfuromonadales*	*Desulfuromonadaceae*	*Desulfuromonas*	4	0.10
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*	*Syntrophus*	3	0.08
Bacteria	*Candidate_division_OP8*					2	0.05
Bacteria	*Chloroflexi*					2	0.05
Bacteria	*Firmicutes*	*Clostridia*				2	0.05
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Incertae_Sedis*	2	0.05
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhodospirillales*			2	0.05
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingopyxis*	2	0.05
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*	*Smithella*	2	0.05
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	2	0.05
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*Synergistaceae*	*Thermanaerovibrio*	2	0.05
Bacteria	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*Chitinophagaceae*	*Sediminibacterium*	1	0.03
Bacteria	*Chloroflexi*	*Caldilineae*	*Caldilineales*	*Caldilineaceae*	*uncultured*	1	0.03
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*			1	0.03
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Anaerobacter*	1	0.03
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Eubacteriaceae*	*Alkalibacter*	1	0.03
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Family_XI_Incertae_Sedis*	*Sedimentibacter*	1	0.03
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*uncultured*	1	0.03
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Veillonellaceae*	*Thermosinus*	1	0.03
Bacteria	*Proteobacteria*					1	0.03
Bacteria	*Proteobacteria*	*Alphaproteobacteria*				1	0.03
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Caulobacterales*	*Caulobacteraceae*	*Brevundimonas*	1	0.03
Bacteria	*Proteobacteria*	*Betaproteobacteria*				1	0.03
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Herminiimonas*	1	0.03
Bacteria	*Proteobacteria*	*Deltaproteobacteria*				1	0.03
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobulbaceae*	*Desulfobulbus*	1	0.03

Table E-2. Taxonomic distribution of 16S rRNA microbial sequences obtained by pyrotag sequencing analysis in the 1-MN-degrading culture. Total number of reads: 5074

Taxonomic term						Number of reads	% of microbial reads
Domain	**Phylum**	**Class**	**Order**	**Family**	**Genus**		
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosaetaceae*	*Methanosaeta*	1342	26.45
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcinaceae*	*Methanomethylovorans*	47	0.93
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanomicrobiaceae*	*Methanoculleus*	44	0.87
Archaea	*Euryarchaeota*	*Methanobacteria*	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobacterium*	13	0.26
Archaea	*Euryarchaeota*	*Thermoplasmata*			*WCHA1-57*	10	0.20
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*		*Candidatus _Methanoregula*	7	0.14
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*			2	0.04
Archaea	*Euryarchaeota*	*Methanobacteria*	*Methanobacteriales*	Methanobacteriaceae		1	0.02
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanomicrobiaceae*		1	0.02
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*			1	0.02
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	3385	66.71
Bacteria	*Chloroflexi*	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	*uncultured*	29	0.57
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*Synergistaceae*		28	0.55
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfuromonadales*	*Geobacteraceae*	*Geobacter*	23	0.45
Bacteria	*Firmicutes*					18	0.36
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*		17	0.34
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobulbaceae*	*Desulfobulbus*	14	0.28
Bacteria	*Actinobacteria*	*Actinobacteria*	*Coriobacteriales*	*Coriobacterineae*	*Coriobacteriaceae*	13	0.26
Bacteria	*Spirochaetes*	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	*uncultured*	13	0.26
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*	12	0.24
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*	*Smithella*	9	0.18

Bacteria	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Proteiniphilum*	8	0.16
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Anaerobacter*	6	0.12
Bacteria						4	0.08
Bacteria	*Spirochaetes*	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	*Spirochaeta*	3	0.06
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*Synergistaceae*	*Thermanaerovibrio*	3	0.06
Bacteria	*Actinobacteria*	*Actinobacteria*				2	0.04
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Clostridiaceae*		2	0.04
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Papillibacter*	2	0.04
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophorhabdaceae*	*Syntrophorhabdus*		2	0.04
Bacteria	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacteriales*	*Chitinophagaceae*	*Sediminibacterium*	1	0.02
Bacteria					*Candidate_division_OP8*	1	0.02
Bacteria	*Chloroflexi*	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*		1	0.02
Bacteria	*Chloroflexi*					1	0.02
Bacteria	*Firmicutes*	*Clostridia*				1	0.02
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*			1	0.02
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Family_XI_Incertae_Sedis*	*Sedimentibacter*	1	0.02
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Fastidiosipila*	1	0.02
Bacteria	*Proteobacteria*	*Betaproteobacteria*				1	0.02
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobacteraceae*	*uncultured*	1	0.02
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfuromonadales*	*Desulfuromonadaceae*	*Desulfuromonas*	1	0.02
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophaceae*	*Syntrophus*	1	0.02
Bacteria	*Verrucomicrobia*				*OPB35*	1	0.02

APPENDIX F: REVIEW ARTICLE: SYNTROPHIC BIODEGRADATION OF HYDROCARBON CONTAMINANTS

Gieg, L.M., Fowler, S.J., and Berdugo-Clavijo C. (2014). Syntrophic biodegradation of hydrocarbon contaminants, *Curr. Opin. Biotechnol.* 27, 21-29.

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Syntrophic biodegradation of hydrocarbon contaminants

Lisa M Gieg, S Jane Fowler and Carolina Berdugo-Clavijo

Anaerobic environments are crucial to global carbon cycling wherein the microbial metabolism of organic matter occurs under a variety of redox conditions. In many anaerobic ecosystems, syntrophy plays a key role wherein microbial species must cooperate, essentially as a single catalytic unit, to metabolize substrates in a mutually beneficial manner. Hydrocarbon-contaminated environments such as groundwater aquifers are typically anaerobic, and often methanogenic. Syntrophic processes are needed to biodegrade hydrocarbons to methane, and recent studies suggest that syntrophic hydrocarbon metabolism can also occur in the presence of electron acceptors. The elucidation of key features of syntrophic processes in defined co-cultures has benefited greatly from advances in 'omics' based tools. Such tools, along with approaches like stable isotope probing, are now being used to monitor carbon flow within an increasing number of hydrocarbon-degrading consortia to pinpoint the key microbial players involved in the degradative pathways. The metagenomic sequencing of hydrocarbon-utilizing consortia should help to further identify key syntrophic features and define microbial interactions in these complex communities.

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Introduction

Syntrophy, simply meaning 'feeding together', can be more specifically defined as a thermodynamically interdependent, mutually beneficial metabolic process that occurs between organisms wherein the metabolism of a given compound by one organism only occurs when the end products are maintained at low concentrations via consumption by a second organism [1••,2,3]. Syntrophy occurs widely in methanogenic ecosystems where anaerobic electron acceptors are limited or absent, but can also occur in higher redox environments or in the presence of electron acceptors [4•]. In our age of fossil energy reliance, the exploration, recovery, and transportation of hydrocarbon fuels have led to the contamination of many pristine surface and shallow subsurface environments that rapidly become anaerobic and in many cases, methanogenic. Thus, understanding how anaerobic syntrophic consortia participate in hydrocarbon metabolism is crucial to understanding the natural attenuation of such contaminants and to develop effective bioremediation strategies and monitoring tools.

Key features of syntrophic metabolism

The underlying principles of syntrophy have been comprehensively described in recent reviews [1••,2,3,5] thus are only briefly described here. In syntrophic environments, the initial substrate-utilizers consist of fermentative organisms (syntrophs) that initiate the metabolism of compounds like fatty acids, alcohols, sugars, amino acids, and aromatic compounds to produce intermediates such as acetate, formate, and/or H_2 [1••]. The anaerobic oxidation of the initial substrate is thermodynamically unfavorable under standard conditions but becomes favorable when partners such as methanogens consume the intermediates, keeping them at low concentrations (Table 1). In these reactions, relatively low amounts of energy are produced and that energy must be shared amongst all partners; as such syntrophy has been deemed an extreme microbial lifestyle [1••,5]. Although syntrophy is most frequently characterized by partnerships of fermentative bacteria (syntrophs) with archaeal methanogens [1••,4•,6•,7•], syntrophic interactions can also occur in the absence of methanogens, particularly in systems characterized by higher redox potentials [8]. Some bacteria, such as sulfate-reducing *Desulfovibrio* spp., are metabolically versatile in that they respire sulfate when it is available, but switch to syntrophic metabolism in its absence [9•]. Other syntrophs are obligate in that they can only metabolize substrates in the presence of an electron-accepting partner, such as some *Pelotomaculum* spp. [1••,3].

Interspecies electron transfer mechanisms underlie thermodynamically favorable syntrophic processes. The best understood electron transfer mechanism is via H_2 or formate exchange between the syntrophic partners. Physiological studies and genomic sequencing of several syntrophs have shown that multiple enzymes and/or membrane-bound complexes function during H_2/formate-based syntrophic processes, and include reverse electron transfer mechanisms when energy input is required [1••]. The close association of syntrophic partners via aggregation has been shown to be important for

Table 1

Stoichiometry and Gibbs free energy for reactions involved in syntrophic hydrocarbon degradation coupled to methane production

		$\Delta G^{\circ\prime}$ (kJ/mol)	$\Delta G'$ (kJ/mol)[b]
Methane-producing reactions[a]			
Acetotrophic	$CH_3COO^- + H_2O \rightarrow HCO_3^- + CH_4$	−31.0	−15.6
Hydrogenotrophic	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	−135.6	−38.5
Syntrophic hydrocarbon oxidations to acetate and H_2			
Toluene	$C_7H_8 + 7H_2O \rightarrow 3.5CH_3COO^- + 3.5H^+ + 4H_2$	+113.6	−40.9
Naphthalene	$C_{10}H_8 + 10H_2O \rightarrow 5CH_3COO^- + 5H^+ + 4H_2$	+101.1[c]	−90.2
Hexadecane	$C_{16}H_{34} + 16H_2O \rightarrow 8CH_3COO^- + 8H^+ + 17H_2$	+470.8[c]	−91.3
Overall conversion of hydrocarbons to methane[d]			
Toluene	$C_7H_8 + 7.5\ H_2O \rightarrow 4.5CH_4 + 2.5HCO_3^- + 2.5H^+$	−130.5	−134.1
Naphthalene	$C_{10}H_8 + 10\ H_2O \rightarrow 6CH_4 + 4HCO_3^- + 4H^+$	−189.5	−206.9
Hexadecane	$C_{16}H_{34} + 16\ H_2O \rightarrow 12.25CH_4 + 3.75HCO_3^- + 3.75H^+$	−353.5	−380.0

[a] From data in [46].
[b] Calculated with the following values as suggested in [5]: H_2, 10 Pa; bicarbonate, 50 mM; acetate, 50 μM; CH_4, 50 kPa; substrates, 100 μM and based on the formula: $\Delta G' = \Delta G^{\circ\prime} + RT\ln[(C)^c(D)^d/(A)^a(B)^b]$, where $R = 0.00831$ kJ K^{-1} mol^{-1} and $T = 298$ K.
[c] Mass balances and Gibbs free energy values under standard conditions are from [24•,25].
[d] Assumes product removal via both acetotrophic and hydrogenotrophic methanogenesis.

electron transfer via H_2 and formate [3]. Aligning with this concept, alternate mechanisms for electron transfer can also occur via direct cell-to-cell contact (direct interspecies electron transfer) or pili [8,10] or via flagella [11]. In natural anaerobic environments comprised of diverse microbial communities and dynamic geochemical surroundings, all of these electron transfer mechanisms are likely occurring.

Tools for unraveling syntrophic processes

Aside from a co-culture-based approach, several methods exist for studying microbial interactions. These include a variety of 'omics'-based approaches (metagenomics, metatranscriptomics, metaproteomics), assessing the flow of carbon through a complex microbial community to identify key substrate degraders via stable isotope probing (SIP) techniques, via spectroscopic methods that are based on fluorescent *in situ* hybridization (FISH) (Magneto-FISH, CARD-FISH, MAR-FISH, Raman-FISH), and high resolution mass spectral approaches such as NanoSIMS [2,12,13,14]. The coupling of cell sorting with single cell whole genome sequencing, recently shown as an effective method for obtaining genetic information for uncultured organisms [15•], is another cutting-edge approach that can potentially be applied to help understand syntrophic processes. Genomics and transcriptomics have allowed for advances in the understanding of key genes involved in syntrophic interactions in well-defined co-cultures [1••,7•,9•,16,17•], thus extending these approaches to mixed hydrocarbon-degrading methanogenic cultures will help determine syntrophic potential among more complex communities. Metagenomic information recently released for hydrocarbon-degrading cultures and resource environments [18•,19•] can now be used to mine for 'syntrophy' genes to help inform the design of further studies. In a field setting, deploying biotraps laced with ^{13}C-labeled hydrocarbons has yielded taxonomic and functional data regarding organisms associated with anaerobic hydrocarbon biodegradation [4•]; such an approach coupled with meta-omics and FISH-based analyses could help resolve *in situ* syntrophic interactions within dynamic hydrocarbon-contaminated aquifers [12].

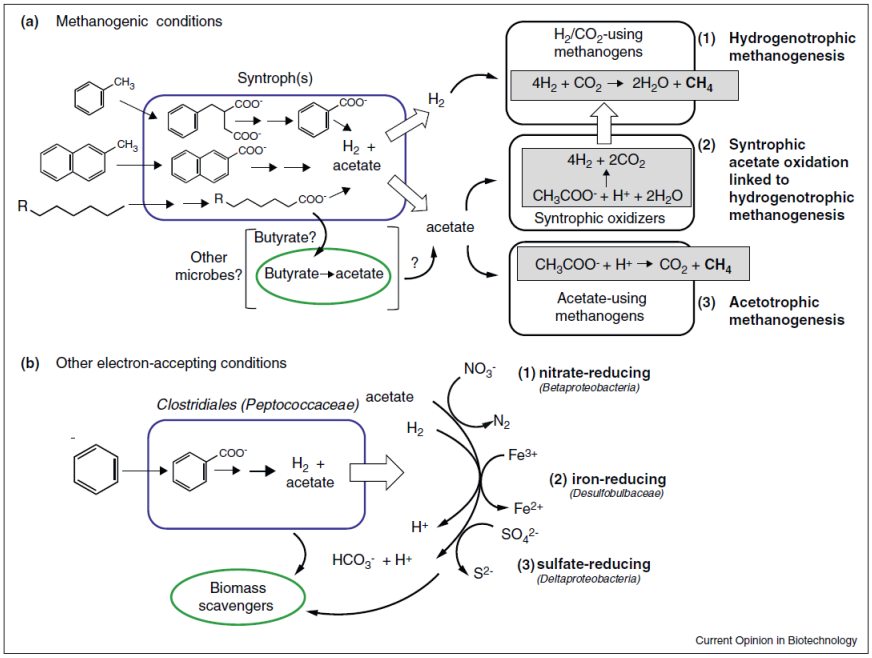
Hydrocarbon-contaminated environments and syntrophic consortia

Many shallow subsurface sediment environments, such as groundwater aquifers, rapidly become anaerobic following hydrocarbon fuel contamination and often become methanogenic (e.g., [20,21•]). Hydrocarbons are considered to be 'difficult' substrates since they are relatively inert but it is now well known that they can be metabolized anaerobically. Although methanogenic biodegradation occurs for *n*-alkanes, alkenes, monoaromatic hydrocarbons, and polycyclic aromatic hydrocarbons (PAHs) [22,23], relatively little is understood about the metabolic pathways or syntrophic interactions in such systems. Syntrophic metabolism is required for hydrocarbon mineralization to CH_4 and CO_2 and this process can involve multiple microbes and pathway variations en route to methane production (Figure 1a). Several different possibilities by which hydrocarbons can be syntrophically degraded to methane have been described [24•,25]; in particular, determining the fate of acetate during hydrocarbon degradation has been of great interest [26•,27•,28•,29•]. Thermodynamics, environmental conditions (e.g., acetate, CO_2, and H_2 concentrations; temperature), and microbial community composition likely determine which metabolic pathway is dominant in different methanogenic hydrocarbon-containing ecosystems [21•,24•,25,28•].

Syntrophy in hydrocarbon-degrading methanogenic cultures

Syntrophic hydrocarbon degradation by co-cultures has only rarely been reported [5]. Recently, Callaghan *et al.*

Figure 1



Working conceptual models for the syntrophic biodegradation of contaminant hydrocarbons under **(a)** methanogenic conditions (in the absence of electron acceptors) and **(b)** in the presence of electron acceptors such as nitrate, Fe(III), or sulfate. Under methanogenic conditions, toluene degradation has been found to proceed via fumarate addition [41], while carboxylic acid metabolites have been detected during the degradation of alkyl PAHs [42] and *n*-alkanes [43,56]. Hydrocarbon metabolism by syntrophs presumably yields H_2 that can be consumed by hydrogenotrophic methanogens (a1) [29•] and acetate that can be used directly by acetotrophic methanogens (a3) [28•,29•] or undergo syntrophic acetate oxidation coupled with hydrogenotropic methanogenesis (a2) [26•,27•]. Other syntrophic interactions may also occur under methanogenic conditions [33••,34]. In the presence of electron acceptors, syntrophic benzene degradation by *Clostridiales* may be coupled with (b1) nitrate reduction [39], (b2) iron reduction [35•], or (b3) sulfate reduction [36,37••,38].

[30••] showed that *Desulfatibacillum alkenivorans* AK-01, a sulfate-reducer capable of *n*-alkane biodegradation via fumarate addition, produces methane from *n*-hexadecane in the absence of sulfate when co-cultured with a H_2/formate-using methanogen. The AK-01 genome revealed key genes related to electron transfer mechanisms found in other syntrophs, and genes for a membrane associated complex known to be involved in syntrophic lactate metabolism by sulfate-reducers. Hydrogenase genes associated with H_2 production were not found, but genes for three formate dehydrogenases were found in the AK-01 genome, suggesting that interspecies formate transfer may be key in this syntrophic pairing [30••].

In mixed methanogenic hydrocarbon-degrading consortia, evidence for syntrophic processes has been gleaned using a variety of approaches (Table 2). Many phylogenetic-based studies have revealed that diverse microbes comprise methanogenic hydrocarbon-degrading consortia or hydrocarbon-laden environments. In a survey of 26 bacterial clone libraries constructed from hydrocarbon-contaminated sites and petroleum reservoirs, Gray *et al.* [6•] showed that 96% of the available bacterial sequences affiliated with 15 different phyla including *Firmicutes* and all classes of *Proteobacteria*, among others. Kleinsteuber *et al.* [4•] provided a summary of the key bacterial taxa associated with syntrophic aromatic hydrocarbon-degrading consortia. Phylotypes related to *Pelotomaculum*,

Table 2

Summary of recent reports (since 2008) describing mixed methanogenic hydrocarbon-degrading cultures and experimental approaches used to assess syntrophic processes

Reference	Source of methanogenic enrichment; hydrocarbon substrate	Approaches to assess syntrophic processes				
		Demonstration of hydrocarbon loss coupled with CH_4 production	Microbial community analysis	qPCR or T-RFLP	Stable isotope probing (SIP)	Other method(s)
Jones *et al.* [47]	River sediments; crude oil	✓	✓			Carbon isotopic measurements of gas and oil
Gieg *et al.* [48]	Contaminated aquifer sediments; crude oil	✓	✓			
Sakai *et al.* [31•]	Lotus field soil; benzene	✓	✓	✓	DNA-SIP	
Gieg *et al.* [49]	Oilfield; crude oil	✓	✓			
Feisthauer *et al.* [50]	Several sites, *n*-hexadecane	Isotopic fractionation of CH_4 and CO_2 produced relative to controls				Stable isotope fractionation
Mayumi *et al.* [26•]	Oilfield; crude oil	CH_4 production relative to controls	✓	✓		^{14}C-labeling to determine dominant methanogenic pathway
Siegert *et al.* [51]	Harbor sediments; *n*-hexadecane, ethylbenzene, naphthalene	CH_4 and CO_2 production relative to controls	✓			Enhanced methanogenesis via the addition of iron(III) or sulfate
Gray *et al.* [27•]	River sediments; crude oil	✓[a]	✓	✓		^{13}C-assay to determine dominant methanogenic pathway
Siddique *et al.* [52]	Oil sands tailings ponds; C_{14}, C_{16}, C_{18} *n*-alkanes	✓	✓			
Zhang *et al.* [32]	Landfill leachate contaminated sediments; anthracene	✓			DNA-SIP	
Wang *et al.* [53]	Oilfield; C_{15}–C_{20} *n*-alkanes	CH_4 production relative to controls	✓			
Siddique *et al.* [54]	Oil sands tailings ponds; C_{14}, C_{16}, C_{18} *n*-alkanes, naphtha, BTEX	✓	✓			
Fowler *et al.* [41]	Contaminated aquifer sediments; toluene	✓	✓			
Berdugo-Clavijo *et al.* [42]	Contaminated aquifer sediments; naphthalenes	✓	✓			
Mbadinga *et al.* [55]	Oilfield; C_{15}–C_{20} *n*-alkanes	CH_4 production relative to controls	✓			Acetate and formate measurements
Morris *et al.* [29•]	Contaminated aquifer sediments; *n*-hexadecane, *n*-hexadecanoic acid	Isotopic fractionation of CH_4 and CO_2 produced relative to controls	✓[a]		Protein-SIP	Stable isotope fractionation, metaproteomics
Zhou *et al.* [56]	Oilfield; C_{15}–C_{20} *n*-alkanes	CH_4 production relative to controls	✓			
Cheng *et al.* [57]	Oilfield waste disposal area; *n*-hexadecane	CH_4 production relative to controls	✓	✓		
Tan *et al.* [18•]	Oil sands tailings pond; C_6–C_{10} *n*-alkanes	✓	✓			Sequenced metagenome

[a] Performed in an earlier study with the same culture.

Peptococcaceae, *Clostridium*, *Deltaproteobacteria*, *Syntrophus*, and *Sporotomaculum* have been reported as anaerobic hydrocarbon-degraders in methanogenic environments, while *Chloroflexi*, *Anaerolineae*, and *Bacteroidetes* (among several others) are thought to be secondary degraders that scavenge dead biomass or hydrocarbon intermediates along with acetate and H_2-using methanogens [4•]. Members of the *Syntrophaceae* (*Syntrophus*, *Smithella*) have been identified in multiple hydrocarbon-impacted environments and are prevalent in *n*-alkane or crude oil-degrading cultures, implicating them as key players in syntrophic hydrocarbon metabolism [6•,27•]. While microbial community analysis has been frequently used to *infer* syntrophy (Table 2), these data alone do not provide definitive evidence for the involvement of the various identified taxa in syntrophic hydrocarbon metabolism.

In a study examining the methanogenic crude oil biodegradation by river sediment communities, Gray *et al.* [27•] used qPCR to show that bacteria most closely related to *Smithella* grew most predominantly in parallel to alkane loss and methane production, suggesting that these organisms were the main hydrocarbon-degrading syntrophs. Assays showed that acetate consumption was due more to syntrophic acetate oxidation coupled with hydrogenotrophic methanogensis rather than acetotrophic methanogenesis in the culture. Mayumi *et al.* [26•] also reached a similar conclusion by using radiotracer assays to examine methanogenic crude oil biodegradation under high temperature and pressure conditions. The known syntrophic acetate-oxidizing thermophile *Thermacetogenium* and the H_2-using *Methanothermobacter* were the dominant organisms identified in the enrichment via 16S rRNA gene sequencing [26•]. In a subsequent study conducted with similar oilfield fluids but in the presence of higher CO_2 concentrations, acetotrophic methanogenesis was favored instead [28•].

Sakai *et al.* [31•] used DNA-SIP to study methanogenic $^{13}C_6$-benzene degradation. The closest cultured member to the dominant organism assimilating ^{13}C (named Hasda-A) was *Syntrophus gentianae* (85% identity). The phylotype increased in abundance during the course of benzene degradation as revealed using qPCR, and both acetate and H_2-using methanogens were found in the enrichment [34]. Also using DNA-SIP, Zhang *et al.* [32] implicated *Methylibium*, *Legionella*, and unclassified *Rhizobiales* in the methanogenic degradation of anthracene, taxa that are typically classified as aerobes and not associated with anaerobic processes. However, the cultures were derived from landfill leachate material; the presence of diverse contaminants in leachate may promote unique microbial interactions compared to environments only impacted by hydrocarbons. Morris *et al.* [29•] used protein-SIP, metaproteomics, and stable isotopic fractionation of CO_2 and CH_4 to demonstrate that acetotrophic and hydrogenotrophic methanogenesis occurred to a relatively equal extent during the mineralization of *n*-hexadecane by a culture known to degrade crude oil, showing that in some systems no single downstream methanogenic pathway is dominant.

Studies conducted with a methanogenic terephthalate-degrading culture have shown that multiple syntrophic interactions can occur in contaminant-degrading communities [33••,34]. Through metagenomic and metaproteomic analyses, *Pelotomaculum* was identified as the key terephthalate degrader via initial decarboxylation, ultimately producing intermediates like butyrate, acetate, and H_2/CO_2. The latter compounds were presumably coupled with methanogenesis (*Methanosaeta*, *Methanolinea*) while secondary syntrophs affiliating with *Thermotogae* and OP5 (*Candidatus* Cloacamonas acidaminovorans) were thought to be involved in the oxidation of butyrate to acetate and H_2. In addition, *Caldiserica* (WWE1) was proposed to fix CO_2/H_2 to butyrate, feeding the butyrate-oxidizers [33••,34]. Although not a hydrocarbon, experiments conducted with terephthalate provided new insights into complex syntrophic interactions that may help inform methanogenic studies being conducted with hydrocarbons. Figure 1a shows a proposed model of syntrophic hydrocarbon degradation coupled to CH_4 production based on studies conducted thus far.

Syntrophy in hydrocarbon-degrading cultures in the presence of electron acceptors

Recently it has been suggested that syntrophic processes can occur during hydrocarbon biodegradation (mainly for benzene) even in the presence of electron acceptors, such as nitrate, Fe(III), or sulfate (Figure 1b). Using an Fe(III)-reducing benzene-degrading enrichment culture, Kunapuli *et al.* [35•] used DNA-SIP with $^{13}C_6$-benzene to identify the key benzene-degrading bacteria. On the basis of the results, the authors proposed that benzene was degraded via syntrophic interactions between *Peptococcaceae* and *Desulfobulbaceae* phylotypes wherein the former coupled benzene oxidation to iron reduction, producing H_2 that served as an energy source for the latter. Other labeled organisms were postulated to act acetogenically, or use dead biomass or secondary fermentation products.

Using BTEX-contaminated aquifer sediments incubated under sulfate-reducing conditions, Herrmann *et al.* [36] also used DNA-SIP with $^{13}C_6$-benzene in a time course experiment to characterize the benzene-degrading community. From the results, the authors proposed that a dominant *Cryptanaerobacter*/*Pelotomaculum* phytotype initiated the attack on benzene, degrading it incompletely to intermediates such as acetate and/or H_2 that could be consumed by methanogens or sulfate-reducers [36]. Subsequent metagenomic-based protein-SIP analyses with the culture (using $^{13}C_6$-benzene and ^{13}C-CO_2) revealed three major differentially labeled protein

groups, likely derived from three functional groups of organisms metabolizing different carbon sources [37••]. Notably, a group of proteins associated primarily with the *Clostridiales* (*Peptococcaceae*, *Cryptanaerobacter*/*Pelotomaculum*) included almost the entire benzoyl CoA pathway (lower part of pathway involved in the anaerobic metabolism of aromatic hydrocarbons) in addition to proteins involved in CO_2 fixation and dissimilatory sulfate reduction; this finding was consistent with the previous reports that this phylotype was the primary benzene degrader [36]. In a separate study, H_2 and acetate additions to the enrichment were found to inhibit benzene degradation; this would not be expected to occur if benzene degradation was only directly linked to sulfate reduction [38].

Finally, syntrophic benzene degradation was also reported to occur under nitrate-reducing conditions [39]. Even after cultivation in a chemostat for 8 years, a biofilm-associated microbial community carrying out nitrate-dependent benzene degradation was found to be very diverse. When H_2 was added to the chemostat, the rate of benzene degradation was reduced suggesting that H_2 transfer may play a role. A time-dependent DNA-SIP analysis with $^{13}C_6$-benzene showed that *Peptococcaceae* and *Betaproteobacteria* phylotypes were enriched in heavy fractions. The authors postulated that the *Peptococcaceae* was degrading benzene to H_2 and acetate that were then being consumed by the *Betaproteobacteria*, with both processes coupled to the reduction of nitrate [39].

Dozens of bacteria that can metabolize hydrocarbons under varying electron-accepting conditions have been isolated [22,23,40], thus these observations represent a shift away from the idea that only single organisms are metabolizing hydrocarbons when electron acceptors are available. While the thermodynamic reasons for this are not clear, the syntrophic metabolism of other substrates like butyrate has been reported in other mixed systems in the presence of electron acceptors such as sulfate, so there is precedence for these observations [21•]. Interestingly, in all of the benzene-amended enrichments described above, *Peptococcaceae* phylotypes were identified as the primary degraders and it is known that some strains within this class (e.g., *Pelotomaculum*) are obligate syntrophs that require metabolic partners to carry out favorable reactions [1••,3]. These studies demonstrate that the composition of a given microbial community, along with the metabolic abilities of the microbial members, can greatly influence the pathway of carbon flow through an ecosystem [21•,38].

Hydrocarbon activation mechanisms under syntrophic conditions

The mechanisms by which hydrocarbon substrates are activated under syntrophic conditions are not clear. Fumarate addition is the most frequently reported mechanism of anaerobic hydrocarbon activation, as it occurs for the activation of methyl substituted aromatics and alkanes under nitrate-reducing and sulfate-reducing conditions [40]. Under methanogenic conditions, only toluene has been shown to undergo fumarate addition (e.g., [41]). Fumarate addition metabolites have not been detected during the methanogenic degradation of methyl-substituted PAH [42] or *n*-alkanes [43] even though the requisite genes (e.g., *assA* encoding alkylsuccinate synthase) have been detected in several crude oil-degrading methanogenic enrichments [44]. Interestingly, in a short chain alkane-degrading methanogenic culture where the *assA* gene was detected in the metagenome, Tan *et al.* [18•] detected fumarate addition products from branched alkanes, but not from *n*-alkanes. Confirming hydrocarbon activation mechanisms by detecting the requisite metabolites during syntrophic metabolism remains difficult, possibly due to tight syntrophic interactions that are not conducive to metabolite accumulation. It is not known whether mechanisms of hydrocarbon activation other than fumarate addition occur during methanogenic hydrocarbon metabolism [43], however, Abu Laban *et al.* [45] detected a putative benzene carboxylase (via proteomics) in the Fe(III)-reducing culture implicated in syntrophic benzene metabolism [35•]. Thus, syntrophic hydrocarbon-degrading consortia do appear to harbor some genes for known anaerobic hydrocarbon activating enzymes though confirming their activity is difficult. Other approaches, such as transcriptomics, qRT-PCR, or proteomics can potentially aid in detecting the expression of hydrocarbon activation genes in mixed systems.

Conclusions and future directions

Mutually beneficial interactions between syntrophic microorganisms occur widely during the cycling of organic matter, including in hydrocarbon-containing environments. While it is known that methanogenic hydrocarbon metabolism occurs, defining syntrophic partnerships in such systems is far from being understood. That syntrophic hydrocarbon metabolism occurs even in the presence of electron acceptors like nitrate, Fe(III), or sulfate shows that microbial community interactions are not straightforward and that multiple metabolic strategies exist in the natural environment. Genomic, transcriptomic, and mutant studies conducted with defined co-cultures are yielding new information about how microbes interact and benefit energetically via different mechanisms of interspecies electron transfer. Future work with the AK-01/methanogen co-culture [30••] will likely serve as a paradigm for syntrophic hydrocarbon metabolism and the isolation of other microbes with similar capabilities is a clear research need. Understanding syntrophic hydrocarbon degradation has clear implications for bioremediation applications, but new knowledge can be used to develop biotechnology applications related to microbially enhanced energy recovery from fossil

energy reservoirs, biofilm control (e.g., related to oil transmission pipeline corrosion), and electromicrobiology [6•,8]. With the recent release of new metagenomic information from hydrocarbon-associated environments [18•,19•], determining the microbial players, metabolic pathways, and interactions among the microbes involved in the syntrophic metabolism of contaminants offers the promise of new discovery.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. •• Sieber JR, McInerney MJ, Gunsalus RP: **Genomic insights into syntrophy: the paradigm for anaerobic metabolic cooperation**. *Ann Rev Microbiol* 2012, **66**:429-452.
A comprehensive review describing the principles of syntrophy, including features recently elucidated via genomic analysis. Provides a starting point for the examination of syntrophy genes in a variety of genomes and metagenomes.

2. Morris BEL, Henneberger R, Huber H, Moissl-Eichinger C: **Microbial syntrophy: interaction for the common good**. *FEMS Microbiol Rev* 2013, **37**:384-406.

3. Stams AJM, Plugge CM: **Electron transfer in syntrophic communities of anaerobic bacteria**. *Nat Rev Microbiol* 2009, **7**:568-577.

4. • Kleinsteuber S, Schleinitz KM, Vogt C: **Key players and team play: anaerobic microbial communities in hydrocarbon-contaminated aquifers**. *Appl Microbiol Biotechnol* 2012, **94**:851-873.
Comprehensive overview of the microbial ecology associated with anaerobic hydrocarbon-contaminated environments.

5. McInerney MJ, Sieber SJ, Gunsalus RP: **Syntrophy in anaerobic global carbon cycles**. *Curr Opin Biotechnol* 2009, **20**:623-632.

6. • Gray ND, Sherry B, Hubert C, Dolfing J, Head IM: **Methanogenic degradation of petroleum hydrocarbons in subsurface environments: remediation, heavy oil formation, and energy recovery**. *Adv Appl Microbiol* 2010, **72**:137-161.
Comprehensive overview of applications related to hydrocarbon methanogenesis; also provides an analysis showing the taxa most commonly associated with oil-containing environments.

7. • Walker CB, Redding-Johanson AM, Baidoo EE, Rajeev L, He Z, Hendrickson EL, Joachimiak MP, Stolyar S, Arking AP, Leigh JA *et al.*: **Functional responses of methanogenic archaea to syntrophic growth**. *ISME J* 2012, **6**:2045-2055.
Using transcriptional and proteomic analyses, the functional responses of a H_2-using methanogen during syntrophic and non-syntrophic growth was assessed to pinpoint important genes expressed by H_2-consuming partners involved in syntrophic interactions.

8. Lovley DR: **Electromicrobiology**. *Ann Rev Microbiol* 2012, **66**:391-409.

9. • Meyer B, Kuehl J, Deutschbauer AM, Price MN, Arkin AP, Stahl DA: **Variation among *Desulfovibrio* species in electron transfer systems used for syntrophic growth**. *J Bacteriol* 2013, **195**:990-1004.
This study highlights the expression of syntrophy-associated genes in two different *Desulfovibrio* species, showing that there can be multiple genetic responses related to energy conservation during syntrophic growth even within the same genus.

10. Kato S, Hashimoto K, Watanabe K: **Methanogenesis facilitated by electric syntrophy via (semi)conductive iron-oxide minerals**. *Environ Microbiol* 2012, **14**:1646-1654.

11. Shimoyama T, Kato S, Ishii S, Watanabe K: **Flagellum mediates symbiosis**. *Science* 2009, **323**:1574.

12. Orphan VJ: **Methods for unveiling cryptic microbial partnerships in nature**. *Curr Opin Biotechnol* 2009, **12**:231-237.

13. Hazen TC, Rocha AM, Techtmann SM: **Advances in monitoring environmental microbes**. *Curr Opin Biotechnol* 2013, **24**:526-533.

14. von Bergen M, Jehmlich N, Taubert M, Vogt C, Bastida F, Herbst FA, Schmidt F, Richnow HH, Seifert J: **Insights from quantitative metaproteomics and protein-stable isotope probing into microbial ecology**. *ISME J* 2013. doi: 10.1038/ismej.2013.78..

15. • Rinke C, Schwientek P, Sczyrba A, Ivanova NN, Anderson IJ, Cheng JF, Darling A, Malfatti S, Swan BK, Gies EA *et al.*: **Insights into the phylogeny and coding potential of microbial dark matter**. *Nature* 2013, **499**:431-437.
Combined cell sorting with single-cell whole genome amplification to reveal genomic information about uncultured organisms, a tool that can help reveal genomic information about syntrophic organisms that have been difficult to obtain in pure culture.

16. Rotaru AE, Shrestha PM, Liu F, Ueki T, Nevin K, Summers ZM, Lovely DR: **Interspecies electron transfer via hydrogen and formate rather than direct electron connections in cocultures of *Pelobacter carbinolicus* and *Geobacter sulfurreducens***. *Appl Environ Microbiol* 2012, **78**:7645-7651.

17. • Shrestha PM, Rotaru AE, Summers ZM, Shrestha M, Liu F, Lovley DR: **Transcriptomic and genetic analysis of direct interspecies electron transfer**. *Appl Environ Microbiol* 2013, **79**:2397-2404.
Shows that transcriptomic analyses can help deduce interspecies electron transfer processes that occur by H_2/formate transfer or via cell-to-cell contact.

18. • Tan B, Dong X, Sensen CW, Foght JM: **Metagenomic analysis of an anaerobic alkane-degrading microbial culture: potential hydrocarbon-activating pathways and inferred roles of community members**. *Genome* 2013. 10.1139/gen-2013-0069.
First published metagenomic analysis of a methanogenic hydrocarbon-degrading mixed culture.

19. • An D, Caffrey SM, Soh J, Agrawal A, Brown D, Budwill B, Dong X, Dunfield P, Foght JM, Gieg LM *et al.*: **Metagenomics of hydrocarbon resource environments indicates aerobic taxa and genes to be unexpectedly common**. *Environ Sci Technol* 2013, **47**:10708-10717.
Presents a taxonomic survey of 160 hydrocarbon-associated environmental samples and a high level analysis of 12 metagenomes from deep oil sands, coal beds, oil reservoirs, and oil sands tailings ponds, data from which can now be used to mine for metabolic features in hydrocarbon environments including genes related to syntrophy.

20. Kleikemper J, Pombo SA, Schroth HM, Sigler WV, Pesaro M, Zeyer J: **Activity and diversity of methanogens in a petroleum hydrocarbon-contaminated aquifer**. *Appl Environ Microbiol* 2005, **71**:149-158.

21. • Struchtemeyer CG, Duncan KE, McInerney MJ: **Evidence for syntrophic butyrate metabolism under sulfate-reducing conditions in a hydrocarbon-contaminated aquifer**. *FEMS Microbiol Ecol* 2011, **76**:289-300.
Conducted physiological studies with aquifer sediments to show that syntrophic butyrate metabolism occurs even in the presence of available electron acceptors such as sulfate, demonstrating a precedence for this metabolism in natural environments.

22. Foght JM: **Anaerobic biodegradation of aromatic hydrocarbons: pathways and prospects**. *J Mol Microbiol Biotechnol* 2008, **15**:93-120.

23. Mbadinga SM, Wang LY, Zhou L, Liu JF, Gu JD, Mu BZ: **Microbial communities involved in anaerobic degradation of alkanes**. *Int Biodeter Biodegrad* 2011, **65**:1-13.

24. • Dolfing J, Larter SR, Head IM: **Thermodynamic constraints on methanogenic crude oil biodegradation**. *ISME J* 2008, **2**:442-452.

Describes the thermodynamic constraints that play a role in determining the different routes used in the downstream pathways for methanogenic hydrocarbon degradation.

25. Dolfing J, Xu A, Gray ND, Larter SR, Head IM: **The thermodynamic landscape of methanogenic PAH degradation**. *Microb Biotechnol* 2009, **2**:566-574.

26. • Mayumi D, Mochimaru H, Yoshioka H, Sakata S, Maeda H, Miyagawa Y, Ikarashi M, Takeuchi M, Kamagata Y: **Evidence for syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis in the high-temperature petroleum reservoir of Yabase oil field (Japan)**. *Environ Microbiol* 2011, **13**:1995-2006.

Showed that hydrocarbon degradation was coupled to syntrophic acetate oxidation and hydrogenotrophic methanogenesis rather than via acetoclastic methanogenesis during crude oil biodegradation under high temperature and pressure conditions as would be expected in an oil reservoir.

27. • Gray ND, Sherry A, Grant RJ, Rowan AK, Hubert CRJ, Callbeck CM, Aitken CM, Jones DM, Adams JJ, Larter SR, Head IM: **The quantitative significance of *Syntrophaceae* and syntrophic partnerships in methanogenic degradation of crude oil alkanes**. *Environ Microbiol* 2011, **13**:2957-2975.

A phylotype affiliating with *Syntrophaceae (Smithella*) was shown to increase in abundance concurrently with alkane degradation and methane production suggesting its importance as a key oil degrader. In addition, the fate of acetate was shown to occur via syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis.

28. • Mayumi D, Dolfing J, Sakata S, Maeda H, Miyagawa Y, Ikarashi M, Tamaki H, Takeuchi M, Nakatsu CH, Kamagata Y: **Carbon dioxide concentration dictates alternative methanogenic pathways in oil reservoirs**. *Nat Commun* 2013, **4** article 1998, doi: 10.1038/ncomms2998..

Demonstrates that environmental factors, such as CO_2 concentrations, can alter the pathway of methane formation (e.g., fate of acetate) during oil biodegradation.

29. • Morris BEL, Herbst FA, Bastida F, Seifert J, von Bergen M, Richnow HH, Suflita JM: **Microbial interactions during residual oil and *n*-fatty acid metabolism by a methanogenic consortium**. *Environ Microbiol Rep* 2012, **4**:297-306.

Using approaches such as protein-SIP and isotopic gas measurements, this study shows that both acetotrophic and hydrogenotrophic methanogenesis can occur during crude oil biodegradation.

30. •• Callaghan AV, Morris BEL, Pereira IAC, McInerney MJ, Austin RN, Groves JT, Kukor JJ, Suflita JM, Young LY, Zylstra GJ *et al.*: **The genome sequence of *Desulfatibacillum alkenivorans* AK-01: a blueprint for anaerobic alkane oxidation**. *Environ Microbiol* 2012, **14**:101-113.

Genome sequencing of an alkane-degrading sulfate reducer/facultative syntroph that could serve as a model organism for elucidating key features of syntrophic hydrocarbon degrading partnerships.

31. • Sakai N, Kurisu F, Yagi O, Nakajima F, Yamamoto K: **Identification of putative benzene-degrading bacteria in methanogenic enrichment cultures**. *J Biosci Bioeng* 2009, **108**:501-507.

Identified a presumed benzene-degrading phylotype by DNA-SIP and showed an increase in abundance of the phylotype during methanogenic benzene degradation.

32. Zhang S, Wang Q, Xie S: **Stable isotope probing identifies anthracene degraders under methanogenic conditions**. *Biodegradation* 2012, **23**:221-230.

33. •• Lykidis A, Chen CL, Tringe SG, McHardy AC, Copeland A, Kyrpides NC, Hugenholtz P, Macarie H, Olmos A, Monroy O, Liu WT: **Multiple syntrophic interactions in a terephthalate-degrading methanogenic consortium**. *ISME J* 2011, **5**:122-130.

Using metagenomic analysis, multiple syntrophic interactions were proposed to occur in a methanogenic contaminant-degrading enrichment culture.

34. Wu JH, Wu FY, Chuang HP, Chen WY, Huang HJ, Chen SH, Liu WT: **Community and proteomic analysis of methanogenic consortia degrading terephthalate**. *Appl Environ Microbiol* 2013, **79**:105-112.

35. • Kunapuli U, Lueders T, Meckenstock RU: **The use of stable isotope probing to identify key iron-reducing microorganisms involved in anaerobic benzene degradation**. *ISME J* 2007, **1**:643-653.

First report to suggest that syntrophic processes are involved in hydrocarbon metabolism under non-methanogenic anaerobic conditions, and that Gram-positive organisms can be key anaerobic benzene degraders.

36. Herrmann S, Kleinsteuber S, Chatzinotas A, Kuppardt S, Lueders T, Richnow HH, Vogt C: **Functional characterization of an anaerobic benzene-degrading enrichment culture by DNA stable isotope probing**. *Environ Microbiol* 2010, **12**:401-411.

37. •• Taubert M, Vogt C, Wubet T, Kleinsteuber S, Tarkka MT, Harms H, Buscot F, Richnow HH, von Bergen M, Seifert J: **Protein-SIP enables time-resolved analysis of the carbon flux in a sulfate-reducing, benzene-degrading microbial consortium**. *ISME J* 2012, **6**:2291-2301.

In a culture proposed to syntrophically degrade benzene in the presence of sulfate, the use of protein-SIP allowed for the assignment of protein groups to the microbial phylotypes involved in assimilating carbon from benzene or CO_2 to trace the flow of carbon through the community.

38. Rakoczy J, Schleinitz KM, Muller N, Richnow HH, Vogt C: **Effects of hydrogen and acetate on benzene mineralization under sulphate-reducing conditions**. *FEMS Microbiol Ecol* 2011, **77**:238-247.

39. van der Zaan BM, Saia FT, Stams AJM, Plugge CM, de Vos WM, Smidt H, Langenhoff AA, Gerritse J: **Anaerobic benzene degradation under denitrifying conditions: *Peptococcaceae* as dominant benzene degraders and evidence for a syntrophic processes**. *Environ Microbiol* 2012, **14**:1171-1181.

40. Callaghan AV: **Metabolomic investigations of anaerobic hydrocarbon-impacted environments**. *Curr Opin Biotechnol* 2013, **24**:506-515.

41. Fowler SJ, Dong X, Sensen CW, Suflita JM, Gieg LM: **Methanogenic toluene metabolism: community structure and intermediates**. *Environ Microbiol* 2012, **14**:754-764.

42. Berdugo-Clavijo C, Dong X, Soh J, Sensen CW, Gieg LM: **Methanogenic biodegradation of two-ringed polycyclic aromatic hydrocarbons**. *FEMS Microbiol Ecol* 2012, **81**:124-133.

43. Aitken CM, Jones DM, Maguire MJ, Gray ND, Sherry A, Bowler BFJ, Ditchfield AK, Larter SR, Head IM: **Evidence that crude oil activation proceeds by different mechanisms under sulfate-reducing and methanogenic conditions**. *Geochim Cosmochim Acta* 2013, **109**:162-174.

44. Callaghan AV: **Enzymes involved in the anaerobic oxidation of *n*-alkanes: from methane to long-chain paraffins**. *Front Microbiol* 2013, **4** article 89, doi: 10.3389/fmicb.2013.00089..

45. Abu Laban N, Selesi D, Rattei T, Tischler P, Meckenstock RU: **Identification of enzymes involved in anaerobic benzene degradation by a strictly anaerobic iron-reducing enrichment culture**. *Environ Microbiol* 2010, **12**:2783-2796.

46. Thauer RK, Jungermann K, Decker K: **Energy conservation in chemotrophic anaerobic bacteria**. *Bacteriol Rev* 1977, **41**:100-180.

47. Jones DM, Head IM, Gray ND, Adams JJ, Rowan AK, Bennett B, Huang A, Brown A, Bowler BFJ, Oldenburg T, Erdmann M, Larter SR: **Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs**. *Nature* 2008, **451**:176-180.

48. Gieg LM, Duncan KE, Suflita JM: **Bioenergy production via microbial conversion of residual oil to natural gas**. *Appl Environ Microbiol* 2008, **74**:3022-3029.

49. Gieg LM, Davidova IA, Duncan KE, Suflita JM: **Methanogenesis, sulfate reduction and crude oil biodegradation in hot Alaskan oilfields**. *Environ Microbiol* 2010, **12**:3755-3762.

50. Feisthauer S, Siegert M, Seidel M, Richnow HH, Zengler K, Gründger F, Krüger M: **Isotopic fingerprinting of methane and CO_2 formation from aliphatic and aromatic hydrocarbons**. *Org Geochem* 2010, **41**:482-490.

51. Siegert M, Cichocka D, Herrmann S, Gründger F, Feisthauer S, Richnow HH, Springael D, Krüger M: **Accelerated methanogenesis from aliphatic and aromatic hydrocarbons**

under iron- and sulfate-reducing conditions. *FEMS Microbiol Lett* 2011, **315**:6-16.

52. Siddique T, Penner T, Semple K, Foght JM: **Anaerobic biodegradation of longer-chain *n*-alkanes coupled to methane production in oil sands tailings**. *Environ Sci Technol* 2011, **45**:5892-5899.

53. Wang LY, Gao CX, Mbadinga SM, Zhou L, Liu JF, Gu JD, Mu BZ: **Characterization of an alkane-degrading methanogenic enrichment culture from production water of an oil reservoir after 274 days of incubation**. *Int Biodeter Biodegrad* 2011, **65**:444-450.

54. Siddique T, Penner T, Klassen J, Nesbø C, Foght JM: **Microbial communities involved in methane production from hydrocarbons in oil sands tailings**. *Environ Sci Technol* 2012, **49**:9802-9810.

55. Mbadinga SM, Li KP, Zhou L, Wang LY, Yang SZ, Liu JF, Gu JD, Mu BZ: **Analysis of alkane-dependent methanogenic community derived from production water of a high-temperature petroleum reservoir**. *Appl Microbiol Biotechnol* 2012, **96**:531-542.

56. Zhou L, Li KP, Mbadinga SM, Yang SZ, Gu JD, Mu BZ: **Analyses of *n*-alkanes degrading community dynamics of a high temperature methanogenic consortium enriched from production water of a petroleum reservoir by a combination of molecular techniques**. *Ecotoxicology* 2012, **21**:1680-1691.

57. Cheng L, Rui J, Li Q, Zhang H, Lu Y: **Enrichment and dynamics of novel syntrophs in a methanogenic hexadecane-degrading culture from a Chinese oilfield**. *FEMS Microbiol Ecol* 2013, **83**:757-766.