Anaerobic biodegradation of hydrocarbons in different oil sands tailings ponds: key microbial players and main activation pathway of hydrocarbon biodegradation

by

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Abstract

Anaerobic biodegradation of hydrocarbons is an important process in oil sands tailings ponds, which directly affects consolidation of tailings and extent of greenhouse gas emissions from tailings ponds. Since tailings ponds from different operators differ due to extraction process, age of tailings deposition in ponds, and tailings management techniques employed, biodegradability of entrained residual hydrocarbons and structure and activities of hydrocarbon-degrading microbial communities may differ from one tailings ponds to another. In this research, we examined methanogenic biodegradation of different extraction solvents (hydrocarbons used in bitumen extraction) and their major components (*n*-alkanes and *iso*-alkanes) in mature fine tailings (MFT) collected from two oil sands operators (Shell Albian Sands and CNRL). We examined biodegradation of recalcitrant hydrocarbons such as *iso*-alkanes under iron-, nitrate- and sulfate-reducing conditions in Albian MFT to determine if presence of higher reduction potential molecules/electron acceptors would accelerate biodegradation of these recalcitrant hydrocarbons. We characterized microbial communities and functional genes during the biodegradation process to identify key microbial players and main activation pathway of hydrocarbon biodegradation.

The methanogenic cultures established from Albian and CNRL MFT exhibited unique hydrocarbon degradation patterns. Albian and CNRL MFT amended with paraffinic solvent and naphtha separately were incubated for ~1600 d. Albian and CNRL MFT exhibited ~400 and ~800 d lag phases, respectively, for paraffinic solvent (C_5-C_6) biodegradation after which *n*-alkanes were preferentially metabolized to CH_4 over *iso*-alkane. A shorter lag phase (~100 d) was observed for naphtha (primarily ~C_6-C_{10}) biodegradation in both Albian and CNRL MFT. Both Albian and CNRL MFT sequentially biodegrade *n*-, *iso*- and *cyclo*-alkane compounds from

naphtha during the incubation. MFT cultures amended with sole *n*-alkanes (C_5-C_{10}) and incubated for ~600 d revealed preferential biodegradation of shorter *n*-alkanes (C_5-C_8) in Albian MFT versus longer *n*-alkanes (C_8-C_{10}) in CNRL MFT though all *n*-alkanes were eventually biodegraded in both MFT. Interestingly when Albian and CNRL MFT were amended with shorter (mixture of three C_5-C_6 compounds) and longer (mixture of five C_6-C_9 compounds) *iso*-alkanes and incubated under methanogenic conditions, Albian MFT, after a lag period of ~200 d, biodegraded mixture of C_6 *iso*-alkanes during ~1500 d of incubation. CNRL MFT also biodegraded C_6 *iso*-alkanes after a lag phase of ~660 but it also displayed biodegradation of C_6-C_9 *iso*-alkanes after a lag phase of ~1200 d where *iso*-alkanes were sequentially biodegraded in the order of decreasing carbon-chain length. C_6 *iso*-alkanes were studied under other reducing conditions. Only partial degradation of a C_6 *iso*-alkane was observed under sulfate-reducing condition and no degradation was observed under iron- and nitrate-reducing conditions. These results suggest that iron- and nitrate-reducers indigenous to Albian MFT may not carry the appropriate genes for *iso*-alkanes degradation.

The 16S rRNA gene pyrosequencing revealed that bacterial sequence reads related to *Peptococcaceae* were enriched during biodegradation of the hydrocarbons in all methanogenic cultures implicating the role of *Peptococcaceae* as the primary hydrocarbon-degraders in methanogenic MFT. Interestingly, the archaeal communities in all methanogenic cultures exhibited codominance of acetoclastic (*Methanosaetaceae*) and hydrogenotrophic ("*Candidatus* Methanoregula"), emphasizing important roles of both methanogens as acetate and hydrogen consumers, respectively, which renders the anaerobic degradation process in MFT a thermodynamically feasible reaction.

The metabolite (intermediary biodegradation products) and functional gene analyses performed on all cultures during active degradation process revealed that fumarate addition might potentially be the primary anaerobic hydrocarbon activation pathway in both Albian and CNRL tailings ponds. The fumarate-added metabolites detected in methanogenic Albian and CNRL cultures were the transient products. However, under sulfate-reducing conditions, the fumarate-added metabolites persisted in the culture even after prolonged incubation, implying absence of microbial players capable of oxidizing these intermediary compounds. Subunit of alkylsuccinate/methylalkylsuccinate synthase (*assA/masD*) genes amplified in all the cultures further substantiates the postulation of fumarate addition as the primary anaerobic hydrocarbon activation pathway. The results demonstrate that microbial communities indigenous to tailings ponds have the potential to biodegrade structurally diverse hydrocarbons; however, the biodegradation pattern differs in different oil sands tailings ponds even though similar key bacterial and archaeal taxa were enriched during active biodegradation of the hydrocarbons. This work provides an insight into how microbial communities in oil sands tailings respond to the influx of complex hydrocarbons under different redox conditions, which could impact future tailings management and reclamation strategies.

Preface

The experimental setup was designed by myself, with the assistance of Associate Professor Dr. T. Siddique and Professor Emeritus Dr. J. M. Foght. All data collection and analysis in this thesis is my original work.

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List of abbreviation

assA	catalytic subunit of alkylsuccinate synthase gene
bssA	catalytic subunit of benzylsuccinate synthase gene
BSTFA	*N,O,*bis-(trimethylsilyl)trifluoroacetamide
CNRL	Canada Natural Resources Limited
FFT	fluid fine tailings
GC-FID	gas chromatography- flame ionization detector
GC-MS	gas chromatography-mass spectrometer
masD	catalytic subunit of methylalkylsuccinate synthase gene
MFT	mature fine tailings
MLSB	Mildred Lake Settling Basin
Naph	CNRL Naphtha
nmsA	catalytic subunit of naphthylmethylsuccinate synthase gene
Par	Albian paraffinic solvent
PCR	polymerase chain reaction
PONAU	paraffins, olefins, naphthenes, aromatics, unknowns
ppm	parts per million
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SCADC	short chain alkane degrading culture
TEA	terminal electron acceptor
TMS	trimethylsilyl
TP6	Suncor Tailing Pond 6
x g	relative centrifugal force

1 Introduction

1.1 Oil sands industry

1.1.1 Oil sands in Alberta

Alberta harbors one of the largest oil sands deposits in the world, estimated over 1.7 trillion barrels of bitumen (Fedorak & Coy, 2006). However, only ~10% of the estimated deposits are recoverable using existing technologies. Oil sands consist of quartz sand surrounded by a layer of water and clay and then covered by a slick of heavy oil known as bitumen. The proportion of each component of oil sands varies considerably with bitumen ranging from 0-19 wt% (weight percent), water ranging from 3-6 wt% and mineral content ranging from 84-86 wt% (Chalaturnyk, Scott, & Özüm, 2002). Since bitumen is very viscous in nature, the mining and extraction of bitumen from oil sands cannot be performed using oil well as used in the recovery of conventional crude oil. Currently, there are two major commercially economical methods that have been employed to mine and extract bitumen from the oil sands: 1) surface mining and 2) in-situ techniques.

Surface mining is only performed if the oil sands deposits are within ~75 meters deep from the surface. The deposit is mined after the overburden of muskeg, glacial till and Cretaceous bedrock (Chalaturnyk et al., 2002) are removed and the oil sands are excavated using power shovel and transported using dump trucks to the bitumen extraction plant. The Athabasca oil sands region is the only region in the world where the oil sands deposit is close enough to the surface, which is suitable for large-scale surface mining. Large-scale surface mining is presently the major method for oil sands extraction in the Athabasca oil sands region. There are currently five oil sands operators that operate oil sands mining projects in Athabasca region: 1) Syncrude Canada Ltd. (Syncrude), 2) Suncor Energy (Suncor), 3) Shell Albian Sands (Albian), 4) Canada Natural Resources Ltd. (CNRL) and 5) Imperial Oil Ltd (Imperial).

For other deeper deposits (more than 75 meters deep), the bitumen can be extracted using in-situ methods. Presently, the most common in-situ techniques used in commercial projects are steam-assisted gravity drainage (SAGD) and cyclic steam stimulation (CSS). These techniques involve drilling several wells into the deposits then pressurized steam is injected into the underground to liquefy the bitumen so that it can flow in the well and be pumped to the surface like conventional crude oil. In-situ methods, however, require high amount of natural gas and no

single method can be applied universally to all oil sands deposits across Athabasca oil sands regions since the content of bitumen varies considerably within each deposit and between different deposits. Therefore, several in-situ technologies, aimed at improving the extraction efficiencies and performances, are currently being developed and tested.

Compared to *in situ* methods, large-scale surface mining leaves greater environmental footprint. Oil sands excavation in surface mining cause disturbance on large area of land and the bitumen extraction process produces huge volume of slurry waste (Clark, 1931), which consists of water, sand, silt, clay, trace elements and residual hydrocarbons (Fedorak et al., 2003). Under Alberta zero-discharge policy for oil sands (Giesy, Anderson, & Wiseman, 2010), the slurry waste, also known as tailings, has to be stored on the mining site pending reclamation. The large volumes of tailings produced daily necessitate an extensive area (excavated mine pits) for containment. These pits act as settling basins for settling of fine particles and recovery of porewater for recycling in bitumen extraction process.

1.1.2 Extraction process

The current extraction process of surface-mined oil sands for bitumen is based on oil sands separation technique introduced by Dr. Karl Clark of the Alberta Research Council in the 1920s (Chalaturnyk et al., 2002) (Figure 1.1). After excavation of the oil sands ore, the first step of commercial bitumen extraction from the ore is conditioning, where the large boulders of oil sands ore are crushed and mixed with water to form a thick mixture known as slurry (Chalaturnyk et al., 2002). The slurry is then transported via hydrotransport pipelines and additional hot water is added to the slurry before it is fed into the primary separation vessel (Chalaturnyk et al., 2002). The slurry is left to settle while the bitumen froth that floats at the top is sent to extraction plant for further processing and upgradation. The sand sinks to the bottom and the sand and water mixture is pumped into tailings ponds (Chalaturnyk et al., 2002). In the secondary separation, the middling (middle portion between floating froth and sinking sand) is aerated to induce further frothing and increase recovery of bitumen (Chalaturnyk et al., 2002). The bitumen froth is heated to de-aerate and sent to froth treatment.

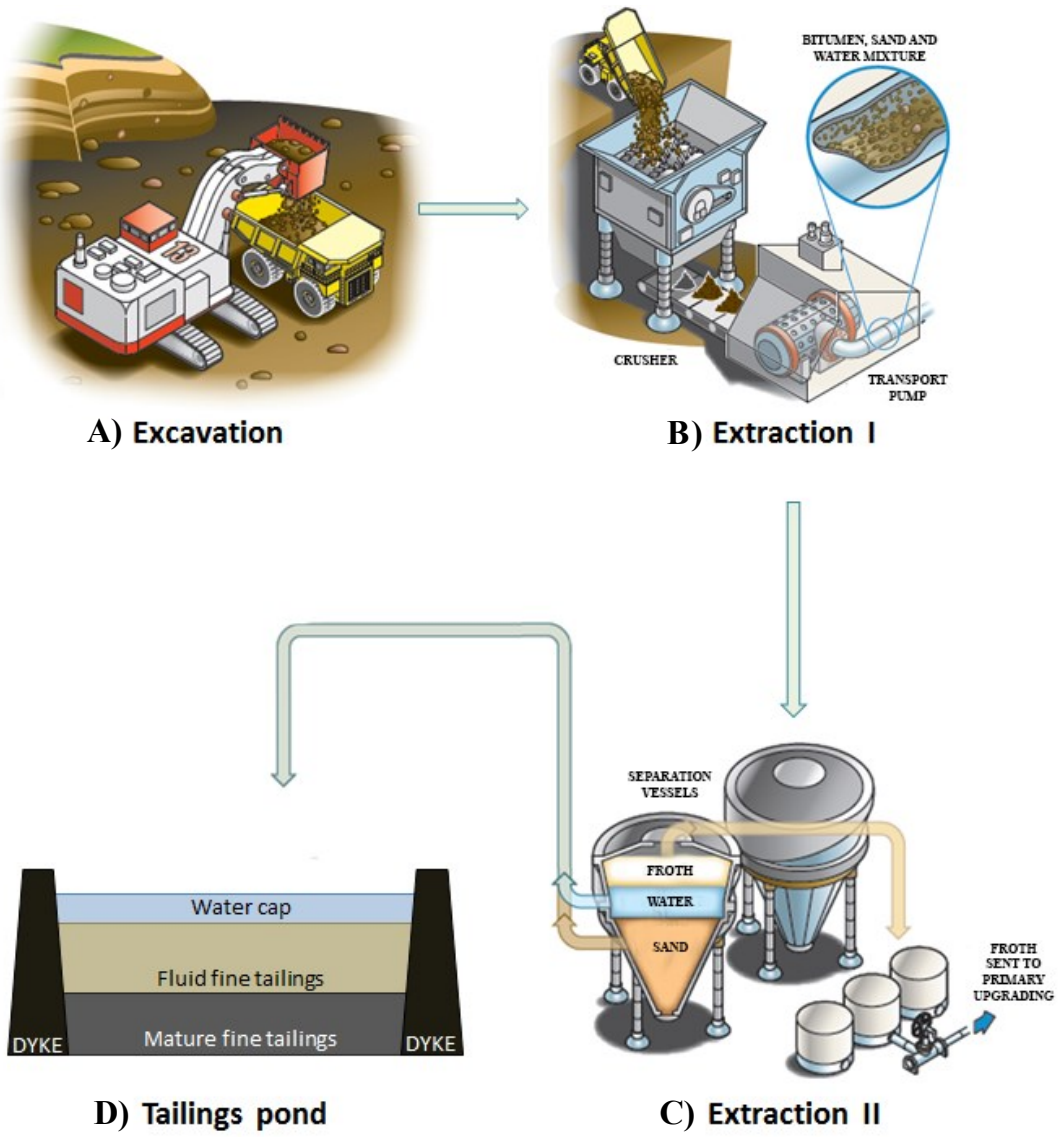

Figure 1.1: Schematic illustration of commercial oil sands mining and extraction procedures based on by Dr. Karl Clark's oil sands separation techniques. Briefly, oil sands were (A) excavated using mining shovels and sent to, (B) crusher, where oil sands were crushed and mixed with caustic water. The slurry mixture is then transported to, (C) the bitumen extraction plant. The slurry will go through frothing process in the bitumen extraction plant where bitumen froth is mixed with solvent and sent to upgrading facilities. The sand and water by-products are then sent to, (D) the tailings ponds to allow the fine particles to sediment, permitting the released water to be recycled for extraction procedure. Images are adapted with permission from CNRL (R. Barron, CNRL, personal communication, January 8, 2015).

The remaining water and solids in the de-aerated bitumen froth are removed and the bitumen is then diluted with extraction solvent, which is a mixture of low molecular weight hydrocarbons (Chalaturnyk et al., 2002). In this step, all oil sands operators use naphtha (mixture of low molecular weight aliphatic and aromatic hydrocarbons) as their extraction solvent except Albian (Li, 2010) and Imperial (Dr. T. Siddique, University of Alberta, personal communication, June 16, 2016), which uses mixtures of aliphatic C_5 and C_6 hydrocarbons as its extraction solvent. The diluted bitumen is sent through inclined plate settlers for particles to settle and thereafter centrifuged to spin out heavier constituents (Chalaturnyk et al., 2002). The extraction process is completed after the froth treatment and the cleaned bitumen is sent to the upgrader for further treatment (Chalaturnyk et al., 2002). The tailings from froth treatment are processed first to recover remaining extraction solvent from the tailings before they are discharged into tailings ponds.

1.1.3 Tailings management

The primary method used by all operators for managing the tailings is via discharging the tailings into tailings ponds. Since tailings has low flocculation, some operators pre-treat their tailings before deposition with additives to enhance consolidation of tailings solids in the ponds. For example, Albian adds both trisodium citrate and organic synthetic polymer flocculants to its tailings (Li, 2010) while CNRL injects carbon dioxide into its tailings (http://www.cosia.ca/carbon-dioxide-amended-tailings). Once the tailings are deposited, the sand particles in the discharged tailings will settle quickly in the tailings forming stable sediment. The cap water will then be recycled and reused in the extraction process (Chalaturnyk et al., 2002). The finer clay particles, however, will take several years to settle and thus they accumulate in the tailings ponds forming fluid fine tailings (FFT). The fine particles will further settle and consolidate over one or two years to reach 30 wt% solid, which is referred to as mature fine tailings (MFT) (Chalaturnyk et al., 2002). Further consolidation after formation of MFT was predicted to take more than a hundred years (Eckert, Masliyah, Gray, & Fedorak, 1996).

For mine closure, two reclamation proposals for the oil sands tailings have been suggested: (1) “wet-landscape” reclamation and, (2) “dry-landscape” reclamation (List & Lord, 1997). “Wet-landscape” reclamation is realized by water capping the MFT in mined-out pits to create end-pit lakes, where natural aquatic ecosystem is expected to develop over time for

sustained aquatic life (List & Lord, 1997). Currently, a commercial scale demonstration is being tested at Syncrude's Base Mine Lake Project to assess the feasibility of the method and to verify the potential benefits and risks associated with end-pit lakes (www.cosia.ca/pit-lake-research). The "dry-landscape" reclamation involves utilization of dried MFT that has enough strength to support sand capping, return of the overburden and original soil horizon, which will eventually be re-established as typical boreal forest (Kasperski & Mikula, 2011). The dried MFT may be formed via various methods where the MFT are either chemically and/or physically processed via the following methods: (1) consolidated or composite tailings, (2) rim-ditching, (3) thin-lift dewatering and, (4) centrifugation (Kasperski & Mikula, 2011; Sobkowicz, 2012). These MFT processing technologies are currently being assessed for viability and cost-effectiveness at pilot scale.

Currently (as of 2013), there are approximately 975 million m^3 of tailings (Siddique et al., 2015) stored in tailings ponds, which covers a liquid surface area of ~185 km^2 (http://osip.alberta.ca/map/). The volume of tailings, however, is estimated to further increase, exceeding one billion m^3 by 2020 (Chalaturnyk et al., 2002). Besides the high volume of tailings and low consolidation rate of tailings' fine particles (Eckert et al., 1996; Siddique, Kuznetsov, Kuznetsova, Li, et al., 2014), tailings also pose other challenges which include high concentration of contaminants (salts, residual hydrocarbons, naphthenic acids and heavy metals) (Siddique, Penner, Semple, & Foght, 2011) and release of greenhouse gasses (methane and carbon dioxide) to the atmosphere (Holowenko, MacKinnon, & Fedorak, 2000). Therefore, tailings management/reclamation has been one of the main concerns for the oil sands operators as well as for the public and regulatory authorities. Nevertheless, the effects of chemical and/or physical interventions in tailings ponds to expedite the remediation of the tailings by accelerating the consolidation of fine particles and removing the contaminants from the tailings are not fully comprehended. Hence, it is important to understand the biogeochemical properties of tailings to formulate effective tailings management practice, which consequently will assist in reducing tailings inventory, improving current tailings reclamation strategies and potentially uncovering new tailings reclamation options.

1.2 Biogeochemical processes in tailings ponds

1.2.1 Methanogenesis in tailings ponds

MFT in the tailings ponds is mostly anoxic. The high temperature used in the extraction process and the depth of the MFT from the surface contributes to the loss of the O_2 in the tailings. However, in the early 1990s, methane (CH_4) gas release was observed in Syncrude's Mildred Lake Settling Basin (MLSB; estimated ~40 million L d^{-1}) after more than a decade of operation (Holowenko et al., 2000). The CH_4 release was due to methanogenesis, which is a process driven by microbial metabolic activities under methanogenic conditions where available substrates in tailings ponds are metabolized to CH_4 and carbon dioxide (CO_2) as the final products of the process. CH_4 release observed in MLSB indicate that tailings ponds are indeed non-sterile and that they must harbor diverse microorganisms since methanogenesis is relatively a complex process (Figure 1.2). Thus, in the past decade, we observed burgeoning interest in examining tailings ponds' key microbial players and their function in oil sands tailings system and studying the effects of their activities on other geochemical processes in tailings ponds and physicochemical properties of oil sands tailings.

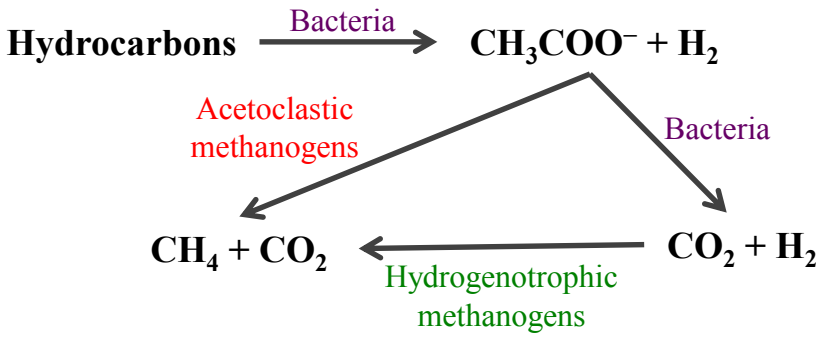

Figure 1.2: Schematic representation of methanogenic hydrocarbons biodegradation pathway.

1.2.1.1 Carbon source(s) for methanogenesis in MFT

Mature fine tailings are generally low in important nutrients, such as nitrogen and phosphate, which are essential for microbial growth (Fedorak et al., 2003; Penner & Foght, 2010). However, CH_4 emission observed in tailings ponds indicates that there are enough resources in tailings ponds to support microbial growth under methanogenic conditions. A recent study (Collins, Foght, & Siddique, 2016) has reported that nitrogen fixation occurred

concurrently with methanogenesis in oil sands tailings. The co-occurring nitrogen fixation process provides important fixed nitrogen as nutrient for sustenance of complex methanogenic microbial communities in tailings ponds. As for the carbon source, during the extraction of bitumen from oil sands, unrecovered bitumen and residual extraction solvent escape with tailings and end up in the tailings ponds, and make up ~5% and ~0.5% of the weight of MFT, respectively (MacKinnon, 1989; Siddique, Penner, Klassen, Nesbø, & Foght, 2012). However due to the fact that bitumen is a complex mixture of high molecular weight hydrocarbons, it is highly improbable for bitumen to be utilized as an immediate carbon source by the indigenous microbial communities in the tailings ponds. Therefore, unrecovered extraction solvent in the tailings ponds, which are composed of simple low molecular weight hydrocarbons, was speculated to provide the microorganisms with more favorable carbon sources (Siddique, Fedorak, & Foght, 2006).

To identify the potential carbon and energy sources which are utilized by the microbial communities in the tailings ponds for methanogenesis, Siddique, Fedorak and Foght (2006) spiked MFT retrieved from MLSB at Syncrude Canada with a mixture of C_6-C_{10} *n*-alkanes, which were major components of Syncrude's extraction solvent (naphtha), and incubated at room temperature under methanogenic conditions for a year. The researchers reported complete biodegradation of the spiked *n*-alkanes indicating components of extraction solvent were the likely source of methanogenesis in tailings ponds. Subsequent studies (Siddique, Fedorak, MacKinnon, & Foght, 2007; Siddique et al., 2011) also demonstrated complete biodegradation of major components of naphtha including monoaromatic compounds (toluene, ethylbenzene, *m*- and *o*-xylene), and longer chain (C_{14}-C_{18}) *n*-alkanes in MLSB MFT under methanogenic conditions. However, other components of naphtha including *iso*- and *cyclo*-alkanes were found to be recalcitrant (Siddique et al., 2007) during a year of incubation. Nevertheless, recent reports (Abu Laban, Dao, Semple, & Foght, 2014; Siddique et al., 2015; Tan, Dong, Sensen, & Foght, 2013; Tan, Semple, & Foght, 2015) have revealed that microbial communities in enrichment cultures derived from MLSB MFT are capable of metabolizing certain *iso*-and *cyclo*-alkanes into CH_4.

Since anaerobic microorganisms only degrade a very restricted range of hydrocarbons (Widdel, Knittel, & Galushko, 2010), specific hydrocarbons in tailings may only be degraded by specific microbial players. Thus, the availability and concentration of certain fractions of the

unrecovered hydrocarbons in the tailings may directly affect the microbial community structure. Besides the availability and concentration of hydrocarbons, other physicochemical properties of tailings, which differ from one tailings pond to another, may also affect the microbial communities' structures and activities. Therefore, it is important to identify the key microbial players involved in biodegradation of different groups of hydrocarbons and to determine how the microbial communities indigenous to oil sands tailings ponds are affected by changes in physicochemical properties of tailings ponds.

1.2.1.2 Microbial communities in the MFT

Initial evaluation of microbial communities via five-tube most probable number (MPN) assay revealed that tailings samples, collected from MLSB, harbor a wide variety of anaerobic microorganisms and exhibited activities from methanogens, denitrifiers and sulfate-reducing bacteria albeit the iron-reducing bacteria were lower in abundance compared to other groups of microorganisms (Holowenko et al., 2000; Penner & Foght, 2010; Salloum, Dudas, & Fedorak, 2002). MPN assay, however, is only a crude analysis to enumerate different types of microorganisms based on their activities and will not characterize the microbial community composition and structure. With the advent of more sophisticated and elaborate molecular analyses, detailed characterization of microbial community composition in MFT was made possible. In the early study (Penner & Foght, 2010), microbial communities in MFT collected from two Syncrude's tailings deposits, MLSB and West In-Pit (WIP), were examined by constructing clone libraries of archaeal and bacterial 16S rRNA genes. The bacterial communities were found to be diverse with *Proteobacteria* being the dominant phylum (~55%) while other bacteria related to nitrate-, iron-, and sulfate-reducing bacteria occupied the rest of the bacterial communities; whereas the archaeal communities were found to be exclusively related to methanogens with acetoclastic *Methanosaeta* spp. being the dominant genus (Penner & Foght, 2010).

However, under anaerobic conditions, different microbial players are involved during biodegradation of different types and/or ranges of hydrocarbons. Therefore, to identify key hydrocarbon-degrading microbial players in oil sands tailings ponds, several studies have been undertaken where MFT retrieved from tailings ponds were amended with various hydrocarbons. In one such study, Siddique et al. (2012) reported that composition of microbial communities in MLSB MFT amended separately with different hydrocarbons differed strikingly. In C_6-C_{10} *n*-

alkanes amended MFT, *Clostridiales* (*Desulfotomaculum*) were enriched while in benzene, toluene, ethylbenzene and xylene (BTEX) and naphtha amended MFT, *Clostridiales* (*Desulfotomaculum*) and *Syntrophobacterales* (*Syntrophus*) dominated the bacterial population (Siddique et al., 2012). The archaeal communities were dominated by acetoclastic *Methanosaetaceae* and hydrogenotrophic *Methanomicrobiales* in MFT amended with *n*-alkanes and BTEX, respectively; while in naphtha amended MFT, codominance of both acetoclastic and hydrogenotrophic methanogens was observed (Siddique et al., 2012). When compared to C_6-C_{10} *n*-alkanes amended MFT, different microbial communities were observed in methanogenic MLSB MFT amended with longer-chain *n*-alkanes (C_{14}-C_{18}) (Siddique et al., 2011); *Deltaproteobacteria* (*Syntrophus*) occupied the majority of the bacterial community whereas the archaeal community exhibited codominance of both acetoclastic and hydrogenotrophic methanogens (*Methanosarcinales* and *Methanomicrobiales*, respectively). Additionally, recent reports on methanogenic biodegradation of *iso*-alkanes (C_7-C_8) in MFT (Abu Laban et al., 2014; Siddique et al., 2015; Tan, Semple, & Foght, 2015) also unveiled different microbial communities where 16S rRNA gene pyrosequencing analysis revealed enrichment of *Peptococcaceae*-related sequences in the bacterial community whereas the archaeal community was dominated by sequences related to *Methanosaetaceae* and *Methanomicrobiaceae*.

Besides availability of carbon sources for microbial metabolism, composition of microbial communities are also affected by change in availability of terminal electron acceptors (TEA), such as sulfate, which has been suggested to inhibit methanogenesis in tailings ponds (Fedorak, Coy, Salloum, & Dudas, 2002; Salloum et al., 2002). In recent studies (An et al., 2013; Ramos-Padrón et al., 2011), where MFT retrieved from Suncor's gypsum-treated tailings pond 6 (TP6) were examined, the 16S rRNA genes pyrosequencing analysis revealed that the microbial community in TP6 was highly diverse with *Proteobacteria, Chloroflexi, Firmicutes* and *Euryarchaeota* representing the dominant phyla within the microbial population. Routine gypsum treatment in TP6 provides an elevated concentration of sulfate as TEA for the microorganisms in the MFT, which sequentially, led to the enrichment of syntrophs (*Pelotomaculum, Syntrophus* and *Smithella* spp.) and sulfate- and/or sulfur-reducing bacteria (*Desulfocapsa* and *Desulfovibrio* spp.) (Ramos-Padrón et al., 2011).

We can conclude from the above-mentioned studies that the microbial community compositions and activities in tailings ponds were indeed affected by the physicochemical

properties (ie: availability of carbon substrates and TEA) of the oil sands tailings system. Furthermore, the process of hydrocarbon biodegradation under anaerobic conditions, especially under methanogenic conditions, is still not fully understood. Therefore, characterization of key microbial players involved in the biodegradation of various types of hydrocarbons and determination of the pathway(s) by which the hydrocarbons are biodegraded in different oil sands tailings systems would improve our understanding of methanogenesis in tailings ponds. The information would not only contribute to the improvement of the current tailings management and reclamation strategies but would also advance our overall understanding of anaerobic hydrocarbons biodegradation in other hydrocarbon-impacted anoxic environmental systems.

1.2.1.3 Biodegradation pathway(s) of hydrocarbons in MFT

The C-H bond dissociation energies of hydrocarbons are high. To overcome this high energetic barrier, a highly reactive species is required. In aerobic microbes, the C-H bond dissociation energy of hydrocarbon is overcome by using highly reactive oxygen species via mono- or dioxygenase reactions (Boll & Heider, 2010). However, under anaerobic conditions, due to lack of oxygen gas (O_2), biodegradation of hydrocarbons cannot be initiated via mono- or dioxygenase reactions (Boll & Heider, 2010), thus, the initial activation of hydrocarbons under anaerobic conditions must proceed via different mechanisms. In the past two decades, numerous studies have been undertaken to elucidate the activation pathways of hydrocarbons under anaerobic conditions and several mechanisms have been postulated based on evidence from metabolites and functional genes analyses. The postulated mechanisms include: (1) fumarate addition, (2) hydroxylation, (3) methylation and (4) carboxylation (Boll & Heider, 2010; Foght, 2008; Mbadinga et al., 2011) (Figure 1.3).

The most reported mechanism of hydrocarbon activation under different anaerobic conditions is fumarate addition (Boll & Heider, 2010; Callaghan, Gieg, Kropp, Suflita, & Young, 2006; Callaghan, 2013; Foght, 2008; Mbadinga et al., 2011; von Netzer et al., 2013; Zedelius et al., 2011). Activation via fumarate addition was first discovered in anaerobic toluene-degrading cultures (Beller, Reinhard, & Grbic-galic, 1992; Biegert, Fuchs, & Heider, 1996; Evans, Ling, Goldschmidt, Ritter, & Young, 1992) where benzylsuccinate was identified as the first metabolite produced in the reaction. Later studies (Annweiler et al., 2000; Elshahed, Gieg, McInerney, & Suflita, 2001; Gieg & Suflita, 2002; Kropp, Davidova, & Suflita, 2000; So,

Phelps, & Young, 2003; So & Young, 1999; Wilkes et al., 2002) also showed that fumarate addition was the primary initial activation in anaerobic cultures amended with other hydrocarbon compounds including xylenes, ethylbenzene, 2-methylnaphthalene, *n*-hexanes and *n*-dodecane. Fumarate addition involves activation of a C-H bond where the alkyl chains of the hydrocarbons are reacted with fumarate to yield substituted succinates (Boll & Heider, 2010) The overall reaction of fumarate addition is exergonic, therefore, activation of hydrocarbons via this pathway was deemed favorable (Rabus et al., 2001). The enzyme catalyzing toluene fumarate addition, benzylsuccinate synthase (Bss), has been purified and well characterized (Leuthner et al., 1998). However, similar Bss-like enzymes have not yet been purified and characterized for other hydrocarbon compounds, although analogous genes with high similarity to the catalytic subunit of Bss (*bssA*) have been detected in cultures amended with alkanes (Callaghan, Wawrik, Ní Chadhain, Young, & Zylstra, 2008; *assA,* and Grundmann et al., 2008; *masD*) and 2-methylnaphthalene (Selesi et al., 2010; *nmsA*). The fumarate-adding genes have since become specific functional marker genes for determining anaerobic hydrocarbon biodegradation pathway. The genes encoding alkylsuccinate synthase have been detected in various hydrocarbon-impacted anaerobic terrestrial and aquatic systems (Aitken et al., 2013; Mbadinga et al., 2012; Tan et al., 2013, 2015; von Netzer et al., 2013; Wang et al., 2012). However, despite the detection of *assA* genes in these studies, the corresponding metabolite was either not identified or detected at low concentration, strengthening the hypothesis that the fumarate-added metabolites do not accumulate under slow-growing conditions and/or possibility of other activation mechanisms may also exist and play an important role in anaerobic biodegradation (Aitken et al., 2013).

Another recently postulated anaerobic hydrocarbon activation pathway is hydroxylation. Due to the high C-H bond dissociation energy of hydrocarbons, anaerobic hydroxylation of hydrocarbons under standard conditions is also considered thermodynamically unfavorable. However, hydroxylation has been demonstrated with monoaromatic compound, ethylbenzene, under nitrate- and sulfate-reducing conditions and the corresponding enzyme, ethylbenzene dehydrogenase, has been well characterized (Boll & Heider, 2010; Foght, 2008). Inversely, concrete evidence for activation of alkanes via hydroxylation is still absent; albeit, recent study (Head et al., 2010) has identified genes encoding for alcohol and aldehyde dehydrogenase from total genomic DNA of methanogenic crude-oil degrading enrichment cultures. Consequently,

Head et al. (2010) postulated hydroxylation as one of the possible pathways for activation of alkanes under methanogenic conditions. In another study (Zedelius et al., 2011), based on the metabolites and genes analyses of denitrifying isolate strain HdN1 grown on *n*-tetradecane, the authors suggested that HdN1 might have used O_2 derived from dismutation of NO_2^- to hydroxylate alkane via putative alkane monooxygenase. The authors also suggested that N-O species might play role as strong oxidant that generates a reactive state of a factor involved in the activation of the alkane or the N-O species might be directly involved in alkane activation (Zedelius et al., 2011). Although activation via hydroxylation has been proven for ethylbenzene (Boll & Heider, 2010; Foght, 2008), enzyme corresponding to alkane dehydrogenase has not been characterized and the full mechanism remains unknown. Further proteomic and metabolomics investigations will be required to further elucidate the mechanism of alkane activation via hydroxylation under various anaerobic conditions.

Methylation is another recently proposed mechanism of anaerobic activation reactions. This activation mechanism, however, is only proposed for anaerobic biodegradation of aromatic hydrocarbons, which is followed by fumarate addition (Foght, 2008; Safinowski & Meckenstock, 2006). Safinowski and Meckenstock (2006) examined biodegradation of naphthalene and 2-methylnaphthalene with sulfate-reducing enrichment culture N47, which demonstrated the capability of culture N47 to utilize naphthalene or 2-methylnaphthalene as sole substrate individually. The metabolite detected from these cultures revealed similar fumarate addition pathway metabolites predicted by the 2-methylnaphthalene pathway, suggesting naphthalene is methylated to form 2-methylnaphthalene first before undergoes fumarate addition (Safinowski & Meckenstock, 2006).

Finally, the last postulated hydrocarbon activation pathway is carboxylation. A study by Kunapuli, Griebler, Beller and Meckenstock (2008) provided initial evidence for direct carboxylation using iron-reducing enrichment culture amended with stable-isotope benzene and bicarbonate, which resulted in the detection of benzoic acid. The evidence for carboxylation was supported by the fact that the iron-reducing enrichment culture could not degrade toluene nor phenol which represented initial intermediates for benzene activations via methylation and hydroxylation, respectively (Kunapuli et al., 2008; Musat & Widdel, 2008). A metatranscriptomic study of an anaerobic benzene-degrading, nitrate-reducing enrichment culture provide further evidence for carboxylation of benzene, where two subunits of a proposed

benzene-carboxylating enzyme were transcribed when the culture was amended with benzene but in benzoate-degrading cultures (Luo et al., 2014). Carboxylation was also suggested for activation of alkanes by So et al. (2003) who presented evidence from stable isotope studies where *Desulfococcus oleovorans* Hxd3 grown on $NaH^{13}CO_3$ and hexadecane resulted in carboxylic acid with one carbon shorter than the parent alkane and ^{13}C formed its carboxyl group. The results indicate that subterminal carboxylation occurred at the C-3 position of the alkane and elimination of C1 and C2 carbon atoms (So et al., 2003). A subsequent study (Callaghan et al., 2006) provided further evidence that alkane might be activated via carboxylation. However, recent biochemical analysis on *D. oleovorans* Hxd3, suggested that carboxylation might actually occur following alkane activation via hydroxylation, which resulted in a fatty acid with one carbon shorter than the parent alkane (Callaghan, 2013; Musat, 2015). Genes encode for putative carboxylase has also been identified in cultures amended with benzene (Abu Laban, Selesi, Rattei, Tischler, & Meckenstock, 2010; Holmes, Risso, Smith, & Lovley, 2011) and naphthalene (Mouttaki, Johannes, & Meckenstock, 2012), however, the reaction by which the carboxylase can directly activate hydrocarbons without energy input like ATP hydrolysis is still unknown. Thus, activation of hydrocarbons via carboxylation under anaerobic conditions has been widely disputed since the corresponding carboxylated intermediates observed is not a solid evidence for the assumed carboxylation reactions (Boll & Heider, 2010) and also due to the fact that the reaction is thermodynamically unfavorable ($\Delta G^{o'}$ = +28 kJ/mol) (Mbadinga et al., 2011).

Few studies have been undertaken to determine hydrocarbon activation pathways in oil sands tailings system. A metagenomic study (An et al., 2013) has revealed that microbial communities in tailings retrieved from TP6 harbored diverse functional genes involved in activation of monoaromatic compounds via carboxylation and fumarate addition pathways. In another study (Tan et al., 2013) where short-chain alkane-degrading enrichment culture derived from MFT was investigated, homologs of putative genes encode for hydrocarbon succinate synthase were detected indicating fumarate addition may be the dominant pathway of alkane activation. Homologs of other putative genes encode for benzene carboxylase and ethylbenzene dehydrogenase (involve in activation via carboxylation and hydroxylation, respectively) were also detected however the evidence was not conclusive (Tan et al., 2013).

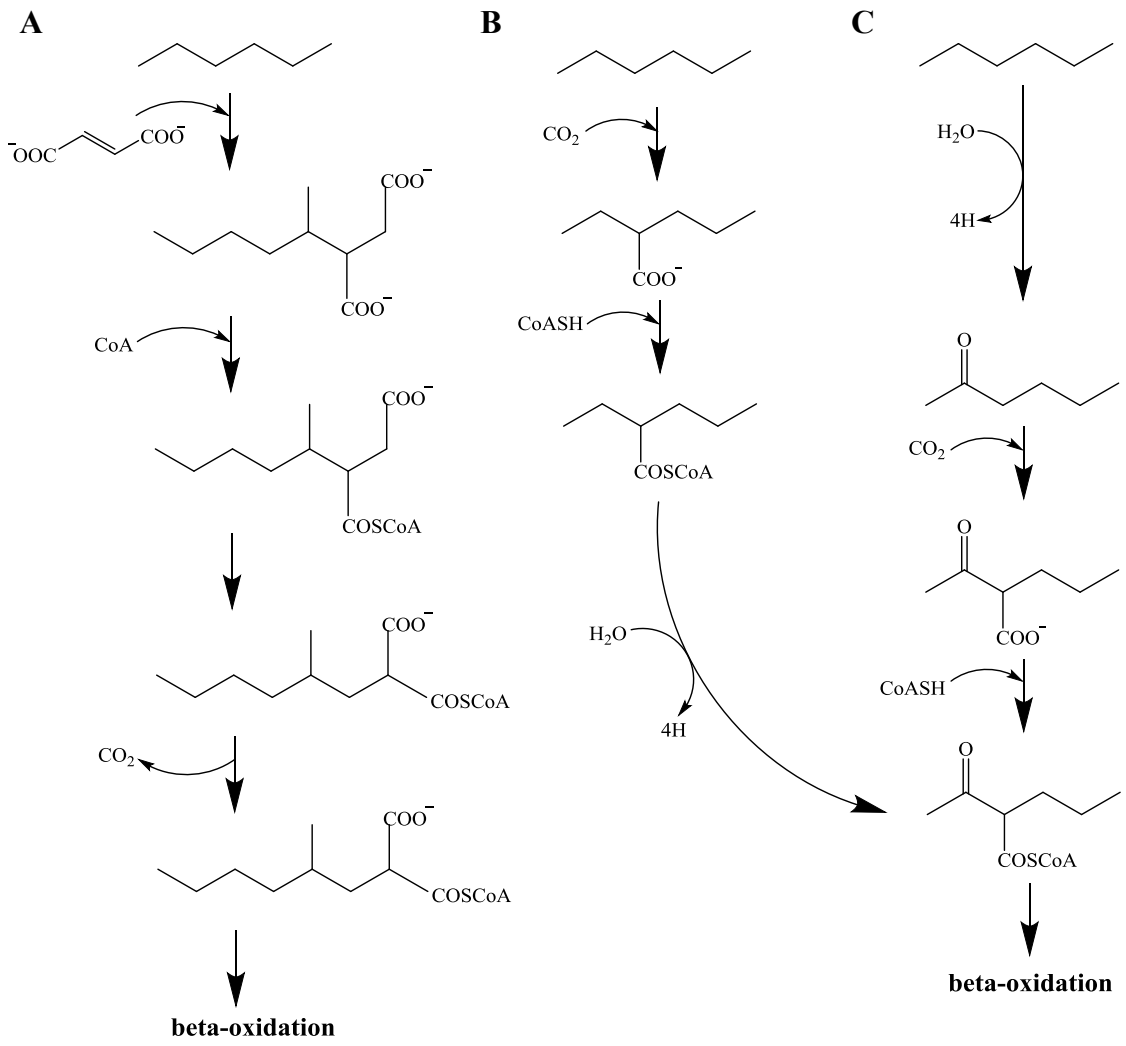

Figure 1.3: Proposed pathway for hydrocarbons degradation under anaerobic conditions. *n*-Hexane was used as model hydrocarbon to illustrate activation of hydrocarbon via (A) fumarate-addition, (B) carboxylation and, (C) hydroxylation pathways. Figure adapted from Heider & Schuhle (2013).

Recent studies (Abu Laban et al., 2014; Tan et al., 2015) have also reported, based on evidence from functional genes (*assA*- and *bssA*-like genes) and metabolite analyses, that activation of *iso*-alkanes in MLSB MFT proceed via fumarate addition. These recent findings (Abu Laban et al., 2014; Tan et al., 2015) are the foremost reports on biodegradation of *iso*-alkanes under methanogenic conditions and these findings suggest that fumarate addition is a universal activation pathway for various hydrocarbon compounds.

Draft genome sequences of *Smithella* (Tan, Rozycki, & Foght, 2014) and *Peptococcaceae* (Tan, Charchuk, Li, Laban, & Foght, 2014b) from methanogenic oil sands

tailings pond enrichment cultures also revealed that bacteria affiliated with *Smithella* and/or *Peptococcaceae*, which were enriched during biodegradation of monoaromatics or alkanes in MFT, carried putative genes encoding for fumarate-addition AssA. From these cumulative findings, it can be inferred that the main activation pathway of hydrocarbons in oil sands tailings system (alkanes and monoaromatics) is fumarate addition. However, previous studies have reported simultaneous stimulation and operation of multiple biodegrading pathways in a single microorganism (Kühner et al., 2005) and enrichment cultures (Callaghan et al., 2006); thus, it is also conceivable that hydrocarbons may be activated via multiple mechanisms simultaneously in oil sands tailings system. Therefore, additional investigations will provide further evidence for the postulated hydrocarbon activation pathway(s) besides improving our understanding of microbial activities indigenous to oil sands tailings system.

1.2.2 Effect of methanogenesis on consolidation of tailings

Because densification (settling of suspended clay particles) of tailings is important to accelerate recycling of recovered water for bitumen extraction and reclaim densified / consolidated tailings, CH_4 produced in tailings ponds was initially thought to interrupt the densification process of fine particles. In contrast to that initial thought, however, Fedorak et al. (2003) reported that methanogenesis enhanced the densification rate of fine particles in MFT when compared to non-methanogenic MFT. The enhanced sedimentation of the fine particles can improve the water recovery from the tailings ponds for recycling in the bitumen extraction process while reducing the dependence on fresh water. Densification would also reduce the tailings inventory (Bordenave et al., 2010) for effective management. Recent studies (Arkell, Kuznetsov, Kuznetsova, Foght, & Siddique, 2015; Siddique, Kuznetsov, Kuznetsova, Arkell, et al., 2014; Siddique, Kuznetsov, Kuznetsova, Li, et al., 2014) conducted to understand the role of methanogenesis in the densification of MFT have suggested two densification pathways. Pathway I demonstrates that the biogenic CO_2 production during methanogenesis lowers the pH of MFT that leads to the dissolution of carbonate minerals present in MFT (Figure 1.4). Divalent cations such as calcium and magnesium (Ca^{2+} and Mg^{2+}) released during the dissolution of carbonate minerals increase the ionic strength of the porewater, reduce the thickness of diffuse double layer and exchange sodium (Na) from exchange surfaces of clay particles (Figure 1.4). These processes increase settling and consolidation of clays in MFT. Pathway II reveals that the microbial metabolism under methanogenic conditions transforms more crystalline Fe^{III} minerals

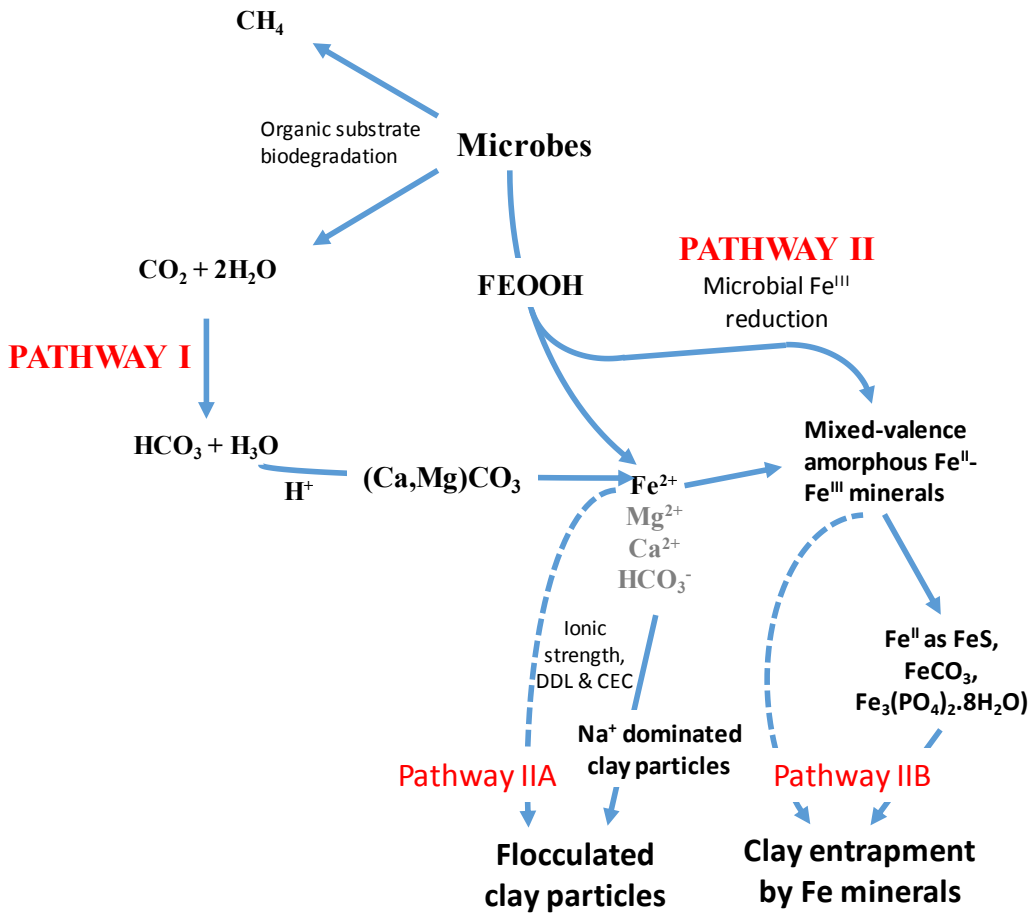

Figure 1.4: Proposed model for geochemical pathways of clay consolidation mediated by microbes. Figure adapted from Siddique, Kuznetsov, Kuznetsova, Li, et al. (2014).

in the MFT to more amorphous Fe^{II} minerals, which mask the clay surfaces and reduce their charge potential (Figure 1.4). This phenomenon improves the aggregation of clay particles and increases consolidation of tailings, enhancing the porewater recovery (Siddique, Kuznetsov, Kuznetsova, Li, et al., 2014). Microbially-accelerated consolidation is suggested as an initial pretreatment for dewatering of fresh fluid fine tailings in a controlled environment where produced CH_4 could be captured for onsite use and densified material will be used for further consolidation using other chemical/physical approaches. Therefore, investigating the performance of the methanogenic community in different tailings ponds is important to evaluate the potential use of this process for accelerated consolidation of oil sands tailings.

1.2.3 Effect of methanogenesis on partitioning of contaminants between aqueous and solid phases of tailings in end-pit lakes

In the "wet-landscape" approach for reclamation of mined-out pits to create end-pit lakes, MFT is placed into the pits and are capped with water. Since MFT will still have recalcitrant hydrocarbons such as *iso*- and *cyclo*-alkanes, long term effects of eventual methanogenic degradation of these recalcitrant compounds needs to be taken into account. Therefore, studies on biodegradation of different hydrocarbons will assist in predicting the carbon source of methanogenesis in the MFT of those in end-pit lakes.

A mesocosm study revealed that MFT amended with hydrolyzed canola meal had higher concentrations of some trace metals (As, V and Sr) than the unamended MFT after incubation (data not published). Based on the mesocosm study, subsequent study (Kuznetsova et al., in preparation) was conducted using 5-L bioreactor and the results revealed that indeed, some ions ($Ca^{2+,}$ Mg^{2+} and HCO_3^-) and trace metals (Ar, Co, Mn, Ni, Sr, and V) increased in concentrations in the porewater recovered from amended tailings compared to the unamended tailings after incubation for 5 weeks under methanogenic conditions. However, concentrations of other trace metals (Cu, Cd and Zn) decreased in the porewater recovered from the amended tailings (Kuznetsova et al., in preparation). During methanogenesis, Fe^{III} clay minerals that were transformed into Fe^{II} minerals of amorphous nature and carbonate minerals that were dissolved might have affect mobilization and immobilization of the trace metals in the tailings into the porewater.

Thus, long continuance of methanogenesis in the end-pit lakes can potentially release these toxic trace metals into the cap water, eventually affecting the quality of the lakes. Further experimentations are currently in-progress to study these processes to evaluate the performance of end-pit lakes as potential reclamation strategy.

1.2.4 Prediction of greenhouse gas emission from tailings ponds based on methanogenic biodegradation of hydrocarbons

Flux chambers are used for measuring gas emissions from the surface of tailings ponds; however, they can only cover a small area at a time for measurement. Other methods for measuring emissions of greenhouse gasses from the surface of tailings ponds have also been suggested including eddy covariance and remote sensing combined with micrometeorological

models (Small, Cho, Hashisho, & Ulrich, 2015). Nevertheless, these methods cannot measure greenhouse gasses produced deep in the tailings ponds, which are not able to escape to the surface due to the overburden pressure. Therefore, the measurement of greenhouse gasses from the surface of tailings ponds does not represent the true extent of greenhouse gasses produced in tailings ponds.

Hence, to complement the data generated from flux chambers, zero- and first-order kinetic models that include lag phase, rate of hydrocarbon biodegradation and conversion to CH_4 to predict CH_4 generation from MLSB were developed (Siddique, Gupta, Fedorak, MacKinnon, & Foght, 2008). Although the kinetic models did not take into account the carbon assimilation into microbial biomass, the parameter was compensated by applying stoichiometric and conversion efficiency factors. The values of the predicted CH_4 generation, estimated using the kinetic models, in naphtha-amended microcosms were comparable to the measured CH_4 and the kinetic models also predicted well the CH_4 generation in MFT amended with C_6-C_{10} *n*-alkanes or monoaromatics (Siddique et al., 2008). With the new findings of anaerobic biodegradation of wider range of hydrocarbons (*iso*- and *cyclo*-alkanes) and anaerobic biodegradation of hydrocarbons in tailings from different operators, the existing model can be further improved for more realistic prediction of CH_4 generation in all different tailings ponds.

1.3 Thesis overview

1.3.1 Research scope

Previous study (Siddique et al., 2007) has shown that the primary carbon source sustaining methanogenesis in tailings ponds is the entrained extraction solvent. Most oil sands operators (Suncor, Syncrude and CNRL) used naphtha as their extraction solvent in their bitumen extraction process; however, other oil sands operators, namely Albian and Imperial, used paraffinic solvent. Anaerobic biodegradation of whole naphtha and its individual hydrocarbon fractions (*n*- and *iso*-alkanes and monoaromatics) in MLSB MFT have been thoroughly studied (Abu Laban et al., 2014; Siddique et al., 2015, 2006, 2007; Boonfei Tan, Semple, et al., 2015). These studies, which have been conducted for ~300-400 days, have revealed that the *n*-alkanes and monoaromatics were completely metabolized by the indigenous microbial community in MLSB MFT; however, only a few compounds of the *iso*-alkane fraction were completely oxidized while some *iso*-alkanes were cometabolized. Because these studies

were only conducted for short period, complete range of hydrocarbons that might be susceptible to biodegradation, ranging from labile to recalcitrant compounds, cannot be determined, which beg the question if prolonged incubation would facilitate the determination of the complete range of hydrocarbons that can be metabolized. Additionally, no study on biodegradation of whole paraffinic solvent has been reported; hence, it is important to investigate how paraffinic solvent is biodegraded in MFT and which compounds within the paraffinic solvent are labile or recalcitrant. It is also important to examine how extraction solvents and their major hydrocarbon fractions are biodegraded in MFT from different tailings ponds since few or no study has been conducted using MFT from the newer oil sands operators such as Albian and CNRL, which have different oil sands composition and tailings age and have experienced different extraction process and tailings management practices. In this thesis research, we were interested to determine how different hydrocarbons including recalcitrant hydrocarbons such as *iso*- and *cyclo*-alkanes present in complex extraction solvents (both naphtha and paraffinic solvent) are biodegraded in MFT from different tailings ponds when incubated for prolonged time because previous studies conducted using MLSB MFT samples were incubated for only short period (~300-400 days), where only *n*-alkanes and certain monoaromatics were biodegraded. We were also interested in determining the pathways by which the hydrocarbons are activated in the biodegradation process in different MFT, which will contribute to the overall understanding of hydrocarbons biodegradation in an anaerobic environment. This research was designed to perform laboratory studies to cover a broad spectrum of investigations using MFT retrieved from Albian and CNRL tailings ponds. Albian and CNRL MFT were selected since very few reports have been published on microbial communities indigenous to Albian and CNRL tailings ponds and their functions for the sustenance of methanogenesis in these tailings ponds. MFT from Albian and CNRL tailings ponds were also of interest because these ponds are relatively younger and different in chemical compositions compared to Suncor and Syncrude tailings ponds which were extensively studied.

1.3.2 Research objectives

The main objective of this thesis research is to investigate the anaerobic biodegradation of extraction solvents and their major hydrocarbon components (*n*-and *iso*-alkanes) in MFT from different tailings ponds. To accomplish the main objective, the following specific objectives were investigated:

- Assessment of biodegradation pattern of whole extraction solvents in Albian and CNRL MFT under methanogenic conditions incubated for an extended period of time (Chapter 2).
- Examination of preferential biodegradation of different hydrocarbon groups (*n*- and *iso*-alkanes) in Albian and CNRL MFT (Chapter 3 and 4)
- Investigation of effects of terminal electron acceptors [nitrate, iron (III), and sulfate] on biodegradation of recalcitrant hydrocarbons (*iso*-alkanes) in MFT (Chapter 4)
- Exploration of main activation pathway(s) of hydrocarbons in Albian and CNRL MFT (Chapter 5).

1.3.3 Thesis outline

Chapter 1: Introduction

Chapter 2: Sequential methanogenic biodegradation of complex hydrocarbons in MFT from two different oil sands tailings

This chapter discusses biodegradation pattern of complex hydrocarbons present in Albian paraffinic solvent and CNRL naphtha in MFT collected from Albian and CNRL tailings ponds and incubated for an extended period (several years). Here, the indigenous microbial communities involved in methanogenic degradation of the solvents in both Albian and CNRL tailings ponds, which have not been studied previously, were also characterized via 16S rRNA gene pyrotag sequencing. Because Albian and CNRL tailings ponds are different in their methods of bitumen extraction and tailings treatment, the differences beg the question of whether microbial communities in each pond have adapted to efficiently biodegrade only their cognate solvents, or are sufficiently metabolically flexible that they can adapt to degrade other solvent as well. The answer would be useful for predicting onset and duration of greenhouse gas production from oil sands tailings ponds operated under different conditions than MLSB.

Chapter 3: Preferential methanogenic biodegradation of short-chain *n*-alkanes in MFT by microbial communities from two different oil sands tailings ponds

Because extraction solvents contain different types of hydrocarbons, this chapter focuses on biodegradation of *n*-alkanes, which constitute a major portion of extraction

solvents. This study was conducted using Albian and CNRL MFT. Key microbial players involved in methanogenic biodegradation of the *n*-alkanes were identified to postulate their roles during biodegradation process. Our previous study has shown that *n*-alkanes (C_6-C_{10}) representing naphtha-range hydrocarbons were degraded preferentially in the order: $C_{10} > C_8 > C_7 > C_6$ (Siddique et al., 2006). This study is designed to address the question whether such chain length discrimination is unique to MLSB microbes, or whether preferential degradation of alkane suites is more widespread in oil sands tailings ponds.

Chapter 4: Biodegradation of *iso*-alkanes in MFT under methanogenic and sulfate-reducing conditions

iso-Alkanes also constitute a major portion of extraction solvents used in bitumen recovery from oil sands ores. These hydrocarbons are considered relatively recalcitrant and a few reports reveal their biodegradation under anaerobic conditions. This chapter describes biodegradability of recalcitrant *iso*-alkanes and microbial players involved in the biodegradation process in MFT from Albian and CNRL tailings ponds. The effects of different terminal electron acceptors on biodegradation of *iso*-alkanes and microbial community structure were also studied. This study is important to understand the biodegradation of recalcitrant hydrocarbons under different redox conditions that can prevail in other suboxic and anoxic environments.

Chapter 5: Insights into anaerobic biodegradation pathway of alkanes in MFT from two oil sands tailings ponds

This chapter describes activation pathway of alkanes in primary cultures from Chapter 2, 3 and 4 via metabolite and functional gene analyses. This information will advance our overall understanding of biodegradation of hydrocarbons in anaerobic environments.

Chapter 6: Summary and synthesis

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2 Sequential methanogenic biodegradation of complex hydrocarbons in MFT from two different oil sands tailings ponds[1]

2.1 Abstract

Biodegradation of entrained hydrocarbons supports methane emissions from tailings ponds. To investigate biodegradability of complex hydrocarbons, mature fine tailings (MFT) collected from Albian and CNRL ponds were amended with Albian paraffinic solvent or CNRL naphtha separately and incubated anaerobically. For paraffinic solvent (C_5-C_6) biodegradation, Albian and CNRL MFT exhibited ~400 and ~800 d lag phases, respectively after which *n*-alkanes (*n*-pentane and *n*-hexane) were preferentially metabolized to methane over *iso*-alkane where only 2-methylpentane was biodegraded during ~1300 d of incubation. A shorter lag phase (100 d) was observed for naphtha (primarily ~C_6-C_{10}) biodegradation in both Albian and CNRL MFT. Both Albian and CNRL MFT preferentially biodegraded alkanes in the order: *n*-alkanes > *iso*-alkanes > *cyclo*-alkanes. Complete biodegradation of all *n*-alkanes (except *n*-octane in CNRL MFT) and several *iso*-alkanes (2-methylpentane, 3-methylhexane, *iso*-octanes, *iso*-nonanes and 2-methylnonane) was observed during ~1600 d of incubation. Partial biodegradation of the rest of *iso*-alkanes and few *cyclo*-alkanes was also observed during the incubation. 16S rRNA gene pyrosequencing showed dominance of *Anaerolineaceae* and *Methanosaetaceae* during initial stage and *Peptococcaceae* and "*Candidatus* Methanoregula" during later stage of solvent biodegradation in both MFT except CNRL MFT amended with paraffinic solvent where only *Peptococcaceae* dominated the bacterial population during biodegradation. The results may impact future strategies of tailings reclamation and management of greenhouse gas emissions.

[1]A version of this chapter has been modified and submitted for publication in Environmental Science and Technology.

2.2 Introduction

Recent expansion in oil sands surface mining in northern Alberta, Canada has resulted in increased volumes of fluid tailings generated during the process of bitumen extraction from oil sands ores. Presently, settling basins (tailings ponds) contain ~1 billion m^3 of tailings that cover a total area of ~185 km^2 (http://osip.alberta.ca/map/). Besides other issues such as large inventory, slow consolidation, and presence of organic (residual hydrocarbons, naphthenic acids) and inorganic (heavy metals) contaminants, deposited tailings emit large quantities of methane (CH_4) and carbon dioxide (CO_2) to the atmosphere. Mildred Lake Settling Basin (MLSB), the largest and one of the oldest oil sands tailings ponds, has been estimated to emit ~43 million L CH_4 day^{-1} (Holowenko et al., 2000; Siddique et al., 2008). Studying the source of greenhouse gas emissions and mechanisms of natural attenuation of contaminants are important from global climate change and contaminant remediation perspectives.

Oil sands tailings deposited in ponds contain residual concentrations of fugitive extraction solvent, which is the major substrate sustaining methanogenesis in tailings ponds (Siddique et al., 2007). Syncrude Canada Ltd. (Syncrude) uses naphtha as a solvent for bitumen extraction which comprises aliphatic and aromatic hydrocarbons (*n*-, *iso*- and *cyclo*-alkanes and monoaromatics) primarily of C_6-C_{10}. Initial study conducted for a year using MFT retrieved from Syncrude's MLSB revealed that only labile fractions of naphtha (*n*-alkanes and monoaromatics) served as carbon sources for methanogenesis because the other components such as *iso*- and *cyclo*-alkanes remained undegraded during a year-long incubation (Siddique et al., 2007). However, subsequent studies reported biodegradation of certain *iso*- and *cyclo*-alkanes (metabolically or co-metabolically) in primary cultures and enrichment cultures derived from MLSB MFT and amended with *iso*- and/or *cyclo*-alkanes (Abu Laban et al., 2014; Siddique et al., 2015; Tan, Semple, et al., 2015). Though these findings demonstrated that indigenous microbial communities in MFT were able to adapt to degrade a wider range of hydrocarbons in MLSB (Abu Laban et al., 2014; Siddique et al., 2012, 2011; Tan, Semple, et al., 2015), no study has been conducted for extended period of time to comprehend biodegradability of different hydrocarbon fractions (from labile to recalcitrant) when tailings ponds receive complex fugitive solvents.

Tailings from different operators are unalike in many aspects such as the age of deposited tailings and tailings management strategies. Compared to Syncrude MLSB (~40 years old), Shell

Albian Sands Inc. (Albian)'s Muskeg River Mine pond (~14 years old) and the Canadian Natural Resources Ltd. (CNRL)'s Horizon pond (~7 years old) are much younger and receive tailings containing residual hydrocarbons from their respective extraction solvents. Albian uses paraffinic solvent (C_5–C_6; *n*- and *iso*-alkanes) for bitumen extraction and amends tailings with trisodium citrate and organic polymer flocculants for consolidation before deposition (Li, 2010), whereas CNRL uses naphtha as an extraction solvent and CO_2 to enhance consolidation of tailings in the pond (http://www.cnrl.com/). Consequently, the composition of the indigenous microbial communities and the hydrocarbon biodegradation behavior of MFT from Albian and CNRL tailings ponds may be different. Our recent studies revealed different patterns of preferential biodegradation of *n*-alkanes in Albian and CNRL MFT (Mohamad Shahimin, Foght, & Siddique, 2016) and metabolism/co-metabolism of certain *iso*-alkanes in Albian MFT (Siddique et al., 2015) when these MFT were amended with a mixture of *n*- or *iso*-alkanes. These observations raise the question on the biodegradability of individual constituents when present in a complex solvent, because almost all previous studies tested biodegradation of individual hydrocarbon groups. Therefore, examining the biodegradation pattern of hydrocarbons in complex solvents in different oil sands tailings ponds would provide a more realistic scenario and contribute to the overall understanding about the onset and duration of methanogenesis that helps improve our existing kinetic model (Siddique et al., 2008) to predict greenhouse gas emission from tailings ponds. Additionally, methanogenesis drives major geochemical processes that increase dewatering of oil sands tailings (Siddique, Kuznetsov, Kuznetsova, Arkell, et al., 2014; Siddique, Kuznetsov, Kuznetsova, Li, et al., 2014); therefore, it is important to evaluate methanogenic activities in Albian and CNRL tailings ponds, which has not previously been studied in detail.

In our present study, we examined methanogenic biodegradation of solvent hydrocarbons in MFT retrieved from Albian and CNRL tailings ponds. We also exposed each MFT to both solvents (paraffinic and naphtha) separately to examine the adaptability and flexibility of indigenous microbial communities in Albian and CNRL MFT to biodegrade hydrocarbons that are different from the cognate hydrocarbons present in their native environments. This is the first study that provides insight into biodegradation of complex extraction solvents by microbial communities indigenous to Albian and CNRL tailings ponds, which contributes to the overall

understanding of methanogenesis in oil sands tailings ponds and its implications in tailings management.

2.3 Materials and methods

2.3.1 Chemicals and materials

The Albian and CNRL extraction solvents tested in this study for their biodegradability and samples of methanogenic MFT were collected and provided by respective oil sands operators (Albian and CNRL). MFT comprised a thick water slurry of silt and clays (usually ≥30% solids), unextracted bitumen (usually ~5 vol %) and unrecovered solvent (≤1 vol%).

The Albian MFT were collected in bulk from the Muskeg River Mine Tailings Pond at 7 m below the water surface in 2008 (coordinates, 0465371E 6342304N; solids, 25 wt.%; bitumen, 0.87 wt.%; pH 8.07; conductivity 3.1 dS m^{-1}) and stored in the dark at 4°C under a cap of tailings pond water, to maintain anaerobic conditions in the tailings sediments, for use in 2011. We have found in numerous previous experiments that storage of MFT for several years under these conditions does not impair its ability to biodegrade hydrocarbons at a rate similar to that of fresh MFT samples. The CNRL MFT was collected in bulk from Horizon tailings ponds in 2011 (coordinates, 446156E 6356933.1N; solids, 28 wt. %; bitumen, 0.97 wt %; pH 8.6; conductivity 2.0 dS m^{-1}) and also stored in the dark at 4°C until used as inoculum in the same year.

2.3.2 Establishment of cultures for solvent biodegradation

The anaerobic microcosms were prepared using 50 mL each of methanogenic medium and Albian or CNRL MFT in 158-mL serum bottles with a headspace of 30% CO_2 balance N_2 as previously described (Siddique et al., 2006). The methanogenic medium contained inorganic salts (NaCl, CaCl, NH_4Cl, $MgCl_2$, $(NH_4)6Mo_7O_{24}$, $ZnSO_4$, H_3BO_3, $FeCl_2$, $CoCl_2$, $MnCl_2$, $NiCl_2$, $AlK(SO_4)_2$, $NaHCO_3$), vitamins (pyridoxine, thiamine, nicotinic acid, pantothenic acid, cyanocobalamin, *p*-aminobenzoic acid), sodium sulfide (reducing agent) and resazurin (redox indicator) as described by Fedorak & Hrudey (1984). The microcosms were pre-incubated at room temperature in the dark for 2 weeks for microbial acclimation and consumption of residual hydrocarbons and any alternative electron acceptors in MFT (Siddique et al., 2006). Prior to amending the microcosms with extraction solvents, the headspace of all microcosms was flushed with 30% CO_2 balance N_2 to remove any CH_4 produced during pre-incubation. Both Albian and CNRL microcosms were amended separately with ~0.2 wt% (~2000 mg L^{-1}) CNRL naphtha and

~0.1 wt% (~1000 mg L^{-1}) Albian paraffinic solvent to achieve environmentally relevant final concentrations (Dr. T. Siddique, University of Alberta, personal communication, November 1, 2011) in MFT. Triplicate microcosms were prepared for each treatment. Duplicate abiotic controls (heat-killed microcosm) were prepared in parallel by autoclaving (121 ^{o}C, 20 psi, 60 min) for four consecutive days prior to hydrocarbon amendment to account for abiotic degradation. Duplicate viable baseline controls (unamended microcosms) were also prepared to account for CH_4 production from any residual endogenous substrates in the MFT. Immediately after the amendment, samples were collected from all the microcosms to determine initial (day 0) status of MFT for hydrocarbons and microbial community structure. The microcosms were incubated statically in the dark at room temperature and headspace analysis was performed periodically to monitor CH_4 production in the microcosms. Culture samples were also taken periodically from the microcosms for hydrocarbon determination and microbial community characterization (see below).

2.3.3 Analytical approaches to monitor methane production and hydrocarbon depletion

For measuring CH_4 periodically in the headspace of each microcosm as an indication of biodegradation of the added extraction solvents, 50 μL headspace gas was injected directly into a Hewlett Packard 5700A gas chromatography equipped with flame ionization detector (GC-FID) fitted with a 0.3 cm, 2 m packed column (Tenax GC 60X80 mesh). The carrier gas (N_2), air and H_2 were set as 50, 300 and 35 mL min^{-1}, respectively. The injector and oven temperatures were set at 40 ^{o}C and detector temperature was set at 200 ^{o}C.

For the determination of hydrocarbons in MFT to monitor biodegradation, three analytical approaches were employed over ~1600 d of incubation. To quantify whole solvent (paraffinic and naphtha) concentrations (F1 fraction; C_5-C_{10}), GC-FID equipped with purge and trap system was used for analyses performed at 0, ~600 and ~1300 d. Individual concentrations of certain major hydrocarbons in extraction solvents were also identified using the same analysis. Because naphtha is a complex mixture of hydrocarbons, PONAU (paraffins, olefins, naphthenes, aromatics, and unknown components) analysis was used to resolve and identify all individual constituents and F1 fraction of naphtha at ~1600 d. Individual components of paraffinic solvent were determined by headspace analyses using GC equipped with mass spectrometer (MS).

For determination of F1 fraction1 mL sample was withdrawn from the microcosms and the hydrocarbons were extracted from the samples by shaking on mechanical shaker for 30 min

at room temperature with 10 mL methanol (Fisher Scientific). After the extraction procedure, the fine particles were allowed to sediment for 30 min at 4°C and 1 mL of the supernatant was transferred into 44 mL EPA glass vial, filled completely with ultrapure water (Milli-Q Water System) and capped immediately avoiding any headspace. All prepared vials sonicated for 10 min to homogenize the samples prior to analysis on a GC-FID (Hewlett Packard HP 6890) equipped with a purge and trap system. The purge and trap line temperature was set at 180 ^{o}C. The purged volatiles were desorbed for 4 min at 225 ^{o}C and kept at 225 ^{o}C for 10 min before introduced into the GC with a split ratio 50:1. The capillary column used was a 30 m DB-1 (0.53 mm diameter and 1.5 μm thickness). The oven temperature was held at 36 ^{o}C for 4 min and increased at 15 ^{o}C min^{-1} to 350 ^{o}C. The carrier gas helium was set at 7.4 ml min^{-1}. The front inlet and FID temperatures were kept at 200 and 250 ^{o}C, respectively. Because most or all hydrocarbons in both extraction solvents were within F1 fraction, individual peaks from C_5 to C_{10} hydrocarbons were integrated using modified method of Canadian Council of Ministers of the Environment for petroleum hydrocarbons (Canadian Council of Ministers of the Environment, 2001) to quantify whole solvent. In addition to F1 fraction, the same purge-trap GC-FID run was used to calculate depletion of certain major hydrocarbons (identified by external standards) in the extraction solvent using cyclopentane (recalcitrant component in both Albian and CNRL extraction solvents) as the internal standard. Briefly, the percent biodegradation of the known hydrocarbons was calculated using the following equation (Prince & Suflita, 2007):

$$\% \text{ depletion: } 100 - [(A_{sample}/C_{sample})/(A_{sterile\ control}/C_{sterile\ control}) \times 100] \quad (2.1)$$

where A and C represent abundance of target compound and internal standard, respectively.

To verify the results obtained using purge-trap GC-FID and to identify the biodegradation of unknown hydrocarbons in complex naphtha, Albian heat-killed culture and live Albian and CNRL cultures amended with naphtha and incubated for ~1600 d were analyzed for complete spectrum of hydrocarbons using PONAU method (CAN/CGSB-3.0 No. 14.3) on a high-resolution gas chromatography at Alberta Innovates-Technology Future (Fuels and Lubricants), Edmonton, Alberta, Canada. The concentrations of all naphtha components (C_5-C_{10}) in the initial live Albian and CNRL MFT were calculated by comparing the mass ratio of all naphtha components within F1 fraction in heat-killed Albian MFT determined by PONAU analysis (assuming no abiotic loss) with the initial concentrations of F1 fraction (determined by purge-

trap GC-FID) in the live Albian and CNRL cultures. Mass ratios of all naphtha components in live Albian and CNRL MFT at ~1600 d were compared with mass ratio of heat-killed Albian MFT to determine percent biodegradation (Eq. 2.1) of individual hydrocarbons using recalcitrant hydrocarbons (methylcyclohexane and 1,1,3-trimethylcyclohexane present in naphtha) as the internal standards. The initial concentrations of live Albian and CNRL MFT estimated with PONAU analysis and values of percent biodegradation for individual hydrocarbons were used for stoichiometric calculation.

For analysis of individual components of paraffinic solvent at ~1600 d, 100 µL of headspace was analyzed by direct injection into a TRACE 1300 gas chromatograph equipped with a TraceGold TG-5MS GC column (30 m by 0.25 mm internal diameter; Thermo Scientific) in split mode. The injector temperature was 250 ºC with helium as carrier gas at a split ratio of 1:10. The initial temperature of the oven was held at 35 °C for 7 min, increased at 10 °C min^{-1} to 100 °C and held for 5 min. The over temperature was then increased at 5 °C min^{-1} to 280 °C and then held at 280 °C for 15 min. The mass spectra of individual alkanes within the paraffinic solvent were obtained using ISQ LT Single Quadrupole mass spectrometer (Thermo Scientific), and the data was acquired in the scan mode from 50 to 600 mass units. The percent biodegradation of individual components of paraffinic solvent was calculated using Eq. 2.1.

The concentrations of all hydrocarbons remaining in the microcosms were used to quantify their biodegraded masses which were incorporated into modified Symons and Buswell equation (Symons & Buswell, 1933) to calculate theoretical maximum CH_4 production:

$$C_cH_h + (c - \frac{h}{4})\, H_2O \rightarrow (\frac{c}{2} - \frac{h}{8})\, CO_2 + (\frac{c}{2} + \frac{h}{8})\, CH_4 \qquad (2.2)$$

For stoichiometric calculation, we assumed complete oxidation of each known hydrocarbons to CH_4 and CO_2 without any incorporation of the carbon into microbial biomass or production of dead-end metabolites. The sum of theoretical CH_4 production from the consumed concentrations of all the known hydrocarbons was compared to the measured CH_4 production in the headspace on the day when hydrocarbon analysis was performed to confirm biodegradation of hydrocarbons.

2.3.4 Characterization of microbial communities

MFT cultures collected at day 0 (when incubation started after hydrocarbon amendment) and after biodegradation of the added hydrocarbons were used to study shift in microbial

community composition. Total genomic DNA was extracted from triplicate 300-µL samples using bead-beating method as previously described (Mohamad Shahimin et al., 2016). Briefly, DNA was extracted from 300-µL samples in screw-cap microcentrifuge tubes containing 1 g of 2.5 mm and 0.1 mm zirconia-silica beads (1:1 w/w) and equal volumes (300 µL) of phosphate buffer (100 mM NaH_2PO_4, pH 8.0), lysis buffer (100 mM NaCl, 500 mM Tris pH 8.0, 10% sodium dodecyl sulfate), and chloroform–isoamyl alcohol (24:1). The samples were processed at 3400 revolutions per minute for 45 s in PowerLyzer™ 24 Bench Top Bead-based Homogenizer (MO BIO Laboratories Inc., Carlsbad, CA). The samples were then spun in a microcentrifuge at 21130 x g for 5 min at ~22 °C to sediment the particles and the supernatant was recovered for subsequent DNA recovery. The extraction procedure was repeated on the same sample, without pooling the supernatants. For each supernatant, 7 M ammonium acetate (CAS# 631-61-8; Fisher Scientific) was added to 2.5 M final concentration, mixed gently and centrifuged to precipitate proteins. The supernatant was transferred to a new sterile 1.5 mL microcentrifuge tube with 0.6 volumes of isopropanol (CAS# 67-63-0; Fisher Scientific) and incubated overnight at 4 °C. DNA was precipitated by microcentrifuge at 15000 rpm for 30 min. After decanting the isopropanol, the DNA pellet was air-dried for 60 min and dissolved in 30 µL nuclease-free water (Integrated DNA Technologies). The extracted DNA from triplicate samples from each microcosm was then pooled and analyzed for concentration and purity using Nanodrop-1000 Spectrophotometer before its use as template in PCR amplification.

To characterize the microbial communities in all treatments, the V6-V8 regions of the archaeal and bacterial 16S rRNA genes were amplified for 454 pyrotag sequencing using the primer set 454T-RA and 454T-FB (Golby, Ceri, Marques, & Turner, 2013). Touchdown thermocycling was performed for the 16S rRNA genes amplification for each sample in 25 µL PCR reaction with the following temperature program: 94°C for 3 min, followed by 10 cycles of 94°C for 30 s, 60°C for 30 s (decreasing by 0.5°C/cycle) and 72°C for 30 s, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, followed by final extension at 72°C for 10 min. The PCR reaction consist of 12.5 µL AccuStart II PCR ToughMix (Quanta Biosciences Inc., Gaithersburg, MD), 2.5 µl of 1.0 µM upstream and downstream primers, 1 µl DNA template (~10-20 ng μl^{-1}) and balanced with nuclease-free water. Negative controls, which contained only PCR reagents and nuclease-free water, were included with every set of samples as PCR amplification quality control. The amplicons (~450 bp) were verified via agarose gel

electrophoresis. All amplicons with the right size were purified using Qiagen's QIAquick PCR purification kit according to the manufacturer's procedure. The purified 16S rRNA gene amplicons from different replications within each treatment were pooled before sequencing except for samples of the triplicate CNRL MFT amended with paraffinic solvent, where each microcosm was sequenced individually because of observed differences in CH_4 production and hydrocarbon biodegradation. The amplicons were sent to McGill University Génome Québec Innovation Centre, Canada, for sequencing using a GS FLX Titanium Series Kit XLR70 (Roche). The Phoenix 2 pipeline (Soh, Dong, Caffrey, Voordouw, & Sensen, 2013) were used for analysis of all raw pyrosequencing data. Quality-controlled raw sequences, ranging from ~5400 to 12200 reads per sample except Albian and CNRL MFT's initial community which ranged from ~900 to 5300 reads, were submitted to NCBI Sequence Read Archive under SRA number SRP064971.

2.4 Results

2.4.1 Methane production in MFT amended with extraction solvents

CH_4 production was monitored periodically in all the microcosms for ~1600 d and insignificant amount of CH_4 ($\leq$ 0.2 mmol) was observed in unamended Albian and CNRL MFT microcosms (Fig. 2.1). In paraffinic solvent-amended microcosms (Fig. 2.1A), Albian MFT produced CH_4 gradually after a lag phase of ~400 d, and exponential increase in CH_4 was observed after ~440 d. CH_4 production continued to increase but plateaued after ~ 930 d (2.28±0.05 mmol). CNRL MFT exhibited a longer lag phase (~800 d) and only one replicate initially started producing CH_4 higher than the unamended microcosms. However shortly after the first replicate, the other two replicates also generated h amount of CH_4 and all replicates yielded comparable results (average ~2.98 mmol CH_4) by ~1600 d. One of the abiotic controls (heat-killed amended microcosms) produced CH_4 at ~1600 d (0.77±0.95 mmol) that caused higher standard deviation (Fig. 2.1A). CH_4 production in abiotic microcosm indicates the survival and re-establishment of hydrocarbon degrading microbial community in MFT.

In CNRL naphtha-amended microcosms (Fig. 2.1B), Albian MFT showed a lag phase of ~100 d before it released CH_4 exponentially in the headspace of microcosms. CH_4 production

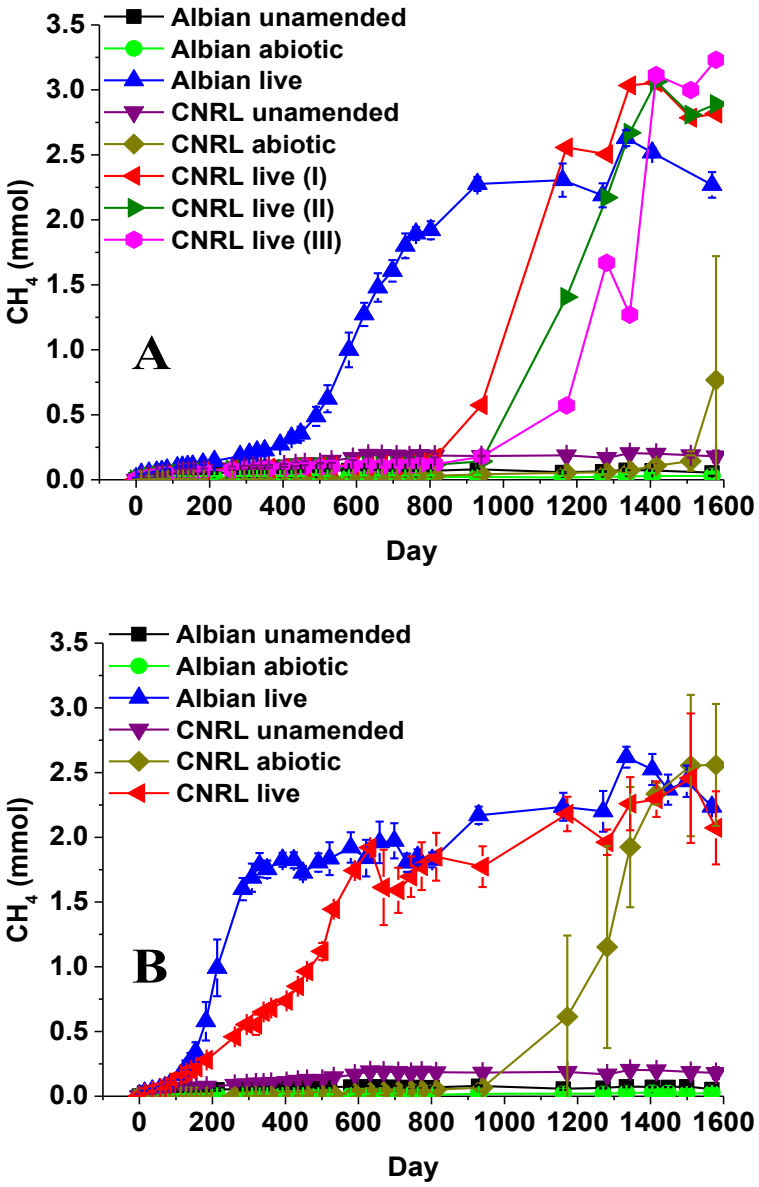

Figure 2.1: Cumulative methane production in (A) paraffinic solvent amended MFT and (B) naphtha amended MFT. Unamended controls consist of MFT without amendment of solvent. The abiotic controls were amended with the respective extraction solvents. With the exception of CNRL MFT amended with paraffinic solvent, which was represented individually, all other symbols represent the mean value from triplicate microcosms and error bars (where visible) represent one standard deviation.

started to plateau by ~330 d (1.78±0.09 mmol) with a small gradual increase to 2.24±0.04 mmol by ~1600 d. In CNRL MFT, CH_4 was generated at a steady rate rather than exponentially after a

lag phase of ~100 d similar to the lag phase observed in Albian MFT. During sampling at day ~670, some headspace from one replicate microcosm was accidentally released, which resulted in higher standard deviation. However, cumulative CH_4 reached 1.77±0.16 mmol by ~950 d and 2.07±0.28 mmol by ~1600 d. Here, we also observed that abiotic controls produced high CH_4 when analysis was performed at ~1200 d, implying recovery of hydrocarbon degraders in the abiotic controls. The cumulative CH_4 in abiotic controls also reached a value of 2.56±0.47 mmol by ~1600 d (Fig. 2.1B).

2.4.2 Biodegradation of the extraction solvents in MFT

Concentrations of paraffinic solvent (F1 fraction) in Albian and CNRL MFT during ~1300 d incubation are shown in Fig. 2.2 (panels A and B). In all abiotic controls, solvent concentrations did not change substantially from their initial (day 0) concentrations. In live (biotic) Albian MFT, the initial solvent concentration (1242±82 mg L^{-1}) at day 0 decreased to 460±58 mg L^{-1} by ~600 d. The solvent was slightly depleted with further incubation and its concentration reduced to 235±6 mg L^{-1} by ~1300 d (Fig. 2.2A). In live CNRL MFT, no major depletion in the solvent concentrations was observed till ~600 d when compared to its initial concentration (1314±27 mg L^{-1}). However, more than half of the solvent was biodegraded (535±142 mg L^{-1}) by ~1300 d (Fig. 2.2B). Because five major hydrocarbons including *n*-pentane (*n*C_5: 24 wt.%), *n*-hexane (*n*C_6: 11 wt.%), 2-methylbutane (2-MC_4: 12 wt.%), 2-methylpentane (2-MC_5: 24 wt.%) and 3-methylpentane (3-MC_5: 13 wt.%) constituted ~85% of the Albian paraffinic solvent, the degradation pattern of these compounds were also monitored (Table 2.1). Both Albian and CNRL MFT completely and preferentially biodegraded *n*-alkanes (*n*C_5 and *n*C_6) over *iso*-alkanes. Among *iso*-alkanes, only 2-MC_5 was completely biodegraded while 2-MC_4 and 3-MC_5 were slightly depleted (partially biodegraded).

Concentrations of naphtha (F1 fraction) in Albian and CNRL MFT during ~1600 d incubation are shown in Fig. 2.2 (panels C and D). Abiotic Albian and CNRL MFT did not biodegrade naphtha except CNRL MFT which showed biodegradation during analysis performed at ~1300 d that corresponded to CH_4 production in those microcosms. In Albian MFT, ~450 mg L^{-1} of naphtha was biodegraded from the initial concentration of 1833±78 mg L^{-1} by ~600 d of incubation (Fig. 2.2C). The concentration of naphtha remained in Albian MFT was ~874 mg L^{-1} by ~1600 d (Fig. 2.2C). CNRL MFT also biodegraded similar amount of naphtha (~460 mg L^{-1}) from initial concentration (2351±103 mg L^{-1}) during ~600 d of incubation (Fig. 2.2D).

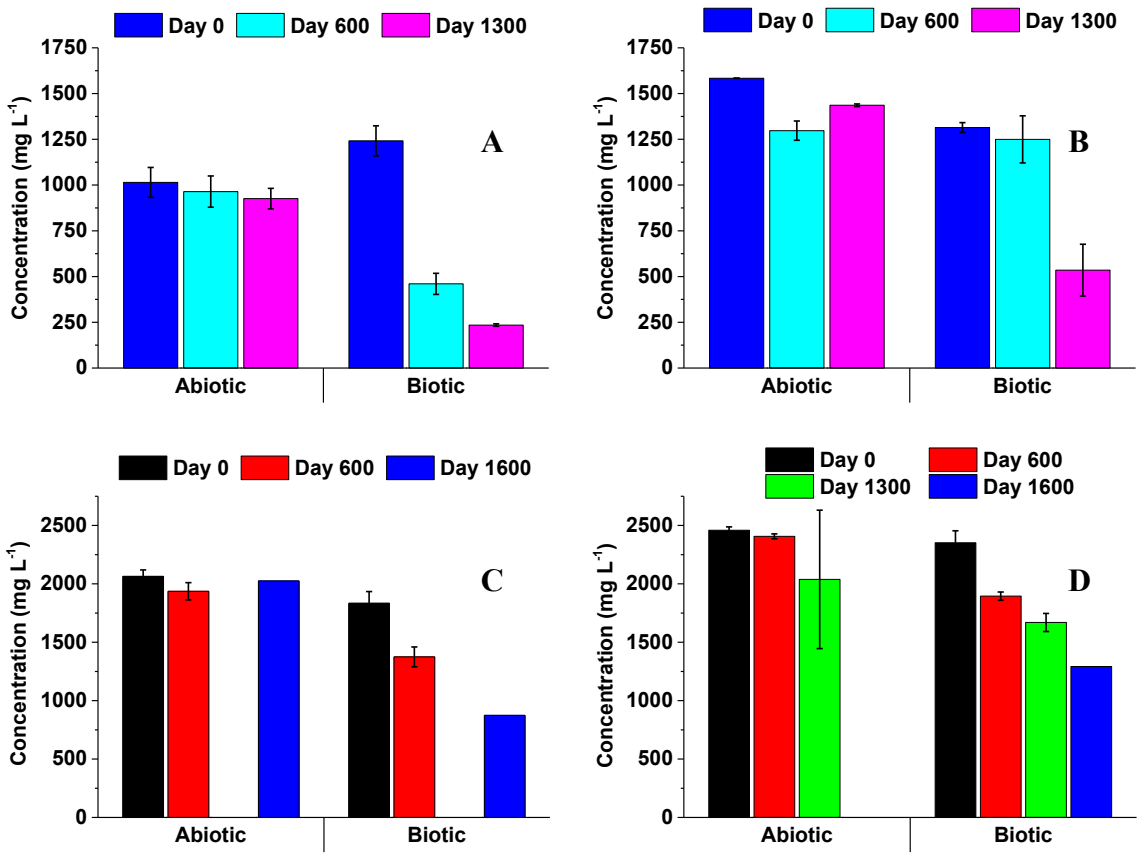

Figure 2.2: Changes in concentrations of paraffinic solvent in (A) Albian MFT and (B) CNRL MFT during ~1300 d or naphtha in (C) Albian MFT and (D) CNRL MFT during ~1600 d incubation. Concentrations of whole solvent (F1 fraction) were determined by GC-FID equipped with purge-trap using CCME (Canadian Council of the Ministers for Environment) integration method except final data point (day 1600) for naphtha amended MFT, where F1 was estimated using data from PONAU analysis. Except PONAU data retrieved from single replicate from each treatment, the bars represent mean value of triplicate microcosms and error bars represent one standard deviation. Data collection for Albian amended with naphtha at 1300 d was missed. The concentration of naphtha in CNRL abiotic control was not determined for day 1600 since all replicates became biotic, exhibiting biodegradation of naphtha as in amended live cultures. Time course biodegradation of known compounds in paraffinic solvent and naphtha determined by GC-FID equipped with purge and trap system is provided in Tables 2.1 and 2.2. Complete spectrum of naphtha hydrocarbons and their biodegradation at day ~1600 determined by PONAU are presented in Table 2.3 and Appendix Table A1.

Approximately 1292 mg L^{-1} of naphtha remained in CNRL MFT after ~1600 d incubation. Individual major hydrocarbon analyses at ~600 and ~1300 d in Albian and CNRL MFT revealed a general trend of preferential biodegradation of short-chain *n*-alkanes followed by *iso*-alkanes (Table 2.2). Complete naphtha analysis for its all component hydrocarbons at ~1600 d revealed complete biodegradation of all *n*-alkanes (nC_6-C_{10}), major *iso*-alkanes (2-MC_5, 3-MC_6: 3-methylhexane, 2-MC_7: 2-methylheptane, 4-MC_7: 4-methylheptane, (2-, 3-, and 4-MC_8: *iso*-nonanes and 2-MC_9: 2-methylnonane) and some *cyclo*-alkanes (compounds of *cyclo*-pentane and *cyclo*-hexane) (Table 2.3). These results suggest a pattern of preferential biodegradation of *n*-alkanes followed by *iso*- and *cyclo*-alkanes. Other *iso*- and *cyclo*-alkanes were either partially degraded or remained undegraded during ~1600 d incubation (Appendix Table A1).

Table 2.1: Percent biodegradation of major hydrocarbons in paraffinic solvent-amended live Albian and CNRL MFT. The initial (day 0) concentrations of the known hydrocarbons in paraffinic solvent added to MFT were calculated using individual external standard curves and presented in parentheses. Hydrocarbon biodegradation was calculated (Eq. 2.1) using recalcitrant cyclopentane as an internal standard.

Hydrocarbon (mg L^{-1})	Albian			CNRL						
	Day									
	600	1300	1600⊥	600			1300			1600⊥
				(I)	(II)	(III)	(I)	(II)	(III)	
***n*-Alkanes**										
n-Pentane (~211-308)	80 ± 6	100 ± 0	100 ± 0	7	3	3	99	98	93	100 ± 0
n-Hexane (~97-141)	99 ± 2	100 ± 0	100 ± 0	4	2	4	100	100	100	100 ± 0
***iso*-Alkanes**										
2-Methylbutane (~106-154)	20 ± 0	15 ± 2	21 ± 0	6	6	5	15	20	19	7 ± 1
2-Methylpentane (~211-308)	42 ± 6	95 ± 0	100 ± 0	4	1	3	94	92	51	100 ± 0
3-Methylpentane (~114-167)	5 ± 3	19 ± 4	14 ± 0	2	1	2	8	13	5	1 ± 0

The decimals have been rounded to the nearest number. Values represent the mean from analysis of triplicate microcosms (± 1 standard deviation) except for CNRL replicates sampled at 600 and 1300 d, which were represented individually.

⊥ The percent biodegradation of hydrocarbons sampled at 1600 d was determined by headspace analysis on gas chromatograph equipped with mass spectrometer and calculated using peak areas as a percentage of the amended abiotic cultures after normalizing to the peak representing recalcitrant cyclopentane (Eq. 2.1).

Table 2.2: Percent biodegradation of known hydrocarbons in naphtha-amended live Albian and CNRL MFT determined using GC-FID equipped with purge and trap system during ~1300 d incubation. The initial (day 0) concentrations of the known hydrocarbons in naphtha added to MFT were calculated using individual external standard curves and presented in parentheses. Hydrocarbon biodegradation was calculated (Eq. 2.1) using recalcitrant cyclopentane as an internal standard.

Hydrocarbon (mg L^{-1})	Albian	CNRL	
	Day		
	600	600	1300
***n*-Alkanes**			
n-Hexane (~91-134)	100 ± 0	98 ± 3	100 ± 0
n-Heptane (~121-152)	100 ± 0	97 ± 4	100 ± 0
n-Octane (~105-126)	57 ± 1	54 ± 2	53 ± 1
n-Decane (~15-21)	3 ± 7	32 ± 6	0 ± 0
***iso*-Alkanes**			
2-Methylbutane (~8-12)	13 ± 1	21 ± 2	17 ± 1
2-Methylpentane (~18-24)	82 ± 0	49 ± 0	89 ± 1
3-Methylpentane (~29-39)	8 ± 1	4 ± 0	5 ± 2
2-Methylhexane (~74-92)	44 ± 1	37 ± 2	48 ± 1
3-Methylhexane (~58-72)	87 ± 0	53 ± 3	94 ± 0
2 + 4-Methylheptane (~35-42)	31 ± 1	18 ± 2	93 ± 1
3-Ethylhexane (~79-102)	17 ± 1	13 ± 1	30 ± 1
2-Methyloctane (~20-24)	100 ± 0	100 ± 0	100 ± 0
***cyclo*-Alkanes**			
Cyclohexane (~46-65)	3 ± 1	-3 ± 0	0 ± 0
Methylcyclohexane (~72-92)	1 ± 1	-3 ± 1	0 ± 0
Ethylcyclopentane (~19-30)	11 ± 1	7 ± 8	22 ± 4
Ethylcyclohexane (~13-18)	11 ± 2	5 ± 2	12 ± 2

The decimals have been rounded to the nearest number. Percent degradation values represent the mean from analysis of triplicate microcosms (± 1 standard deviation).

To determine the carbon flow from hydrocarbons to CH_4 via microbial metabolism, the theoretical CH_4 was compared with the measured CH_4 in experimental microcosms. The measured CH_4 production in Albian and CNRL MFT amended with paraffinic solvent varied between 68-88% of the predicted theoretical maximum CH_4 whereas MFT amended with naphtha yielded 43-76% of the predicted theoretical CH_4 (Appendix Table A2).

Table 2.3: Percent biodegradation of full spectrum of hydrocarbons in naphtha-amended live Albian and CNRL MFT that exhibited noteworthy biodegradation (≥ 50%) determined by PONAU analysis at ~1600 d of incubation. Values in parentheses are the concentrations (mg L^{-1}) before biodegradation, calculated by comparing mass ratios of hydrocarbons in heat-killed MFT (abiotic control) determined by PONAU analysis with initial naphtha concentrations (determined by GC-FID equipped with purge and trap system) in live Albian and CNRL MFT. Percent biodegradation of hydrocarbons was qualitatively determined (Eq. 2.1) by comparing mass ratios of individual hydrocarbon in heat-killed MFT with the mass ratios present in live MFT determined by PONAU at ~ 1600 d using recalcitrant methylcyclohexane and 1,1,3-trimethylcyclohexane as internal standards.

Composition (% weight)	**Components**	**Percent Biodegradation (%)**	
		Albian MFT	**CNRL MFT**
n-Alkanes C_6-C_{10} (17.96)	*n*-Hexane	100 (65)	100 (77)
	n-Heptane	100 (157)	100 (187)
	n-Octane	100 (78)	65 (93)
	n-Nonane	100 (29)	100 (34)
	n-Decane	100 (28)	100 (33)
iso-Alkanes C_5-C_{10} (39.01)	2-Methylpentane	100 (21)	100 (25)
	2-Methylhexane	54 (89)	52 (106)
	3-Methylhexane	100 (85)	100 (101)
	2,2,4-Trimethylpentane	47 (145)	70 (172)
	2-Methylheptane	100 (64)	100 (77)
	4-Methylheptane	100 (24)	100 (25)
	3-Methylheptane	71 (32)	70 (38)
	2-Methyl-4-Ethylhexane	100 (5)	100 (6)
	2-Methyloctane	100 (17)	100 (20)
	3-Methyloctane	100 (9)	100 (11)
	4-Methyloctane	100 (9)	100 (11)
	3,3-Dimethyloctane	100 (14)	0 (17)
	2-Methylnonane	100 (49)	79 (58)
cyclo-Alkanes C_6-C_9 (34.88)	Methylcyclopentane	91 (59)	0 (71)
	Ethylcyclopentane	79 (37)	32 (44)
	cis-1,4-Dimethylcyclohexane	0 (24)	100 (28)
	trans-1-Ethyl-4-Methylcyclohexane	100 (6)	0 (7)
	1 -Methyl-2-Propylcyclopentane	100 (18)	11 (22)
Aromatics C_9-C_{10} (2.52)		100 (32)	8 (39)
Alkenes C_8 (1.58)		50 (30)	49 (40)

Other components that showed ≤ 50% degradation are presented in Appendix Table A1. Masses of all degraded components (calculated from the percent biodegradation of the initial concentration) were fit into Eq. 2.2 to calculate the theoretical maximum methane production in Albian and CNRL MFT amended with naphtha (Appendix Table A2).

2.4.3 Composition of prokaryotic community during biodegradation of the extraction solvents

To identify the key microbial players involved in the biodegradation of solvents, partial pyrosequencing of 16S rRNA genes was performed using unamended and amended MFT at different time intervals. Bacterial DNA reads in all unamended and amended MFT only constituted 2-29% of the total prokaryotic reads yielding the rest to Archaea, except Albian MFT at day 0 where bacterial reads constituted 74-87% of the total prokaryotic community DNA reads (Appendix Fig. A1). It is important to note that day 0 analysis for CNRL MFT amended with paraffinic solvent was unavailable due to low amplicon yield or inhibition in downstream DNA processing.

The initial bacterial communities in Albian MFT exhibited domination of *Hydrogenophilaceae* (85-93% of total bacterial reads) whereas CNRL MFT showed more diverse community comprised of major bacterial families; *Coriobacteriaceae*, *Anaerolineaceae, Peptococcaceae*, and *Syntrophaceae* with large contribution from 'rare' OTUs (individually abundant at ≤ 5% of the total bacterial reads) (Fig. 2.3; Appendix Tables A3 and A4). Though the initial microbial community in CNRL MFT amended with paraffinic solvent could not be determined, we expected similar result because the same MFT was used for all the treatments. Upon long incubation (~1100 d), bacterial community in unamended CNRL MFT did not change (Fig. 2.3B); however, unamended Albian MFT was enriched with *Anaerolineaceae* (27%) followed by *Syntrophaceae* (15%) and *Hydrogenophilaceae* (8%) (Fig. 2.3A).

During paraffinic solvent biodegradation producing exponential CH_4 (~600 d) in Albian MFT, the bacterial community was enriched with *Anaerolineaceae* (52%) followed by *Peptococcaceae* (10%), however, *Peptococcaceae*-affiliated reads became enriched (63%) when CH_4 production had plateaued (~1100 d) (Fig. 2.3C). CNRL MFT started metabolizing paraffinic solvent to CH_4 after long lag phase (~800 d). Because different extent of CH_4 was produced in replicate microcosms, microbial communities in individual microcosms were analyzed at ~1100 d. The replicates (I and II) that exhibited exponential CH_4 production showed enrichment of

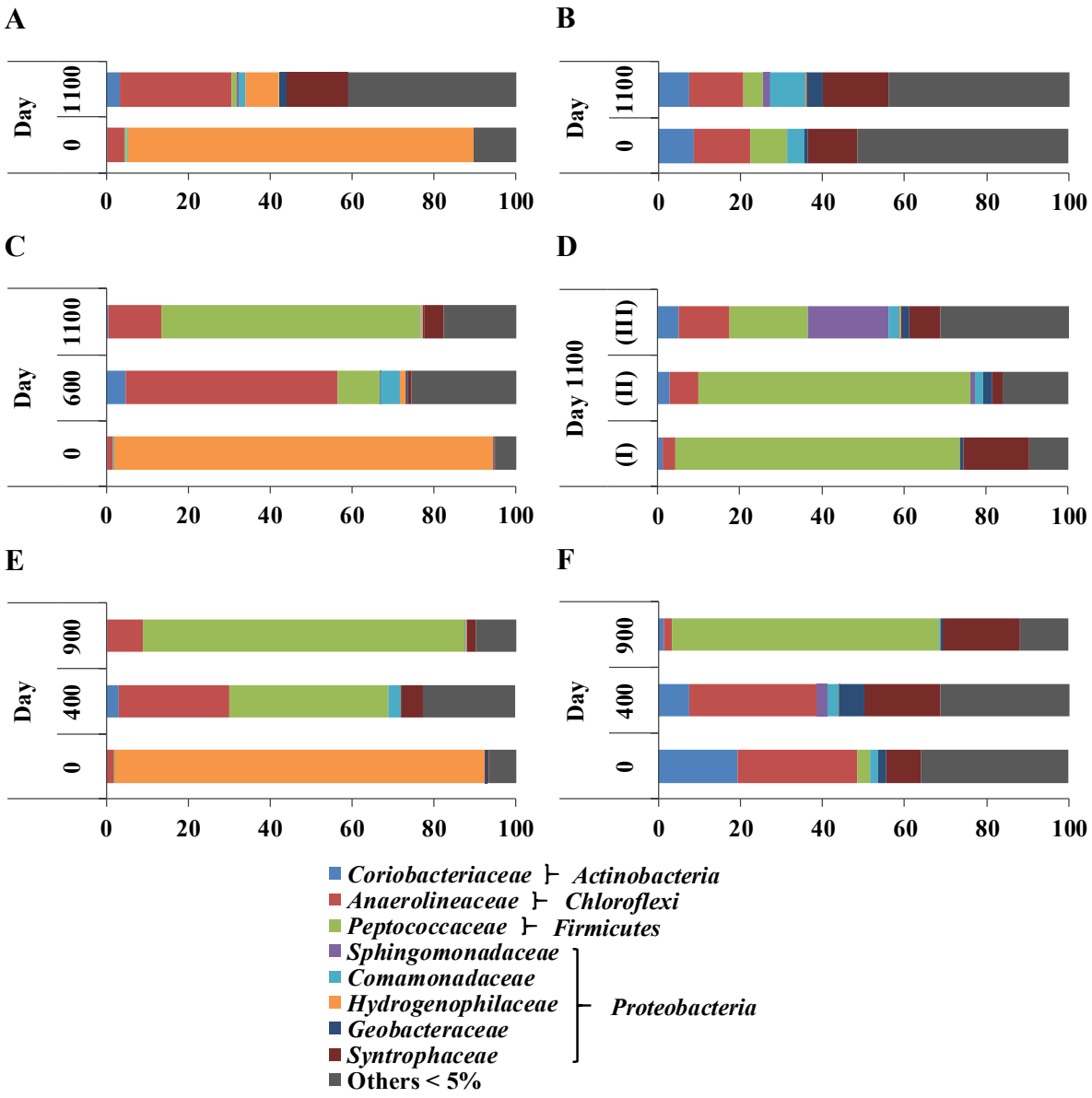

Figure 2.3: Bacterial compositions based on 16S rRNA gene analysis during incubation of unamended MFT and MFT amended with naphtha or paraffinic solvent. (A) Unamended Albian MFT, (B) Unamended CNRL MFT, (C) Albian MFT, paraffinic solvent, (D) CNRL MFT, paraffinic solvent (E) Albian MFT, naphtha, and (F) CNRL MFT, naphtha. The replicates in CNRL amended with paraffinic solvent was analyzed individually due to the difference in CH_4 production between the replicates. The sequences were clustered at 5% distance cut-off and expressed as percentage of total bacterial reads. "Others <5%" is the sum of all taxa individually abundant at <5% of the total bacterial reads. Detailed results including the number of taxa grouped under "Others <5%" are shown in Appendix Tables A3 and A4.

Peptococcaceae (~67-69%); whereas replicate III, that did not produce much CH_4 production, had bacterial composition similar to unamended CNRL MFT except for *Sphingomonadaceae*-affiliated sequences which were more enriched (20% of the total bacterial sequence reads) in replicate III (Fig. 2.3D; Appendix Table A4).

For naphtha biodegradation, Albian MFT harbored bacterial community codominated with *Peptococcaceae* and *Anaerolineaceae* (39% and 27%, respectively) during the exponential production of CH_4 (~400 d), but *Peptococcaceae* dominated (79%) after CH_4 production plateaued (~900 d) (Fig. 2.3E). Interestingly in CNRL MFT during initial biodegradation of naphtha (~400 d), the bacterial community resembled day 0 with a slight increase in *Anaerolineaceae* (Fig. 2.3F). After ~900 d (when CH_4 production had plateaued), *Peptococcaceae* (65%) and *Syntrophaceae* (19%) dominated the community (Fig. 2.3F).

The initial archaeal communities in Albian MFT were dominated by acetoclastic methanogens; *Methanosaetaceae* (~63-67%) and *Methanosarcinaceae* (~14-17%) (Figs. 2.4A, C and E). However, long incubation (~1100 d) even changed unamended Albian MFT community by increasing *Methanosarcinaceae* (52%) and "*Candidatus* Methanoregula" (15%) (Fig. 2.4A). Initial archaeal community in CNRL MFT was co-dominated by "*Candidatus* Methanoregula" (hydrogenotrophic methanogen) and *Methanosaetaceae* (Figs. 2.4B and F). The proportion of "*Candidatus* Methanoregula" increased (84%) in unamended CNRL MFT observed at ~1100 d (Fig. 2.4B).

During active biodegradation of paraffinic solvent in Albian MFT (~600 d), *Methanosaetaceae* and *Methanosarcinaceae* remained dominant; however, "*Candidatus* Methanoregula" (87%) dominated the community when CH_4 production plateaued (~1100 d). Interestingly, all replicates of CNRL MFT amended with paraffinic solvent exhibited similar codomination of "*Candidatus* Methanoregula" and *Methanosaetaceae* during active biodegradation (~1100 d) albeit each replicate showed different extent of biodegradation. In naphtha amended MFT, both Albian and CNRL MFT exhibited similar observations where *Methanosaetaceae* (~61-81%) and "*Candidatus* Methanoregula" (~17-32%) dominated archaeal communities during the active production of CH_4 (~400 d). However, "*Candidatus* Methanoregula" overwhelmingly dominated (~66%-91%) when CH_4 production had plateaued (~900 d incubation) (Figs. 2.4E and F).

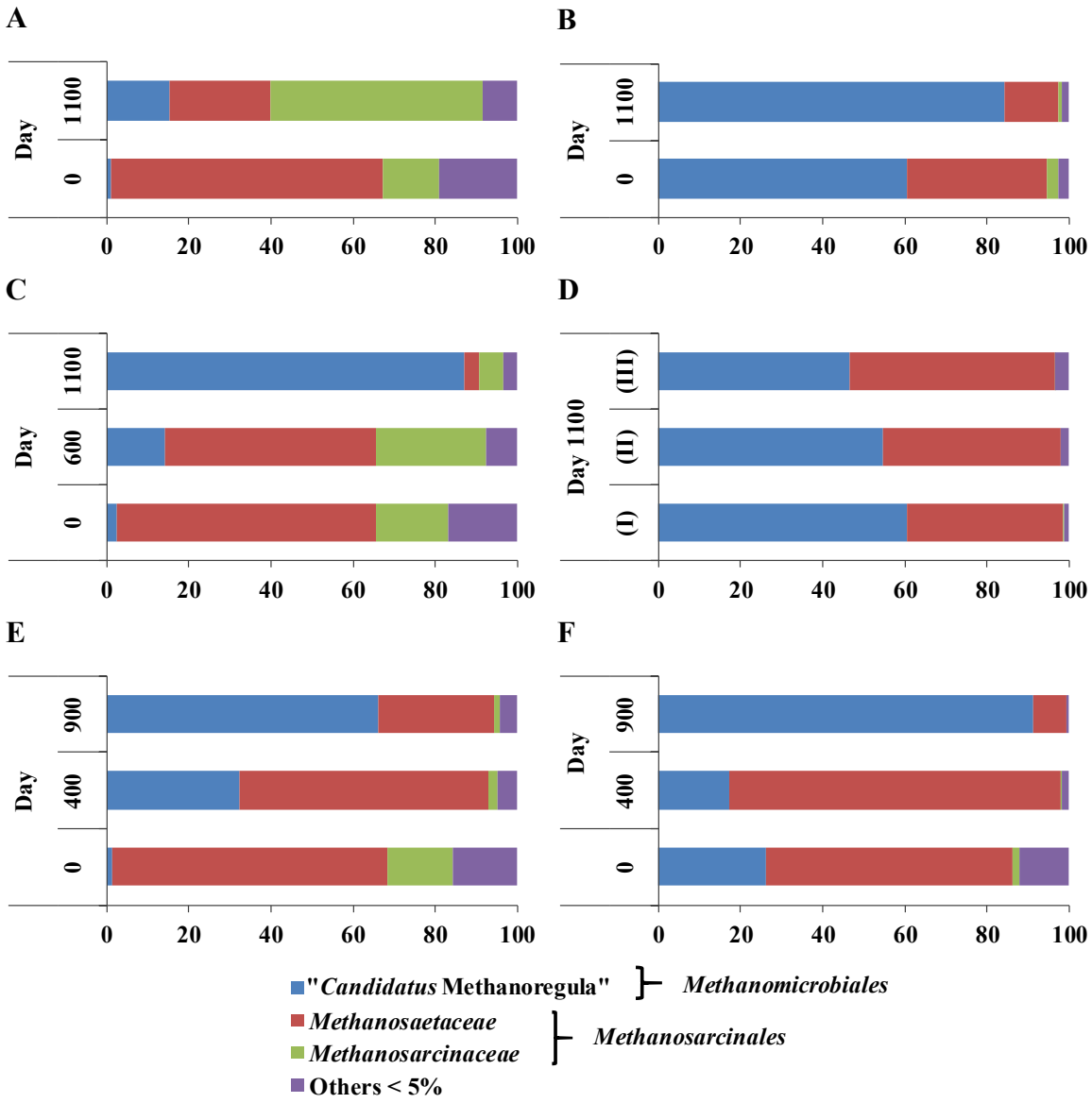

Figure 2.4: Archaeal compositions based on 16S rRNA gene analysis during incubation of unamended MFT and MFT amended with naphtha and paraffinic solvent. (A) Unamended Albian MFT, (B) Unamended CNRL MFT, (C) Albian MFT, paraffinic solvent, (D) CNRL MFT, paraffinic solvent (E) Albian MFT, naphtha, and (F) CNRL MFT, naphtha. The replicates in CNRL amended with paraffinic solvent was analyzed individually due to the difference in methane production between the replicates. The sequences were clustered at 5% distance cut-off and expressed as percentage of total archaeal reads. "Others <5%" is the sum of all taxa individually abundant at <5% of the total archaeal reads. Detailed results including the number of taxa grouped under "Others <5%" are shown in Appendix Tables A5 and A6.

2.5 Discussion

Methanogenesis in oil sands tailings ponds not only causes surface greenhouse gas emissions from tailings ponds but also alters porewater and clay surface chemistry (Siddique, Kuznetsov, Kuznetsova, Arkell, et al., 2014; Siddique, Kuznetsov, Kuznetsova, Li, et al., 2014) that can affect phase transfer of contaminants in tailings. This is particularly important in the creation of end-pit lakes, a viable option for reclaiming tailings ponds, where methanogenesis in underlying MFT can mobilize/transport contaminants and affect the quality of overlying cap water. Here, we present a comprehensive investigation into the biodegradation of two extraction solvents (paraffinic and naphtha) into CH_4 in two different oil sands tailings. Biodegradation of paraffinic solvent began earlier (after ~400 d) in Albian MFT than CNRL MFT (~800 d) indicating the adaptation of indigenous microbial communities in Albian MFT to their cognate Albian paraffinic solvent. However, both Albian and CNRL MFT displayed much shorter lag phase (~100 d) for naphtha biodegradation (Fig. 2.1). Longer lag phases for paraffinic solvent biodegradation might be due to higher concentrations of soluble and more bioavailable short-chain hydrocarbons in the solvent which rendered toxicity to microorganisms (Sikkema, de Bont, & Poolman, 1995) or inhibited their growth and activities requiring a longer time for acclimation. Naphtha, however, comprised a wider range of hydrocarbons; therefore, even though higher concentration (double the mass of Albian solvent) of naphtha was added into each microcosm, the concentrations of individual bioavailable short-chain hydrocarbon were low eliminating long acclimation for indigenous microbial communities. Thus, biodegradation of hydrocarbons in MFT amended with naphtha occurred relatively quickly. In our previous hydrocarbon biodegradation studies, we have also observed that if a hydrocarbon mixture contains higher numbers of compounds, it causes shorter lag phase compared to the one with fewer compounds even though the concentrations of individual compounds remain the same in each mixture (Siddique et al., 2011).

After the lag phase, rapid biodegradation of extraction solvents occurred inferred from the exponential increase in CH_4 production and depletion of hydrocarbons. Albian and CNRL MFT biodegraded major portion (81 and 59%, respectively) of added solvent during ~1300 d (Fig. 2.2) where *n*-alkanes (*n*C_5 and *n*C_6) were completely and preferentially biodegraded over *iso*-alkanes (Table 2.1). This biodegradation pattern was expected because *n*-alkanes are more readily biodegradable than *iso*-alkanes (Siddique et al., 2015, 2007). Among three major *iso*-alkanes, only 2-MC_5 was completely biodegraded while 2-MC_4 and 3-MC_5 remained partially

biodegraded. No further biodegradation of these partially biodegraded *iso*-alkanes into CH_4 during the incubation suggested cometabolism of 2-MC_4 and 3-MC_5 during oxidation of 2-MC_5 (Siddique et al., 2015). This biodegradation pattern of *n*- and *iso*-alkanes from paraffinic solvent was also observed in previous studies conducted using MFT from Syncrude MLSB (Siddique et al., 2015; Tan, Semple, et al., 2015).

Albian and CNRL MFT biodegraded 52 and 45% of naphtha, respectively, during ~1600 d incubation (Fig. 2.2). The lower amount of naphtha biodegraded compared to paraffinic solvent in both Albian and CNRL MFT might be attributed to the composition of naphtha which is more complex than the paraffinic solvent; thus, more compounds in naphtha remained undegraded. In both MFT, preferential biodegradation of *n*-alkanes followed by *iso*-alkanes and then *cyclo*-alkanes was observed among the biodegraded compounds (Tables 2.2 and 2.3; Appendix Table A1). In *n*-alkane fraction of naphtha, shorter *n*-alkanes (nC_6 and nC_7) were preferentially metabolized by both MFT. This pattern is contradictory to our previous findings where CNRL MFT preferentially metabolized nC_8 and nC_{10} versus preferential biodegradation of nC_5 and nC_6 in Albian MFT when amended with a mixture of nC_5-C_{10} (Mohamad Shahimin et al., 2016). The reason for this behavior of CNRL MFT cannot be explained except that this study used the whole naphtha instead of a mixture of *n*-alkanes. Both Albian and CNRL MFT also exhibited complete biodegradation of certain *iso*-alkanes; some of which (2-MC_5, 3-MC_6 and 4-MC_7) have been reported previously (Abu Laban et al., 2014; Siddique et al., 2015; Tan, Semple, et al., 2015) while others (2-MC_7, 2-MC_8, 3-MC_8, 4-MC_8, and 2-MC_9) have never been reported before for their biodegradability. Other *iso*-alkanes, however, were only partially biodegraded implying cometabolic biodegradation. In previous studies using Syncrude and Albian MFT (Abu Laban et al., 2014; Siddique et al., 2015; Tan, Semple, et al., 2015), 2-MC_4, 3-MC_5 and 2-MC_6, were only biodegraded when incubated in a mixture of *iso*-alkanes suggesting their cometabolic biodegradation. Abu Laban et al.(Abu Laban et al., 2014) reported recalcitrance of 3-EC_6 to degradation, however, this compound was partially degraded in our naphtha amended cultures, perhaps through cometabolism. A few *cyclo*-alkanes (compounds of cyclopentane and cyclohexane) were also considerably biodegraded mostly by Albian MFT. Stoichiometric calculations provide an ease in understanding hydrocarbon metabolism in a complex mixture like extraction solvents (Appendix Table A2). Because major hydrocarbons (nC_5, nC_6 and 2-MC_5) in paraffinic solvent were completely metabolized into CH_4 by microbes leaving a few (2-MC_4 and 3-MC_5) for partial biodegradation through cometabolism, the measured CH_4 in the paraffinic

solvent amended Albian and CNRL microcosms yielded expected ~68-88% of the predicted theoretical maximum CH_4 production, which was in agreement with our previous findings (Siddique et al., 2006, 2007). The difference between measured and predicted CH_4 is attributed to incomplete oxidation of complex substrates to recalcitrant metabolites and/or high carbon assimilation to biomass (Fowler, Dong, Sensen, Suflita, & Gieg, 2012; Mohamad Shahimin et al., 2016; Tan et al., 2015). However, in the case of naphtha, MFT initially (by ~ 600 d) produced 70-76% of theoretical maximum CH_4 during complete biodegradation of *n*-alkanes and major *iso*-alkanes but these values decreased to 43-55% afterward suggesting cometabolic partial biodegradation of other *iso*- and *cyclo*-alkanes during rest of the incubation. Cometabolism of *iso*- and *cyclo*-alkanes indicates the enzymes involved in the oxidation of some alkanes have relaxed substrate specificity (Abu Laban et al., 2014) allowing degradation of other alkanes.

Microbial community composition in both Albian and CNRL MFT changed during biodegradation of extraction solvents. The initial bacterial communities in all Albian MFT microcosms were dominated by *Thiobacillus* (*Hydrogenophilaceae*) (Appendix Table A3), which were classified as strict aerobic bacteria except *Thiobacillus denitrificans*, a facultative anaerobe (Beller et al., 2006). During the long storage of bulk Albian MFT in pail since 2008, periodic exposure to air during sampling might have turned a portion of the MFT aerobic, allowing high growth of *Hydrogenophilaceae.* The pre-incubation of Albian MFT with methanogenic medium for two weeks may not be long enough to allow growth of methanogenic bacterial communities, thus, shows domination of *Hydrogenophilaceae* in initial (day 0) bacterial communities. During biodegradation of the extraction solvents, only a few bacterial families were enriched, namely *Anaerolineaceae* and *Peptococcaceae* in Albian MFT, and *Anaerolineaceae*, *Peptococcaceae* and *Syntrophaceae* in CNRL MFT. *Anaerolineaceae* were enriched in all cultures during exponential CH_4 production except in CNRL MFT amended with paraffinic solvent. Enrichment of *Anaerolineaceae* suggested their involvement in biodegradation process in oil sands tailings. Indeed, a recent study (Liang et al., 2015) has implicated *Anaerolineaceae* as the primary degraders of *n*-alkanes in an anaerobic enrichment culture, however, *Anaerolineaceae* were also suggested to function as scavengers of intermediary products of incomplete hydrocarbon fermentation (Kleinsteuber, Schleinitz, & Vogt, 2012). Therefore, it is possible that *Anaerolineaceae* were directly involved both in the initial and intermediary steps during degradation of solvent hydrocarbons in MFT. Besides *Anaerolineaceae*, *Peptococcaceae*-affiliated bacteria were also enriched in both Albian and

CNRL MFT. Previous studies have also reported enrichment of *Peptococcaceae* in primary and enrichment cultures derived from Albian, CNRL and Syncrude MFT during active biodegradation of short-chain (C_5-C_8) *n*- and *iso*-alkanes (Abu Laban et al., 2014; Mohamad Shahimin et al., 2016; Siddique et al., 2015; Tan et al., 2015), implicating *Peptococcaceae* as the primary alkanes-degraders in MFT. Recent genomic study of an uncultivated *Peptococcaceae* isolated from short-chain alkane degrading cultures (Tan, Charchuk, Li, Laban, & Foght, 2014) revealed that *Peptococcaceae* might be capable of activating alkanes via fumarate addition, a known anaerobic hydrocarbon activation pathway, solidifying the proposition of *Peptococcaceae* as primary alkanes-degraders in different MFT. Exclusively in CNRL MFT, *Syntrophaceae* were also enriched along with *Anaerolineaceae* and *Peptococcaceae* during biodegradation of the solvents. Draft genome sequences from *Syntrophaceae*-affiliated bacteria (*Smithella* spp.) obtained from methanogenic alkane-degrading culture and oil field produced water revealed that the *Smithella* spp. genomes harbored genes encoding sequences homologous to the catalytic subunit of alkylsuccinate synthase genes (*assA*: an enzyme involved in anaerobic alkanes activation via fumarate addition pathway), highlighting capability of members of *Syntrophaceae* as potential primary degraders of hydrocarbons. Other studies have also implicated members of *Syntrophaceae* as primary degraders of various hydrocarbons (Cheng et al., 2013; Gray et al., 2011; Siddique et al., 2012, 2011; Tan, Rozycki, & Foght, 2014; Zengler, Richnow, Rosselló-Mora, Michaelis, & Widdel, 1999), providing further evidence supporting the postulation of *Syntrophaceae* as one of primary hydrocarbon degraders in CNRL MFT.

In all the amended cultures (Albian and CNRL MFT amended with paraffinic solvent and naphtha), acetoclastic *Methanosaetaceae* were enriched during active biodegradation of solvents, highlighting acetoclastic methanogenesis as primary methanogenic pathway during biodegradation of solvents. Other studies have also reported similar domination of acetoclastic methanogens during methanogenic biodegradation of *n*-alkanes (Mohamad Shahimin et al., 2016; Siddique et al., 2012). However, after CH_4 production plateaued, "*Candidatus* Methanoregula" became enriched in all amended cultures. Enrichment of "*Candidatus* Methanoregula" occurred simultaneously with enrichment of *Peptococcaceae*. Similar observations have also been reported in primary and enrichment cultures derived from MFT incubated with various hydrocarbon mixtures (Abu Laban et al., 2014; Mohamad Shahimin et al., 2016; Siddique et al., 2015, 2012; Boonfei Tan, Semple, et al., 2015). Some members of *Peptococcaceae* have been implicated in syntrophic relationship with hydrogenotrophs

(Stackebrandt, 2014), therefore, overwhelming domination of *Peptococcaceae* after the active phase of biodegradation might have favored the growth of hydrogenotrophic "*Candidatus* Methanoregula" in both Albian and CNRL MFT. Additionally, methanogenic biodegradation of hydrocarbons by syntrophic hydrocarbon-degrading bacteria produces hydrogen or formate. Therefore, for methanogenic biodegradation of hydrocarbons to be thermodynamically favorable, hydrogen-consuming microbes (hydrogenotrophs) must be present, explaining the presence of hydrogenotrophic methanogens in all our cultures. Overall, our data on archaeal communities in Albian and CNRL MFT underline the importance of both acetoclastic and hydrogenotrophic methanogenesis in oil sands tailings.

Our study reveals that indigenous microbial communities in both Albian and CNRL MFT are capable of biodegrading complex mixtures of hydrocarbons. Albian and CNRL MFT exhibited similar biodegradation pattern for narrow-range hydrocarbons (paraffinic solvent); however, biodegradation pattern of wide-range hydrocarbons (naphtha) was different in Albian and CNRL MFT. Our findings also highlight the possibility of cometabolism as an important mechanism that affects biodegradation of different groups of hydrocarbons, especially the recalcitrant *iso*- and *cyclo*-alkanes, in oil sands tailings ponds. These results are important in improving the existing kinetic models for greenhouse gas emissions from oil sands tailings ponds (Siddique et al., 2008) and in refining the strategic management practice of greenhouse gas emissions and remediation of oil sands tailings ponds and other anaerobic environments impacted by hydrocarbons.

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3 Preferential methanogenic biodegradation of short-chain *n*-alkanes by microbial communities in MFT from two different oil sands tailings ponds[1]

3.1 Abstract

Oil sands tailings ponds harbor diverse anaerobic microbial communities capable of methanogenic biodegradation of solvent hydrocarbons entrained in the tailings. Mature fine tailings (MFT) from two operators (Albian and CNRL) that use different extraction solvents were incubated with mixtures of either two (*n*-pentane and *n*-hexane) or four (*n*-pentane, *n*-hexane, *n*-octane and *n*-decane) *n*-alkanes under methanogenic conditions for ~600 d. Microbes in Albian MFT began methane production by ~80 d, achieving complete depletion of *n*-pentane and *n*-hexane in the two-alkane mixture and their preferential biodegradation in the four-alkane mixture. Microbes in CNRL MFT preferentially metabolized *n*-octane and *n*-decane in the four-alkane mixture after a ~80 d lag but exhibited a lag of ~360 d before commencing biodegradation of *n*-pentane and *n*-hexane in the two-alkane mixture. 16S rRNA gene pyrosequencing revealed *Peptococcaceae* members as key bacterial *n*-alkane degraders in all treatments except CNRL MFT amended with the four-alkane mixture, in which *Anaerolineaceae*, *Desulfobacteraceae* (*Desulfobacterium*) and *Syntrophaceae* (*Smithella*) dominated during nC_8 and nC_{10} biodegradation. *Anaerolineaceae* sequences increased only in cultures amended with the four-alkane mixture and only during *n*-octane and *n*-decane biodegradation. The dominant methanogens were acetoclastic *Methanosaetaceae*. These results highlight preferential *n*-alkane biodegradation by microbes in oil sands tailings from different producers, with implications for tailings management and reclamation.

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3.2 Introduction

The oil sands industry in northern Alberta, Canada has expanded rapidly in recent years. Surface mining of oil sands ore and its treatment with hot water for bitumen extraction produces enormous volumes of fluid tailings that are deposited in large basins ('tailings ponds') where they are retained indefinitely, pending reclamation. The volume of tailings is projected to grow from the current volume of ~975 million m^3 (osip.alberta.ca/map/) to >1.2 billion m^3 by 2030 (Houlihan & Hale, 2011). Most oil sands tailings ponds are methanogenic and produce large volumes of methane (CH_4), a potent greenhouse gas, as well as carbon dioxide (CO_2). Mildred Lake Settling Basin (MLSB), the largest and one of the oldest oil sands tailings ponds, has been estimated to emit ~43 million L CH_4 day^{-1} (Holowenko et al., 2000; Siddique et al., 2008). Methanogenesis drives many biogeochemical processes in such tailings ponds. For example, recent studies have shown that indigenous microbial communities in mature fine tailings (MFT) from MLSB accelerate pore water recovery and densification of tailings solids by changing pore water and solid phase chemistry while metabolizing organic substrates to CH_4 (Arkell et al., 2015; Siddique, Kuznetsov, Kuznetsova, Arkell, et al., 2014; Siddique, Kuznetsov, Kuznetsova, Li, et al., 2014). The dewatering and densification of tailings can potentially reduce the tailings inventory and speed up the reclamation of MFT, hence the interest in methanogenesis in these ponds.

Recovery of bitumen from surface-mined oil sands ores involves hydrocarbon solvent extraction, a small proportion of which (<1 vol%) cannot be recovered for reuse, and enters the ponds with fresh tailings. This fugitive solvent is the major substrate sustaining methanogenesis in the tailings ponds (Siddique et al., 2007). The solvent used by Syncrude Canada Ltd. is naphtha, comprising aliphatic and aromatic hydrocarbons primarily of ~C_6-C_{10}. Laboratory biodegradation studies have revealed that *n*-alkanes, monoaromatics (BTEX; benzene, toluene, ethylbenzene and xylene isomers), C_7 and C_8 *iso*-alkanes and a C_6 *cyclo*-alkane (methylcyclopentane) present in naphtha can be degraded under methanogenic conditions by indigenous microorganisms in MFT (Abu Laban et al., 2014; Siddique et al., 2006, 2007, 2012; Tan et al., 2015). Short-chain *n*-alkanes (C_6-C_{10}) representing naphtha-range hydrocarbons were degraded preferentially in the order: $C_{10} > C_8 > C_7 > C_6$ (Siddique et al., 2006), albeit no such preferential biodegradation was observed with longer-chain *n*-alkanes (C_{14}, C_{16}, C_{18}) (Siddique et al., 2011). The question arises whether such chain length discrimination is unique to MLSB

microbes, or whether preferential degradation of alkane suites is more widespread in oil sands tailings ponds. The answer would be useful for predicting onset and duration of greenhouse gas production from oil sands tailings ponds operated under different conditions than MLSB.

Thus, we examined alkane biodegradation by microbes in MFT from two oil sands companies, Albian Sands Inc. (Albian) and Canadian Natural Resources Ltd. (CNRL). Their tailings ponds are much younger than MLSB, allowing less time for anaerobic hydrocarbon-degrading microbial communities to be enriched: Albian's Muskeg River Mine pond is ~14 years old and the CNRL Horizon pond is ~7 years old, versus Syncrude's ~40-year-old MLSB pond. Tailings management also differs between the sites, with Syncrude and CNRL using naphtha containing C_6–C_{10} *n*-, *iso*- and *cyclo*-alkanes and monoaromatics, but Albian using a predominantly aliphatic solvent (C_5–C_6 *n*- and *iso*-alkanes). Processes used to enhance consolidation of tailings solids in the ponds also differ, with Albian adding both trisodium citrate and organic polymer flocculants to tailings before deposition (Li, 2010) versus CNRL injecting CO_2 into tailings (www.cnrl.com/). These differences beg the question of whether microbial communities in each pond have adapted to efficiently biodegrade only the components of their cognate solvent, or are sufficiently metabolically flexible that they can adapt to degrade other solvent components. The anaerobic solvent biodegradation capability and key microbial players in Albian and CNRL tailings, which have not previously been published in detail, are of particular interest.

Because methanogenesis apparently drives some major geochemical processes in some oil sands tailings ponds (Siddique, Kuznetsov, Kuznetsova, Arkell, et al., 2014; Siddique, Kuznetsov, Kuznetsova, Li, et al., 2014) and little is known about methanogenic processes in Albian (Siddique et al., 2015) and CNRL MFT, it is important to evaluate the metabolic flexibility of their indigenous microbial communities. Therefore, in this study, we assessed the preferential biodegradation of two mixtures of short-chain *n*-alkanes (*n*C_5 and *n*C_6, or *n*C_5, *n*C_6, *n*C_8 and C_{10}, representing Albian and CNRL solvents, respectively) by microbes in Albian and CNRL MFT. We measured CH_4 production, determined hydrocarbon depletion and characterized microbial communities using pyrosequencing of 16S rRNA genes to infer the roles of different microbial groups in the methanogenic community during the biodegradation of these *n*-alkanes mixtures. This study contributes to the overall understanding of methanogenesis in oil sands tailings and its implications in tailings management.

3.3 Materials and methods

3.3.1 Chemicals and materials

n-Pentane (≥99%; CAS # 109-66-0), *n*-hexane (≥96%; CAS # 110-54-3) and *n*-octane (≥99%; CAS # 111-65-9) were purchased from Fisher Scientific, Ontario, Canada. *n*-Decane (≥99%; CAS # 124-18-5) was purchased from Sigma-Aldrich, Ontario, Canada.

Samples of methanogenic MFT were collected by respective oil sands operators (Albian and CNRL). MFT is a thick slurry of sediment (usually ≥30% solids), water, unextracted bitumen (usually ~5 vol %) and unrecovered solvent (≤1 vol%). Albian MFT was collected in bulk from the Muskeg River Mine Tailings Pond at 7 m below the water surface in 2008 (0465371E 6342304N**;** solids, 25 wt %; bitumen, 0.87 wt %; pH 8.07; conductivity 3.1 dS m^{-1}) and stored in the dark at 4ºC under a cap of tailings pond water, to maintain anaerobic conditions in the tailings sediments, for use as inoculum in 2011. We have found in numerous previous experiments that storage of MFT for several years under these conditions does not impair its ability to biodegrade hydrocarbons at a rate similar to that of fresh MFT samples. The CNRL MFT was collected from the Horizon tailings pond (446156E 6356933.1N) in 2011 (solids, 28 wt %; bitumen, 0.97 wt %; pH 8.6; conductivity 2.0 dS m^{-1}) and also stored in the dark at 4ºC until used as inoculum in the same year.

3.3.2 Culture conditions for hydrocarbon biodegradation

The experiment was conducted using anaerobic microcosms prepared in 158-mL sealed serum bottles. Fifty milliliters of Albian or CNRL MFT was added into the serum bottles, sealed with butyl rubber stopper and crimped with aluminum cap in an anaerobic chamber. The headspace was flushed with 30% O_2-free CO_2, balance N_2 and the microcosms were stored in the dark at room temperature for two months, after which, each microcosm filled with either Albian or CNRL MFT received 50 mL of anaerobic methanogenic medium lacking organic carbon (as detailed in Chapter 2; Fedorak & Hrudey, 1984). The headspace of each microcosm was again flushed with 30% O_2-free CO_2, balance N_2, at a slight overpressure, to prevent incursion of atmospheric oxygen. The microcosms were pre-incubated in the dark at room temperature for 2 weeks to allow for microbial acclimation and consumption of residual hydrocarbons and alternative electron acceptors in MFT prior to amendment with new substrates (Siddique et al., 2006). Immediately prior to hydrocarbon addition, the microcosm headspace was flushed again

with 30% CO_2, balance N_2, to remove any CH_4 produced during pre-incubation. The microcosms were then amended with either a two-alkane mixture of *n*-pentane (nC_5) and *n*-hexane (nC_6) or a four-alkane mixture of *n*-pentane (nC_5), *n*-hexane (nC_6), *n*-octane (nC_8) and *n*-decane (nC_{10}) at volumes corresponding to ~40 mg of each individual compound (nominally representing individual concentrations of ~400 mg L^{-1} of total culture volume) at room temperature. To confirm the initial concentrations of individual alkanes in the microcosms, samples were analyzed immediately after alkane addition (see below). Each treatment was prepared in triplicate. Duplicate viable baseline controls (unamended microcosms) were prepared to account for CH_4 production from any residual endogenous substrates in the MFT. To account for abiotic degradation, heat-killed (sterilized) controls were also prepared in the same way as the live cultures but autoclaved (121 ^{O}C, 20 psi, 60 min) four times on four consecutive days prior to alkane addition. The heat-killed microcosms were prepared in triplicate and amended with the four-alkane mixture. All microcosms were incubated at room temperature in the dark. The microcosms were subjected to headspace gas analyses to monitor CH_4 production over time and samples were retrieved periodically from the microcosms for hydrocarbon and 16S rRNA gene analyses.

3.3.3 Chemical analyses

All manipulations of the sealed microcosms were performed using small bore sterile needles and syringes. CH_4 production was measured by removing 50 μL headspace for direct injection into a gas chromatograph equipped with a flame ionization detector (GC-FID) as detailed in Chapter 2. If high pressure developed in the microcosms due to CH_4 production, it was reduced by removing 30 mL or 60 mL headspace gas from two-alkane- and four-alkane-amended microcosms, respectively. The gas volume removed was taken into account when calculating the total mass of CH_4 produced.

To monitor biodegradation, concentrations of the residual alkanes were determined at intervals by removing 1-mL samples from hand-mixed microcosms and extracting hydrocarbons with 10 mL methanol (Fisher Scientific) in a 20-mL EPA glass vial. The vials were shaken for 30 min at 20°C and the solids in the vials were allowed to settle for 30 min. One milliliter of the supernatant was transferred to a 44-mL EPA glass vial, filled completely with ultrapure water (Milli-Q Water System) to avoid any headspace and capped. All prepared vials were analyzed

using a gas chromatograph fitted with a flame ionization detector (GC-FID) and equipped with a purge and trap system, as described in Chapter 2.

3.3.4 Stoichiometry of hydrocarbon biodegradation to cumulative methane production

The concentrations of *n*-alkanes remaining in the microcosms were determined at the final sampling point and used to calculate theoretical maximum CH_4 production using stoichiometric equations derived from the modified Symons and Buswell equation (Symons & Buswell, 1933) assuming complete metabolism of hydrocarbons to CH_4 and CO_2 under methanogenic conditions:

n-pentane (*n*C_5) $C_5H_{12} + 2.0H_2O \rightarrow 1.00CO_2 + 4.00CH_4$ (3.1)

n-hexane (*n*C_6) $C_6H_{14} + 2.5H_2O \rightarrow 1.25CO_2 + 4.75CH_4$ (3.2)

n-octane (*n*C_8) $C_8H_{18} + 3.5H_2O \rightarrow 1.75CO_2 + 6.25CH_4$ (3.3)

n-decane (*n*C_{10}) $C_{10}H_{22} + 4.5H_2O \rightarrow 2.25CO_2 + 7.75CH_4$ (3.4)

The theoretical maximum CH_4 values were compared with the measured CH_4 values in the microcosm headspace to confirm the biodegradation of hydrocarbons.

3.3.5 Characterization of microbial communities

To study the microbial community structure, genomic DNA was extracted from MFT at day 0 (on the day the hydrocarbon mixtures were spiked in the microcosms) and after the biodegradation of the added hydrocarbons, by using the protocol previously described in Chapter 2 (Foght et al., 2004). The DNA from both extractions of each sample was then pooled. The pooled DNA was analyzed for purity and concentrations using Nanodrop-1000 Spectrophotometer before used for PCR amplification.

Bacterial and archaeal 16S rRNA genes were amplified for 454 pyrotag sequencing using the universal primer set 454T-RA/454T-FB targeting the V6-V8 regions of the 16S rRNA gene for both Bacteria and Archaea (Berdugo-Clavijo, Dong, Soh, Sensen, & Gieg, 2012). 16S rRNA gene amplification was performed in an S1000™ Thermal Cycler (BIO RAD) using the thermocycling temperature program described in Chapter 2. Each 25-µL PCR reaction contained 2.5 µL (10 µM) of each primer, 5 µL 5X KAPA2G reaction buffer A (Kapa Biosystems, Woburn MA), 5 µL KAPA enhancer 1 solution, 1.25 µL 100% dimethylsulfoxide, 0.1 µL DNA polymerase (KAPA2G), 0.5 µL dNTP mix (10mM, KAPA2G), 2 µL $MgCl_2$ (25mM), 1 µL

pooled genomic DNA extract (~50 ng μL^{-1}) and 4.65 µL sterile nuclease-free water. Three PCR replications were prepared for each sample and pooled after the PCR amplification. Negative controls consisting of only PCR reagents and nuclease-free water were included with every set of samples for quality control during PCR amplification. PCR products were examined for quality using agarose gel electrophoresis and purified using a QIAquick PCR purification kit (Qiagen). All purified PCR products from different replications within each treatment were pooled before sequencing except for samples of the triplicate Albian four-alkane cultures, where each microcosm was sequenced individually because of observed differences in hydrocarbon biodegradation.

16S rRNA gene amplicons were sequenced using a GS FLX Titanium Series Kit XLR70 (Roche) at McGill University Génome Québec Innovation Centre, Montreal, Canada. The raw pyrosequencing data were analyzed using the Phoenix 2 pipeline.(Soh et al., 2013) All pyrotag data sets (~1500-11500 reads per sample) have been submitted to NCBI Sequence Read Archive (SRA) under accession number SRP050020.

3.4 Results and discussion

3.4.1 Methane production during *n*-alkane biodegradation

Cumulative CH_4 production was monitored during incubation by analyzing the headspace of replicate microcosms (Fig. 3.1). No substantial CH_4 was produced by any unamended baseline control cultures during the incubation. After a lag phase of ~80 d, CH_4 production from amended Albian MFT exceeded the baseline control and increased exponentially to 1.75±0.07 and 2.79±0.25 mmol in two-alkane and four-alkane mixtures, respectively by ~200 d. Thereafter, CH_4 increased gradually to 1.99±0.12 mmol in two-alkane mixture by ~300 d and 3.94±0.37 mmol in four-alkane mixture by ~600 d (Fig. 3.1A).

The mass of CH_4 produced by CNRL MFT microcosms was similar to that produced by Albian MFT, but the patterns differed (Fig. 3.1B). A long lag phase (~360 d) was observed in the two-alkane CNRL microcosms, after which CH_4 increased to 2.21±0.05 mmol by ~600 d, similar to the mass produced by Albian MFT with these two substrates. CNRL MFT amended with the four-alkane mixture produced CH_4 after a short lag phase (~90 d), followed by exponential CH_4 production until ~180 d (2.92±0.24 mmol) then a plateau for approximately ~300 d, after which CH_4 production resumed, reaching 4.75±0.30 mmol by ~600 d, the final sampling time. The

latter pattern resembles diauxie, where a preferred substrate is metabolized first, followed by other substrate(s).

To track the carbon flow from substrates to greenhouse gasses via microbial metabolism, we calculated the theoretical maximum CH_4 production using Eqs. 3.1-3.4 to compare with the measured CH_4 in experimental microcosms. CH_4 measured in the headspace ranged from 67-74% of the predicted (calculated) CH_4 (Appendix Table B1), slightly lower than the values we reported previously (77-79% of predicted CH_4) using MFT from MLSB (Siddique et al., 2006). The difference between measured and predicted CH_4 could be associated with microbial carbon assimilation, production of recalcitrant intermediary compounds (metabolites) and/or residual alkane loss through sampling (see below) (Fowler et al., 2012; Zengler et al., 1999).

3.4.2 Preferential biodegradation of *n*-alkanes

In all microcosms, the concentrations of nC$_5$ measured immediately after amendment (day 0) were lower (~200 mg L^{-1}) than predicted (~400 mg L^{-1}). This was likely due to volatility losses of nC$_5$, the smallest and most volatile substrate, during preparation of the alkane mixtures. However, analysis of the heat-killed control microcosms accounted for such abiotic losses and allowed calculation of alkane biodegradation.

GC-FID measurement of residual *n*-alkanes in the Albian MFT microcosms during incubation revealed preferential biodegradation of the smaller alkanes (C$_5$-C$_6$) corresponding to the composition of its aliphatic extraction solvent (also C$_5$-C$_6$), (Figs. 3.2 and 3.3). In Albian MFT amended with the two-alkane mixture, nC$_5$ and nC$_6$ (initially 216±1.8 and 394±39 mg L^{-1}, respectively) were completely depleted by ~300 d versus only a slight decrease in the abiotic control microcosms (Fig. 3.2A). Analysis of the four-alkane-amended Albian MFT microcosms also showed complete depletion of nC$_5$ (initially 202±3.5 mg L^{-1}), nC$_6$ (411±9.1 mg L^{-1}) and nC$_8$ (381±19 mg L^{-1}), with nC$_{10}$ being the only alkane still detected in Albian MFT at ~300 d (Fig. 3.3A). Complete removal of nC$_{10}$ was observed in only one of the three Albian MFT replicates by day ~400 (Appendix Fig. B1), and the other two microcosms still showed no degradation of nC$_{10}$ by ~500 d; this difference resulted in very large standard deviation values for these four-alkane Albian MFT microcosms (Fig. 3.3A). nC$_{10}$ was eventually depleted in the two replicates by ~900 d when the headspace was analyzed (data not shown). Sterilized Albian MFT (abiotic control microcosms) did not show any considerable loss of the four amended alkanes during ~500 d incubation (Fig. 3.3A).

CNRL preferentially degraded C_8 and C_{10}, reflecting the higher average molecular weight of its naphtha solvent (C_6-C_{10}). Analysis of the two-alkane-amended microcosms revealed that *n*C_5 and *n*C_6 were only slightly depleted (residual concentrations of 222±15 and 300±19 mg L^{-1}, respectively) from their initial concentrations (260±1.8 and 386±17 mg L^{-1}) (Fig. 3.2B) by ~300 d and were completely biodegraded by ~500 d. In the four-alkane-amended CNRL microcosms, complete biodegradation of *n*C_8 (initial concentration; 394±21 mg L^{-1}) and *n*C_{10} (initial concentration; 459±4.2 mg L^{-1}) had occurred by day 294 whereas *n*C_5 and *n*C_6 (residual concentrations of 169±5.5 mg L^- and 248±12 mg L^{-1}, respectively) were only partially depleted compared to their initial concentrations (204± 10 and 343±14 mg L^{-1}, respectively) (Fig. 3.3B). GC-FID analysis performed at day ~400 still showed appreciable concentrations of *n*C_5 (132±3.6 mg L^{-1}) and *n*C_6 (215±5.3 mg L^{-1}) present in the cultures, with eventual depletion by day ~500. Sterilized *n*-alkane-amended CNRL MFT showed no substantial loss of amended alkanes during incubation (Fig. 3.3B).

Thus, microbes from two different ponds preferentially degraded components of their cognate solvents even though the range of alkane chain lengths differed by a maximum of five methylene bridges. The preferential biodegradation of longer chain *n*-alkanes in CNRL MFT is similar to sequential biodegradation by MLSB tailings of *n*-alkanes in the order of decreasing carbon chain length (C_{10}>C_8>C_7>C_6) (Siddique et al., 2006). However, Albian and CNRL MFT exhibited a longer lag phase (~80 d) prior to CH_4 production from short-chain *n*-alkanes (C_6-C_{10}) than previously reported for Syncrude MFT (~35 d) (Siddique et al., 2006). This may be due to the fact that MLSB is older than the Albian and CRNL ponds, and over time its microbial community may have become more acclimatized to hydrocarbon biodegradation. Additionally, there are other factors such as different proportion of key microbial players involved in biodegradation of *n*-alkanes in different MFT and different signaling/response of the microbial players to availability of *n*-alkanes, which we did not investigate, that might have led to the longer lag phase in the Albian and CNRL primary cultures. It is possible that sequential transfer of the Albian and CNRL primary cultures and addition of fresh hydrocarbon substrate would result in shortened lag times.

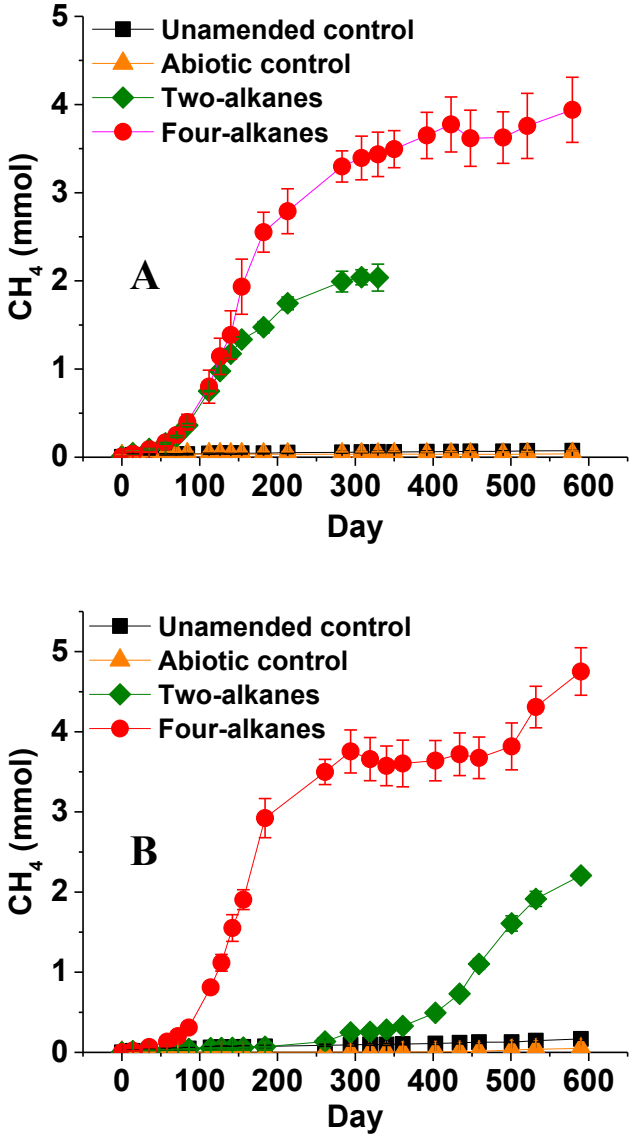

Figure 3.1: Cumulative methane production in microcosms containing (A) Albian MFT or (B) CNRL MFT amended with either a two- or four-alkane mixture and incubated for ~600 days. The two- and four-alkane mixtures comprised *n*-pentane (nC$_5$) and *n*-hexane (nC$_6$), or *n*-pentane (nC$_5$), *n*-hexane (nC$_6$), *n*-octane (nC$_8$) and *n*-decane (nC$_{10}$), respectively. Unamended controls comprised MFT incubated without alkane addition. The abiotic controls were heat-killed and amended with the four-alkane mixture. Symbols represent the mean value from duplicate microcosms for unamended controls and triplicate microcosms for the rest of treatments and error bars, where visible, represent one standard deviation.

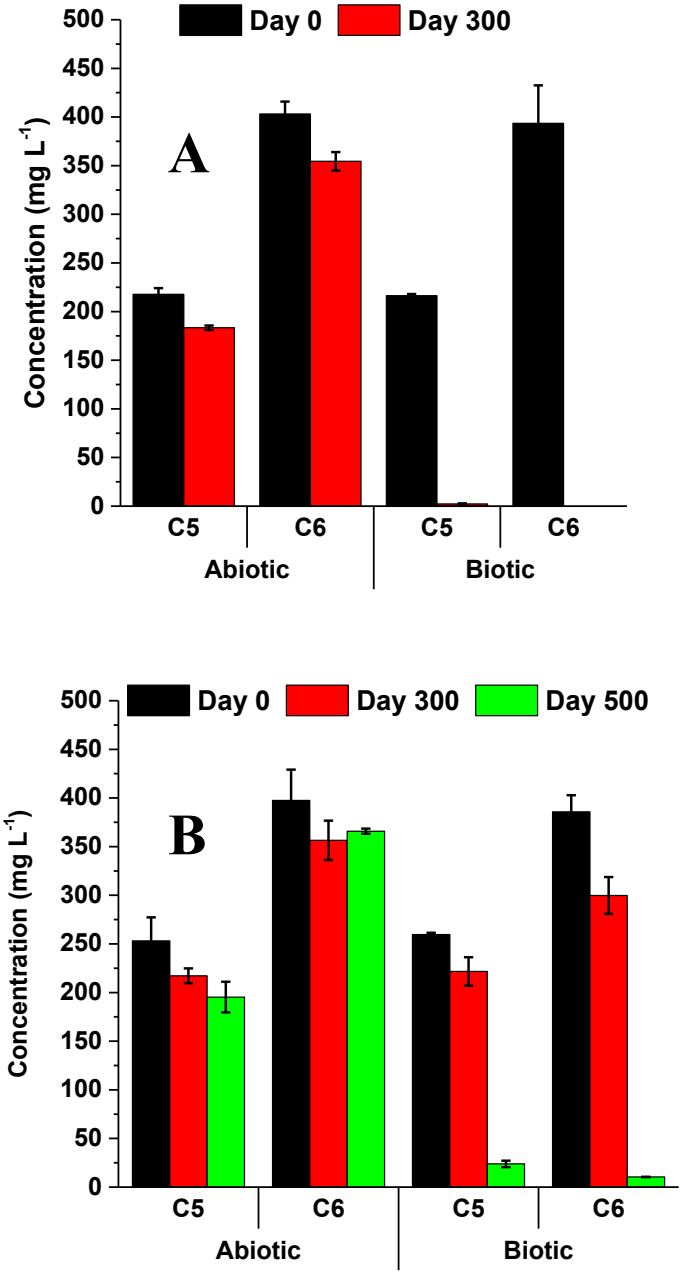

Figure 3.2: Concentrations of individual residual *n*-alkanes in microcosms during incubation of (A) Albian amended with the two-alkane mixture, and (B) CNRL MFT amended with the two-alkane mixture. The bars represent the mean value of triplicate microcosms and error bars, where visible, represent one standard deviation.

The differences in biodegradation patterns exhibited by Albian and CNRL microcosms might be due to one or more of the following explanations: (1) the presence and abundance of different key microbes (primary hydrocarbon degrading species) enriched on the predominant endogenous hydrocarbons in each pond, each having a restricted range of hydrocarbon substrates (Widdel et al., 2010); (2) different and/or selective hydrocarbon uptake mechanisms in different primary hydrocarbon degrading species (Kim, Foght, & Gray, 2002; Widdel & Grundmann, 2010), associated with water solubility of those *n*-alkanes (ranging from 40 mg L^{-1} for nC_5 to 0.7 mg L^{-1} for nC_8 and insolubility for nC_{10}); (3) the activity of enzymes involved in alkanes degradation having different specificity or affinity for alkanes of particular chain length (Acosta-Gonzalez, Rossello-Mora, & Marques, 2013); and (4) the presence of syntrophic partners for such primary degraders in correct proportions, including both bacteria and methanogens (Widdel et al., 2010). Responding to a shift in predominant alkane substrates might involve recruitment and enrichment of competent species and/or syntrophs from the 'rare' microbiota, or acquisition of appropriate genes for expanded substrate range; the current study cannot distinguish between these two possibilities. However, in Albian MFT amended with four alkanes, nC_5, nC_6 and nC_8 were simultaneously biodegraded, suggesting that at least some of the initial nC_5- and nC_6-degraders might be suited to nC_8 metabolism. Biodegradation of nC_{10} in one of the four-alkane amended Albian replicates at day ~500 (Appendix Fig. B1) and very late biodegradation of nC_5-C_6 in CNRL MFT imply the enrichment of appropriate hydrocarbon degraders in response to the change in substrate composition.

3.4.3 Prokaryotic community structure during *n*-alkane biodegradation

The prokaryotic community in Albian and CNRL MFT cultures was monitored during incubation by pyrosequencing of partial 16S rRNA genes. The bacterial reads constituted 17-86% of the total quality-controlled reads at day 0, but during metabolism of *n*-alkanes the archaeal sequence reads became enriched, representing 91-98% of the total prokaryotic reads in all alkane-amended microcosms (Appendix Fig. B2).

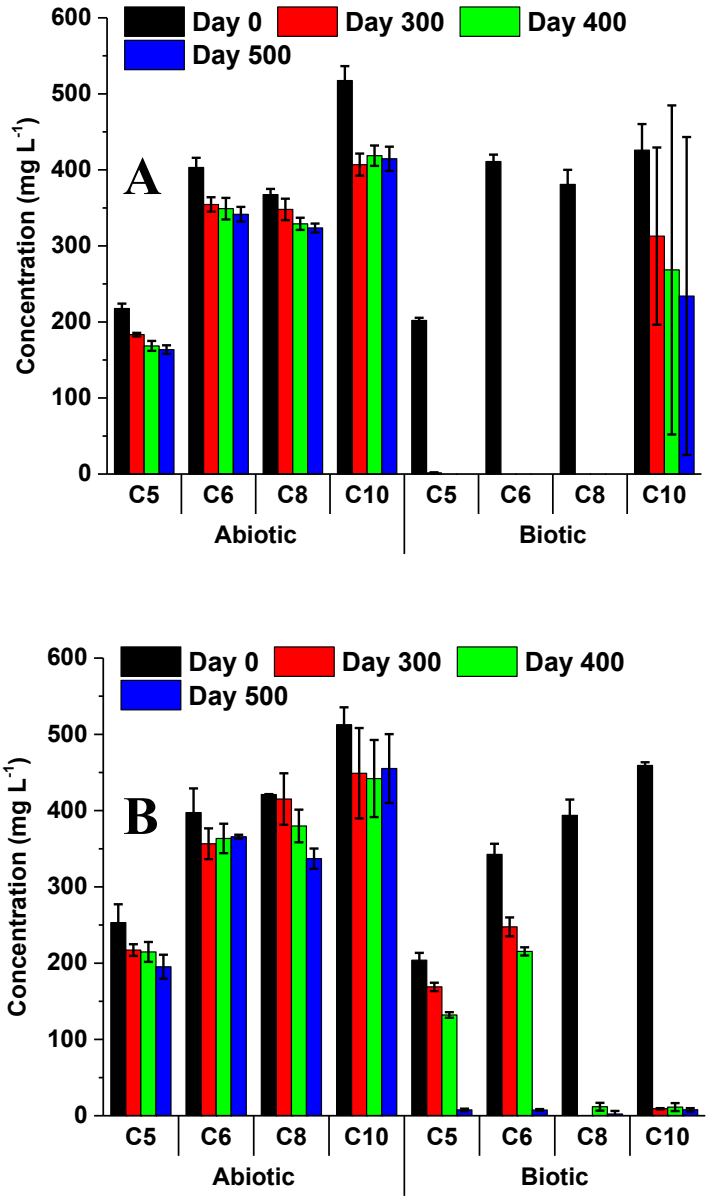

Figure 3.3: Concentrations of individual residual *n*-alkanes in microcosms during incubation of (A) Albian MFT amended with the four-alkane mixture, and (B) CNRL MFT amended with the four-alkane mixture. The bars represent the mean value of triplicate microcosms and error bars, where visible, represent one standard deviation. Hydrocarbon analysis of individual replicates from four-alkane-amended Albian MFT is provided in Appendix Fig. B1.

For Albian MFT microcosms, two DNA sampling intervals (0 and ~300 d) were selected to represent, respectively, the initial community structure and communities degrading the two- or four-alkane mixtures. The initial bacterial communities in two- and four-alkane-amended Albian MFT (constituting 39% and 62% of total prokaryotic reads, respectively) were similar in structure and comprised *Proteobacteria* (~33-38% of bacterial reads, with >22% *Hydrogenophilaceae*); *Chloroflexi* (~7-9% *Anaerolineaceae*); *Nitrospirae* (~7-9% *Nitrospiraceae*); *Actinobacteria* (~4-6% *Coriobacteriaceae*); and *Firmicutes* (~3-4% *Peptococcaceae*) (Figs. 3.4A and B; Appendix Table B2). After ~300 d incubation with alkanes, the bacterial sequences represented only 7-8% of total reads (Appendix Figs. B2A-D). Furthermore, the bacterial communities became less diverse, with the detected number of 'rare' operational taxonomic units (OTUs individually present at ≤5% abundance) decreasing from as many as 145 'rare' OTUs at time 0 to as few as 9 OTUs by 308 d for Albian MFT (Appendix Table B2). This decrease in rare taxa occurred as *Peptococcaceae*-related sequences increased to constitute ~78% and 54% of bacterial reads in two- and four-alkane-amended Albian MFT, respectively (Figs. 3.4A and B), and members of the *Chloroflexi* increased to ~12% in two-alkane- and ~26% in four-alkane-amended MFT. Because nC$_{10}$ was not degraded by two out of three replicates of four-alkane-amended Albian MFT, we analyzed the communities in these microcosms individually (Appendix Table B2). Although the community structure shown in Fig. 3.4B is from the replicate exhibiting nC$_{10}$ biodegradation, in fact the bacterial sequences were similar in all three replicates, with bacteria constituting 7-9% of total prokaryotic reads and *Peptococcaceae* (~54-63%) and *Anaerolineaceae* (~26%) being the dominant members (Appendix Table B2). This similarity in community structure of the replicates likely reflects the sequence of biodegradation of the smaller *n*-alkanes (nC$_5$, nC$_6$, and nC$_8$,) (Appendix Fig. B1): had the microcosms been sampled later (e.g., at ~500 d incubation when nC$_{10}$ had been depleted in one of the replicates), differences might have been observed in community structure between that microcosm and the two that did not degrade nC$_{10}$ until much later. That is, the potential to biodegrade nC$_{10}$ could not be predicted from the Albian MFT community profiles at 0 d or at ~300 d.

In CNRL MFT at day 0, the bacterial communities comprised 24% and 17% of total prokaryotic reads in two- and four-alkane-amended microcosms, respectively and, as expected, the communities were similar (Figs. 3.4C and D). However, the initial community structure in

CNRL MFT differed from that in Albian MFT: the dominant *Proteobacteria* in Albian MFT (*Hydrogenophilaceae*) were replaced in CNRL MFT by *Syntrophaceae* (~15-17% of total bacterial reads), along with greater proportions of *Chloroflexi* (14-17%) and *Actinobacteria* (11-13%) (Appendix Table B3). CNRL MFT incubated with the two-alkane mixture was sampled at ~500 d, at which time CH_4 production was just beginning (Fig. 3.1B). At this point, the bacterial population represented only 9% of total prokaryotic reads (Appendix Fig. B2E). During incubation, sequences related to *Peptococcaceae* increased to constitute ~80% of bacterial reads (Fig. 3.4C) whereas all other bacterial taxa decreased in proportion compared with the initial CNRL community and the number of 'rare' OTUs decreased from 81 at 0 d to as few as 5 OTUs by ~500 d (Appendix Table B3). For the four-alkane-amended CNRL MFT microcosms, two DNA sampling times were used: at ~300 d during a plateau in CH_4 production (Fig. 3.1B) and at ~500 d when a second phase of methanogenesis was underway. Bacterial reads represented 5% and 2% of the total prokaryotic reads at these sampling points, respectively (Appendix Fig. B2F). At day ~300 corresponding to depletion of nC$_8$ and nC$_{10}$ but only partial degradation of nC$_5$ and nC$_6$, the bacterial community was enriched in members of the proteobacterial families *Syntrophaceae* (~29%) and *Desulfobacteraceae* (~18%). *Anaerolineaceae* within the *Chloroflexi* (Figs. 3.4C and D) were also enriched from ~13% at 0 d to 34% at ~300 d, but *Peptococcaceae*-related sequences had become undetectable by this sample time. The community composition changed substantially again by ~500 d incubation when nC$_5$ and nC$_6$ were finally depleted in the four-alkane amended MFT. At this sampling point, the members of *Peptococcaceae* had become greatly enriched (~57% of bacterial reads) in the presence of *Syntrophaceae* (~28%) and *Desulfobacteraceae* (~9%) whereas *Anaerolineaceae* reads had decreased to ~2% of the bacterial sequences (Appendix Table B3).

The tremendous increase (up to 28-fold; Appendix Tables B2 and B3) in the proportion of sequences related to *Peptococcaceae* in the alkane-amended treatments, except CNRL MFT amended with four-alkane mixture in which *Peptococcaceae*-related sequences declined during biodegradation of C$_8$-C$_{10}$ but later dominated during the biodegradation of nC$_5$ and nC$_6$, suggest that *Peptococcaceae* members are the primary oxidizers of nC$_5$ and nC$_6$ and may play a lesser role in mineralization of nC$_8$ and nC$_{10}$ in CNRL MFT. The dominance of *Peptococcaceae* have also been observed during methanogenic biodegradation of *iso*-alkanes (2-methylbutane, 2-methylpentane and 3-methylpentane) in Albian and Syncrude MFT (Siddique et al., 2015). Other

reports have implicated members of *Peptococcaceae* as potential primary degraders of monoaromatic (Abu Laban et al., 2010; Herrmann et al., 2010; Kunapuli, Lueders, & Meckenstock, 2007; van der Zaan et al., 2012; Winderl, Penning, Netzer, Meckenstock, & Lueders, 2010) and aliphatic (Kniemeyer et al., 2007; Rios-Hernandez, Gieg, & Suflita, 2003) compounds under various anaerobic conditions. Transcript mapping of a *Peptococcaceae*-affiliated spp. genome (Tan et al., 2013) demonstrated high expression of genes involved in activation of alkanes via fumarate addition pathway and beta-oxidation, further supporting the proposal of *Peptococcaceae* as primary nC$_5$- and nC$_6$ degraders in Albian and CNRL MFT. The enrichment of *Anaerolineaceae* sequences in microcosms amended with the four-alkane mixture suggests that members of the *Anaerolineaceae* may either be directly involved in activation and biodegradation of nC$_8$ and nC$_{10}$ or may act as scavengers of metabolic intermediates, as suggested by Kleinsteuber et al (2012). Sequences affiliated with *Desulfobacterium* (*Desulfobacteraceae*) and *Smithella* (*Syntrophacea*e) were enriched only in CNRL MFT amended with four-alkanes during degradation of nC$_8$ and nC$_{10}$, implying a role in biodegradation of longer chain *n*-alkanes. These observations agree with other studies where cultures grown on long-chain *n*-alkanes (C$_{12}$-C$_{20}$) became enriched with *Desulfobacteraceae* (Aeckersberg, Rainey, & Widdel, 1998; Siddique et al., 2012; So & Young, 1999) and/or *Syntrophaceae* (Cheng et al., 2013; Gray et al., 2011; Siddique et al., 2012, 2011; Tan, Nesbø, & Foght, 2014d; Tan et al., 2014). The single Albian MFT replicate to degrade nC$_{10}$ by 308 d incubation did not show enrichment of *Desulfobacteraceae* and *Syntrophaceae,* and unfortunately we have no community analysis at a later time to determine whether these taxa are also responsible for longer chain alkanes in Albian MFT.

The archaeal communities in Albian MFT initially were dominated by acetoclastic methanogens belonging to the family *Methanosaetaceae* (~61-65% of archaeal reads), followed by hydrogenotrophic methanogens belonging to "*Candidatus* Methanoregula" (22-26%) (Figs. 3.4A and B; Appendix Table B4). The proportion of *Methanosaetaceae* reads increased to ~81-84% of archaeal sequences during biodegradation of both two- and four-alkane mixtures by day ~300. In CNRL MFT, the initial (0 d) archaeal communities were dominated by members of "*Candidatus* Methanoregula" (~61-68%) and *Methanosaetaceae* (~28-35%) (Figs. 3.4C and D; Appendix Table B5). These proportions shifted to domination by *Methanosaetaceae* (~87-93%) during biodegradation of the two-alkane mixture by ~500 d and by ~300 d in the four-alkane

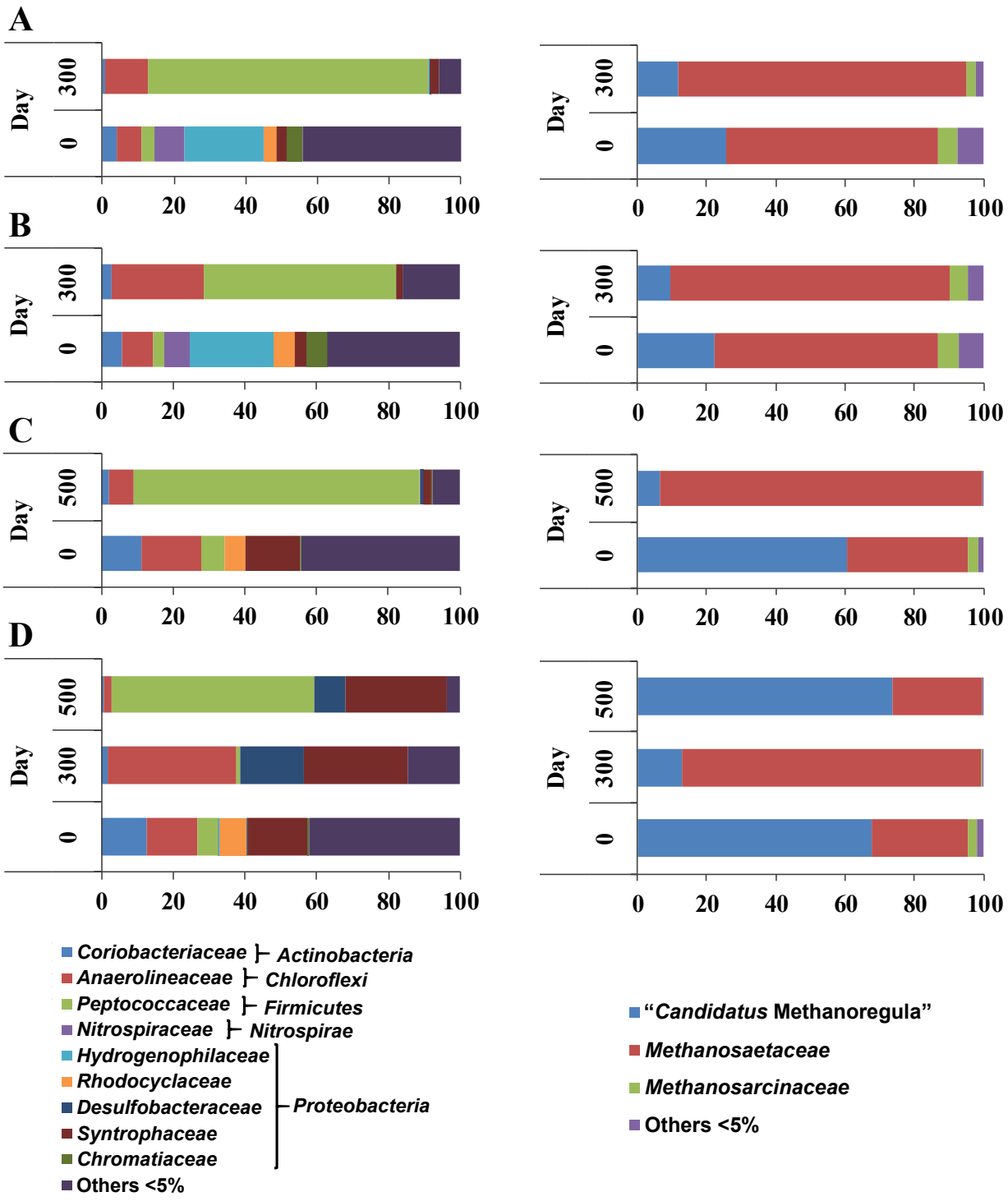

Figure 3.4: Bacterial (left) and archaeal (right) community compositions based on analysis of 16S rRNA gene pyrosequences before and during incubation of MFT with two- and four-alkane mixtures. (A) Albian MFT, two alkanes; (B) Albian MFT, four alkanes; (C) CNRL MFT, two alkanes; and (D) CNRL MFT, four alkanes. The results represent

pooled amplicons of triplicate cultures, with the exception of Panel B that represents the single microcosm that was degrading decane. Quality-controlled pyrosequences were clustered at ≤5% distance and expressed as a percentage of total bacterial or archaeal reads. "Others <5%" is the sum of all taxa individually abundant at <5% of the total bacterial or archaeal reads. Detailed results including the number of taxa grouped under "Others <5%" are shown in Appendix Tables B2-B5.

amended MFT. The proportions of Archaea in the latter microcosms subsequently shifted back towards "*Candidatus* Methanoregula" (~74%) and *Methanosaetaceae* (~26%) by day ~500.

The results suggest that acetoclastic methanogenesis is the major pathway active during *n*-alkane biodegradation, as observed in previous *n*-alkane studies (Siddique et al., 2012; Zengler et al., 1999). Nonetheless, the importance of hydrogenotrophic methanogens cannot be ruled out, particularly in CNRL MFT. Syntrophic biodegradation of alkanes by alkane-degrading bacteria (*Peptococcaceae* and/or *Syntrophaceae*) produces hydrogen or formate under methanogenic conditions (J. R. Sieber, J.McInerney, Plugge, Schink, & P.Gunsalus, 2010; Jessica R Sieber, McInerney, & Gunsalus, 2012; Tan, Charchuk, et al., 2014b) and there must be a hydrogen-consuming partner (hydrogenotrophs) to drive this process thermodynamically, which explains the presence of hydrogenotrophs in our study. Hydrogenotrophic methanogenesis has been shown to be the primary methanogenic pathway in cultures amended with crude oil (Gray et al., 2011; Mayumi et al., 2011), long-chain *n*-alkanes (C_{15}-C_{20}) (Zhou et al., 2012) and monoaromatics (BTEX) (Siddique et al., 2012). Other studies have reported relatively equal occurrence of acetoclastic and hydrogenotrophic methanogenesis in a crude-oil degrading culture (Morris et al., 2012) and MFT (Siddique et al., 2015, 2012, 2011).

3.5 Conclusions

The current study demonstrates that indigenous microorganisms in Albian and CNRL MFT preferentially metabolize *n*-alkanes present in their respective tailings ponds but now have been shown to be capable of expanding their substrate range to include 'unfamiliar' *n*-alkanes by changing bacterial and archaeal community compositions. Such shifts may require lag times of up to 1 year. These results are important for understanding the nature of anaerobic hydrocarbon degradation processes that could aid in devising appropriate strategies for management of

greenhouse gas emissions and remediation of oil sands tailings ponds, in addition to providing insights into methanogenic degradation of alkanes in other contaminated environments.

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4 Biodegradation of *iso*-alkanes in MFT under methanogenic and sulfate-reducing conditions[1]

4.1 Abstract

iso-Alkanes constitute major fraction of extraction solvents used in the bitumen extraction from oil sands ores. These hydrocarbons are generally considered relatively recalcitrant under anaerobic conditions. In this study, we investigated biodegradation of *iso*-alkanes in mixtures of either three (2-methylbutane, 2-methylpentane, 3-methylpentane) or five (2-methylbutane, 2-methylpentane, 2-methylhexane, 2-methylheptane, 2-methyloctane) *iso*-alkanes representing paraffinic solvent and naphtha, respectively under methanogenic conditions. Mature fine tailings (MFT) from two operators (Shell Albian and CNRL) that use different extraction solvents were amended with *iso*-alkanes and incubated for ~1600 d. In addition, another set of Albian MFT was amended with three *iso*-alkanes under sulfate-reducing conditions. Under methanogenic conditions, indigenous microbes in all three-*iso*-alkane amended Albian MFT began degrading the hydrocarbons after ~200 d; however, in CNRL MFT, ~700 and ~1200 d lag phases were observed in three- and five-*iso*-alkane amended cultures, respectively. Complete biodegradation of 2-methylpentane and partial biodegradation of 2-methylbutane and 3-methylpentane in three-*iso*-alkane mixture were observed in Albian and CNRL MFT. Initial results obtained in CNRL MFT amended with five-*iso*-alkane mixture exhibited sequential biodegradation in the order of decreasing carbon-chain length. Under sulfate-reducing conditions in Albian MFT, only partial biodegradation of 2-methylpentane was observed. Microbial community analyses revealed *Peptococcaceae* as key *iso*-alkane degraders in methanogenic Albian and CNRL MFT while *Anaerolineaceae, Syntrophaceae, Peptococcaceae, Desulfobacteraceae* and *Desulfobulbaceae* co-dominated the bacterial community in sulfate-reducing Albian MFT. The archaeal communities in methanogenic and sulfate-reducing Albian MFT exhibited codominance of acetoclastic (*Methanosaetaceae*) and hydrogenotrophic (*Methanobacteriaceae*, "*Candidatus* Methanoregula", *Methanolinea*) methanogens whereas in methanogenic CNRL MFT, hydrogenotrophic "*Candidatus* Methanoregula" dominated the archaeal population. This study highlights the capability of

indigenous microbes in different oil sands tailings ponds to biodegrade recalcitrant *iso*-alkanes under anaerobic conditions.

[1]A version of this chapter was modified for publication to establish biodegradation patterns of i*so*-alkane mixtures and key microbial players involved in the biodegradation of the *iso*-alkanes in Albian MFT under methanogenic and sulfate-reducing conditions and in CNRL MFT under methanogenic condition.

4.2 Introduction

Crude oil and its derivative products are examples of common organic contaminants in the environment, which occur naturally or anthropogenically. The majority of the components of crude oil and its derivative products are alkanes, which belong to the least reactive organic compounds due to the presence of apolar sigma bonds (Rabus, Hansen, & Widdel, 2013). Despite the inert nature of alkanes, microbial utilization of alkanes as sole carbon and energy sources under aerobic condition has been demonstrated and well-characterized since a century ago (Sierra-Garcia & Oliveira, 2013), albeit microbial metabolism of alkanes under anaerobic conditions has only been convincingly demonstrated in the last two decades (Anderson & Lovley, 2000; Callaghan, 2013; Jones et al., 2008; Mbadinga et al., 2011; So & Young, 2001). Unlike the well-characterized aerobic alkane degradation pathway, the biochemistry of anaerobic degradation pathways of alkanes has only been studied to some extent. Numerous studies on anaerobic biodegradation of *n*-alkanes have been published in recent years; however, reports on anaerobic biodegradation of *iso*- and *cyclo*-alkanes are still scarce due to their relative recalcitrance compared to the *n*-alkanes. Nonetheless, studies on biodegradability of *iso*- and *cyclo*-alkanes under anaerobic conditions are equally important since these alkanes are also major components of crude oil and other organic contaminants.

Oil sands tailings ponds are examples of man-made (engineered) environments that are impacted by alkanes. The unrecovered extraction solvent, which consists primarily of alkanes, that ends up in tailings ponds along with water, sand, silt, clay and unextracted bitumen, was found to sustain the indigenous methanogenic microbial communities in the tailings ponds (Siddique et al., 2006). Initial studies (Fedorak et al., 2002; Siddique et al., 2006, 2011) have revealed that *n*-alkanes (major components of the unrecovered extraction solvent) were the major substrates utilized by the indigenous methanogenic microbial community in mature fine tailings (MFT) (OSRIN, 2010) retrieved from Mildred Settling Lake Basin (MLSB); while other major components, such as *iso*- and *cyclo*-alkanes, showed no or very little degradation (Siddique et al., 2007). Our initial study on *iso*-alkanes was focussed on methanogenic biodegradation of simple C_5-C_6 *iso*-alkanes in MLSB MFT and Shell Albian Sands (Albian) MFT, which only exhibited degradation of *iso*-alkanes after prolonged incubation of ~900-1800 d (Siddique et al., 2015). Using the initial information from the long-term *iso*-alkanes study, we prepared similar mixture

of three-*iso*-alkanes (2-methylbutane, 2-methylpentane and 3-methylpentane) as a treatment using methanogenic MFT retrieved from Albian and Canada Natural Resources Limited (CNRL) tailings ponds to expand our understanding of the biodegradation pattern of *iso*-alkanes in different tailings ponds, which are different in age and physicochemical properties as detailed in Chapter 2 and 3. We also expand this study to include a wider range (C_5-C_9) of *iso*-alkanes (five-*iso*-alkane mixture; 2-methylbutane, 2-methylpentane, 2-methylhexane, 2-methylheptane and 2-methyloctane), representing *iso*-alkanes in naphtha, to examine if microbes preferentially biodegrade different carbon chains of same methyl substituted carbon under methanogenic conditions.

Because oxide minerals such as iron oxides are present in the solid phase of MFT (Siddique, Kuznetsov, Kuznetsova, Li, et al., 2014) and sulfate, in the form of gypsum ($CaSO_4$), is known to be used as an amendment to treat and consolidate MFT (Fedorak et al., 2002; Salloum et al., 2002), these oxidizing agents can potentially be used as terminal electron acceptors (TEA) during biodegradation of alkanes. Therefore, we also prepared similar three-*iso*-alkanes treatment for Albian MFT under iron-, nitrate- and sulfate-reducing conditions to determine if *iso*-alkanes oxidation in MFT can be coupled with reduction of TEA and if the presence of TEA can accelerate the *iso*-alkanes degradation process. However, throughout the duration of this study, we did not observe noteworthy reduction of iron and nitrate in treatments under iron- and nitrate-reducing conditions, respectively, and no depletion of any *iso*-alkane compound in the treatments; therefore, the data from these treatments were not included in the thesis.

Since *iso*-alkanes constitute major fractions of hydrocarbons in all diluent solvents used by different oil sands operators, evaluation of *iso*-alkanes biodegradation in MFT from different tailings ponds will provide crucial information on how the indigenous microbial communities from different tailings ponds respond to the availability of carbon sources. The findings in this study will provide further insights into anaerobic *iso*-alkane biodegradation process. The information will also be useful in improving existing model for greenhouse gas release in tailings ponds (Siddique et al., 2008) and redevising the appropriate strategies, not only for oil sands tailings management and reclamation but also management and reclamation of other anaerobic hydrocarbon impacted-environments.

4.3 Materials and methods

4.3.1 Chemicals and materials

2-Methylbutane (2-MC_4; >99%; CAS#78-78-4) and 2-methylpentane (2-MC_5; >99%; CAS#107-83-5) were purchased from Alfa Aesar, Massachusetts, USA. 2-Methylhexane (2-MC_6: >99%; CAS#591-76-4) and 2-methylheptane (2-MC_7: >99%; CAS#592-27-8) were purchased from Acros Organics, New Jersey, USA. 2-Methyloctane (2-MC_8: > 99%; CAS#34464-40-9) was purchased from MP Biomedicals, California, USA. 3-Methylpentane (3-MC_5: >99%; CAS#96-14-0) was purchased from Sigma-Aldrich, Missouri, USA.

Methanogenic Albian and CNRL MFT were collected from Muskeg River Mine Tailings Pond and Horizon Tailings Ponds by the respective oil sands operators. The detailed description on coordinates, MFT storage and physical and chemical properties of the MFT are given in Chapter 2.

4.3.2 Preparation of microcosms

All microcosms were prepared using 158-mL serum bottle in an anaerobic chamber to maintain anaerobic condition. Microcosms under methanogenic conditions were prepared as described in Chapter 2. Briefly, fifty-milliliter methanogenic medium was added into the microcosms containing 50 mL of Albian or CNRL MFT and the headspace was purged with 30% CO_2 balanced N_2 prior to the amendment of the *iso*-alkane mixtures. The hydrocarbon mixtures were added to each microcosm immediately after the headspace was purged. The experimental methanogenic microcosms were amended with a mixture of three (M-3I) *iso*-alkanes (2-MC_4, 2-MC_5 and 3-MC_5), or five (M-5I) *iso*-alkanes (2-MC_4, 2-MC_5, 2-MC_6, 2-MC_7 and 2-MC_8) at volumes equivalent to 20-70 mg per 100 mL of culture in each microcosm to make up final concentrations of ~200-700 mg L^{-1} for each compound. The experimental sulfate-reducing microcosms with Albian MFT amended with a mixture of three *iso*-alkanes (S-3I) were also prepared in the same manner as methanogenic microcosms except using modified sulfate-reducing medium (So & Young, 1999). The sulfate-reducing medium contained inorganic salts (NaCl, KCl, MgCl, $CaCl_2$, NH_4Cl, KH_2PO_4, Na_2SO_4, $NaHCO_3$), trace elements ($CoCl_2$, $CuCl_2$, $FeCl_2$, H_3BO_3, $MnCl_2$, Na_2MoO_4, $NiCl_2$, $ZnCl_2$), vitamins (pyridoxine, thiamine, nicotinic acid, *D*-(+)-biotin, cyanocobalamin, *p*-aminobenzoic acid, calcium *D*-(+)-pantothenate, riboflavin, thioctic acid), Na_2S (reducing agent) and resazurin (redox indicator) with the pH of the medium

adjusted to 8 with 1 M NaOH. To account for abiotic degradation, heat-killed sterilized (abiotic) controls were also prepared in the same manner as the experimental cultures but the MFT was first autoclaved (121 ^{o}C; 20 psi; 60 min) four times on four consecutive days prior to addition of medium and amendment of *iso*-alkane mixture. The heat-killed controls were prepared in triplicate and amended with the same mixture at the same concentration. Duplicate unamended methanogenic and sulfate-reducing microcosms were also prepared as baseline controls to account for metabolism of residual endogenous substrates in the MFT, which may result in methane (CH_4) production or sulfate-reduction, respectively. All microcosms were incubated at ~20 ^{o}C in the dark. Periodic analyses of CH_4, sulfate and hydrocarbons were performed to monitor degradation of *iso*-alkanes under methanogenic and sulfate-reducing conditions.

4.3.3 Chemical analyses

CH_4 production was measured in all methanogenic microcosms by removing 50 μL of headspace periodically from each microcosm and analyzed on gas chromatography with flame ionization detector (GC-FID) as previously described in Chapter 2 (Holowenko, MacKinnon, & Fedorak, 2000). CH_4 in unamended and abiotic controls cultures were concurrently measured to take into account biodegradation of endogenous organic compounds and abiotic losses of added hydrocarbons, respectively. The concentration of sulfate was determined in all sulfate-reducing microcosms by removing 1 mL of the culture from each microcosm and extracted with 9 mL of ultrapure water (Milli-Q) in 15 mL centrifuge tubes. The tubes were then shaken for 30 min at room temperature on a bench top shaker and then, centrifuged to sediment the sand and clay particles. The supernatant was filtered with 0.22 μm PTFE filter and analyzed on Dionex Ion Chromatography DX 600 at Natural Resources Analytical Laboratory (NRAL) in the Department of Renewable Resources at the University of Alberta.

The concentration of the *iso*-alkanes in all microcosms was determined by removing 1 mL of culture from well-shaken microcosms and extracted with 10 mL methanol in 20-mL EPA glass vial. The vials were shaken for 30 min at room temperature. The particles in the vials were allowed to settle for 30 min. One milliliter of the supernatant was transferred to 44 mL EPA glass vial, filled completely with ultrapure water and capped while avoiding any air bubble. The vials were sonicated for 10 min prior to analysis. All prepared vials were analyzed using purge and trap GC-FID as described previously in Chapter 2 (Siddique et al., 2006).

4.3.4 Stoichiometry of *iso*-alkanes mineralization under methanogenic and sulfate-reducing conditions

Under methanogenic conditions, complete oxidation of *iso*-alkanes will result in the production of CH_4 and CO_2 as final products. To determine the theoretical CH_4 and CO_2 generated from complete oxidation of *iso*-alkanes in all methanogenic cultures, the calculation was made based on modified Symons and Buswell simple stoichiometric equation (Symons & Buswell, 1933), assuming complete mineralization of *iso*-alkanes to CH_4 and CO_2 without any incorporation of the carbon into biomass. The calculated theoretical CH_4 was then compared to the measured CH_4. The general equations are as described as follows:

2-methylbutane (2-MC_4) $C_5H_{12} + 2H_2O \rightarrow CO_2 + 4CH_4$ (4.1)

2-methylpentane (2-MC_5) $C_6H_{14} + 2.5H_2O \rightarrow 1.25CO_2 + 4.75CH_4$ (4.2)

3-methylpentane (3-MC_5) $C_6H_{14} + 2.5H_2O \rightarrow 1.25CO_2 + 4.75CH_4$ (4.3)

2-methylhexane (2-MC_6) $C_7H_{16} + 3H_2O \rightarrow 1.\ 5CO_2 + 5.5CH_4$ (4.4)

2-methylheptane (2-MC_7) $C_8H_{18} + 3.5H_2O \rightarrow 1.75CO_2 + 6.25CH_4$ (4.5)

2-methyloctane (2-MC_8) $C_9H_{20} + 4H_2O \rightarrow 2CO_2 + 7CH_4$ (4.6)

Under sulfate-reducing conditions, sulfate functions as the terminal electron acceptor during complete oxidation of *iso*-alkanes, therefore, sulfate was reduced to hydrogen sulfide (HS^-) as the *iso*-alkanes were oxidized to CO_2. To determine the theoretical consumption of sulfate during complete oxidation of *iso*-alkanes, the theoretical calculation for sulfate reduction was made based on the following general equations:

2-methylbutane (2-MC_4) $C_5H_{12} + 4SO_4 + 5H^+ \rightarrow 5CO_2 + 4HS^- + 6H_2O$ (4.7)

2-methylpentane (2-MC_5) $C_6H_{14} + 4.75SO_4 + 6H^+ \rightarrow 6CO_2 + 4.75HS^- + 7H_2O$ (4.8)

3-methylpentane (3-MC_5) $C_6H_{14} + 4.75SO_4 + 6H^+ \rightarrow 6CO_2 + 4.75HS^- + 7H_2O$ (4.9)

The theoretical consumption of sulfate was then compared to the measured sulfate in all sulfate-reducing cultures.

4.3.5 Nucleic acid extraction and purification

DNA extraction and purification were performed on all culture samples retrieved from three-*iso*-alkanes amended MFT under both methanogenic and sulfate-reducing conditions. The DNA extraction and purification was not performed on cultures from five-*iso*-alkanes amended MFT because biodegradation in these cultures started very late (~1200 d). The microbial community analysis will be performed on cultures from five-*iso*-alkanes amended MFT in a subsequent batch of samples. Sampling for microbial community analysis was performed at day 0 (initial microbial community) and after substantial *iso*-alkanes degradation has occurred (indicated in Results section) using the method as described in Chapter 2.

4.3.6 PCR amplification and bioinformatics

All purified DNA were subjected to polymerase chain reaction (PCR) amplification using the primer set 454T-RA/454T-FB targeting the V6-V8 regions of the 16S rRNA gene universal for Bacteria and Archaea (Berdugo-Clavijo et al., 2012; Fowler et al., 2012; Golby et al., 2013). The PCR reaction and amplification and the subsequent purification method were performed as described in Chapter 3 (Mohamad Shahimin, Foght, & Siddique, 2016). The 16S rRNA gene amplicons were pyrosequenced using GS FLX Titanium Series Kit XLR70 (Roche) at McGill University Génome Québec Innovation Centre, Canada. The raw pyrosequencing data were analyzed using the Phoenix 2.0 pipeline (Soh et al., 2013) and we used the taxonomic annotation results generated with the SILVA database using average neighbor clustering at 5% maximum distance cut-off. All raw pyrosequences (~1700-6500 reads per sample) have been submitted to NCBI Sequence Read Archive under SRA number SRP052814.

4.4 Results

4.4.1 Biodegradation of *iso*-alkanes under methanogenic conditions

Two mixtures of *iso*-alkanes were tested for their biodegradability under methanogenic conditions. The experiment was conducted for ~1600 d and periodically monitored for CH_4 production and hydrocarbon biodegradation. During the ~1600 d of incubation, the heat-killed amended microcosms (abiotic control) and unamended microcosms (baseline control) in both methanogenic Albian and CNRL MFT did not produce high (< 0.2 mmol) amount of CH_4 (Fig. 4.1). In Albian MFT amended with three-*iso*-alkanes mixture under methanogenic conditions (M-3I), a lag phase of ~110 d was observed before an exponential CH_4 production started, which

plateaued after ~300 d of incubation and reached ~0.91 mmol by ~400 d (Fig. 4.1A). CH_4 in Albian MFT M-3I increased slowly afterward to 1.20±0.03 mmol by day ~1200 (Fig. 4.1). In CNRL MFT M-3I, however, longer lag time (~660 d) was observed before high CH_4 production was observed reaching 1.61±0.05 mmol by ~1200 d (Fig. 4.1B). Likewise, CNRL MFT amended with five-*iso*-alkanes mixture under methanogenic conditions (M-5I) had a very long lag phase of ~1200 d, after which high CH_4 was produced, reaching 2.45±0.25 mmol by day ~1600 (Fig. 4.1B).

The headspace of both Albian and CNRL M-3I microcosms was flushed with 30% CO_2 balanced N_2 after ~1400 d before re-amended with additional 2-MC_5 to maintain the active *iso*-alkane degrading microbial communities. No lag phase was observed in both Albian and CNRL M-3I after re-amendment of 2-MC_5 indicating 2-MC_5 degrading communities are still active in both cultures albeit after long incubation without replenishment of 2-MC_5 especially in Albian M-3I (Fig. 4.1).

Measurement of residual *iso*-alkanes in both Albian and CNRL MFT amended with M-3I revealed a similar pattern of biodegradation, where 2-MC_5 was almost completely biodegraded while 2-MC_4 and 3-MC_5 were only slightly depleted after ~1200 d incubation (Figs. 4.2A and B). Approximately 30% of 2-MC_4 was depleted after ~1200 d from the initial concentrations, 133±18 and 186±14 mg L^{-1}, in Albian and CNRL MFT, respectively. Similarly, Albian and CNRL MFT exhibited partial (~25-33%) depletion of 3-MC_5, from the initial concentrations 269±8 and 368±16 mg L^{-1}, respectively (Figs. 4.2A and B). In CNRL amended with M-5I, however, we observed preferential biodegradation of *iso*-alkanes in sequence: 2-MC_8>2-MC_7>2-MC_6>2-MC_5>2-MC_4 (Fig. 4.3C; Table 4.1). During the incubation of ~1500 d, 2-MC_8 was almost completely biodegraded (14±2 mg L^{-1}) from the initial concentration of 737±52 mg L^{-1} whereas ~41% of 2-MC_7 was depleted from the initial concentration (740±94 mg L^{-1}) (Fig. 4.3C). 2-MC_6, 2-MC_5 and 2-MC_4 only exhibited ~26-29% depletion from their initial concentrations of 522±70, 431±31 and 228±9 mg L^{-1}, respectively (Fig. 4.3C).

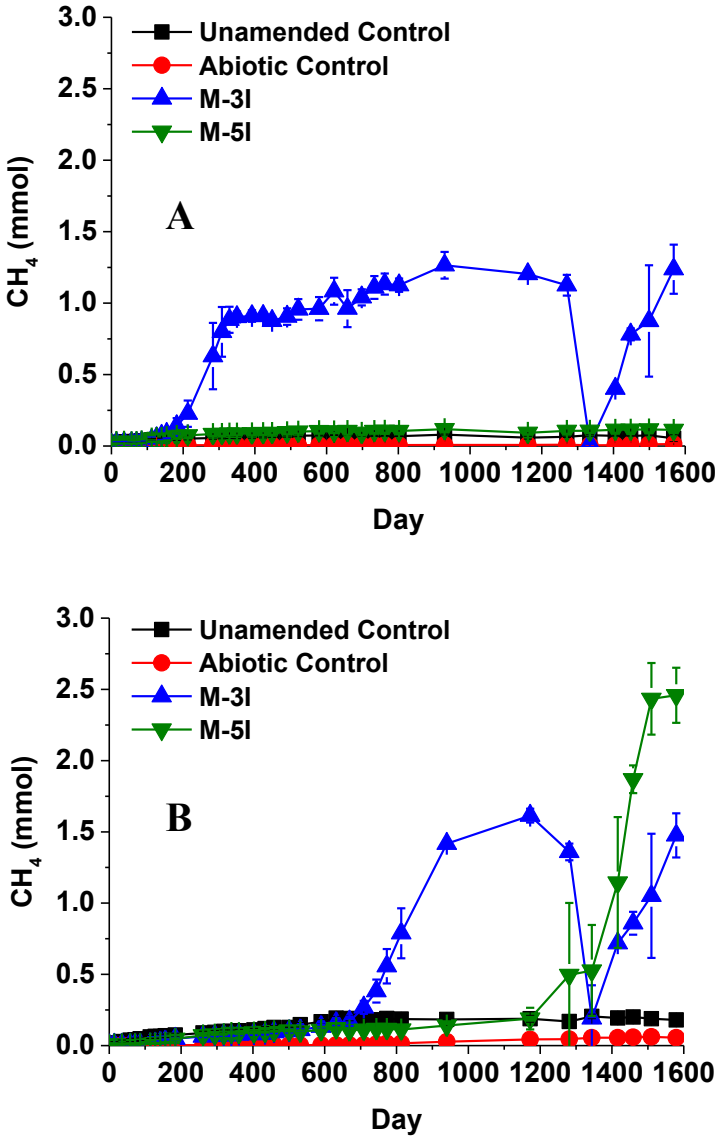

Figure 4.1: Cumulative methane production by microbes in A) Albian MFT and B) CNRL MFT amended with *iso*-alkanes under methanogenic condition and incubated for ~1600 d, respectively. The three-*iso*-alkane mixture (M-3I) comprised 2-methylbutane (2-MC_4), 2-methylpentane (2-MC_5) and 3-methylpentane (3-MC_5) whereas five-*iso*-alkane mixture (M-5I) comprised 2-MC_4, 2-MC_5, 2-methylhexane (2-MC_6), 2-methylheptane (2-MC_7) and 2-methyloctane (2-MC_8). The headspace in both Albian and CNRL M-3I was flushed on day ~1400 and the cultures were re-amended with 2-MC_5 to maintain active 2-MC_5 degrading communities in both cultures. Symbols represent the mean value from triplicate microcosms and error bars, where visible, represent one standard deviation.

The masses of *iso*-alkanes depleted in the cultures were translated into CH_4 using the modified Symons and Buswell stoichiometric equations (Eqs. 4.1-4.6) to track the carbon flow from substrates to greenhouse gasses via microbial metabolism (Table 4.1). The measured CH_4 in Albian MFT amended with M-3I represents 50% of the theoretical maximum CH_4 yield whereas in CNRL MFT amended with M-3I, the measured CH_4 represents 55% of the predicted CH_4 value (Table 4.1). The measured CH_4 in CNRL M-5I only represents 27% of the predicted CH_4 yield (Table 4.1).

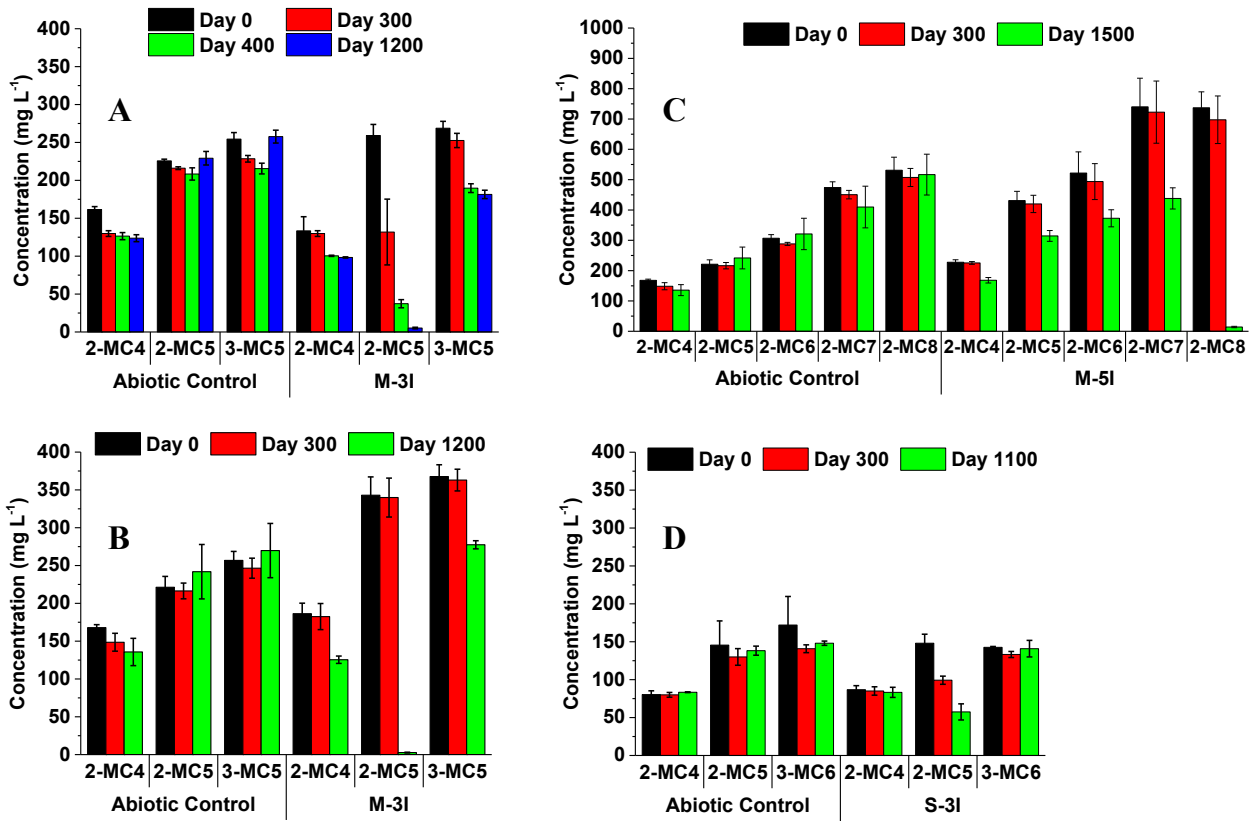

Figure 4.2: Concentrations of individual residual *iso*-alkanes in cultures during incubation. (A) three *iso*-alkanes amended Albian MFT under methanogenic conditions; (B) three *iso*-alkanes amended CNRL MFT under methanogenic conditions; (C) five *iso*-alkanes amended CNRL MFT under methanogenic conditions; and (D) three *iso*-alkanes amended Albian MFT under sulfate-reducing conditions. The bars represent mean value of triplicate microcosms and error bars, where visible, represent one standard deviation.

Table 4.1: Predicted and measured methane production and sulfate-reduction in mature fine tailings with the degradation of *iso*-alkanes after incubation[ǂ].

		Incubation time (d)	Substrate consumed (mmol) [ǂ]	Predicted methane yield (mmol) [1]	Measured methane yield (mmol)	Percent of theoretical production
Albian M-3I	2-MC4	1200	0.12 ± 0.02	2.42 ± 0.18	1.20 ± 0.03	49.6
	2-MC5		0.27 ± 0.01			
	3-MC5		0.14 ± 0.01			
CNRL M-3I	2-MC4	1200	0.08 ± 0.01	2.93 ± 0.23	1.61 ± 0.05	55.0
	2-MC5		0.39 ± 0.03			
	3-MC5		0.16 ± 0.01			
CNRL M-5I	2-MC4	1500	0.09 ± 0.00	6.94 ± 0.86	1.87 ± 0.10	27.0
	2-MC5		0.20 ± 0.02			
	2-MC6		0.21 ± 0.04			
	2-MC7		0.26 ± 0.05			
	2-MC8		0.50 ± 0.03			
		Incubation time (d)	**Substrate consumed (mmol) [ǂ]**	**Predicted sulfate reduced (mmol)**	**Measured sulfate reduced (mmol)**	**Percent of measured reduction**
Albian S-3I	2-MC4	1100	0.02 ± 0.00	0.66 ± 0.14[2]	0.69 ± 0.03	95.3
	2-MC5		0.11 ± 0.02			
	3-MC5		0.02 ± 0.01			

[ǂ] Calculation was made based on the difference of measured day 0 concentrations, taking into account abiotic losses, and residual alkane concentrations at incubation time indicated.

[1] Calculation based on Eqs. 4.1-4.6 using masses (GC quantitation) of the consumed *iso*-alkanes. Values represent the mean from analysis of triplicate microcosms (±1 standard deviation).

[2] Calculation based on Eqs. 4.7-4.9 using masses (GC quantitation) of the consumed *iso*-alkanes. Values represent the mean from analysis of triplicate microcosms (±1 standard deviation).

4.4.2 Biodegradation of *iso*-alkanes under sulfate-reducing conditions

Biodegradation of *iso*-alkanes in Albian MFT amended with three-*iso*-alkanes under sulfate-reducing condition (S-3I) was determined periodically by observing the depletion of *iso*-alkanes and sulfate concentration. Hydrocarbon analysis revealed that only 2-MC_5 was partially (~61%) biodegraded from the initial concentration of 148±12 mg L^{-1} during ~1100 d of

incubation (Fig. 4.2D). 2-MC_4 and 3-MC_5 were not depleted from their initial concentrations (83±1 mg L^{-1} and 148±3 mg L^{-1}, respectively) after incubation for ~1100 d (Fig. 4.2D). The initial concentrations of sulfate in all sulfate-reducing treatments were ~9.95 µM (Fig. 4.3). The concentrations of sulfate in abiotic and unamended controls measured after ~1100 d of incubation were not different from the initial day 0 concentrations (~10.04 and ~8.66 µM, respectively). The concentration of sulfate in Albian S-3I, however, showed considerable depletion after ~200 d and plateaued by 900 d of incubation (Fig. 4.3). The final sulfate concentration measured on ~1100 d was 1.75±0.51 µM (Fig. 4 3). The measured amount of sulfate reduced, calculated based on Eqs. 4.7-4.9 represented 95% of the theoretical concentration of sulfate reduced (Table 4.1).

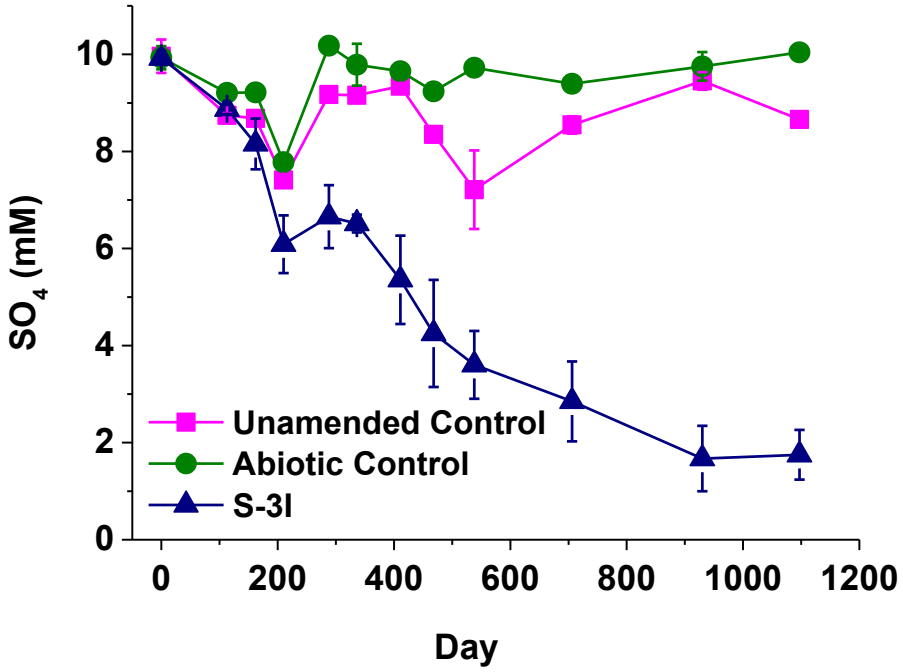

Figure 4.3: Cumulative sulfate reduction by microbes in Albian MFT amended with *iso*-alkanes under sulfate-reducing condition. The *iso*-alkanes were composed of 2-methylbutane (2-MC_4), 2-methylpentane (2-MC_5) and 3-methylpentane (3-MC_5). Symbols represent the mean value from triplicate microcosms and error bars, where visible, represent one standard deviation.

4.4.3 Microbial communities involved in biodegradation of *iso*-alkanes mixture

The shift in microbial community structure in Albian and CNRL MFT cultures was monitored throughout the period of the study by pyrosequencing of partial 16S rRNA genes. Two DNA sampling intervals were selected to represent the initial community structure (day 0) and the active *iso*-alkane degrading communities (sampled during active degradation of the amended *iso*-alkanes). At day 0, the pyrosequencing data revealed that bacterial reads in Albian MFT constituted 78-84% of the total quality-controlled prokaryotic reads whereas, in CNRL MFT, bacterial reads only constituted 18% of the total prokaryotic reads (Appendix Fig. C1). However, during metabolism of *iso*-alkanes, the archaeal sequence reads increased representing 83-92% of the total sequence reads in all the treatments (Appendix Fig. C1). In Albian and CNRL amended with M-3I, we observed a decrease in bacterial diversity as the bacterial sequence reads decreased during biodegradation of *iso*-alkanes (Appendix Table C1). In contrast, Albian amended with S-3I exhibited increased bacterial diversity as the bacterial sequence reads decreased during biodegradation of *iso*-alkanes (Appendix Table C1).

For Albian MFT, the initial (day 0) bacterial composition under methanogenic and sulfate-reducing conditions were similar (Figs. 4.4A and C) and dominated overwhelmingly by bacterial reads related to *Hydrogenophilaceae* (94-96% of the total bacterial population). CNRL MFT, however, exhibited a more diverse initial bacterial composition than Albian MFT, with 'rare' operational taxonomic units (OTUs less than 5% abundance) constituted the majority (49%) of the bacterial composition (Fig. 4.4B; Appendix Table C1). During methanogenic biodegradation of the *iso*-alkanes, the bacterial communities in both Albian and CNRL M-3I (~300 and 800 d, respectively) exhibited similar composition, where sequence reads related to *Peptococcaceae* dominated 62-77% of the total bacterial reads (Figs. 4.4A and B; Appendix Table C1). *Anaerolineaceae*- and *Syntrophaceae*-related sequence reads occupied 6-9% and 5-10% of the total bacterial reads, respectively (Figs. 4.4A and B; Appendix Table C1). The rare OTUs in both Albian and CNRL M-3I, which represented 10-11% of the total bacterial reads, decreased to 11 from 41 and 111 in Albian and CNRL MFT, respectively, during active biodegradation of the *iso*-alkanes (Appendix Table C1). During active biodegradation of *iso*-alkanes in Albian S-3I, the microbial community was enriched with *Anaerolineaceae*-related sequence reads (26%) followed by *Peptococcaceae* (10%), *Desulfobacteraceae* (10%), *Syntrophaceae* (9%) and *Coriobacteriaceae* (6%) (Fig. 4.4C; Appendix Table C1).

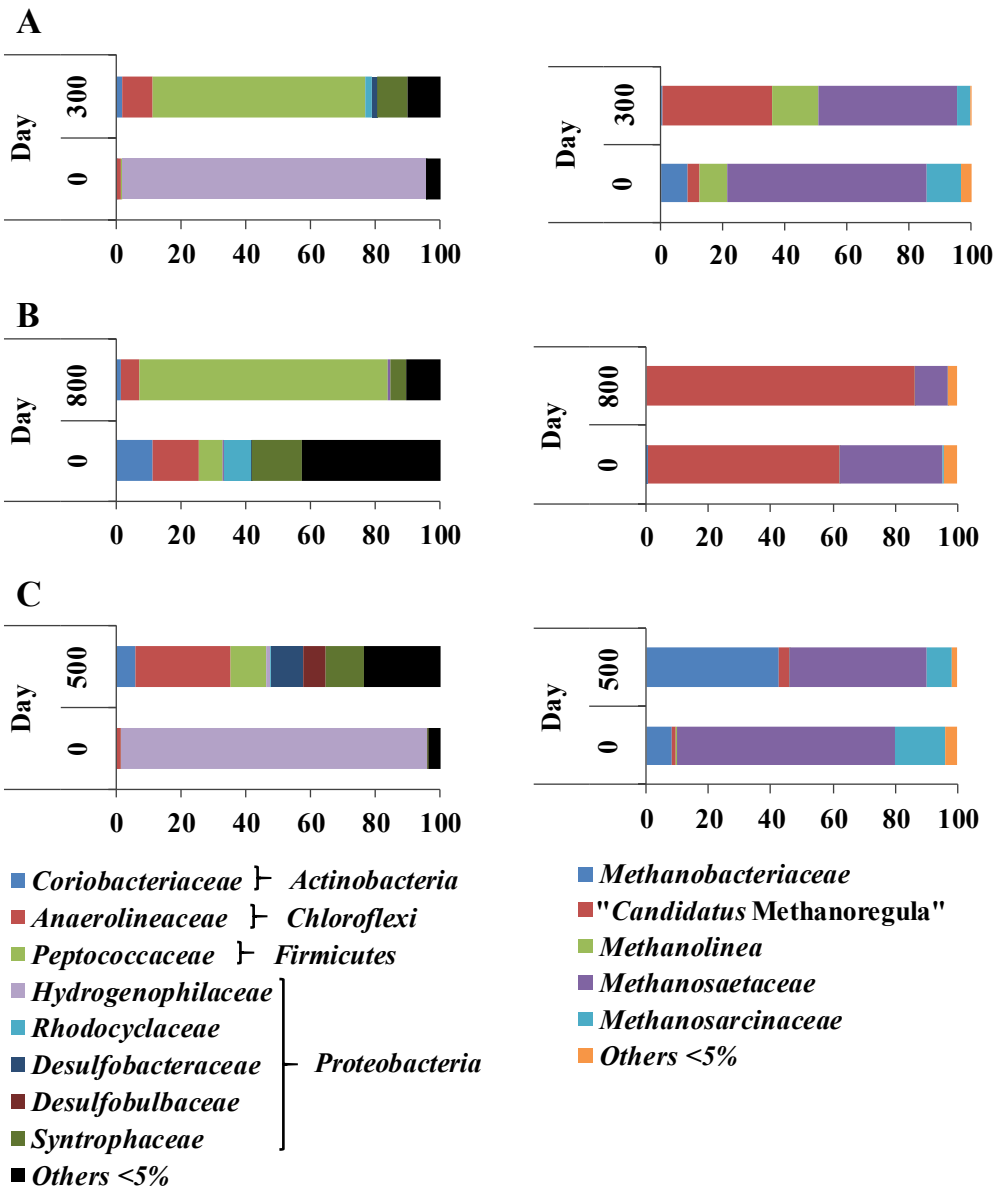

Figure 4.4: Bacterial (left) and archaeal (right) community compositions determined using 16S rRNA gene pyrosequencing before and during incubation of MFT amended with *iso*-alkanes. (A) Albian MFT under methanogenic conditions; (B) CNRL MFT under methanogenic conditions; and (C) Albian MFT under sulfate-reducing conditions. The results represent pooled amplicons of triplicate cultures. Quality-controlled pyrosequences were clustered at ≤5% distance and expressed as a percentage of total archaeal/bacterial reads. "Others <5%" is the sum of all taxa individually abundant at <5% of the total archaeal/bacterial reads. Detailed results including the number of taxa grouped under "Others <5%" are shown in Appendix Tables C1 and C2.

The number of rare OTUs in Albian S-3I increased from 27 to 79 during active biodegradation of *iso*-alkanes and represented 24% of the total bacterial reads (Appendix Table C1).

The archaeal communities in all cultures were strictly dominated by methanogens constituting acetoclastic and hydrogenotrophic methanogens. The initial archaeal communities in Albian MFT were dominated by acetoclastic *Methanosaetaceae* (~64-70% of the total reads) followed by *Methanosarcinaceae* (~11-16%) and *Methanobacteriaceae* (~8-9%) (Figs. 4.4A and C; Appendix Table C2). However, the initial archaeal community in CNRL MFT was dominated by "*Candidatus* Methanoregula" (62%) followed by acetoclastic methanogens belonging to *Methanosaetaceae* (~33%) (Fig. 4.4B; Appendix Table C2). During active biodegradation of *iso*-alkanes, codomination of both acetoclastic and hydrogenotrophic methanogens was observed in Albian M-3I (~300 d). The hydrogenotrophic methanogens in Albian M-3I were represented by members of "*Candidatus* Methanoregula" and *Methanolinea* (~35 and 15%, respectively) while acetoclastic methanogens were represented by *Methanosaetaceae* (45%) (Fig. 4.4A; Appendix Table C2). In CNRL M-3I, however, "*Candidatus* Methanoregula" were further enriched to 86% while *Methanosaetaceae* only occupied 11% of the total archaeal reads during exponential CH_4 production (~800 d) (Fig.4.4B). Similar to Albian M-3I, Albian S-3I also exhibited codomination of both acetoclastic and hydrogenotrophic methanogens during active *iso*-alkane degradation (~500 d), however, the hydrogenotrophic methanogens were represented by *Methanobacteriaceae* (43%) while the acetoclastic methanogens were represented by *Methanosaetaceae* (~44%) (Fig. 4.4C).

4.5 Discussion

Recently, methanogenesis has shown to impact geochemical processes in oil sands tailings, particularly in the consolidation of colloidal MFT solids and recovery of porewater for re-use (Arkell et al., 2015; Fedorak et al., 2003; Siddique, Kuznetsov, Kuznetsova, Arkell, et al., 2014; Siddique, Kuznetsov, Kuznetsova, Li, et al., 2014). However, tailings produced by different operators have experienced unique combinations of extraction conditions, processing agents and depositional histories, which influence the physicochemical properties of the tailings from different tailings ponds. Therefore, understanding anaerobic hydrocarbon metabolism, since it is one of the processes affecting geochemical phenomena in tailings, is important for the management and reclamation of oil sands tailings. In the current study, we examined the

biodegradability of relatively recalcitrant hydrocarbons such as *iso*-alkanes under methanogenic and sulfate-reducing conditions that will enable the accurate prediction of greenhouse gas emissions from tailings ponds and the determination of *iso*-alkanes as potential substrates for methanogenesis in end-pit lakes where methanogenesis can affect the quality of cap water.

Methanogenic biodegradation of the amended three-*iso*-alkanes occurred earlier in Albian MFT than CNRL MFT but biodegradation of five-*iso*-alkanes occurred earlier in CNRL MFT than Albian MFT. This phenomenon could be explained by the fact that the three-*iso*-alkanes were the major *iso*-alkane components of paraffinic solvent whereas majority components of five-*iso*-alkanes (2-MC_8, 2-MC_7 and 2-MC_6) can only be found in naphtha. Therefore, shorter lag phase was observed during methanogenic biodegradation of 'familiar' three- and five-*iso*-alkanes in Albian MFT and CNRL MFT, respectively, and longer lag phase was observed before 'unfamiliar' *iso*-alkanes began to be degraded in the respective MFT. In a recent report (Mohamad Shahimin et al., 2016), longer lag phase was noticed when methanogenic CNRL MFT was amended with *n*-pentane and *n*-hexane (major components of paraffinic solvent) which were absent or present in low concentrations in CNRL tailings ponds. The results imply that acclimation is an important step before the indigenous methanogenic microbial community in MFT oxidizes unfamiliar substrates. Long lag phase was also observed in three-*iso*-alkanes amended Albian MFT under sulfate-reducing conditions. Because Albian MFT is naturally methanogenic and generally low in sulfate (Fedorak et al., 2003), the indigenous methanogenic microbial communities in Albian MFT require acclimation when subjected to sudden high sulfate concentration. Therefore, biodegradation of *iso*-alkanes in Albian S-3I occurred later than in Albian M-3I.

The pattern of *iso*-alkanes biodegradation in Albian and CNRL MFT amended with M-3I was similar to the pattern of these *iso*-alkanes biodegradation in Syncrude MFT (Siddique et al., 2015; Tan et al., 2015) where 2-MC_5 was completely metabolized while 2-MC_4 and 3-MC_5 were partially biodegraded by the indigenous microorganisms in the MFT. Because partial biodegradation of 2-MC_4 and 3-MC_5 occurred only during active biodegradation of 2-MC_5 and no further biodegradation was observed during ~ 2400 d of incubation, the process of cometabolism was suggested for 2-MC_4 and 3-MC_5 partial biodegradation by Siddique et al. (2015). Similar observation was observed in Albian and CNRL M-3I indicating cometabolism might have also occurred in methanogenic Albian and CNRL MFT during biodegradation of the

iso-alkanes. In CNRL MFT amended with M-5I, we observed degradation of *iso*-alkanes in the sequence of decreasing carbon chain length similar to the observation noticed in CNRL MFT amended with *n*-alkanes (C_5-C_{10}) (Mohamad Shahimin et al., 2016). The preferential biodegradation of *iso*-alkanes in CNRL MFT amended with M-5I might be attributed to the lower toxicity of longer-chain *iso*-alkanes compared to the more soluble shorter-chain *iso*-alkanes (Heipieper & Martinez, 2010).

Overall, methanogenic oxidation of *iso*-alkanes to CH_4 in MFT was inefficient in rate and completeness since there is large disagreement between the measured and the theoretical CH_4 yields based on the stoichiometric conversion of the *iso*-alkanes. However, similar stoichiometric values have been reported in recent studies examining methanogenic biodegradation of *iso*-alkanes in MFT (Abu Laban et al., 2014; Siddique et al., 2015; Tan et al., 2015). In contrast to our observation, other studies (Mohamad Shahimin et al., 2016; Siddique et al., 2006, 2011), which examined biodegradation of *n*-alkanes under methanogenic conditions, reported ~60-80% of theoretical CH_4 production. The possible reasons that might have resulted in the low CH_4 yield could be: 1) cometabolism of 2-MC_4 and 3-MC_5 during oxidation of 2-MC_5 in Albian and CNRL MFT amended with M-3I, which sometimes is an incomplete oxidation process that results in accumulation of persistent intermediary products, and/or 2) high incorporation of the substrate carbon to biomass (Tan et al., 2015).

Unlike methanogenic biodegradation of three-*iso*-alkanes in Albian MFT, under sulfate-reducing conditions, Albian MFT only exhibited considerable depletion of 2-MC_5. Additionally, high concentration of 2-MC_5 still remained in Albian S-3I despite still having high sulfate concentration. This phenomenon may be attributed to the accumulation of 'dead-end' metabolites (see Chapter 5), which inhibit further degradation of the parent substrate. Cometabolism of 2-MC_4 and 3-MC_5 was not observed under sulfate-reducing conditions, which might be explained by the difference in microbial community composition (discussed below) and/or strict specificity of the activating enzyme involved in activation of 2-MC_5. The predicted value of sulfate reduction was close to the measured sulfate reduction value in Albian S-3I. Similar stoichiometric values have also been reported in previous reports, which examined biodegradation of *n*-alkanes under sulfate-reducing conditions (Kropp et al., 2000; So & Young, 2001; So & Young, 1999), indicating sulfate as a potential terminal electron acceptor during metabolism of *n*- and *iso*-alkanes in various anaerobic environments.

To determine shift in microbial community composition during biodegradation of *iso*-alkanes and to identify the key microbial players involved in the biodegradation process, we performed microbial community analysis on cultures withdrawn from day 0 (initial community composition) and cultures withdrawn during exponential production of CH_4 (*iso*-alkane-degrading community composition; sampling time indicated in Fig. 4.4). The initial bacterial community in all Albian MFT amended with three-*iso*-alkanes was almost exclusively dominated by *Hydrogenophilaceae* (*Thiobacillus*) (Appendix Table C1), similar to the initial bacterial community in Albian MFT described in Chapter 2. This observation contradicts the observation reported in Chapter 3 (Mohamad Shahimin et al., 2016), which exhibited diverse composition of bacterial community with *Hydrogenophilaceae* -related sequences only occupied ~20% of the total bacterial sequence reads. Since the bulk Albian MFT used in this study was collected in 2008 and stored in pail until use in 2011, a portion of the Albian MFT might have become aerobic, allowing the growth of *Thiobacillus* (Appendix Table C1), a known group of strict aerobic bacteria except *Thiobacillus denitrificans*, a facultative anaerobe (Beller et al., 2006). The overwhelming domination of *Hydrogenophilaceae* at day 0 in Albian MFT amended with three-*iso*-alkanes might be attributed to the lack of long-term incubation of Albian MFT in anaerobic bottles before addition of hydrocarbons as was performed during *n*-alkanes degradation (Chapter 3).

During methanogenic biodegradation of *iso*-alkanes, only sequences related to *Peptococcaceae* were enriched in both Albian and CNRL M-3I. Therefore, *Peptococcaceae* were implicated as the primary 2-MC_5-degrader under methanogenic conditions, inferred from the enrichment of *Peptococcaceae* reads in both Albian and CNRL MFT. Similar observations of *Peptococcaceae* enrichment during methanogenic biodegradation of *iso*-alkanes have also been reported in recent studies (Abu Laban et al., 2014; Siddique et al., 2015; Tan et al., 2015) reinforcing the proposed role of *Peptococcaceae* as the primary 2-MC_5-degraders under methanogenic conditions. In Albian S-3I, however, bacteria related to *Anaerolineaceae, Syntrophaceae, Peptococcaceae, Desulfobacteraceae* and *Desulfobulbaceae* were simultaneously enriched. Some members belonging to *Syntrophaceae, Peptococcaceae, Desulfobacteraceae* and *Desulfobulbaceae* have been verified to carry the appropriate genes for activation of alkanes via fumarate addition pathway (Tan, Charchuk, et al., 2014b; Tan, Nesbø, et al., 2014d; Tan et al., 2014; Tan, Jane Fowler, et al., 2015), however, *Anaerolineaceae* have

not been established to directly involved in alkanes activation but has been implicated as autotrophic carbon fixer and H_2 scavenger (Tan et al., 2013). Therefore, it is possible that under sulfate-reducing conditions, 2-MC_5 was activated by *Syntrophaceae, Peptococcaceae, Desulfobacteraceae* and/or *Desulfobulbaceae* via fumarate addition while *Anaerolineaceae* function as the secondary oxidizers and scavenge for the intermediates.

The archaeal communities in Albian and CNRL M-3I were enriched with hydrogenotrophic methanogens during biodegradation of *iso*-alkanes. Similar observations have also been reported in other methanogenic *iso*-alkane-degrading primary cultures derived from MFT (Abu Laban et al., 2014; Siddique et al., 2015), highlighting importance of hydrogenotrophic methanogenesis in oil sands tailings ponds. However, in contrast to our observation, other methanogenic MFT cultures grown on *n*-alkanes have exhibited enrichment of acetoclastic methanogens during active biodegradation of *n*-alkanes (Mohamad Shahimin et al., 2016; Siddique et al., 2012). These contrasting observations might be attributed to the inefficiency of methanogenic *iso*-alkanes metabolism compared to *n*-alkanes as discussed above, which might have resulted in lower yield of acetate, hence, affecting growth of acetoclastic methanogens while favoring growth of hydrogenotrophic methanogens, which metabolize H_2 as a source of energy. In Albian S-3I, however, the archaeal community was dominated by acetoclastic methanogens throughout the ~1100 d of incubation. Under sulfate-reducing conditions, sulfate-reducing bacteria might have taken over the role of hydrogenotrophic methanogens as H_2 consumers since sulfate-reducing bacteria have higher affinity to H_2 (Winfrey & Zeikus, 1977), hence, hydrogenotrophic methanogens were not enriched in Albian S-3I during active biodegradation of *iso*-alkanes. Nevertheless, the high population of archaea in Albian S-3I was unexpected since methanogenesis is inhibited in the presence of high sulfate concentration (Holowenko et al., 2000). However, a recent study (Ozuolmez et al., 2015) has demonstrated the coexistence of a marine acetoclastic methanogen and a sulfate-reducer under sulfate-reducing conditions similar to our observation in Albian S-3I. Therefore, it is possible that sulfate-reducing bacteria and acetoclastic methanogens in oil sands tailings have similar metabolic flexibility, allowing coexistence of both microbes under sulfate-reducing conditions.

In summary, our study demonstrates that under methanogenic conditions, cometabolism of 2-MC_4 and 3-MC_5 might have simultaneously occurred during complete oxidation of 2-MC_5; whereas under sulfate-reducing conditions, only 2-MC_5 was partially degraded without the

occurrence of cometabolism of other *iso*-alkane isomers. The current study also demonstrates that indigenous microbial community in CNRL MFT preferentially oxidizes *iso*-alkanes in the order of decreasing molecular weights and capable of metabolizing a wide range of *iso*-alkanes. These results are important for understanding the *iso*-alkane degradation process which could improve the current oil sands tailings' greenhouse gas emission model and impact future decisions regarding oil sands remediation and management.

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5 Insights into anaerobic biodegradation pathway of hydrocarbon mixtures in MFT from two oil sands tailings ponds[1]

5.1 Abstract

Alkanes constitute the largest portion of hydrocarbons entrained in oil sands tailings and their biodegradation into CH_4 in Albian and CNRL tailings ponds has never been comprehensively investigated. We used biochemical and functional gene analyses to examine activation pathway of alkanes in Albian and CNRL MFT primary cultures incubated with either sole mixture of *n*-alkanes and *iso*-alkanes, separately or paraffinic solvent (C_5-C_6) or naphtha containing alkanes for ~600-1600 d under methanogenic and sulfate-reducing conditions. The cumulative findings revealed that fumarate addition might potentially be the primary activation pathway of alkanes in Albian and CNRL tailings ponds. Transient succinylated 2-methylpentane and 2-methylbutane metabolites were detected only during initial methanogenic biodegradation of *iso*-alkanes and paraffinic solvent in Albian and CNRL primary cultures but these metabolites were not detected after extended incubation. However, under sulfate-reducing conditions, succinylated 2-methylpentane persisted throughout incubation period of ~1100 d, implying the metabolites were dead-end products under sulfate-reducing conditions. No metabolite was detected in *n*-alkanes and naphtha amended primary cultures in any sampling points. Nevertheless, *assA/masD* genes closely related to *Peptococcaceae* were amplified in all primary cultures including cultures which did not exhibit metabolite detection. Our findings indicate that different oil sands tailings ponds have the similar capability to catalyze fumarate activation of alkanes under methanogenic and sulfate-reducing conditions.

[1]A version of this chapter was modified for publication to demonstrate potential activation pathways of hydrocarbons in mixtures using biochemical and functional gene analyses on primary cultures from different mature fine tailings incubated with various hydrocarbons mixtures.

5.2 Introduction

Alkanes constitute major components of crude oil and its derived products, which impacted many environments, naturally or anthropogenically. Alkanes are inert in nature and have high C-H bond dissociation energy (Blanksby & Ellison, 2003). Under aerobic condition, aerobic microorganisms utilize highly reactive oxygen species to overcome the high C-H bond dissociation energies of alkanes via mono and/or dioxygenase reactions (Boll & Heider, 2010). However, under anaerobic conditions, anaerobic microorganisms have to activate alkanes via alternative mechanisms. For the last two decades, fumarate addition has been implicated as the main hydrocarbon activation pathway in many anaerobic cultures amended with: 1) *n*-alkanes (Bian et al., 2015; Callaghan et al., 2008; Khelifi et al., 2014; Zedelius et al., 2011; Zhou et al., 2012), 2) *iso*-alkanes (Abu Laban et al., 2014; Tan et al., 2015), 3) monoaromatics (Beller & Spormann, 1998; Bozinovski et al., 2012; Fowler et al., 2012; Gieg & Suflita, 2002), and 4) polyaromatics (Annweiler et al., 2000; Berdugo-Clavijo et al., 2012; Gieg & Suflita, 2002; Selesi et al., 2010). Other anaerobic hydrocarbon activation pathways including carboxylation and hydroxylation (Callaghan, 2013) have also been implicated and highly debated but not widely reported.

Oil sands tailings ponds are engineered environments that are impacted by hydrocarbons containing large proportion of alkanes. The unrecovered extraction solvents, comprised majorly of *n*- and *iso*-alkanes, entrained in oil sands tailings ponds have been shown to sustain the metabolism of complex indigenous methanogenic microbial communities (Siddique et al., 2007). In previous studies (Abu Laban et al., 2014; Tan et al., 2015), microbial communities in enrichment cultures derived from mature fine tailings (MFT; OSRIN, 2010) collected from Syncrude's Mildred Lake Settling Basin (MLSB) were grown on various *n*- and *iso*-alkanes. These enrichment cultures exhibited capability to biodegrade a wide-range of alkanes. The metabolite and functional gene analyses from these enrichment cultures revealed possibility of fumarate-addition as the primary activation pathway of *n*- and *iso*-alkanes biodegradation in MLSB (Abu Laban et al., 2014; Tan et al., 2015).

However, little is known about the biodegradation pathway of alkanes in tailings ponds from two relatively new oil sands operators, Shell Albian Sands (Albian) and Canada Natural Resources Ltd. (CNRL). Because the age of tailings, bitumen extraction process and tailings management strategies are different for different oils sands operators, the anaerobic hydrocarbon

biodegradation process (biodegradation pattern, microbial community structure and initial activation mechanism) in different tailings ponds might differ from one site to another. In this study, we characterized Albian and CNRL primary cultures grown on various hydrocarbons for metabolites and functional genes to postulate the main activation pathway employed by the indigenous microbial communities in Albian and CNRL tailings ponds. We employed organic solvent for extraction of metabolites from cultures and their determination using gas chromatography equipped with mass spectrometer (GC-MS). The cultures were also subject to functional gene analysis characterizing alkylsuccinate synthase (*assA*) or methylalkysuccinate synthase (*masD*) genes known to encode enzymes involved in activation of alkanes via fumarate-addition pathway. Our findings will widen our insights into alkane activation mechanisms and advance our overall understanding of anaerobic hydrocarbon biodegradation process in different tailings ponds.

5.3 Materials and methods

5.3.1 Primary cultures description

The primary cultures analyzed in this study have been described in detail in Chapter 2, 3 and 4. Briefly, the primary cultures were established by incubating 50 mL of MFT collected from Albian's Muskeg River Mine tailings pond or CNRL's Horizon tailings pond MFT with 50 mL of methanogenic medium or sulfate-reducing medium in a 158-mL serum bottle. The headspace of microcosms was flushed with 30% CO_2 balance N_2 at slightly above atmospheric pressure. The methanogenic Albian and CNRL cultures were amended with either a mixture of two *n*-alkanes (C_5 and C_6; ~400 ppm each; 2-alkanes), four *n*-alkanes (C_5, C_6, C_8 and C_{10}; ~400 ppm each; 4-alkanes), three *iso*-alkanes (2-methylbutane, 2-methylpentane and 3-methylpentane; ~400 ppm each; M-3I), CNRL naphtha (~2000 ppm; Naph) or Albian paraffinic extraction solvent (~1500 ppm; Par), or were unamended. Two separate cultures, one set of triplicate cultures received three *iso*-alkanes (2-methylbutane, 2-methylpentane and 3-methylpentane; ~400 ppm each) and another set of duplicate cultures were unamended, were prepared using Albian MFT and incubated under sulfate-reducing conditions (Albian S-3I). Triplicate abiotic controls (heat-killed at 121^{o}C, 20 psi, 60 min for 4 times on 4 consecutive days prior to the amendment of hydrocarbons) were also prepared for each treatment in parallel with the experimental microcosms. All cultures were incubated at room temperature (~ 20^{o}C) statically in

the dark until CH_4 production or sulfate-reduction plateaued. CH_4 was measured periodically with a gas chromatography fitted with flame ionization detector (GC-FID) as described in Chapter 2, sulfate concentration was determined with ion chromatography (IC) as described in Chapter 4 and the residual hydrocarbons in the cultures were determined using GC-FID equipped with purge and trap system as described in Chapter 2.

5.3.2 Metabolite analysis

The cultures were sampled for metabolite analysis at two time points: 1) during the exponential production of CH_4 or reduction of sulfate, and 2) after CH_4 production or sulfate reduction plateaued. Two to four milliliters of cultures were drawn from all replicates of each treatment and pooled in 20 mL EPA vials (Fisher Scientific). One microgram of 4- fluoro-1-naphthoic acid (4F1N; CAS#573-03-5; Alfa Aesar) was then added to all samples and into extraction-derivatization control reactions containing only ultrapure (MilliQ) water as a surrogate standard (So et al., 2003). Using concentrated hydrochloric acid (CAS#7647-01-0; Sigma Aldrich), all samples were acidified to pH <2 (Kropp et al., 2000). The acidified samples were extracted three times with 15 mL of ethyl acetate (CAS#141-78-6; Fisher Scientific) and dried overnight in a fume hood. The dried samples were dissolved in 1.5 mL ethyl acetate and transferred into 2.0 mL GC vials. The solution was concentrated to ~100 µL under N_2 flow and derivatized with 100 µL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA; CAS#25561-30-2; Thermo Scientific) at 70 ºC for ~90 min. The derivatized samples were diluted to 500 µL with ethyl acetate before analysis by gas chromatography-mass spectrometry (GC-MS). One microliter of derivatized sample was analyzed by TRACE 1300 gas chromatograph equipped with a TraceGold TG-5MS GC column (30 m by 0.25 mm internal diameter; Thermo Scientific) in splitless mode. The initial temperature of the oven was held at 65 °C for 5 min, increased at 5 °C min^{-1} to 280 °C and then held at 280 °C for 15 min. Mass spectra of metabolites were obtained using ISQ LT Single Quadrupole mass spectrometer (Thermo Scientific), and the data was acquired in the scan mode from 50 to 600 mass units.

5.3.3 Nucleic acid extraction and functional gene amplification

For functional genes (*assA/masD*) analysis, triplicate 300 µL of primary culture was collected from each of three replicate primary cultures described above (two *n*-alkanes, four *n*-alkanes, naphtha and paraffinic solvent amended cultures; total 24 cultures) at the end of

incubation. The genomic DNA was extracted from the cultures using the protocol described in detailed in Chapter 2. The recovered DNA from each replicate of a single treatment was pooled before PCR amplification. The *assA/masD* gene was amplified in triplicate from pooled DNA using primers 1432F (5'-CCNACCACNAAGCAYGG-3') and 1936R (5'-TCRTCATTNCCCCAYTTNGG-3') as described previously (Callaghan et al., 2010). The temperature program was as follow: 95 ^{o}C for 3 min followed by 40 cycles of 96 ^{o}C for 45 s, 52 ^{o}C for 30 s, 72 ^{o}C for 45 s and a final extension step at 72 ^{o}C for 10 min. The PCR reaction (25 µL) contained 12.5 µL AccuStart II PCR ToughMix (Quanta Biosciences, Gaithersburg, MD), 1 µL extracted DNA (~20 ng/µL), 2.5 µL (10 µM) of each primer and 6.5 µL of sterile nuclease-free water. Negative controls containing only PCR reagents and nuclease-free water were also included with every set of samples to maintain quality control. The triplicate amplifications from single treatment were pooled and the amplicons' size was confirmed via 1% agarose gel electrophoresis. All amplicons with the right size were excised and purified using Qiagen's QIAquick Gel Extraction kit according to the manufacturer's procedure.

5.3.4 Construction of *assA/masD* gene clone libraries and phylogenetic analysis

The gel purified amplicons were quantified on NanoDrop-1000 spectrophotometer v3.3 before used for cloning with Invitrogen's TA Cloning Kit (pCR™2.1-TOPO® vector and DH5α-T1^{R} *E. coli* competent cells) according to manufacturer's protocol. All clones were first checked for inserts via PCR amplification with insert-specific primers and resolved by gel electrophoresis. The amplicons were sequenced using T7 primers and BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystem) on an ABI 3730 sequencer (Applied Biosystem, Foster City, CA) Molecular Biology Services Unit (MBSU) at the University of Alberta. Our initial analysis indicates that the *assA/masD* genes in our samples show minimal diversity; therefore, we performed restriction analysis on all amplicons by MspI digestion for restriction fragment pattern similarities to reduce the number of clones for sequencing (Winderl, Schaefer, & Lueders, 2007). The sequences from *assA/masD* clone libraries were trimmed to remove vector sequences. The trimmed sequences were then compared against GenBank Database using BLASTX algorithm to determine closest *assA/masD* -derived amino acid sequences. The *assA/masD* gene sequences were aligned with reference sequences from GenBank using MUSCLE (Edgar, 2004) and manually edited. The sequences were then clustered into OTUs and translated using Geneious R8 (Biomatters Ltd, New Zealand). Maximum likelihood tree with

WAG model and 100 bootstrap replicates was constructed using PhyML (Guindon et al., 2010). All clone sequences were deposited in GenBank under the accession numbers KU840850 - KU840905.

5.4 Results

5.4.1 Detection of succinylated metabolites in primary cultures

During incubation, different primary cultures started biodegrading the amended hydrocarbons at different times. However, we selected two sampling time points for all cultures to determine metabolites: first during the active biodegradation of the amended hydrocarbons and second at the end of incubation to determine if the metabolites detected (if any) persisted after prolonged incubation (sampling times indicated in Table 5.1). In all Albian and CNRL primary cultures amended with naphtha or *n*-alkanes, no metabolite was detected at both sampling points. However, in all Albian and CNRL primary cultures amended with paraffinic solvent or *iso*-alkanes, succinylated metabolites were detected at the first sampling point but the same metabolites were not detected at second sampling point except Albian MFT amended with *iso*-alkanes under sulfate-reducing conditions (Albian S-3I) (Table 5.1). A cluster of two GC-MS peaks at 25.9 and 26.1 min were detected with mass spectra profiles identical to mass spectra expected from fumarate-added 2-methylpentane (Fig. 5.1A). The mass spectra of putative double-derivatized trimethylsilyl (di-TMS) ester of (2-methylpentyl) succinic acid comprised ion with m/z 331 and other key ion fragments (m/z 73, 147, 174, 217 and 262) (Fig. 5.1B) which were similar to previously reported spectra of derivatized fumarate-added 2-methylpentane (or 1,3-dimethylbutylsuccinic acid) (Tan et al., 2015).

Additionally, we also detected another cluster of two peaks at 23.9 and 24.1 min exclusively at the first sampling point of methanogenic Albian MFT amended with three *iso*-alkanes (Albian M-3I) (Fig. 5.1A). The mass spectra profiles of both peaks were similar and the profiles were consistent with derivatized fumarate-added 2-methylbutane with diagnostic $(M-15)^+$ ion fragments of m/z 317 and other key fumarate-added ion fragments (m/z 73, 147, 172, 217 and 262) (Figs. 5.1A and C). The key ion fragments conformed well with fumarate-added ion fragments reported in previous studies (Abu Laban et al., 2014; Callaghan et al., 2006; Davidova et al., 2005; Gieg & Suflita, 2002; Rios-Hernandez et al., 2003; Tan et al., 2015). These peaks were not detected at the second sampling point for Albian M-3I.

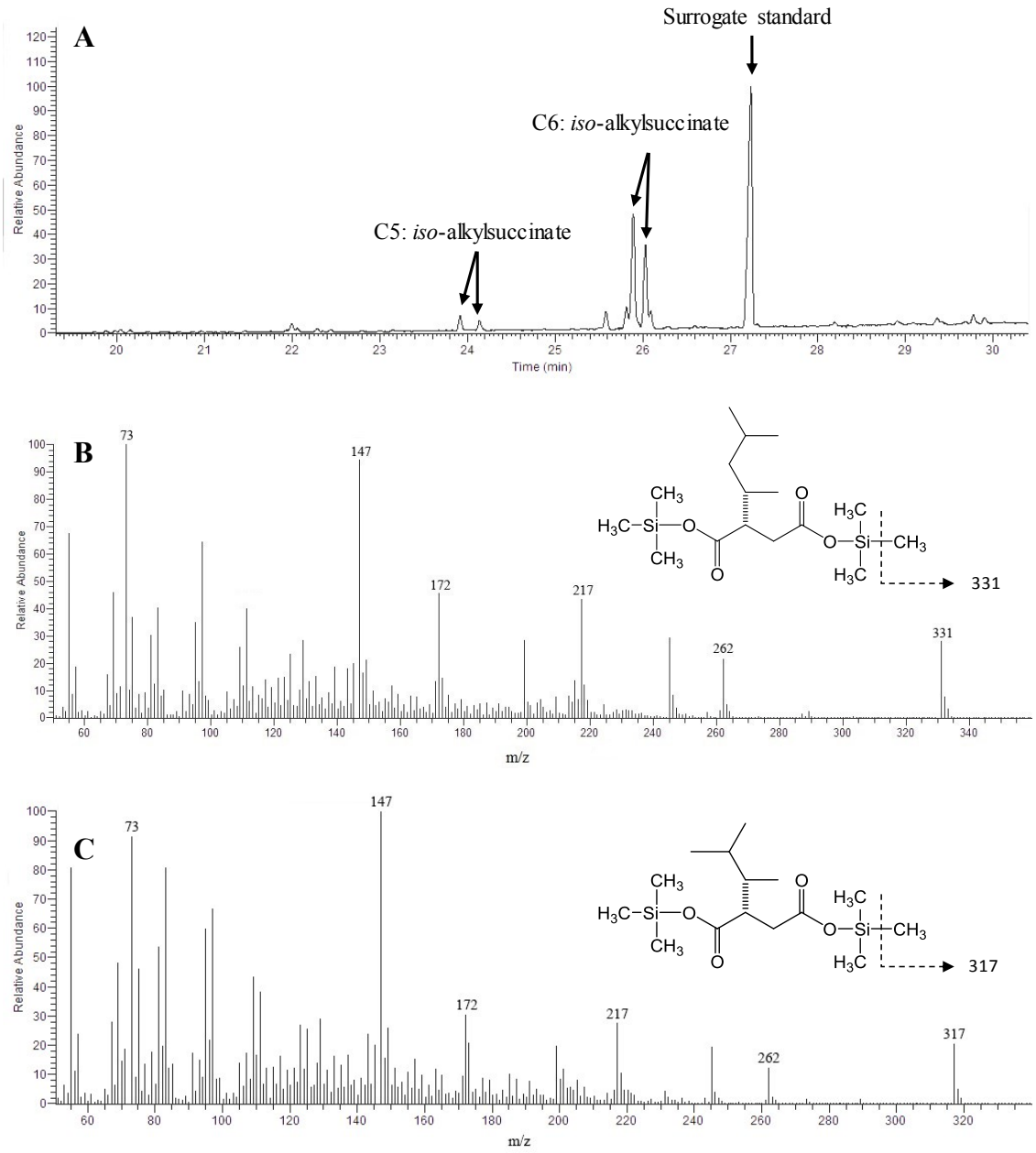

Figure 5.1: Postulated TMS-derivatized metabolites extracted from methanogenic and sulfate-reducing *iso*-alkanes- and paraffinic solvent-degrading cultures detected using GC-MS. (A) Retention times of the postulated TMS-derivatized C_5 and C_6 *iso*-alkylsuccinate. Surrogate standard: 4-fluoro-1-naphthoic acid added in all samples prior to metabolite extraction procedure. (B) Mass spectrum of putative TMS-derivatized 2-methylbutylsuccinic acid. (C) Mass spectrum of putative TMS-derivatized 2-methylpentylsuccinic acid. Inferred putative structures and diagnostic $(M\text{-}15)^+$ ion fragments of m/z 317 and 331 are shown in the insets.

Table 5.1: Silylated putative alkylsuccinate metabolites detected in extraction from methanogenic and sulfate-reducing primary cultures.

Parent compound	Retention times (min)	Selected ions (m/z)	CNRL MFT				Albian MFT					
			Paraffinic Solvent		M-3I		Paraffinic Solvent		M-3I		S-3I	
			Day									
			1200	1400	800	1200	200	600	200	400	400	1100
2-methylbutane	23.9, 24.1	73, 147, 172, 217, 262, 317	-	-	-	-	-	-	+	-	-	-
2-methylpentane	25.9, 26.1	73, 147, 172, 217, 262, 331	+	-	+	-	+	-	+	-	+	+

M-3I, Methanogenic microcosms amended with three *iso*-alkane mixture.

S-3I**,** Sulfate-reducing microcosms amended with three *iso*-alkane mixture.

-, peak not detected; +, peak detected. Detection limit of alkylsuccinate metabolites on GC-MS was not determined in this study, although previous studies (Agrawal & Gieg, 2013; Aitken et al., 2013) have reported detection limit in the nanomolar range for alkylsuccinates.

5.4.2 *assA/masD* gene detection during anaerobic biodegradation of alkanes

Because alkylsuccinate were detected in some of our primary cultures and the fact that the amendments we used in all our treatments were completely or mostly consisted of alkanes, we were interested in looking at genes encoding catalytic subunits of alkylsuccinate synthase (*assA*) or methylalkylsuccinate synthase (*masD*). We performed PCR to amplify partial *assA/masD* genes in all our cultures to support our detection of postulated succinylated metabolites and to ascertain if fumarate addition might be the potential activation pathway in treatments where activated metabolites were not detected.

assA/masD-like genes were identified as key functional genes involved in activation of alkanes via fumarate addition pathway in all our amended primary cultures. However, we did not observe any amplification of *assA/masD*-like genes from unamended cultures, which might indicate low abundance of key bacteria carrying the functional genes in alkane-deficient cultures. The restriction analysis revealed only one gene fragmentation pattern in all amended primary cultures except Albian and CNRL MFT amended with naphtha, which exhibited up to three unique fragmentation patterns (reflected in the number of OTUs present in each treatment; Fig. 5.2), indicating low diversity of *assA/masD*-like genes. Our observation coincided with observations from previous studies (Abu Laban et al., 2014; Tan, 2014) which reported low

diversity of *assA/masD*-like genes in MFT-derived enrichment cultures incubated with various *n*- and *iso*-alkanes amendments.

Clone libraries generated from all Albian and CNRL amended primary cultures revealed that all the cloned sequences were related to *assA/masD* genes (Fig. 5.2). Subsequent analysis performed at the protein level of the *assA/masD* genes to construct maximum-likelihood tree revealed that our translated *assA/masD* fragments were closely related to a putative methylalkylsuccinate subunit (MasD) from an uncultured bacterium recovered from gas seepage-impacted and pristine cold marine sediments and putative AssA from an uncultured *Peptococcaceae* recovered from a methanogenic short-chain alkane-degrading enrichment culture (SCADC) (Tan, Charchuk, Li, Laban, & Foght, 2014; Tan et al., 2015; Fig. 5.2). All AssA/MasD sequences had high similarity at protein level (≥ 98% identity) to each other and to AssA related to *Peptococcaceae* SCADC except Albian-Naph OTU 1, Albian M-3I and CNRL-Naph OTU 3 which had ~94-95% identity to AssA from *Peptococcaceae* SCADC (Fig. 5.2).

5.5 Discussion

In recent years, there has been increasing evidence indicating that hydrocarbons are primarily activated via fumarate activation pathway under anaerobic conditions. Fumarate addition has been demonstrated under various reducing conditions and the corresponding succinylated metabolites have been detected in various hydrocarbon-impacted environments and hydrocarbons-degrading enrichment and pure cultures (Callaghan, 2013; Foght, 2008; Musat, 2015). Fumarate addition has also been demonstrated in MLSB MFT-derived enrichment cultures grown on various alkanes (Abu Laban et al., 2014; Tan et al., 2015). However, no similar study on hydrocarbon activation pathway has been reported in MFT from relatively 'younger' oil sands tailings ponds from Albian and CNRL, which employed different oil sands extraction process and treated their tailings differently for solids consolidation enhancement before deposited into the tailings ponds (Mohamad Shahimin et al., 2016). Since MFT from different oil sands operators exhibited different microbial community structures and unique hydrocarbon biodegradation patterns (Mohamad Shahimin et al., 2016), these differences beg the question whether hydrocarbons in Albian and CNRL tailings ponds were activated differently in the biodegradation process.

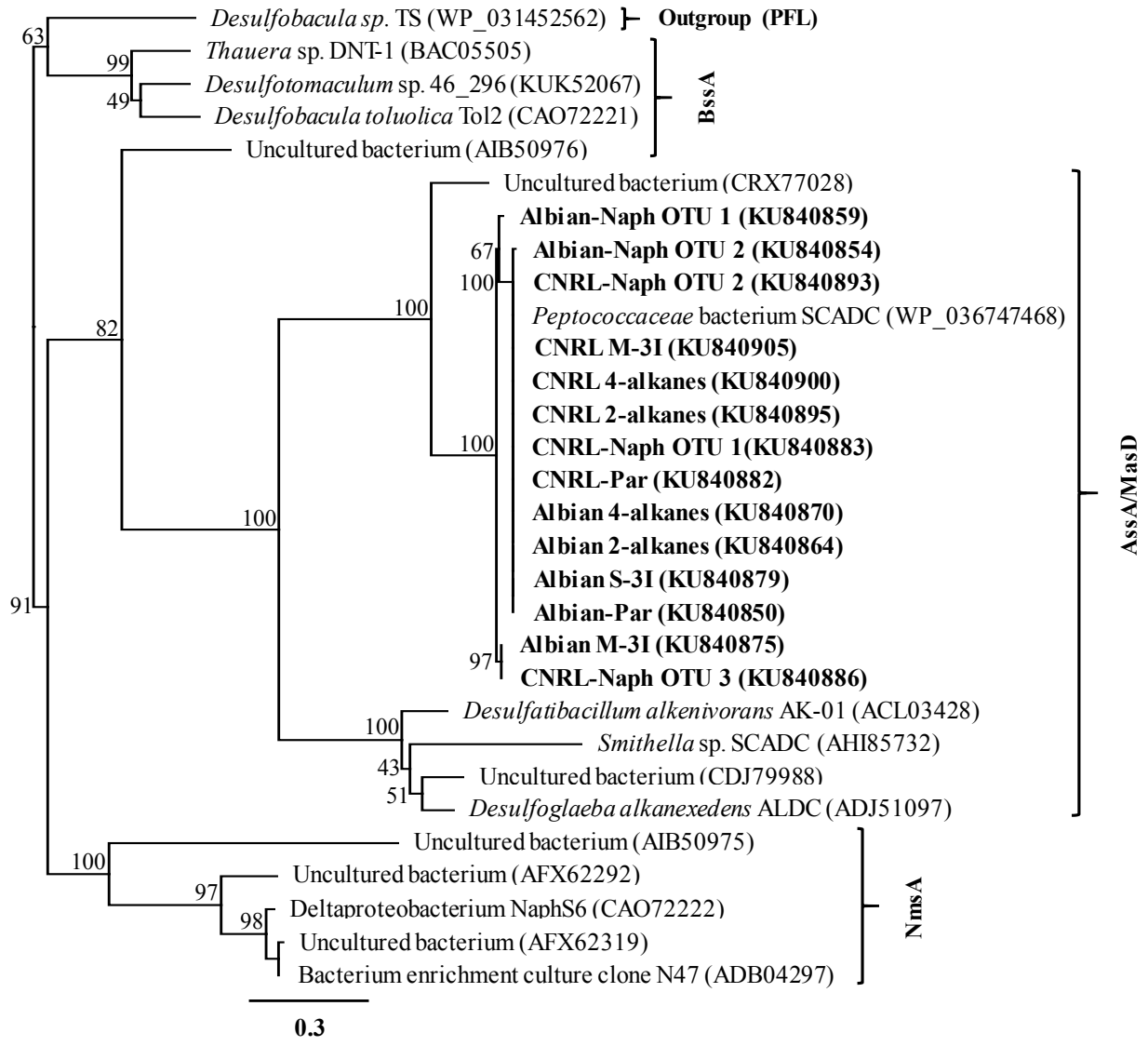

Figure 5.2: Maximum likelihood tree showing the affiliation of translated sequences coding for the alpha subunit of alkylsuccinate/methylalkylsuccinate synthase (*assA*/masD) to selected reference sequences. AssA/MasD-like sequences from the current study (bold) were aligned with closely related AssA/MasD, BssA and NmsA reference sequences recovered from NCBI nr-database through BLASTX searches. Maximum likelihood tree was constructed using PhyML with WAG model and 100 bootstrap replicates. Pyruvate formate lyase sequence from *Desulfobacula* sp. TS was used as the outgroup. The GenBank accession numbers are indicated in parentheses. Naph, methanogenic microcosms amended with naphtha. Par, methanogenic microcosms amended with paraffinic solvent. M-3I, methanogenic microcosms amended with three *iso*-alkane mixture. S-3I**,** sulfate-reducing microcosms amended with three *iso*-alkane mixture.

To identify the metabolites resulted from activation of the amended alkanes and to discover if the detected metabolites persist in the cultures during extended incubation, culture samples retrieved from two sampling points (sampling time indicated in Table 5.1) from all primary cultures were analyzed. We noted that succinylated metabolites were only detected in primary cultures amended with *iso*-alkanes and paraffinic solvent (Table 5.1) but no succinylated metabolite was detected in primary cultures amended with *n*-alkanes and naphtha. The putative metabolites were only detected during active biodegradation of *iso*-alkanes, however, the metabolites appeared to be transient because the metabolites were not detected at second sampling point except Albian S-3I. Similarly, in previous reports which examined methanogenic biodegradation of alkanes, succinylated metabolites were only transiently detected in cultures grown on *iso*-alkanes (Abu Laban et al., 2014; Tan et al., 2015) but not in cultures amended with *n*-alkanes (Aitken et al., 2013; Tan et al., 2015). This phenomenon could be attributed to the recalcitrance nature of *iso*-alkanes over *n*-alkanes. Thus, although under methanogenic condition the succinylated *iso*-alkanes were only transient products, the succinylated *iso*-alkanes were not further oxidized as rapidly as succinylated *n*-alkanes, allowing the metabolic intermediates to transiently accumulate and thus, be detected during exponential *iso*-alkanes degradation phase. The reason for the unsuccessful detection of any metabolites from methanogenic cultures amended with *n*-alkanes, therefore, may be explained by: 1) non-accumulating metabolites resulting from the close link of alkane activation with metabolites turnover, and 2) instrumentation limitation in detecting low yield metabolites.

Nonetheless, *iso*-alkanes also constitute a major portion of naphtha (~39 wt. %; Chapter 2). However, naphtha comprised of a wide range of *iso*-alkanes (C_6-C_{10}) as oppose to paraffinic solvent, which only has three major *iso*-alkane components constituting ~58 wt. % of paraffinic solvent's total mass (Chapter 2). Therefore, the concentration of individual *iso*-alkanes in naphtha is low compared to the concentration of individual *iso*-alkanes in paraffinic solvent; hence, the concentration of any metabolites that might arise from the oxidation of the *iso*-alkanes in naphtha amended cultures may be too low to be detected by GC-MS. Interestingly under sulfate-reducing conditions, the succinylated *iso*-alkane, 1,3-dimethylbutylsuccinic acid persisted throughout ~1100 d incubation even though sulfate concentration has plateaued since day ~900 (Chapter 4). Accumulation of 1,3-dimethylbutylsuccinic acid in Albian S-3I might be attributed to the absence or low growth of microbes carrying the enzymes appropriate for oxidation of 1,3-dimethylbutylsuccinic acid. The accumulation of 1,3-dimethylbutylsuccinic acid might have

resulted in inhibition of further degradation of 2-methylpentane in Albian S-3I despite the fact that high concentration of sulfate still present in the culture (see Chapter 4). The derivatized metabolites from 2-methylbutane and 2-methylpentane exhibited a cluster of two GC-MS peaks. These peaks might have represented diastereomers formed by enzymatic mechanisms as described by Jarling et al., (2012), which have also been reported in previous studies examining anaerobic biodegradation of *iso*-alkanes (Abu Laban et al., 2014; Tan et al., 2015).

Activation of alkanes via fumarate addition pathway is carried out by glycyl radical alkylsuccinate or methylalkylsuccinate synthase (Ass or Mas, respectively). To determine the presence of genes encoding Ass/Mas and diversity of the genes in all our cultures, we performed amplification of *assA/masD* genes, which encode for the catalytic subunit of Ass/Mas, using degenerate *assA/masD* primers. *assA/masD* genes were amplified from all Albian and CNRL amended primary cultures, indicating microbial communities in all our primary cultures have the potential of activating alkanes via fumarate addition pathway despite the expected alkysuccinate metabolites were not detected in all the cultures. The diversity of the translated *assA/masD* genes in all our cultures, however, are low and have high similarity to *Peptococcaceae* SCADC. Incidentally, all our primary cultures exhibited enrichment of *Peptococcaceae* during biodegradation of the amended alkanes (Chapter 2, 3 and 4) and similar enrichment of *Peptococcaceae* has also been reported in *n*- and *iso*-alkane-degrading cultures from MFT (Abu Laban et al., 2014; Mohamad Shahimin et al., 2016; Siddique et al., 2015; Tan, Charchuk, et al., 2014b; Tan et al., 2015), providing further evidence for implication of *Peptococcaceae* as primary degraders of alkanes in oil sands tailings ponds.

Our current metabolites and functional genes profiling data along with findings from previous reports (Abu Laban et al., 2014; An et al., 2013; Tan et al., 2015) suggest that anaerobic biodegradation of hydrocarbons occurs via fumarate addition pathway in all oil sands tailings ponds despite the differences in oil sands extraction procedures and tailings management practices employed by different oil sands operators. However, we cannot disregard the possibility that other metabolic pathways, namely carboxylation and/or hydroxylation, were playing an important role in activation of hydrocarbons in tailings ponds.

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6 Summary and synthesis

6.1 Anaerobic hydrocarbon biodegradation in different oil sands tailings

Oil sands tailings ponds are engineered environments that are highly impacted with salts, heavy metals and residual hydrocarbons. Despite the initial speculation that oil sands tailings ponds cannot support living organisms, recent evidence proves that oil sands tailings ponds harbor various specialized anaerobic microbial communities capable of degrading simple hydrocarbons (Abu Laban et al., 2014; Mohamad Shahimin et al., 2016; Penner & Foght, 2010; Siddique et al., 2015, 2012, 2011, 2007, 2006; Tan, Semple, et al., 2015). Because oil sands tailings from different operators differ from one another due to oil sands compositions, tailings age, and extraction process and tailings management practices, we assessed biodegradation of various hydrocarbons in MFT from Albian and CNRL tailings ponds, which have scarcely been examined before. The objective of this study is to examine how indigenous microbial communities from different tailings ponds biodegrade different hydrocarbons and to identify the key microbial players involved in the biodegradation process. This research also includes examination of other terminal electron acceptors in biodegradation of recalcitrant compounds and the study of anaerobic hydrocarbon activation pathway to better understand the process of anaerobic hydrocarbon biodegradation in oil sands tailings ponds specifically, and more broadly in other hydrocarbon-impacted anaerobic environments such as crude-oil reservoirs and petroleum-contaminated aquifers.

For this thesis project, methanogenic Albian and CNRL primary cultures were prepared with three groups of hydrocarbon treatments, which were amended separately: extraction solvents (paraffinic solvent and naphtha) (Chapter 2), *n*-alkanes (two-alkanes and four-alkanes) (Chapter 3) and *iso*-alkanes (three-*iso*-alkanes and five-*iso*-alkanes) (Chapter 4). In addition to the methanogenic cultures, iron-, nitrate and sulfate-reducing Albian primary cultures were also prepared and amended with three-*iso*-alkanes (2-methylbutane; 2-MC_4, 2-methylpentane; 2-MC_5, and 3-methylpentane; 3-MC_5). The overall findings from these primary cultures revealed that the pattern of hydrocarbon biodegradation is different in MFT from different tailings ponds and that hydrocarbon biodegradation is affected by the presence of terminal electron acceptors.

In cultures amended with extraction solvents, both methanogenic Albian and CNRL MFT exhibited similar preferential biodegradation of solvents' components in the order of *n*-alkanes >

iso-alkanes > *cyclo*-alkanes, albeit Albian MFT biodegraded *iso*- and *cyclo*-alkanes more extensively than CNRL MFT. Partial depletion of some *iso*- and/or *cyclo*-alkanes was also noted in both Albian and CNRL cultures amended with both paraffinic solvents and CNRL, implying cometabolism of these *iso*- and/or *cyclo*-alkanes during complete oxidation of other alkanes (Abu Laban et al., 2014; Siddique et al., 2015; Tan et al., 2015). In Albian and CNRL cultures amended with *n*-alkanes, Albian MFT preferentially biodegraded the short-chain (C_5-C_8) *n*-alkanes whereas, CNRL MFT preferred the longer-chain (C_8-C_{10}) *n*-alkanes. All amended *n*-alkanes, however, were eventually completely biodegraded in both Albian and CNRL MFT amended with both two- (*n*-pentane; *n*C_5 and *n*-hexane; *n*C_6) and four- (*n*C_5, *n*C_6, *n*-octane; *n*C_8, and *n*-decane; *n*C_{10}) alkanes. In methanogenic Albian and CNRL cultures amended with three-*iso*-alkanes, only 2-MC_5 was completely biodegraded whereas 2-MC_4 and 3-MC_5, were only partially depleted, suggesting cometabolism of 2-MC_4 and 3-MC_5 during complete oxidation of 2-MC_5 (Abu Laban et al., 2014; Siddique et al., 2015; Tan et al., 2015). Under sulfate-reducing conditions, however, Albian MFT only demonstrated partial depletion of 2-MC_5 despite still having high sulfate concentration in the cultures. It is important to note that some of the *iso*-alkanes amended cultures did not produce the hypothesized outcomes. The iron- and nitrate-reducing cultures did not biodegrade the amended three-*iso*-alkanes during ~1100 d of incubation. To date, no literature has reported biodegradation of *n*- and *iso*-alkanes under iron-reducing conditions (Agrawal & Gieg, 2013; Callaghan, 2013; Musat & Widdel, 2008), which might imply the possibility that iron-reducers did not carry the appropriate enzymes to degrade *n*- and *iso*-alkanes. Conversely, *iso*-alkane biodegradation under nitrate-reducing conditions has been reported previously (Bregnard, Haner, Hohener, & Zeyer, 1997), however, it is possible that the denitrifiers indigenous to oil sands tailings pond are not capable of oxidizing the specific three-*iso*-alkane mixture or the enzymes required for *iso*-alkane oxidation are absent in the oil sand's indigenous denitrifiers' genetic makeup. Interestingly, in methanogenic cultures amended with five-*iso*-alkanes (2-MC_4, 2-MC_5, 2-methylhexane, 2-methylheptane and 2-methyloctane), only CNRL MFT have recently exhibited preferential biodegradation of the amended *iso*-alkanes (in the sequence: C_9>C_8>C_7>C_6>C_5) albeit Albian MFT did not show any substantial degradation of the amended *iso*-alkanes (Chapter 4). The observations in the methanogenic Albian MFT amended with five-*iso*-alkanes may be attributed to: 1) high concentration of the amended *iso*-alkanes that might retard growth of key microbial players due to toxicity of low molecular

weight hydrocarbons (<C_{10}) in high concentration (Sikkema et al., 1995), and/or 2) late development of the key microbial players that are able to metabolize the *iso*-alkane mixtures. Therefore, further incubation of Albian MFT amended with five-*iso*-alkanes amended might eventually result in the development of the key microbial players capable of biodegrading the amended *iso*-alkanes.

It is worthy of note that the findings from all methanogenic primary cultures amended with solvents and *iso*-alkanes indicate that cometabolism may be an important process occurring in oil sands tailings. Cometabolism, however, was only observed in biodegradation of some *iso*- and *cyclo*-alkanes isomers (Chapter 2 and 4; Abu Laban et al., 2014; Siddique et al., 2015; Tan, Semple, et al., 2015), indicating the enzymes involved in complete oxidation of specific *iso*-alkanes have relaxed active sites, allowing cometabolism of other structurally close-related *iso*- or *cyclo*-alkane isomers. These data are important in improving existing greenhouse gas emission models by taking into account cometabolism of some recalcitrant compounds indigenous to oil sands tailings ponds. Since cometabolism of recalcitrant compounds under methanogenic conditions resulted in lower yield of CH_4 (Chapter 2 and 4), integration of this information into the existing greenhouse gas emission models will result in better prediction of the potential greenhouse gas emitted from different tailings ponds.

To characterize key microbial players involved during biodegradation of hydrocarbons, 16S rRNA gene pyrosequencing analysis was performed on all the primary cultures. The microbial community analysis revealed that only a handful of key microbial players were enriched during biodegradation of the hydrocarbon mixtures, which recurred throughout all the treatments. These enriched taxa were composed of *Anaerolineaceae, Peptococcaceae, Syntrophaceae*, "*Candidatus* Methanoregula", and *Methanosaetaceae*. The proportions of these microbial players, however, differ from one culture to another. Other key bacterial players, however, were enriched only in specific cultures, such as: 1) *Desulfobacteraceae* in CNRL MFT amended with four-alkanes during biodegradation of *n*C_8 and *n*C_{10} (Chapter 3) and, 2) *Desulfobacteraceae* and *Desulfobulbaceae* in Albian MFT amended with three-*iso*-alkanes under sulfate-reducing conditions during biodegradation of 2-MC_5 (Chapter 4). All the enriched bacterial taxa (*Anaerolineaceae, Peptococcaceae, Syntrophaceae, Desulfobacteraceae,* and *Desulfobulbaceae*) in our cultures have been implicated as primary-hydrocarbon degraders in many cultures grown on various hydrocarbons under various reducing conditions (Abu Laban et

al., 2014; Cheng et al., 2013; Fowler, Gutierrez-Zamora, Manefield, & Gieg, 2014; Gray et al., 2011; Grundmann et al., 2008; Liang et al., 2015; Mohamad Shahimin et al., 2016; Siddique et al., 2012, 2011, 2015; Tan et al., 2014, 2015; Zengler et al., 1999), whereas the enriched archaeal taxa have been implicated as important methanogens involved in both acetoclastic and hydrogenotrophic methanogenesis in oil sands tailings ponds (Abu Laban et al., 2014; Siddique et al., 2015, 2012, 2011; Tan et al., 2015).

To elucidate the major activation pathway of alkanes in all the primary cultures, both metabolites and functional genes analyses were performed on all the primary cultures. Succinylated metabolites derived from *iso*-alkanes were only detected in paraffinic solvent and *iso*-alkanes amended cultures during active biodegradation phase. However, the succinylated metabolites were found to be transient intermediary products under methanogenic conditions but under sulfate-reducing conditions, the metabolites persist even after prolonged incubation implying the metabolites under sulfate-reducing conditions were 'dead-end' products. Interestingly, all primary cultures exhibited amplification of genes encoding catalytic subunit of alkylsuccinate/methylalkylsuccinate synthase (*assA/masD*), which are known to encode enzymes involved in fumarate-addition pathway, suggesting fumarate-addition as the potential primary activation pathway in oil sands tailings ponds (Chapter 5). Although succinylated metabolites were not detected in naphtha and *n*-alkanes amended cultures, detection of *assA/masD* genes in these cultures implies genetic potential of these cultures to activate alkanes via fumarate-addition pathway. Fumarate addition as the main alkane activation pathway was also suggested by other researchers who examined biodegradation of various hydrocarbons in oil sands tailings (Abu Laban et al., 2014; An et al., 2013; Tan et al., 2013, 2015). The diversity of *assA/masD* homologs, however, was low throughout all the cultures indicating the probability of only one bacterial species representing each enriched family in all the treatments. Similar low *assA* genes diversity was also reported in hydrocarbon-degrading enrichment cultures derived from MFT (Abu Laban et al., 2014; Tan et al., 2015).

Conclusively, our findings showed that indigenous microbial communities in different oil sands tailings ponds preferentially biodegrade hydrocarbons that are cognate to their tailings ponds. However, microbial communities in different tailings ponds are capable of biodegrading noncognate hydrocarbons with prolonged incubation indicating the flexibility of the microbes to utilize wide-range of hydrocarbons as carbon sources. Interestingly, the presence of terminal

electron acceptors in Albian MFT impedes complete biodegradation of recalcitrant *iso*-alkanes. Cumulative findings from this thesis work prove that oil sands compositions, tailings age, and extraction process and tailings management practices influence hydrocarbon biodegradation pattern and microbial communities' structure and activities in different tailings ponds. Since this thesis work only examines a few aspects of physicochemical properties (ie: MFT source, hydrocarbons amendment and terminal electron acceptors) that influence anaerobic hydrocarbon biodegradation process in oil sands tailings, further studies examining the effects of different physicochemical properties (ie: different pH, temperatures or salt concentrations) on the anaerobic hydrocarbon biodegradation process in different tailings ponds are required to fully comprehend the factors influencing tailings ponds' indigenous microbial communities' structure and functions.

6.2 Future work and commercial biotechnological application potential

Tailings ponds receive a complex mixture of hydrocarbons necessitating investigation of hydrocarbon biodegradation (complete substrate spectrum for the key microbial players involved in degradation of specific range or group of hydrocarbons) to effectively manage tailings ponds. Besides the range of hydrocarbons, impacts of other tailings physicochemical properties such as temperatures, pH range, different salts concentrations, and elevated concentration of heavy metals on the indigenous microbial structure would contribute to the overall understanding of microbial roles in geochemical phenomena in oil sands tailings ponds. Moreover, development of enrichment cultures grown on specific hydrocarbons and other physicochemical conditions could unlock the opportunity for biotechnological implementations such as in commercial development of cultures that can: 1) effectively remediate anaerobic environments impacted by hydrocarbons under various conditions, and 2) commercially produce clean-burning renewable natural gas from petroleum reservoirs (Gieg, Duncan, & Suflita, 2008). Metagenomic coupled with metatranscriptomic analyses from these enrichment cultures can also provide further insights into genetic potential and putative roles of microbial players in anaerobic hydrocarbon degradation process.

Isolation of microorganisms capable of biodegrading hydrocarbons from oil sands tailings ponds under various reducing conditions is also an important project. Although isolation development is laborious and time-consuming, studying isolates would greatly simplify the study of complex and understudied syntrophic anaerobic hydrocarbon degradation process. The

isolates would also simplify studies of anaerobic microbial communication and sensing mechanisms, which is important to identify how microbial communities establish and maintain the community structure.

This thesis work describes the unique degradation pattern and capability of indigenous microbial communities to biodegrade various mixtures of hydrocarbons in different oil sands tailings ponds. The work has revealed that only a few microbial players were enriched during anaerobic biodegradation of different hydrocarbon mixtures, however, roles of these microbial players (ie: primary degraders, secondary degraders or scavengers) remain undefined, emphasizing the molecular work remains to be done to establish the roles of the microbial players. The sample volume and more sampling points in this thesis work were also the main limiting factors for detailed metabolite analysis. Scaling up the current primary cultures would potentially provide suitable sample volume at more sampling times for detailed metabolites analysis which will provide further evidence for better understanding the complete hydrocarbons degradation pathway: from activation step to CH_4 and CO_2 generation. The cumulative findings from these potential future studies will not only widen our understanding on anaerobic hydrocarbons biodegradation process in tailings ponds but also deepen our insights into specific environmental conditions or stimulants that would promote growth of specific key microbial players in tailings ponds, which will be valuable in formulating suitable bioremediation strategies for different oil sands tailings ponds.

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8 Appendix A: Supplementary Data for Chapter 2

Table A1: Hydrocarbons in naphtha-amended live Albian and CNRL MFT that exhibited no or ≤ 50% biodegradation at ~1600 d of incubation determined by PONAU analysis. Values in parentheses are the concentrations (mg L^{-1}) before biodegradation. See more details given in the title of Table 2.2 in main text.

Table A2: Predicted and measured methane (CH_4) yields from MFT incubated with paraffinic extraction solvents or naphtha for ~1600 d.

Table A3: Relative abundance of bacterial 16S rRNA gene pyrosequences grouped at class level in Albian MFT after incubation. Data represent analysis of pooled amplicons for triplicate microcosms. "Others <5%" is the sum of all taxa individually abundant at <5% of the total bacterial reads.

Table A4: Relative abundance of bacterial 16S rRNA gene pyrosequences grouped at class level in CNRL MFT after incubation. Results for paraffinic solvent amended MFT were from analysis of individual microcosms, as the cultures showed different methane production patterns (Fig. 2.1A in main text). Results for all other treatments represent analysis of pooled amplicons for triplicate microcosms. "Others <5%" is the sum of all taxa individually abundant at <5% of the total bacterial reads.

Table A5: Relative abundance of archaeal 16S rRNA gene pyrosequences grouped at order level in Albian MFT after incubation. Data represent analysis of pooled amplicons for triplicate microcosms. "Others <5%" is the sum of all taxa individually abundant at <5% of the total archaeal reads.

Table A6: Relative abundance of archaeal 16S rRNA gene pyrosequences grouped at order level in CNRL MFT after incubation. Results for paraffinic solvent amended MFT were from analysis of individual microcosms, as the cultures showed different methane production patterns (Fig. 2.1A in main text). Results for all other treatments represent analysis of pooled amplicons for triplicate microcosms. "Others <5%" is the sum of all taxa individually abundant at <5% of the total archaeal reads.

Figure A1: Proportion of archaeal and bacterial reads in the microbial community during incubation of unamended MFT and MFT amended with naphtha or paraffinic solvent. (A) Albian MFT, unamended, (B) CNRL MFT, unamended, (C) Albian MFT,

paraffinic solvent, (D) CNRL MFT, paraffinic solvent, (E) Albian MFT, naphtha, and (F) CNRL MFT, naphtha. The results represent pooled amplicons of replicate cultures, with the exception of Panel D that represent the individual replicate microcosms.

Table A1: Hydrocarbons in naphtha-amended live Albian and CNRL MFT that exhibited no or ≤ 50% biodegradation at ~1600 d of incubation determined by PONAU analysis. Values in parentheses are the concentrations (mg L^{-1}) before biodegradation. See more details given in the title of Table 2.2 in main text.

Composition (% weight)	Components[ǂ]	Percent Biodegradation (%)	
		Albian MFT	CNRL MFT
iso-Paraffins C_5-C_{10} (39.01)	2-Methylbutane	11 (6)	39 (7)
	3-Methylpentane	16 (31)	0 (37)
	3,3-Dimethylpentane	41 (11)	28 (13)
	2,3-Dimethylpentane	0 (23)	0 (23)
	2,3-Dimethylhexane	29 (14)	23 (16)
	3-Ethylpentane	31 (16)	28 (19)
	2,4-Dimethylhexane	31 (19)	39 (23)
	2,6-Dimethylheptane	17 (17)	0 (20)
	2,2,5-Trimethylhexane	11 (12)	18 (14)
	3-Ethylhexane	38 (25)	13 (29)
	3,6-Dimethyloctane	0 (10)	15 (12)
Naphthenes C_6-C_9 (34.88)	Cyclohexane	19 (59)	0 (70)
	trans-1,3-Dimethylcyclopentane	17 (39)	0 (46)
	cis-1,3-Dimethylcyclopentane	28 (34)	0 (40)
	trans-1,2-Dimethylcyclopentane	21 (50)	0 (59)
	1,1,3-Trimethylcyclopentane	17 (36)	4 (43)
	trans,cis-1,2,4-Trimethylcyclopentane	0 (25)	0 (29)
	trans,cis-1,2,3-Trimethylcyclopentane	0 (30)	0 (26)
	1,1-Dimethylcyclohexane	28 (23)	8 (27)
	cis-1,4-Dimethylcyclohexane	0 (33)	0 (39)
	trans-1-Ethyl-3-Methylcyclopentane	28 (14)	0 (17)
	trans-1-Ethyl-2-Methylcyclopentane	0 (14)	0 (0)
	trans-1,2-Dimethylcyclohexane	0 (18)	0 (22)
	Ethylcyclohexane	31 (22)	5 (26)
Unknowns C_8-C_{10} (4.97)		0 (99)	0 (117)

[ǂ] Masses of the components that showed degradation (calculated from the percent biodegradation from the initial concentration) were fit into Eq. 2.2 (main text) to calculate the theoretical methane production in Albian and CNRL MFT amended with naphtha.

Table A2: Predicted and measured methane (CH_4) yields from MFT incubated with paraffinic extraction solvents or naphtha for ~1600 d.

		Incubation time (d)	Predicted Methane Yield [1] (mmol)	Measured Methane Yield (mmol)	Percent of theoretical production (%)
Paraffinic Solvent	Albian	600	1.87 ± 0.07	1.27 ± 0.09	68
		1300	2.64 ± 0.24	2.19 ± 0.09	83
		1600	2.71 ± 0.21	2.28 ± 0.10	84
	CNRL (I)		2.88	2.51	87
	CNRL (II)	1300	2.81	2.17	77
	CNRL (III)		2.21	1.67	76
	CNRL	1600	3.29 ± 0.09	2.91 ± 0.10	88
Naphtha	Albian	600	2.43 ± 0.13	1.84 ± 0.14	76
		1600	5.6	2.38	43
	CNRL	600	2.76 ± 0.16	1.92 ± 0.02	70
		1300	3.37 ± 0.13	1.96 ± 0.10	58
		1600	5.5	3	55

[1] Sum of theoretical maximum methane production at ~600 and ~1300 d was predicted (Eq. 2.2) using the masses of known hydrocarbon biodegraded determined by GC-FID equipped with purge and trap system. Theoretical methane production in paraffinic-amended MFT at ~1600 d was calculated using the headspace GC-MS analysis given in Table 2.1. Theoretical methane production in naphtha-amended MFT at ~1600 d was calculated using the PONAU analysis given in Table 2.3 and Appendix Table A1. Eq. 2.2 was used to convert individual hydrocarbon to methane.

All methane values with one standard deviation were calculated from analysis of triplicate microcosms whereas methane values (~1600 d) without standard deviation were calculated from analysis of single microcosms.

Table A3: Relative abundance of bacterial 16S rRNA gene pyrosequences grouped at class level in Albian MFT after incubation. Data represent analysis of pooled amplicons for triplicate microcosms. "Others <5%" is the sum of all taxa individually abundant at <5% of the total bacterial reads.

Taxa, grouped at <5% distance level	Albian MFT							
	Unamended		Paraffinic Solv.			Naphtha		
	Day							
	0	1100	0	600	1100	0	400	900
Actinobacteria								
Coriobacteriales;Coriobacteriaceae	0.2	3.3	0.2	4.6	0.5	0.0	3.1	0.3
Anaerolineae								
Anaerolineales;Anaerolineaceae;Leptolinea	1.4	8.5	0.6	19.6	4.8	0.9	11.4	2.8
Anaerolineales;Anaerolineaceae;uncultured	1.6	18.9	0.4	32.4	8.1	0.5	15.6	5.8
Clostridia								
Clostridiales;Peptococcaceae	0.1	0.6	0.0	4.9	60.4	0.0	35.7	75.9
Clostridiales;Peptococcaceae; Cryptanaerobacter	0.1	0.7	0.0	5.3	2.8	0.1	3.1	2.6
Alphaproteobacteria								
Sphingomonadales;Sphingomonadaceae; Sphingomonas	0.0	0.3	0.0	0.2	0.0	0.0	0.0	0.0
Betaproteobacteria								
Burkholderiales;Comamonadaceae	0.3	1.8	0.2	4.7	0.2	0.2	3.0	0.3
Hydrogenophilales;Hydrogenophilaceae; Thiobacillus	84.5	8.1	92.5	1.5	0.5	90.1	0.2	0.2
Deltaproteobacteria								
Desulfuromonadales;Geobacteraceae; Geothermobacter	0.0	1.9	0.0	0.5	0.2	0.1	0.0	0.0
Syntrophobacterales;Syntrophaceae; Smithella	0.0	7.0	0.0	0.3	1.6	0.0	2.7	0.8
Syntrophobacterales;Syntrophaceae; Syntrophus	0.0	7.9	0.1	0.5	3.2	0.2	2.5	1.7
Others <5% (Number of taxa)	10.2 (33)	41.0 (106)	5.0 (58)	25.6 (69)	17.7 (30)	6.6 (61)	22.6 (58)	9.8 (39)

0% ≤  **≥ 93%**

Table A4: Relative abundance of bacterial 16S rRNA gene pyrosequences grouped at class level in CNRL MFT after incubation. Results for paraffinic solvent amended MFT were from analysis of individual microcosms, as the cultures showed different methane production patterns (Fig. 2.1A in main text). Results for all other treatments represent analysis of pooled amplicons for triplicate microcosms. "Others <5%" is the sum of all taxa individually abundant at <5% of the total bacterial reads.

Taxa, grouped at <5% distance level	CNRL MFT							
	Unamended		Paraffinic Solv.			Naphtha		
			(I)	(II)	(III)			
	Day							
	0	1100	1100			0	400	900
Actinobacteria								
Coriobacteriales;Coriobacteriaceae	8.6	7.5	1.1	3.0	5.0	19.4	7.5	1.2
Anaerolineae								
Anaerolineales;Anaerolineaceae;Leptolinea	3.3	1.9	0.7	3.4	5.4	7.1	7.3	0.2
Anaerolineales;Anaerolineaceae;uncultured	9.6	11.3	2.5	3.4	7.2	16.8	23.8	1.8
Clostridia								
Clostridiales;Peptococcaceae	3.3	4.7	68.7	66.5	18.5	1.9	0.1	65.2
Clostridiales;Peptococcaceae; Cryptanaerobacter	0.0	0.0	0.7	0.0	0.4	0.0	0.0	0.0
Alphaproteobacteria								
Sphingomonadales;Sphingomonadaceae; Sphingomonas	0.0	1.9	0.0	0.9	19.7	0.0	2.6	0.0
Betaproteobacteria								
Burkholderiales;Comamonadaceae	3.6	8.6	0.0	2.1	2.8	1.9	2.6	0.3
Hydrogenophilales;Hydrogenophilaceae; Thiobacillus	0.0	0.4	0.0	0.0	0.4	0.0	0.1	0.0
Deltaproteobacteria								
Desulfuromonadales;Geobacteraceae; Geothermobacter	1.0	3.8	0.7	2.1	1.8	1.3	6.2	0.5
Syntrophobacterales;Syntrophaceae; Smithella	6.7	13.2	13.5	1.7	7.0	4.5	17.8	16.3
Syntrophobacterales;Syntrophaceae; Syntrophus	2.3	2.8	2.5	0.9	0.6	0.0	0.6	2.6
Others <5% (Number of taxa)	51.5 (102)	44.0 (77)	9.6 (14)	15.9 (20)	31.4 (61)	36.1 (35)	31.5 (73)	11.9 (23)

Table A5: Relative abundance of archaeal 16S rRNA gene pyrosequences grouped at order level in Albian MFT after incubation. Data represent analysis of pooled amplicons for triplicate microcosms. "Others <5%" is the sum of all taxa individually abundant at <5% of the total archaeal reads.

Taxa, grouped at <5% distance level	Albian MFT							
	Unamended		Paraffinic Solv.			Naphtha		
	Day							
	0	1100	0	600	1100	0	400	900
Methanomicrobiales								
"*Candidatus* Methanoregula"	0.9	15.3	1.3	14.2	87.0	2.6	32.3	66.2
Methanosarcinales								
Methanosaetaceae;Methanosaeta	66.4	24.5	67.1	51.2	3.8	63.1	60.8	28.1
Methanosarcinaceae;Methanosarcina	12.7	51.8	14.1	26.8	5.7	15.5	2.3	1.4
Others <5% (Number of taxa)	19.1 (12)	8.4 (17)	15.6 (13)	7.7 (16)	3.4 (11)	16.9 (13)	4.7 (10)	4.3 (13)

0% ≤  **≥ 87%**

Table A6: Relative abundance of archaeal 16S rRNA gene pyrosequences grouped at order level in CNRL MFT after incubation. Results for paraffinic solvent amended MFT were from analysis of individual microcosms, as the cultures showed different methane production patterns (Fig. 2.1A in main text). Results for all other treatments represent analysis of pooled amplicons for triplicate microcosms. "Others <5%" is the sum of all taxa individually abundant at <5% of the total archaeal reads.

Taxa, grouped at <5% distance level	CNRL MFT							
	Unamended		Paraffinic Solv.			Naphtha		
			(I)	(II)	(III)			
	Day							
	0	1100	1100			0	400	900
Methanomicrobiales								
"*Candidatus* Methanoregula"	60.6	84.2	60.5	54.6	46.5	26.1	17.3	91.3
Methanosarcinales								
Methanosaetaceae;Methanosaeta	33.9	13.1	38.2	43.3	50.0	60.2	80.6	8.1
Methanosarcinaceae;Methanosarcina	0.2	0.8	0.0	0.0	0.1	0.1	0.4	0.0
Others <5% (Number of taxa)	2.7 (16)	1.8 (13)	1.3 (9)	2.1 (11)	3.4 (12)	12.0 (10)	1.7 (10)	0.5 (9)

0% ≤  **≥ 91%**

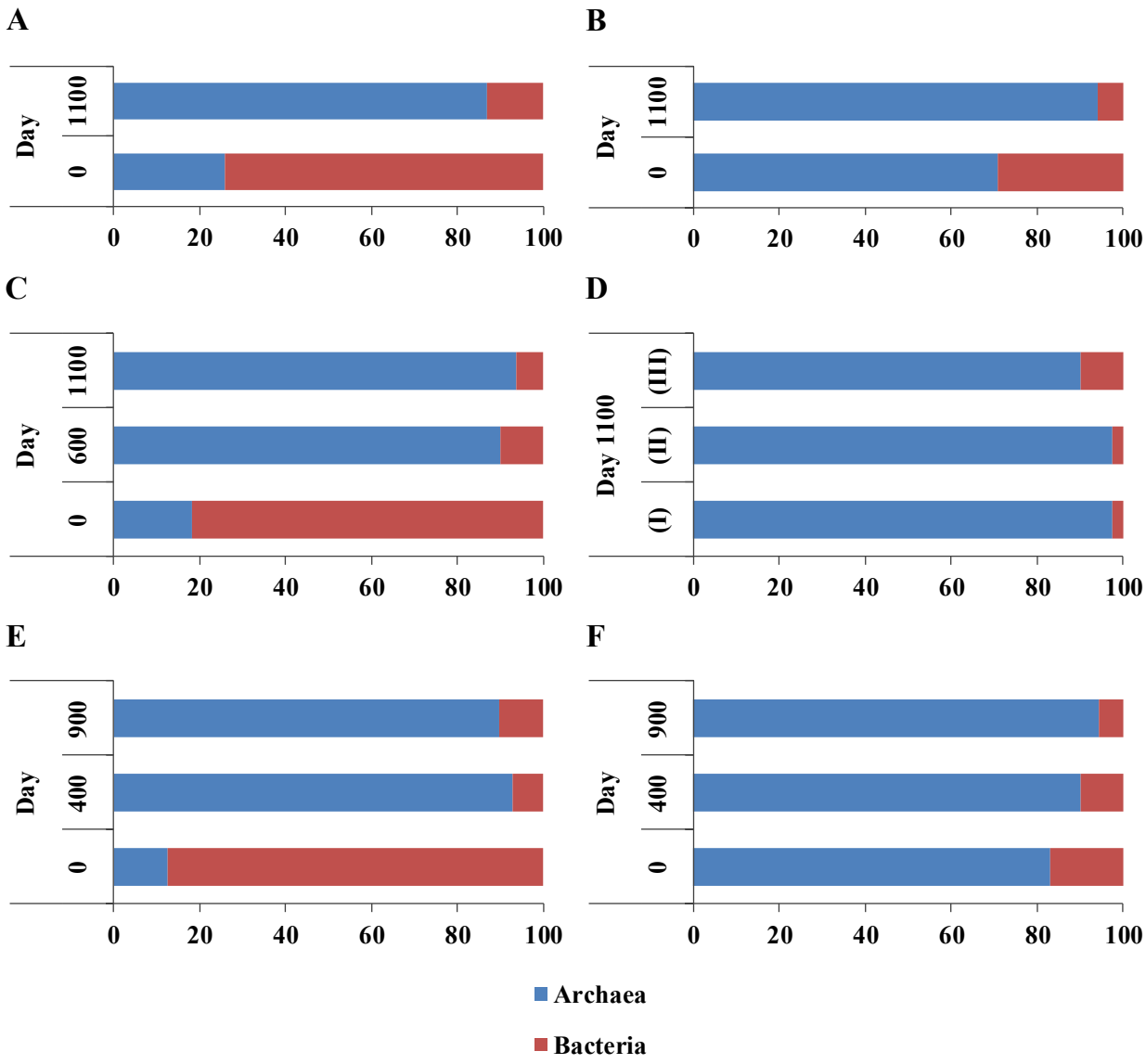

Figure A1: Proportion of archaeal and bacterial reads in the microbial community during incubation of unamended MFT and MFT amended with naphtha or paraffinic solvent. (A) Albian MFT, unamended, (B) CNRL MFT, unamended, (C) Albian MFT, paraffinic solvent, (D) CNRL MFT, paraffinic solvent, (E) Albian MFT, naphtha, and (F) CNRL MFT, naphtha. The results represent pooled amplicons of replicate cultures, with the exception of Panel D that represent the individual replicate microcosms.

9 Appendix B: Supplementary Data for Chapter 3

Table B1: Predicted and measured methane (CH_4) yields from MFT incubated with two- or four-alkane mixtures.

Table B2: Relative abundance of bacterial 16S rRNA gene pyrosequences grouped at class level in Albian MFT before degradation (day 0) and after incubation for 308 d with either the two- or four-alkane mixture. Genus assignations are given where possible. Results from the two-alkane treatment represent analysis of pooled amplicons for triplicate microcosms. Results for the four-alkane treatment are from analysis of individual microcosms, as the cultures showed different biodegradation patterns (Fig. 1 and Fig. B1). "Others <5%" is the sum of all taxa individually abundant at <5% of the total bacterial reads.

Table B3: Relative abundance of bacterial 16S rRNA gene pyrosequences grouped at class level in CNRL MFT before degradation (day 0) and after incubation for 319 d and 532 d with either the two- or four-alkane mixture. Genus assignations are shown where possible. "Others <5%" is the sum of all taxa individually abundant at <5% of the total bacterial reads.

Table B4: Relative abundance of archaeal 16S rRNA gene pyrosequences grouped at order level in Albian MFT before degradation (day 0) and after incubation for 308 d with either the two- or four-alkane mixture. Genus assignations are shown where possible. Results for the four-alkane treatment are from analysis of individual microcosms, as the cultures showed different biodegradation patterns (Fig. 1 and Fig. B1). "Others <5%" is the sum of all taxa individually abundant at <5% of the total archaeal reads.

Table B5: Relative abundance of archaeal 16S rRNA gene pyrosequences grouped at order level in amended CNRL MFT before degradation (day 0) and after incubation for 319 d and 532 d with either the two- or four-alkane mixture. Genus assignations are shown where possible. "Others <5%" is the sum of all taxa individually abundant at <5% of the total archaeal reads.

Figure B1: Measured concentrations of n-alkanes in individual replicate Albian MFT microcosms during 521 d incubation with the four-alkane mixture. Values for the abiotic control are means of triplicate microcosms and the error bars represent one standard deviation.

Figure B2: Archaeal and bacterial community proportions based on analysis of 16S rRNA gene pyrosequences before and during incubation of MFT with two- and four-alkane mixtures. (A) Albian MFT, two alkanes; (B) Albian MFT, four alkanes replicate A; (C) Albian MFT, four alkanes replicate B; (D) Albian MFT, four alkanes replicate C; (E) CNRL MFT, two alkanes; and (F) CNRL MFT, four alkanes. The results represent pooled amplicons of triplicate cultures, with the exception of Panel B, C and D that represents the single microcosm.

Table B1: Predicted and measured methane (CH_4) yields from MFT incubated with two- or four-alkane mixtures.

		Incubation time (d)	Substrate Consumed (mmol) *	Predicted CH_4 Yield (mmol) §	Measured CH_4 Yield (mmol)	Percent of theoretical production
Albian two-alkane	C_5	300	0.40 ± 0.03¶	2.90 ± 0.21	1.99 ± 0.12	68.8
	C_6		0.67 ± 0.08			
Albian four-alkane	C_5	500	0.21 ± 0.02	5.07 ± 0.86	3.76 ± 0.37	74.0
	C_6		0.41 ± 0.05			
	C_8		0.30 ± 0.10			
	C_{10}		0.05 ± 1.06			
CNRL two-alkane	C_5	500	0.27 ± 0.00	2.88 ± 0.12	1.91 ± 0.09	66.5
	C_6		0.40 ± 0.02			
CNRL four-alkane	C_5	500	0.17 ± 0.01	6.27 ± 0.19	4.31 ± 0.26	68.7
	C_6		0.35 ± 0.02			
	C_8		0.27 ± 0.02			
	C_{10}		0.28 ± 0.00			

***** Calculated from the difference of measured initial alkane concentrations, taking into account abiotic losses, and residual alkane concentrations at incubation time indicated.

§ Calculated using Eqs. 4.1-4.4 (main text) and the calculated mass of substrate consumed.

¶ All values represent the mean from analysis of triplicate microcosms (±1 standard deviation).

Table B2: Relative abundance of bacterial 16S rRNA gene pyrosequences grouped at class level in Albian MFT before degradation (day 0) and after incubation for 308 d with either the two- or four-alkane mixture. Genus assignations are given where possible. Results from the two-alkane treatment represent analysis of pooled amplicons for triplicate microcosms. Results for the four-alkane treatment are from analysis of individual microcosms, as the cultures showed different biodegradation patterns (Fig. 3.1 and Appendix Fig. B1). "Others <5%" is the sum of all taxa individually abundant at <5% of the total bacterial reads.

Taxa, assigned at ≤5% sequence difference	Albian two-alkane		Albian four-alkane (A)[1]		Albian four-alkane (B)[2]		Albian four-alkane (C)[2]	
	Day							
	0	300	0	300	0	300	0	300
Actinobacteria								
Coriobacteriales; Coriobacteriaceae	3.9	0.7	5.7	2.7	0.4	3.2	4.9	1.5
Anaerolinea								
Anaerolineales; Anaerolineaceae; Leptolinea	1.8	2.6	2.4	9.8	1.0	6.4	3.0	8.7
Anaerolineales; Anaerolineaceae; Levilinea	1.7	1.1	1.8	2.7	0.3	5.1	1.1	3.2
Anaerolineales; Anaerolineaceae; uncultured	3.3	8.1	4.7	13.4	0.9	14.7	5.5	14.6
Clostridia								
Clostridiales; Peptococcaceae	0.1	77.1	0.4	50.9	0.0	57.1	0.3	57.6
Clostridiales;Peptococcaceae; Cryptanaerobacter	2.7	1.2	2.2	2.7	0.1	0.0	1.7	5.8
Nitrospira								
Nitrospirales; Nitrospiraceae	8.5	0.2	7.0	0.0	0.2	0.0	7.7	0.0
Betaproteobacteria								
Hydrogenophilales; Hydrogenophilaceae; Thiobacillus	22.1	0.1	23.4	0.0	93.6	0.0	24.1	0.0
Rhodocyclales; Rhodocyclaceae; uncultured	3.3	0.0	6.0	0.0	0.4	0.0	5.7	0.0
Gammaproteobacteria								
Chromatiales; Chromatiaceae	4.3	0.0	5.6	0.0	0.1	1.3	5.4	0.2
Others <5% (Number of taxa)	48.1 (90)	8.7 (29)	40.9 (132)	17.9 (9)	3.2 (29)	12.2 (10)	40.5 (145)	8.5 (21)

[1] Replicate that showed complete biodegradation of nC_{10} in four-alkane mixture. These results were used to construct Fig. 4B (left).

[2] Replicates that did not show considerable biodegradation of nC_{10}.

Table B3: Relative abundance of bacterial 16S rRNA gene pyrosequences grouped at class level in CNRL MFT before degradation (day 0) and after incubation for 319 d and 532 d with either the two- or four-alkane mixture. Genus assignations are shown where possible. "Others <5%" is the sum of all taxa individually abundant at <5% of the total bacterial reads.

Taxa, assigned at <5% sequence difference	CNRL two-alkane		CNRL four-alkane		
	Day				
	0	500	0	300	500
Actinobacteria					
Coriobacteriales; Coriobacteriaceae	11.0	2.3	12.6	1.7	0.5
Anaerolinea					
Anaerolineales; Anaerolineaceae; Leptolinea	4.1	2.1	3.6	14.8	0.9
Anaerolineales; Anaerolineaceae; uncultured	12.2	4.2	9.2	18.8	1.4
Clostridia					
Clostridiales; Peptococcaceae	4.1	79.5	3.4	0.0	56.6
Betaproteobacteria					
Rhodocyclales; Rhodocyclaceae; uncultured	5.9	0.1	7.8	0.0	0.0
Deltaproteobacteria					
Desulfobacterales; Desulfobacteraceae; Desulfobacterium	0.0	0.7	0.2	17.7	8.5
Syntrophobacterales; Syntrophaceae; Smithella	8.9	0.7	9.0	28.4	28.3
Others <5% (Number of taxa)	53.8 (80)	10.3 (48)	54.2 (81)	18.6 (26)	3.8 (5)

Table B4: Relative abundance of archaeal 16S rRNA gene pyrosequences grouped at order level in Albian MFT before degradation (day 0) and after incubation for 308 d with either the two- or four-alkane mixture. Genus assignations are shown where possible. Results for the four-alkane treatment are from analysis of individual microcosms, as the cultures showed different biodegradation patterns (Fig. 3.1 and Appendix Fig. B1). "Others <5%" is the sum of all taxa individually abundant at <5% of the total archaeal reads.

Taxa, assigned at <5% sequence difference	Albian two-alkane		Albian four-alkane (A)[1]		Albian four-alkane (B)[2]		Albian four-alkane (C)[2]	
	Day							
	0	300	0	300	0	300	0	300
Methanobacteriales								
Methanobacteriaceae; Methanobacterium	3.9	0.3	3.4	2.4	9.3	0.0	3.9	0.5
Methanomicrobiales								
"*Candidatus* Methanoregula"	25.8	11.9	22.2	9.5	3.2	11.9	24.5	15.4
Methanosarcinales								
Methanosaetaceae; Methanosaeta	61.2	83.2	64.4	80.8	65.1	83.5	64.4	79.1
Methanosarcinaceae; Methanomethylovorans	3.9	0.1	5.1	0.1	1.8	0.2	3.4	0.1
Methanosarcinaceae; Methanosarcina	1.8	2.4	0.9	4.9	17.8	0.1	0.8	2.2
Others <5% (Number of taxa)	3.5 (6)	2.1 (7)	3.9 (7)	2.3 (5)	2.8 (5)	4.3 (4)	2.9 (10)	2.6 (6)

0% ≤  **≥ 84%**

[1] Replicate that showed complete biodegradation of nC_{10} in four-alkane mixture. These results were used to construct Fig. 4B (right).

[2] Replicates that did not show considerable biodegradation of nC_{10}.

Table B5: Relative abundance of archaeal 16S rRNA gene pyrosequences grouped at order level in amended CNRL MFT before degradation (day 0) and after incubation for 319 d and 532 d with either the two- or four-alkane mixture. Genus assignations are shown where possible. "Others <5%" is the sum of all taxa individually abundant at <5% of the total archaeal reads.

Taxa, assigned at <5% sequence difference	CNRL two-alkane		CNRL four-alkane		
	Day				
	0	500	0	300	500
Methanomicrobiales					
"*Candidatus* Methanoregula"	60.7	6.6	67.8	12.7	73.6
Methanosarcinales					
Methanosaetaceae; Methanosaeta	34.9	92.9	27.8	86.6	25.9
Others <5% (Number of taxa)	4.4 (9)	0.5 (7)	4.4 (8)	0.6 (7)	0.5 (9)

0% ≤  **≥ 93%**

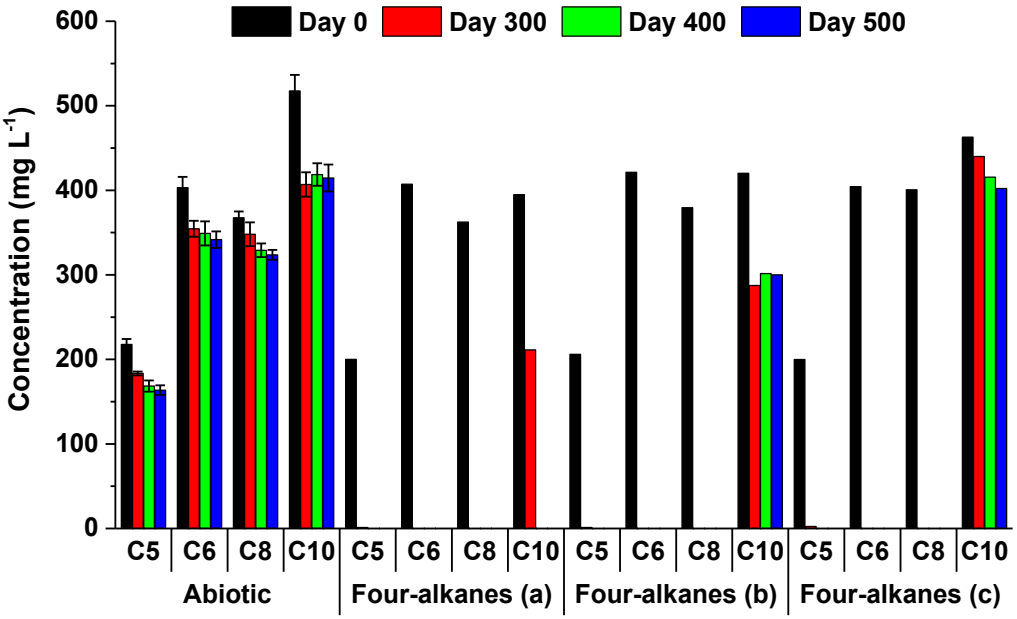

Figure B1: Measured concentrations of *n*-alkanes in individual replicate Albian MFT microcosms during 521 d incubation with the four-alkane mixture. Values for the abiotic control are means of triplicate microcosms and the error bars represent one standard deviation.

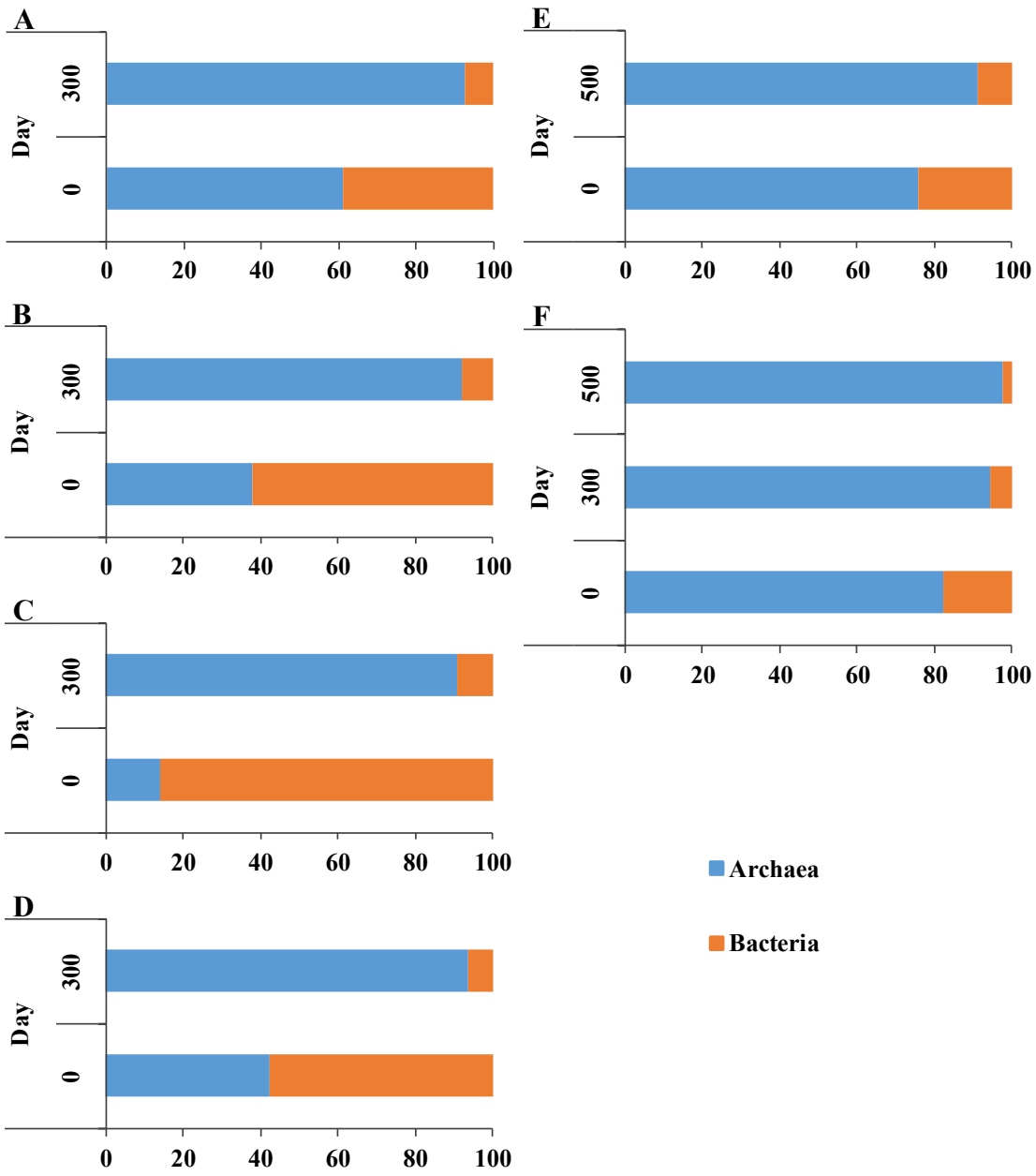

Figure B2: Archaeal and bacterial community proportions based on analysis of 16S rRNA gene pyrosequences before and during incubation of MFT with two- and four-alkane mixtures. (A) Albian MFT, two alkanes; (B) Albian MFT, four alkanes replicate A; (C) Albian MFT, four alkanes replicate B; (D) Albian MFT, four alkanes replicate C; (E) CNRL MFT, two alkanes; and (F) CNRL MFT, four alkanes. The results represent pooled amplicons of triplicate cultures, with the exception of Panel B, C and D that represents the single microcosm.

10 Appendix C: Supplementary Data for Chapter 4

Table C1: Relative abundance of bacterial 16S rRNA gene pyrosequences grouped at class level in Albian MFT under sulfate-reducing and methanogenic conditions and CNRL MFT under methanogenic conditions before degradation (day 0) and after incubation at time indicated with three *iso*-alkanes mixture. Genus assignations are shown where possible. "Others <5%" is the sum of all taxa individually abundant at <5% of the total bacterial reads.

Table C2: Relative abundance of archaeal 16S rRNA gene pyrosequences grouped at order level in Albian MFT under sulfate-reducing and methanogenic conditions and CNRL MFT under methanogenic conditions before degradation (day 0) and after incubation at time indicated with three *iso*-alkanes mixture. Genus assignations are shown where possible. "Others <5%" is the sum of all taxa individually abundant at <5% of the total archaeal reads.

Figure C1: Archaeal and bacterial community proportions based on analysis of 16S rRNA gene pyrosequences before and during incubation of MFT with *iso*-alkanes mixture. (A) Albian MFT under sulfate-reducing conditions; (B) Albian MFT under methanogenic conditions; and (C) CNRL MFT under methanogenic conditions. The results represent pooled amplicons of triplicate cultures.

Table C1: Relative abundance of bacterial 16S rRNA gene pyrosequences grouped at class level in Albian MFT under sulfate-reducing and methanogenic conditions and CNRL MFT under methanogenic conditions before degradation (day 0) and after incubation at time indicated with three *iso*-alkanes mixture. Genus assignations are shown where possible. "Others <5%" is the sum of all taxa individually abundant at <5% of the total bacterial reads.

Taxa, grouped at <5% distance level	Albian S-3I		Albian M-3I		CNRL M-3I	
	Day					
	0	500	0	300	0	800
Actinobacteria						
Coriobacteriales;Coriobacteriaceae	0.2	5.7	0.1	1.7	11.3	1.4
Anaerolineae						
Anaerolineales;Anaerolineaceae;Leptolinea	0.3	10.4	0.6	4.3	3.6	2.8
Anaerolineales;Anaerolineaceae;uncultured	0.6	15.3	0.4	5.1	10.3	2.8
Clostridia						
Clostridiales;Peptococcaceae	0.0	0.4	0.2	62.4	3.6	76.6
Clostridiales;Peptococcaceae;Cryptanaerobacter	0.1	10.1	0.0	1.7	0.0	0.0
Betaproteobacteria						
Hydrogenophilales;Hydrogenophilaceae;Thiobacillus	94.5	1.3	93.8	0.0	0.3	0.7
Rhodocyclales;Rhodocyclaceae;uncultured	0.1	0.6	0.0	0.9	8.7	0.0
Deltaproteobacteria						
Desulfobacterales;Desulfobacteraceae	0.0	9.6	0.0	0.0	0.1	0.0
Desulfobacterales;Desulfobulbaceae;Desulfocapsa	0.1	6.8	0.0	0.0	0.0	0.0
Syntrophobacterales;Syntrophaceae;Smithella	0.0	9.0	0.0	8.5	9.7	1.4
Others <5% (Number of taxa)	4.1 (27)	30.0 (79)	4.6 (41)	15.4 (11)	48.9 (111)	14.1 (11)

S-3I, MFT amended with three *iso*-alkane mixture under sulfate reducing conditions

M-3I, MFT amended with three *iso*-alkane mixture under methanogenic conditions

Table C2: Relative abundance of archaeal 16S rRNA gene pyrosequences grouped at order level in Albian MFT under sulfate-reducing and methanogenic conditions and CNRL MFT under methanogenic conditions before degradation (day 0) and after incubation at time indicated with three *iso*-alkanes mixture. Genus assignations are shown where possible. "Others <5%" is the sum of all taxa individually abundant at <5% of the total archaeal reads.

Taxa, grouped at <5% distance level	Albian S-3I		Albian M-3I		CNRL M-3I	
	Day					
	0	500	0	300	0	800
Methanobacteriales						
Methanobacteriaceae;Methanobacterium	8.2	42.6	8.5	0.6	0.4	0.0
Methanomicrobiales						
"*Candidatus* Methanoregula"	1.2	3.2	3.9	35.3	61.5	86.1
Methanolinea	0.3	0.1	8.9	14.6	0.0	0.1
Methanosarcinales						
Methanosaetaceae;Methanosaeta	70.3	44.2	64.1	44.6	33.3	10.5
Methanosarcinaceae;Methanosarcina	16.2	7.9	11.2	4.6	0.3	0.3
Others <5% (Number of taxa)	3.8 (6)	1.9 (10)	3.3 (8)	0.3 (5)	4.4 (11)	3.0 (5)

0% ≤  **≥ 86%**

S-3I, MFT amended with three *iso*-alkane mixture under sulfate reducing conditions

M-3I, MFT amended with three *iso*-alkane mixture under methanogenic conditions

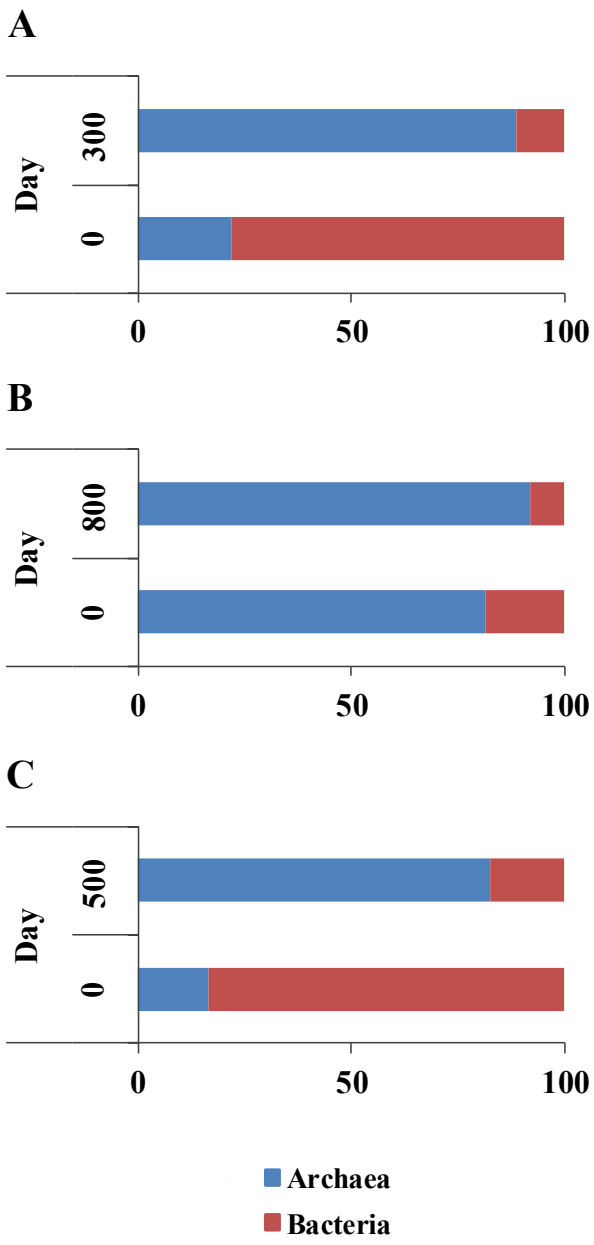

Figure C1: Archaeal and bacterial community proportions based on analysis of 16S rRNA gene pyrosequences before and during incubation of MFT with *iso*-alkanes mixture. (A) Albian MFT under methanogenic conditions; (B) CNRL MFT under methanogenic conditions; and (C) Albian MFT under sulfate-reducing conditions. The results represent pooled amplicons of triplicate cultures.

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Preferential methanogenic biodegradation of short-chain *n*-alkanes by microbial communities from two different oil sands tailings ponds

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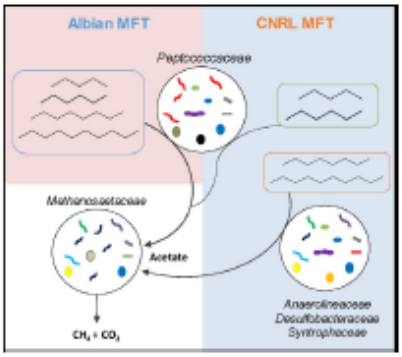
HIGHLIGHTS

- Residual hydrocarbons sustain methanogenesis in oil sands tailings ponds.
- Preferential alkane biodegradation was observed in different tailings.
- *Peptococcaceae* was the dominant bacterial taxa involved in the biodegradation.
- Acetoclastic methanogenesis was the main pathway.

GRAPHICAL ABSTRACT



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ABSTRACT

Oil sands tailings ponds harbor diverse anaerobic microbial communities capable of methanogenic biodegradation of solvent hydrocarbons entrained in the tailings. Mature fine tailings (MFT) from two operators (Albian and CNRL) that use different extraction solvents were incubated with mixtures of either two (*n*-pentane and *n*-hexane) or four (*n*-pentane, *n*-hexane, *n*-octane and *n*-decane) *n*-alkanes under methanogenic conditions for ~600 d. Microbes in Albian MFT began methane production by ~80 d, achieving complete depletion of *n*-pentane and *n*-hexane in the two-alkane mixture and their preferential biodegradation in the four-alkane mixture. Microbes in CNRL MFT preferentially metabolized *n*-octane and *n*-decane in the four-alkane mixture after a ~80 d lag but exhibited a lag of ~360 d before commencing biodegradation of *n*-pentane and *n*-hexane in the two-alkane mixture. 16S rRNA gene pyrosequencing revealed *Peptococcaceae* members as key bacterial *n*-alkane degraders in all treatments except CNRL MFT amended with the four-alkane mixture, in which *Anaerolineaceae*, *Desulfobacteraceae* (*Desulfobacterium*) and *Syntrophaceae* (*Smithella*) dominated during *n*-octane and *n*-decane biodegradation. *Anaerolineaceae* sequences increased only in cultures amended with the four-alkane mixture and only during *n*-octane and *n*-decane biodegradation. The dominant methanogens were acetoclastic *Methanosaetaceae*. These results highlight preferential *n*-alkane biodegradation by microbes in oil sands tailings from different producers, with implications for tailings management and reclamation.

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1. Introduction

The oil sands industry in northern Alberta, Canada has expanded rapidly in the recent years. Surface mining of oil sands ore and its treatment with hot water for bitumen extraction produces enormous volumes of fluid tailings that are deposited in large basins ('tailings ponds') where they are retained indefinitely, pending reclamation. The volume of tailings is projected to grow from the current volume of ~975 million m^3 (http://osip.alberta.ca/map/) to >1.2 billion m^3 by 2030 (Houlihan and Hale, 2011). Most oil sands tailings ponds are methanogenic and produce significant volumes of methane (CH_4), a potent greenhouse gas, as well as carbon dioxide (CO_2). Mildred Lake Settling Basin (MLSB), the largest and one of the oldest oil sands tailings ponds, has been estimated to emit ~43 million L CH_4 day^{-1} (Holowenko et al., 2000; Siddique et al., 2008). Methanogenesis drives many biogeochemical processes in such tailings ponds. For example, recent studies have shown that indigenous microbial communities in mature fine tailings (MFT) from MLSB accelerate pore water recovery and densification of tailings solids by changing pore water and solid phase chemistry while metabolizing organic substrates to CH_4 (Arkell et al., 2015; Siddique et al., 2014a,b). The dewatering and densification of tailings can potentially reduce the tailings inventory and speed up the reclamation of MFT, hence the interest in methanogenesis in these ponds.

Recovery of bitumen from surface-mined oil sands ores involves hydrocarbon solvent extraction, a small proportion of which (<1 vol%) cannot be recovered for reuse, and enters the ponds with fresh tailings. This fugitive solvent is the major substrate sustaining methanogenesis in the tailings ponds (Siddique et al., 2007). The solvent used by Syncrude Canada Ltd. is naphtha, comprising aliphatic and aromatic hydrocarbons primarily of ~C_6–C_{10}. Laboratory biodegradation studies have revealed that *n*-alkanes, monoaromatics (BTEX; benzene, toluene, ethylbenzene and xylene isomers), C_7 and C_8 *iso*-alkanes and a C_6 *cyclo*-alkane (methylcyclopentane) present in naphtha can be degraded under methanogenic conditions by indigenous microorganisms in MFT (Abu Laban et al., 2014; Siddique et al., 2006, 2007, 2012; Tan et al., 2015). Short-chain *n*-alkanes (C_6–C_{10}) representing naphtha-range hydrocarbons were degraded preferentially in the order: $C_{10} > C_8 > C_7 > C_6$ (Siddique et al., 2006), albeit no such preferential biodegradation was observed with longer-chain *n*-alkanes (C_{14}, C_{16}, C_{18}) (Siddique et al., 2011). The question arises whether such chain length discrimination is unique to MLSB microbes, or whether preferential degradation of alkane suites is more widespread in oil sands tailings ponds. The answer would be useful for predicting onset and duration of greenhouse gas production from oil sands tailings ponds operated under different conditions than MLSB.

Thus, we examined alkane biodegradation by microbes in MFT from two surface-mined oil sands companies, Albian Sands Inc. (Albian) and Canadian Natural Resources Ltd. (CNRL). Their tailings ponds are much younger than MLSB, allowing less time for anaerobic hydrocarbon-degrading microbial communities to be enriched: Albian's Muskeg River Mine pond is ~13 years old and the CNRL Horizon pond is ~6 years old, versus Syncrude's ~40-year-old MLSB pond. Tailings management also differs between the sites, with Syncrude and CNRL using naphtha containing C_6–C_{10} *n*-, *iso*- and *cyclo*-alkanes and monoaromatics, but Albian using a predominantly aliphatic solvent (C_5–C_6 *n*- and *iso*alkanes). Processes used to enhance consolidation of tailings solids in the ponds also differ, with Albian adding both trisodium citrate and organic polymer flocculants to tailings before deposition (Li, 2010) versus CNRL injecting CO_2 into tailings (http://www.cnrl.com/). These differences beg the question of whether microbial communities in each pond have adapted to efficiently biodegrade only the components of their cognate solvent, or are sufficiently metabolically flexible that they can adapt to degrade other solvent components. The anaerobic solvent biodegradation capability and key microbial players in Albian and CNRL tailings, which have not previously been published in detail, are of particular interest.

Because methanogenesis apparently drives some major geochemical processes in some oil sands tailings ponds (Siddique et al., 2014a,b) and little is known about methanogenic processes in Albian (Siddique et al., 2015) and CNRL MFTs, it is important to evaluate the metabolic flexibility of their indigenous microbial communities. Therefore in this study, we assessed the preferential biodegradation of two mixtures of short-chain *n*-alkanes (nC_5 and nC_6, or nC_5, nC_6, nC_8 and C_{10}, representing Albian and CNRL solvents, respectively) by microbes in Albian and CNRL MFT. We measured CH_4 production, determined hydrocarbon depletion and characterized microbial communities using pyrosequencing of 16S rRNA genes to infer the roles of different microbial groups in the methanogenic community during the biodegradation of these *n*-alkanes mixtures. This study contributes to the overall understanding of methanogenesis in oil sands tailings and its implications in tailings management.

2. Materials and methods

2.1. Chemicals and materials

n-Pentane (≥99%; CAS # 109-66-0), *n*-hexane (≥96%; CAS # 110-54-3) and *n*-octane (≥99%; CAS # 111-65-9) were purchased from Fisher Scientific, Ontario, Canada. *n*-Decane (≥99%; CAS # 124-18-5) was purchased from Sigma-Aldrich, Ontario, Canada.

Samples of methanogenic MFT were collected by respective oil sands operators (Albian and CNRL). MFT is a thick slurry of sediment (usually ≥30% solids), water, unextracted bitumen (usually ~5 vol%) and unrecovered solvent (≤1 vol%). Albian MFT was collected in bulk from the Muskeg River Mine Tailings Pond at 7 m below the water surface in 2008 (0465371 E 6342304 N; solids, 25 wt%; bitumen, 0.87 wt%; pH 8.07; conductivity 3.1 dS m^{-1}) and stored in the dark at 4 °C under a cap of tailings pond water, to maintain anaerobic conditions in the tailings sediments, for use as inoculum in 2011. We have found in numerous previous experiments that storage of MFT for several years under these conditions does not impair its ability to biodegrade hydrocarbons at a rate similar to that of fresh MFT samples. The CNRL MFT was collected from the Horizon tailings pond (446156 E 6356933.1 N) in 2011 (solids, 28 wt%; bitumen, 0.97 wt%; pH 8.6; conductivity 2.0 dS m^{-1}) and also stored in the dark at 4 °C until used as inoculum in the same year.

2.2. Culture conditions for hydrocarbon biodegradation

The experiment was conducted using anaerobic microcosms prepared in 158-mL sealed serum bottles as described previously (Siddique et al., 2006). Briefly, each microcosm received 50 mL each of Albian or CNRL MFT and anaerobic methanogenic medium lacking organic carbon (Fedorak and Hrudey, 1984), with a headspace of 30% O_2-free CO_2, balance N_2, at a slight overpressure to prevent incursion of atmospheric oxygen. The microcosms were pre-incubated in the dark at room temperature for 2 weeks to allow for microbial acclimation and consumption of residual hydrocarbons and alternative electron acceptors in MFT prior to amendment with new substrates (Siddique et al., 2006). Immediately prior to hydrocarbon addition, the microcosm headspace was flushed again with 30% CO_2, balance N_2, to remove any CH_4 produced during pre-incubation. The microcosms were then amended with either a two-alkane mixture of *n*-pentane (nC_5) and *n*-hexane (nC_6) or a four-alkane mixture of *n*-pentane (nC_5), *n*-hexane (nC_6), *n*-octane (nC_8) and *n*-decane (nC_{10}) at volumes corresponding to ~40 mg of each individual compound (nominally representing individual concentrations of ~400 mg L^{-1} of total culture volume) at room temperature. To confirm the initial concentrations of individual alkanes in the microcosms, samples were analyzed immediately after alkane addition (see below). Each treatment was prepared in triplicate. Duplicate viable baseline controls (unamended microcosms) were prepared to account for CH_4 production from any residual endogenous

substrates in the MFTs. To account for abiotic degradation, heat-killed (sterilized) controls were also prepared in the same way as the live cultures but autoclaved (121 °C, 20 psi, 60 min) four times on four consecutive days prior to alkane addition. The heat-killed microcosms were prepared in triplicate and amended with the four-alkane mixture. All microcosms were incubated at room temperature in the dark. The microcosms were subjected to headspace gas analyses to monitor CH_4 production over time and samples were retrieved periodically from the microcosms for hydrocarbon and 16S rRNA gene analyses.

2.3. Chemical analyses

All manipulations of the sealed microcosms were performed using small bore sterile needles and syringes. CH_4 production was measured by removing 50 μL headspace for direct injection into a gas chromatograph equipped with a flame ionization detector (GC-FID) as previously described (Holowenko et al., 2000). If high pressure developed in the microcosms due to CH_4 production, it was reduced by removing 30 mL or 60 mL headspace gas from two-alkane- and four-alkane-amended microcosms, respectively. The gas volume removed was taken into account when calculating the total mass of CH_4 produced.

To monitor biodegradation, concentrations of the residual alkanes were determined at intervals by removing 1-mL samples from hand-mixed microcosms and extracting hydrocarbons with 10 mL methanol (Fisher Scientific) in a 20-mL EPA glass vial. The vials were shaken for 30 min at 20 °C and the solids in the vials were allowed to settle for 30 min. One milliliter of the supernatant was transferred to a 44-mL EPA glass vial, filled completely with ultrapure water (Milli-Q Water System) to avoid any headspace and capped. All prepared vials were analyzed using a gas chromatograph fitted with a flame ionization detector (GC-FID) and equipped with a purge and trap system, as described previously (Siddique et al., 2006).

2.4. Stoichiometry of hydrocarbon biodegradation to cumulative methane production

The concentrations of *n*-alkanes remaining in the microcosms were determined at the final sampling point and used to calculate theoretical maximum CH_4 production using stoichiometric equations derived from the modified Symons and Buswell equation (Symons and Buswell, 1933) assuming complete metabolism of hydrocarbons to CH_4 and CO_2 under methanogenic conditions:

$$n-\text{pentane } (nC_5)\cdots C_5H_{12} + 2.0H_2O \rightarrow 1.00CO_2 + 4.00CH_4 \quad (1)$$

$$n-\text{hexane } (nC_6)\cdots C_6H_{14} + 2.5H_2O \rightarrow 1.25CO_2 + 4.75CH_4 \quad (2)$$

$$n-\text{octane } (nC_8)\cdots C_8H_{18} + 3.5H_2O \rightarrow 1.75CO_2 + 6.25CH_4 \quad (3)$$

$$n-\text{decane } (nC_{10})\cdots C_{10}H_{22} + 4.5H_2O \rightarrow 2.25CO_2 + 7.75CH_4 \quad (4)$$

The theoretical maximum CH_4 values were compared with the measured CH_4 values in the microcosm headspace to confirm the biodegradation of hydrocarbons.

2.5. Characterization of microbial communities

To study the microbial community structure, genomic DNA was extracted from MFTs at day 0 (on the day the hydrocarbon mixtures were spiked in the microcosms) and after the biodegradation of the added hydrocarbons, by using the protocol previously described (Foght et al., 2004) with slight modification. Briefly, DNA was extracted from triplicate 300-μL samples in screw-cap microcentrifuge tubes (Fisher Scientific) containing 1 g of 2.5 mm and 0.1 mm zirconia silica beads (1:1 w/w), phosphate buffer (100 mM NaH_2PO_4, pH 8.0), lysis buffer (100 mM NaCl, 500 mM Tris pH 8.0, 10% sodium dodecyl sulfate), and chloroform–isoamyl alcohol (24:1), and processed at 3400 revolutions per minute for 45 s in PowerLyzer™ 24 Bench Top Bead-based Homogenizer (MO BIO Laboratories Inc., Carlsbad, CA). The samples were then spun in a microfuge at full speed (15000 rpm) for 5 min to sediment the particles and the supernatant was recovered for subsequent DNA recovery. The extraction procedure was repeated on the same sample, without pooling the supernatants. For each supernatant, ammonium acetate (7 M) was added to 2.5 M final concentration, mixed gently and microcentrifuged to precipitate proteins. The supernatant was transferred to a new tube with 0.6 volumes of isopropanol and incubated overnight at 4 °C. DNA was precipitated by microfuge at full speed for 30 min. After decanting the isopropanol the DNA pellet was air-dried for 60 min and dissolved in 30 μl nuclease-free water (Integrated DNA Technologies). The DNA from both extractions of each sample was then pooled. The pooled DNA was analyzed for purity and concentrations using Nanodrop-1000 Spetrophotometer before used for PCR amplification.

Bacterial and archaeal 16S rRNA genes were amplified for 454 pyrotag sequencing using the universal primer set 454 T-RA/454 T-FB targeting the V6-V8 regions of the 16S rRNA gene for both Bacteria and Archaea (Berdugo-Clavijo et al., 2012). 16S rRNA gene amplification was performed in a S1000™ Thermal Cycler (BIO RAD) using the thermocycling temperature program described by Tan et al. (2013). Each 25-μL PCR reaction contained 2.5 μL (10 μM) of each primer, 5 μL 5X KAPA2G reaction buffer A (Kapa Biosystems, Woburn MA), 5 μL KAPA enhancer 1 solution, 1.25 μL 100% dimethylsulfoxide, 0.1 μL DNA polymerase (KAPA2G), 0.5 μL dNTP mix (10 mM, KAPA2G), 2 μL $MgCl_2$ (25 mM), 1 μL pooled genomic DNA extract (~50 ng μL^{-1}) and 4.65 μL sterile nuclease-free water. Three PCR replications were prepared for each sample and pooled after the PCR amplification. Negative controls consisting of only PCR reagents and nuclease-free water were included with every set of samples for quality control during PCR amplification. PCR products were examined for quality using agarose gel electrophoresis and purified using a QIAquick PCR purification kit (Qiagen). All purified PCR products from different replications within each treatment were pooled before sequencing except for samples of the triplicate Albian four-alkane cultures, where each microcosm was sequenced individually because of observed differences in hydrocarbon biodegradation.

16S rRNA amplicons were sequenced using a GS FLX Titanium Series Kit XLR70 (Roche) at McGill University Génome Québec Innovation Centre, Montreal, Canada. The raw pyrosequencing data were analyzed using the Phoenix 2 pipeline. (Soh et al., 2013) All pyrotag data sets (~1500–11500 reads per sample) have been submitted to NCBI Sequence Read Archive (SRA) under accession number SRP050020.

3. Results and discussion

3.1. Methane production during n-alkane biodegradation

Cumulative CH_4 production was monitored during incubation by analyzing the headspace of replicate microcosms (Fig. 1). No significant CH_4 was produced by any unamended baseline control cultures during the incubation. After a lag phase of ~80 d, CH_4 production from amended Albian MFT exceeded the baseline control and increased exponentially to 1.75 ± 0.07 and 2.79 ± 0.25 mmol in two-alkane and four-alkane mixtures, respectively by 213 d. Thereafter, CH_4 increased gradually to 1.99 ± 0.12 mmol in two-alkane mixture by 283 d and 3.94 ± 0.37 mmol in four-alkane mixture by 579 d (Fig. 1A).

The mass of CH_4 produced by CNRL MFT microcosms was similar to that produced by Albian MFT, but the patterns differed (Fig. 1B). A long lag phase (~360 d) was observed in the two-alkane CNRL microcosms, after which CH_4 increased to 2.21 ± 0.05 mmol by 590 d, similar to the mass produced by Albian MFT with these two substrates. CNRL MFT amended with the four-alkane mixture produced CH_4 after a short lag phase (86 d), followed by exponential CH_4 production until

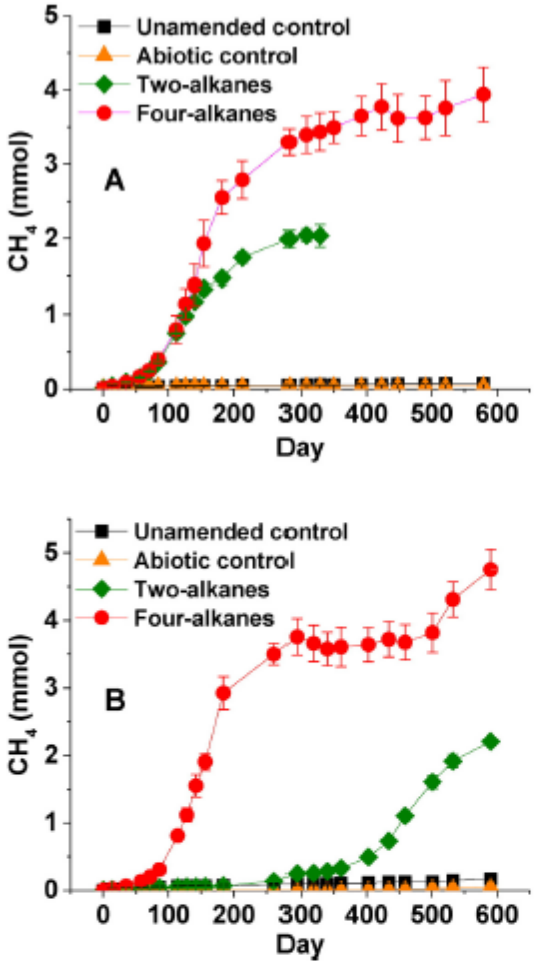

Fig. 1. Cumulative methane production in microcosms containing (A) Albian MFT or (B) CNRL MFT amended with either a two- or four-alkane mixture and incubated for ~600 days. The two- and four-alkane mixtures comprised *n*-pentane (nC_5) and *n*-hexane (nC_6), or *n*-pentane (nC_5), *n*-hexane (nC_6), *n*-octane (nC_8) and *n*-decane (nC_{10}), respectively. Unamended controls comprised MFT incubated without alkane addition. The abiotic controls were heat-killed and amended with the four-alkane mixture. Symbols represent the mean value from duplicate microcosms for unamended controls and triplicate microcosms for the rest of treatments and error bars, where visible, represent one standard deviation.

184 d (2.92 ± 0.24 mmol) then a plateau for approximately 300 d, after which CH_4 production resumed, reaching 4.75 ± 0.30 mmol by 590 d, the final sampling time. The latter pattern resembles diauxie, where a preferred substrate is metabolized first, followed by other substrate(s).

To track the carbon flow from substrates to greenhouse gases via microbial metabolism, we calculated the theoretical maximum CH_4 production using Eqs. (1)–(4) to compare with the measured CH_4 in experimental microcosms. CH_4 measured in the headspace ranged from 67 to 74% of the predicted (calculated) CH_4 (Table S1), slightly lower than the values we reported previously (77–79% of predicted CH_4) using MFT from MLSB (Siddique et al., 2006). The difference between measured and predicted CH_4 could be associated with microbial carbon assimilation, production of recalcitrant intermediary compounds (metabolites) and/or residual alkane loss through sampling (see below) (Fowler et al., 2012; Zengler et al., 1999).

3.2. Preferential biodegradation of n-alkanes

In all microcosms, the concentrations of nC_5 measured immediately after amendment (day 0) were lower (~200 mg L^{-1}) than predicted (~400 mg L^{-1}). This was likely due to volatility losses of nC_5, the smallest and most volatile substrate, during preparation of the alkane mixtures. However, analysis of the heat-killed control microcosms accounted for such abiotic losses and allowed calculation of alkane biodegradation.

GC-FID measurement of residual *n*-alkanes in the Albian MFT microcosms during incubation revealed preferential biodegradation of the smaller alkanes (C_5–C_6) corresponding to the composition of its aliphatic extraction solvent (also C_5–C_6), (Figs. 2 and 3). In Albian MFT amended with the two-alkane mixture, nC_5 and nC_6 (initially 216 ± 1.8 and 394 ± 39 mg L^{-1}, respectively) were completely depleted by 283 d versus only a slight decrease in the abiotic control microcosms (Fig. 2A). Analysis of the four-alkane-amended Albian MFT microcosms also showed complete depletion of nC_5 (initially 202 ± 3.5 mg L^{-1}), nC_6 (411 ± 9.1 mg L^{-1}) and nC_8 (381 ± 19 mg L^{-1}), with nC_{10} being the only alkane still detected in Albian MFT at 283 d (Fig. 3A). Complete removal of nC_{10} was observed in only one of the three Albian MFT replicates by day 392 (Fig. S1), and the other two microcosms still showed no degradation of nC_{10} by 521 d; this difference resulted in very large standard deviation values for these four-alkane Albian MFT microcosms (Fig. 3A). nC_{10} was eventually depleted in the two replicates by 929 d when the headspace was analyzed (data not shown). Sterilized Albian MFT (abiotic control microcosms) did not show any significant loss of the four amended alkanes during 521 d incubation (Fig. 3A).

CNRL preferentially degraded nC_8 and nC_{10}, reflecting the higher average molecular weight of its naphtha solvent (C_6–C_{10}). Analysis of the two-alkane-amended microcosms revealed that nC_5 and nC_6 were only slightly depleted (residual concentrations of 222 ± 15 and 300 ± 19 mg L^{-1}, respectively) from their initial concentrations (260 ± 1.8 and 386 ± 17 mg L^{-1}) (Fig. 2B) by 294 d and were completely biodegraded by 532 d. In the four-alkane-amended CNRL microcosms, complete biodegradation of nC_8 (initial concentration; 394 ± 21 mg L^{-1}) and nC_{10} (initial concentration; 459 ± 4.2 mg L^{-1}) had occurred by day 294 whereas nC_5 and nC_6 (residual concentrations of 169 ± 5.5 mg L^{-1} and 248 ± 12 mg L^{-1}, respectively) were only partially depleted compared to their initial concentrations (204 ± 10 and 343 ± 14 mg L^{-1}, respectively) (Fig. 3B). GC-FID analysis performed at day 403 still showed appreciable concentrations of nC_5 (132 ± 3.6 mg L^{-1}) and nC_6 (215 ± 5.3 mg L^{-1}) present in the cultures, with eventual depletion by day 532. Sterilized *n*-alkane-amended CNRL MFT showed no significant loss of amended alkanes during incubation (Fig. 3B).

Thus, microbes from two different ponds preferentially degraded components of their cognate solvents even though the range of alkane chain lengths differed by a maximum of five methylene bridges. The preferential biodegradation of longer chain *n*-alkanes in CNRL MFT is similar to sequential biodegradation by MLSB tailings of *n*-alkanes in the order of decreasing carbon chain length ($C_{10} > C_8 > C_7 > C_6$) (Siddique et al., 2006). However, Albian and CNRL MFT exhibited a longer lag phase (~80 d) prior to methane production from short-chain *n*-alkanes (C_6–C_{10}) than previously reported for Syncrude MFT (~35 d) (Siddique et al., 2006). This may be due to the fact that MLSB is older than the Albian and CRNL ponds, and over time its microbial community may have become more acclimatized to hydrocarbon biodegradation. It is possible that sequential transfer of the Albian and CNRL primary cultures and addition of fresh hydrocarbon substrate would result in shortened lag times.

The differences in biodegradation patterns exhibited by Albian and CNRL microcosms might be due to one or more of the following explanations: (1) the presence and abundance of different key microbes (primary hydrocarbon degrading species) enriched on the predominant endogenous hydrocarbons in each pond, each having a restricted range

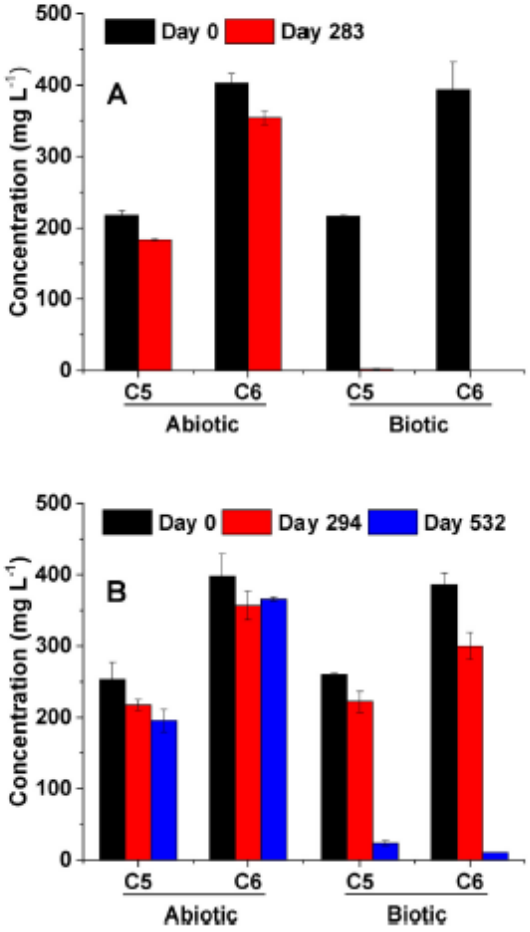

Fig. 2. Concentrations of individual residual *n*-alkanes in microcosms during incubation of (A) Albian amended with the two-alkane mixture, and (B) CNRL MFT amended with the two-alkane mixture. The bars represent the mean value of triplicate microcosms and error bars, where visible, represent one standard deviation.

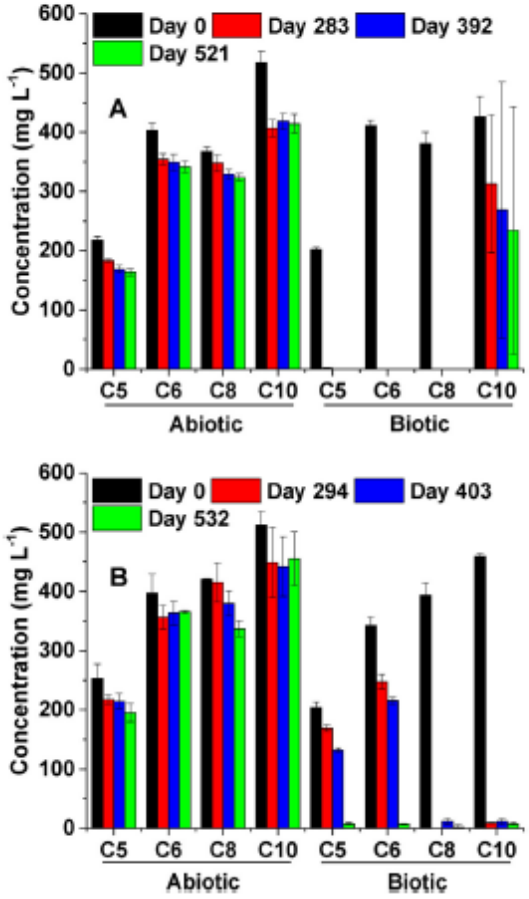

Fig. 3. Concentrations of individual residual *n*-alkanes in microcosms during incubation of (A) Albian MFT amended with the four-alkane mixture, and (B) CNRL MFT amended with the four-alkane mixture. The bars represent the mean value of triplicate microcosms and error bars, where visible, represent one standard deviation. Hydrocarbon analysis of individual replicates from four-alkane-amended Albian MFT is provided in Appendix A (Fig. S1).

of hydrocarbon substrates (Widdel et al., 2010); (2) different and/or selective hydrocarbon uptake mechanisms in such organisms (Kim et al., 2002; Widdel and Grundmann, 2010), associated with water solubility of those *n*-alkanes (ranging from 40 mg L^{-1} for nC_5 to 0.7 mg L^{-1} for nC_8 and insolubility for nC_{10}); (3) the activity of enzymes having different specificity or affinity for alkanes of particular chain length (Acosta-Gonzalez et al., 2013); and (4) the presence of syntrophic partners for such primary degraders in correct proportions, including both bacteria and methanogens (Widdel et al., 2010). Responding to a shift in predominant alkane substrates might involve recruitment and enrichment of competent species and/or syntrophs from the 'rare' microbiota, or acquisition and/or evolution of appropriate genes for expanded substrate range; the current study cannot distinguish between these two possibilities. However, in Albian MFT amended with four alkanes, nC_5, nC_6 and nC_8 were simultaneously biodegraded, suggesting that at least some of the initial nC_5- and nC_6-degraders were suited to nC_8 metabolism. Biodegradation of nC_{10} in one of the four-alkane amended Albian replicates at day 521 (Fig. S1) and very late biodegradation of nC_5-C_6 in CNRL MFT imply the enrichment of appropriate hydrocarbon degraders in response to the change in substrate composition.

3.3. Prokaryotic community structure during *n*-alkane biodegradation

The prokaryotic community in Albian and CNRL MFT cultures was monitored during incubation by pyrosequencing of partial 16S rRNA genes. The bacterial reads constituted 17–86% of the total quality-controlled reads at day 0, but during metabolism of *n*-alkanes the archaeal sequence reads became enriched, representing 91–98% of the total prokaryotic reads in all alkane-amended microcosms (Fig. S2).

For Albian MFT microcosms, two DNA sampling intervals (0 and 308 d) were selected to represent, respectively, the initial community structure and communities degrading the two- or four-alkane mixtures. The initial bacterial communities in two- and four-alkane-amended Albian MFT (constituting 39% and 62% of total prokaryotic reads, respectively) were similar in structure and comprised *Proteobacteria* (~33–38% of bacterial reads, with >22% *Hydrogenophilaceae*); *Chloroflexi* (~7–9% *Anaerolineaceae*); *Nitrospirae* (~7–9% *Nitrospiraceae*); *Actinobacteria* (~4–6% *Coriobacteriaceae*); and *Firmicutes* (~3–4% *Peptococcaceae*) (Fig. 4A and B; Table S2). After 308 d incubation with alkanes, the bacterial sequences represented only 7–8% of total reads (Fig. S2A–S2D). Furthermore, the bacterial communities became less diverse, with the detected number of 'rare' operational taxonomic

units (OTUs individually present at ≤5% abundance) decreasing from as many as 145 'rare' OTUs at time 0 to as few as 9 OTUs by 308 d for Albian MFT (Table S2). This decrease in rare taxa occurred as *Peptococcaceae*-related sequences increased to constitute ~78% and 54% of bacterial reads in two- and four-alkane-amended Albian MFT, respectively (Fig. 4A and B), and members of the *Chloroflexi* increased to ~12% in two-alkane- and ~26% in four-alkane-amended MFT. Because nC_{10} was not degraded by two out of three replicates of four-alkane-amended Albian MFT, we analyzed the communities in these microcosms individually (Table S2). Although the community structure shown in Fig. 4B is from the replicate exhibiting nC_{10} biodegradation, in fact the bacterial sequences were similar in all three replicates, with bacteria constituting 7–9% of total prokaryotic reads and *Peptococcaceae* (~54–63%) and *Anaerolineaceae* (~26%) being the dominant members (Table S2). This similarity in community structure of the replicates likely reflects the sequence of biodegradation of the smaller *n*-alkanes (nC_5, nC_6, and nC_8,) (Fig. S1): had the microcosms been sampled later (e.g., at 521 d incubation when nC_{10} had been depleted in one of the replicates), differences might have been observed in community structure between that microcosm and the two that did not degrade nC_{10} until much later. That is, the potential to biodegrade nC_{10} could not be predicted from the Albian MFT community profiles at 0 d or at 308 d.

In CNRL MFT at day 0, the bacterial communities comprised 24% and 17% of total prokaryotic reads in two- and four-alkane-amended microcosms, respectively and, as expected, the communities were similar (Fig. 4C and D). However, the initial community structure in CNRL MFT differed from that in Albian MFT: the dominant *Proteobacteria* in Albian MFT (*Hydrogenophilaceae*) were replaced in CNRL MFT by *Syntrophaceae* (~15–17% of total bacterial reads), along with greater proportions of *Chloroflexi* (14–17%) and *Actinobacteria* (11–13%) (Table S3). CNRL MFT incubated with the two-alkane mixture was sampled at 532 d, at which time significant CH_4 production was just beginning (Fig. 1B). At this point, the bacterial population represented only 9% of total prokaryotic reads (Fig. S2E). During incubation, sequences related to *Peptococcaceae* increased to constitute ~80% of bacterial reads (Fig. 4C) whereas all other bacterial taxa decreased in proportion compared with the initial CNRL community and the number of 'rare' OTUs decreased from 81 at 0 d to as few as 5 OTUs by 532 d (Table S3). For the four-alkane-amended CNRL MFT microcosms, two DNA sampling times were used: at 319 d during a plateau in methane

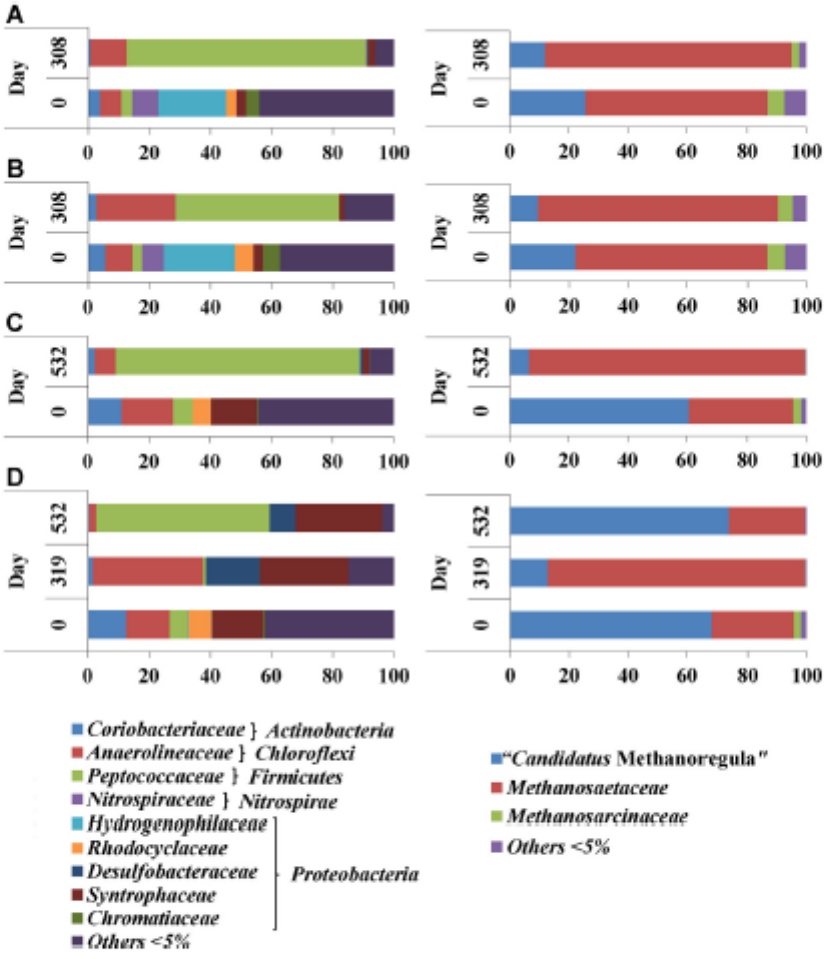

Fig. 4. Bacterial (left) and archaeal (right) community compositions based on analysis of 16S rRNA gene pyrosequences before and during incubation of MFT with two- and four-alkane mixtures. (A) Albian MFT, two alkanes; (B) Albian MFT, four alkanes; (C) CNRL MFT, two alkanes;, and (D) CNRL MFT, four alkanes. The results represent pooled amplicons of triplicate cultures, with the exception of Panel B that represents the single microcosm that was degrading decane. Quality-controlled pyrosequences were clustered at ≤5% distance and expressed as a percentage of total bacterial or archaeal reads. "Others <5%" is the sum of all taxa individually abundant at <5% of the total bacterial or archaeal reads. Detailed results including the number of taxa grouped under "Others <5%" are shown in Appendix A (Tables S2–S5).

production (Fig. 1B) and at 532 d when a second phase of methanogenesis was underway. Bacterial reads represented 5% and 2% of the total prokaryotic reads at these sampling points, respectively (Fig. S2F). At day 319 corresponding to depletion of nC_8 and nC_{10} but only partial degradation of nC_5 and nC_6, the bacterial community was enriched in members of the proteobacterial families *Syntrophaceae* (~29%) and *Desulfobacteraceae* (~18%). *Anaerolineaceae* within the *Chloroflexi* (Fig. 4C and D) were also enriched from ~13% at 0 d to 34% at 319 d, but *Peptococcaceae*-related sequences had become undetectable by this sample time. The community composition changed significantly again by 532 d incubation when nC_5 and nC_6 were finally depleted in the four-alkane amended MFT. At this sampling point, the members of *Peptococcaceae* had become greatly enriched (~57% of bacterial reads) in the presence of *Syntrophaceae* (~28%) and *Desulfobacteraceae* (~9%) whereas *Anaerolineaceae* reads had decreased to ~2% of the bacterial sequences (Table S3).

The tremendous increase (up to 28-fold; Tables S2 and S3) in the proportion of sequences related to *Peptococcaceae* in the alkane-amended treatments, except CNRL MFT amended with four-alkane mixture in which *Peptococcaceae*-related sequences declined during biodegradation of nC_8–C_{10} but later dominated during the biodegradation of nC_5 and nC_6, suggest that *Peptococcaceae* members are the primary oxidizers of nC_5 and nC_6 and may play a lesser role in mineralization of nC_8 and nC_{10} in CNRL MFT. The dominance of *Peptococcaceae* has also been observed during methanogenic biodegradation of *iso*-alkanes (2-methylbutane, 2-methylpentane and 3-methylpentane) in Albian and Syncrude MFT (Siddique et al., 2015). Other reports have implicated members of *Peptococcaceae* as potential primary degraders of monoaromatic (Abu Laban et al., 2010; Herrmann et al., 2010; Kunapuli et al., 2007; van der Zaan et al., 2012; Winderl et al., 2010) and aliphatic (Kniemeyer et al., 2007; Rios-hernandez et al., 2003) compounds under various anaerobic conditions. Transcript mapping of a *Peptococcaceae*-affiliated spp. genome (Tan et al., 2013) demonstrated high expression of genes involved in activation of alkanes via fumarate addition pathway and beta-oxidation, further supporting the proposal of *Peptococcaceae* as primary nC_5 and nC_6 degraders in Albian and CNRL MFT. The significant enrichment of *Anaerolineaceae* sequences in microcosms amended with the four-alkane mixture suggests that members of the *Anaerolineaceae* may either be directly involved in activation and biodegradation of nC_8 and nC_{10} or may act as scavengers of metabolic intermediates, as suggested by Kleinsteuber et al. (2012). Sequences affiliated with *Desulfobacterium* (*Desulfobacteraceae*) and *Smithella* (*Syntrophaceae*) were enriched only in CNRL MFT amended with four-alkanes during degradation of nC_8 and nC_{10}, implying a role in biodegradation of longer chain *n*-alkanes. These observations agree with other studies where cultures grown on long-chain *n*-alkanes (C_{12}–C_{20}) became enriched with *Desulfobacteraceae* (Aeckersberg et al., 1998; Siddique et al., 2012; So and Young, 1999) and/or *Syntrophaceae* (Cheng et al., 2013; Gray et al., 2011; Siddique et al., 2011, 2012; Tan et al., 2014b,c). The single Albian MFT replicate to degrade nC_{10} by 308 d incubation did not show enrichment of *Desulfobacteraceae* and *Syntrophaceae*, and unfortunately we have no community analysis at a later time to determine whether these taxa are also responsible for longer chain alkanes in Albian MFT.

The archaeal communities in Albian MFT initially were dominated by acetoclastic methanogens belonging to the family *Methanosaetaceae* (~61–65% of archaeal reads), followed by hydrogenotrophic methanogens belonging to "*Candidatus* Methanoregula" (22–26%) (Fig. 4A and B; Table S4). The proportion of *Methanosaetaceae* reads increased to ~81–84% of archaeal sequences during biodegradation of both two- and four-alkane mixtures by day 308. In CNRL MFT, the initial (0 d) archaeal communities were dominated by members of "*Candidatus* Methanoregula" (~61–68%) and *Methanosaetaceae* (~28–35%) (Fig. 4C and D; Table S5). These proportions shifted to domination by *Methanosaetaceae* (~87–93%) during biodegradation of the two-alkane mixture by 532 d and by 319 d in the four-alkane amended MFT. The proportions of Archaea in the latter microcosms subsequently shifted back towards "*Candidatus* Methanoregula" (~74%) and *Methanosaetaceae* (~26%) by day 532.

The results suggest that acetoclastic methanogenesis is the major pathway active during *n*-alkane biodegradation, as observed in previous *n*-alkane studies (Siddique et al., 2012; Zengler et al., 1999). Nonetheless, the importance of hydrogenotrophic methanogens cannot be ruled out, particularly in CNRL MFT. Syntrophic biodegradation of alkanes by alkane-degrading bacteria (*Peptococcaceae* and/or *Syntrophaceae*) produces hydrogen or formate under methanogenic conditions (Sieber et al., 2010, 2012; Tan et al., 2014a) and there must be a hydrogen-consuming partner (hydrogenotrophs) to drive this process thermodynamically, which explains the presence of hydrogenotrophs in our study. Hydrogenotrophic methanogenesis has been shown to be the primary methanogenic pathway in cultures amended with crude oil (Gray et al., 2011; Mayumi et al., 2011), long-chain *n*-alkanes (C_{15}–C_{20}) (Zhou et al., 2012) and monoaromatics (BTEX) (Siddique et al., 2012). Other studies have reported relatively equal occurrence of acetoclastic and hydrogenotrophic methanogenesis in a crude-oil degrading culture (Morris et al., 2012) and MFTs (Siddique et al., 2011, 2012, 2015).

4. Conclusions

The current study demonstrates that indigenous microorganisms in Albian and CNRL MFT preferentially metabolize *n*-alkanes present in their respective tailings ponds but now have been shown to be capable of expanding their substrate range to include 'unfamiliar' *n*-alkanes by changing bacterial and archaeal community compositions. Such shifts may require lag times of up to 1 year. These results are important for understanding the nature of anaerobic hydrocarbon degradation processes that could aid in devising appropriate strategies for management of greenhouse gas emissions and remediation of oil sands tailings ponds, in addition to providing insights into methanogenic degradation of alkanes in other contaminated environments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.02.061.

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Appendix A: Supplementary Data

Preferential methanogenic biodegradation of short-chain *n*-alkanes by microbial communities from two different oil sands tailings ponds

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The supplementary data contain 5 tables (Table S1-S5) and 2 figures (Fig. S1 and S2)

Table S1. Predicted and measured methane (CH_4) yields from MFT incubated with two- or four-alkane mixtures.

		Incubation time (d)	Substrate Consumed (mmol) *	Predicted CH_4 Yield (mmol) §	Measured CH_4 Yield (mmol)	Percent of theoretical production
Albian two-alkane	C_5	283	0.40 (± 0.03)¶	2.90 (± 0.21)	1.99 (± 0.12)	68.8
	C_6		0.67 (± 0.08)			
Albian four-alkane	C_5	521	0.21 (± 0.02)	5.07 (± 0.86)	3.76 (± 0.37)	74.0
	C_6		0.41 (± 0.05)			
	C_8		0.30 (± 0.10)			
	C_{10}		0.05 (± 1.06)			
CNRL two-alkane	C_5	532	0.27 (± 0.00)	2.88 (± 0.12)	1.91 (± 0.09)	66.5
	C_6		0.40 (± 0.02)			
CNRL four-alkane	C_5	532	0.17 (± 0.01)	6.27 (± 0.19)	4.31 (± 0.26)	68.7
	C_6		0.35 (± 0.02)			
	C_8		0.27 (± 0.02)			
	C_{10}		0.28 (± 0.00)			

* Calculated from the difference of measured initial alkane concentrations, taking into account abiotic losses, and residual alkane concentrations at incubation time indicated.

§ Calculated using Eqs 1-4 (main text) and the calculated mass of substrate consumed.

¶ All values represent the mean from analysis of triplicate microcosms (±1 standard deviation).

Table S2. Relative abundance of bacterial 16S rRNA gene pyrosequences grouped at class level in Albian MFT before degradation (day 0) and after incubation for 308 d with either the two- or four-alkane mixture. Genus assignations are given where possible. Results from the two-alkane treatment represent analysis of pooled amplicons for triplicate microcosms. Results for the four-alkane treatment are from analysis of individual microcosms, as the cultures showed different biodegradation patterns (Fig. 1 and Fig. S1). "Others <5%" is the sum of all taxa individually abundant at <5% of the total bacterial reads.

Taxa, assigned at ≤5% sequence difference	Albian two-alkane		Albian four-alkane (A)[1]		Albian four-alkane (B)[2]		Albian four-alkane (C)[2]	
	Day							
	0	308	0	308	0	308	0	308
Actinobacteria								
Coriobacteriales; Coriobacteriaceae	3.9	0.7	5.7	2.7	0.4	3.2	4.9	1.5
Anaerolinea								
Anaerolineales; Anaerolineaceae; Leptolinea	1.8	2.6	2.4	9.8	1.0	6.4	3.0	8.7
Anaerolineales; Anaerolineaceae; Levilinea	1.7	1.1	1.8	2.7	0.3	5.1	1.1	3.2
Anaerolineales; Anaerolineaceae; uncultured	3.3	8.1	4.7	13.4	0.9	14.7	5.5	14.6
Clostridia								
Clostridiales; Peptococcaceae	0.1	77.1	0.4	50.9	0.0	57.1	0.3	57.6
Clostridiales;Peptococcaceae; Cryptanaerobacter	2.7	1.2	2.2	2.7	0.1	0.0	1.7	5.8
Nitrospira								
Nitrospirales; Nitrospiraceae	8.5	0.2	7.0	0.0	0.2	0.0	7.7	0.0
Betaproteobacteria								
Hydrogenophilales; Hydrogenophilaceae; Thiobacillus	22.1	0.1	23.4	0.0	93.6	0.0	24.1	0.0
Rhodocyclales; Rhodocyclaceae; uncultured	3.3	0.0	6.0	0.0	0.4	0.0	5.7	0.0
Gammaproteobacteria								
Chromatiales; Chromatiaceae	4.3	0.0	5.6	0.0	0.1	1.3	5.4	0.2
Others <5% (Number of taxa)	48.1 (90)	8.7 (29)	40.9 (132)	17.9 (9)	3.2 (29)	12.2 (10)	40.5 (145)	8.5 (21)

[1] Replicate that showed complete biodegradation of nC_{10} in four-alkane mixture. These results were used to construct Fig. 4B (left).

[2] Replicates that did not show significant biodegradation of nC_{10}.

Table S3. Relative abundance of bacterial 16S rRNA gene pyrosequences grouped at class level in CNRL MFT before degradation (day 0) and after incubation for 319 d and 532 d with either the two- or four-alkane mixture. Genus assignations are shown where possible. "Others <5%" is the sum of all taxa individually abundant at <5% of the total bacterial reads.

Taxa, assigned at <5% sequence difference	CNRL two-alkane		CNRL four-alkane		
	Day				
	0	532	0	319	532
Actinobacteria					
Coriobacteriales; Coriobacteriaceae	11.0	2.3	12.6	1.7	0.5
Anaerolinea					
Anaerolineales; Anaerolineaceae; Leptolinea	4.1	2.1	3.6	14.8	0.9
Anaerolineales; Anaerolineaceae; uncultured	12.2	4.2	9.2	18.8	1.4
Clostridia					
Clostridiales; Peptococcaceae	4.1	79.5	3.4	0.0	56.6
Betaproteobacteria					
Rhodocyclales; Rhodocyclaceae; uncultured	5.9	0.1	7.8	0.0	0.0
Deltaproteobacteria					
Desulfobacterales; Desulfobacteraceae; Desulfobacterium	0.0	0.7	0.2	17.7	8.5
Syntrophobacterales; Syntrophaceae; Smithella	8.9	0.7	9.0	28.4	28.3
Others <5% (Number of taxa)	53.8 (80)	10.3 (48)	54.2 (81)	18.6 (26)	3.8 (5)

Table S4. Relative abundance of archaeal 16S rRNA gene pyrosequences grouped at order level in Albian MFT before degradation (day 0) and after incubation for 308 d with either the two- or four-alkane mixture. Genus assignations are shown where possible. Results for the four-alkane treatment are from analysis of individual microcosms, as the cultures showed different biodegradation patterns (Fig. 1 and Fig. S1). "Others <5%" is the sum of all taxa individually abundant at <5% of the total archaeal reads.

Taxa, assigned at <5% sequence difference	Albian two-alkane		Albian four-alkane (A)[1]		Albian four-alkane (B)[2]		Albian four-alkane (C)[2]	
	Day							
	0	308	0	308	0	308	0	308
Methanobacteriales								
Methanobacteriaceae; Methanobacterium	3.9	0.3	3.4	2.4	9.3	0.0	3.9	0.5
Methanomicrobiales								
"*Candidatus* Methanoregula"	25.8	11.9	22.2	9.5	3.2	11.9	24.5	15.4
Methanosarcinales								
Methanosaetaceae; Methanosaeta	61.2	83.2	64.4	80.8	65.1	83.5	64.4	79.1
Methanosarcinaceae; Methanomethylovorans	3.9	0.1	5.1	0.1	1.8	0.2	3.4	0.1
Methanosarcinaceae; Methanosarcina	1.8	2.4	0.9	4.9	17.8	0.1	0.8	2.2
Others <5% (Number of taxa)	3.5 (6)	2.1 (7)	3.9 (7)	2.3 (5)	2.8 (5)	4.3 (4)	2.9 (10)	2.6 (6)

[1] Replicate that showed complete biodegradation of nC_{10} in four-alkane mixture. These results were used to construct Fig. 4B (right).

[2] Replicates that did not show significant biodegradation of nC_{10}.

Table S5. Relative abundance of archaeal 16S rRNA gene pyrosequences grouped at order level in amended CNRL MFT before degradation (day 0) and after incubation for 319 d and 532 d with either the two- or four-alkane mixture. Genus assignations are shown where possible. "Others <5%" is the sum of all taxa individually abundant at <5% of the total archaeal reads.

Taxa, assigned at <5% sequence difference	CNRL two-alkane		CNRL four-alkane		
	Day				
	0	532	0	319	532
Methanomicrobiales					
"*Candidatus* Methanoregula"	60.7	6.6	67.8	12.7	73.6
Methanosarcinales					
Methanosaetaceae; Methanosaeta	34.9	92.9	27.8	86.6	25.9
Others <5% (Number of taxa)	4.4 (9)	0.5 (7)	4.4 (8)	0.6 (7)	0.5 (9)

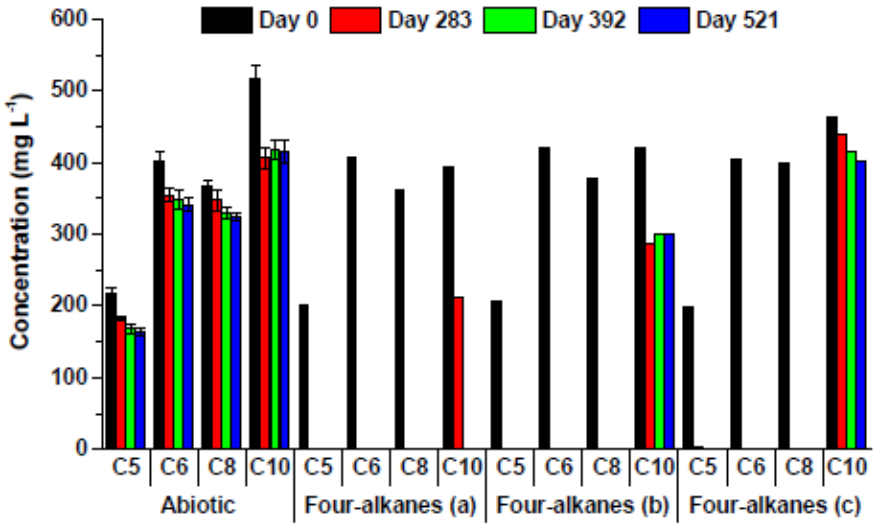

Fig. S1. Measured concentrations of *n*-alkanes in individual replicate Albian MFT microcosms during 521 d incubation with the four-alkane mixture. Values for the abiotic control are means of triplicate microcosms and the error bars represent one standard deviation.

Title: Long-Term Incubation Reveals Methanogenic Biodegradation of C5 and C6 iso-Alkanes in Oil Sands Tailings

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Long-Term Incubation Reveals Methanogenic Biodegradation of C_5 and C_6 *iso*-Alkanes in Oil Sands Tailings

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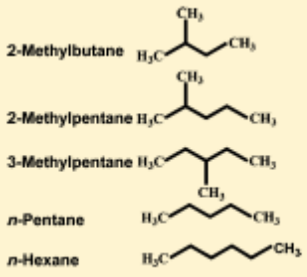
Supporting Information

ABSTRACT: *iso*-Alkanes are major components of petroleum and have been considered recalcitrant to biodegradation under methanogenic conditions. However, indigenous microbes in oil sands tailings ponds exposed to solvents rich in 2-methylbutane, 2-methylpentane, 3-methylpentane, *n*-pentane, and *n*-hexane produce methane in situ. We incubated defined mixtures of *iso*- or *n*-alkanes with mature fine tailings from two tailings ponds of different ages historically exposed to different solvents: one, ~10 years old, receiving C_5–C_6 paraffins and the other, ~35 years old, receiving naphtha. A lengthy incubation (>6 years) revealed *iso*-alkane biodegradation after lag phases of 900–1800 and ~280 days, respectively, before the onset of methanogenesis, although lag phases were shorter with *n*-alkanes (~650–1675 and ~170 days, respectively). 2-Methylpentane and both *n*-alkanes were completely depleted during ~2400 days of incubation, whereas 2-methylbutane and 3-methylpentane were partially depleted only during active degradation of 2-methylpentane, suggesting co-metabolism. In both cases, pyrotag sequencing of 16S rRNA genes showed codominance of *Peptococcaceae* with acetoclastic (*Methanosaeta*) and hydrogenotrophic (*Methanoregula* and *Methanolinea*) methanogens. These observations are important for predicting long-term greenhouse-gas emissions from oil sands tailings ponds and extend the known range of hydrocarbons susceptible to methanogenic biodegradation in petroleum-impacted anaerobic environments.



INTRODUCTION

Methanogenic biodegradation of hydrocarbons has been an area of great interest in recent years due to its implication in the formation of heavy oils, its potential for enhanced recovery of petroleum from marginally productive oil fields, and the remediation of hydrocarbon-contaminated anoxic environments.[1] Oil sands tailings ponds in northern Alberta, Canada are engineered environments where indigenous microbes have the potential to biodegrade residual hydrocarbons in oil sands tailings under methanogenic conditions. Methanogenesis sustained by hydrocarbon metabolism drives many biogeochemical processes in tailings ponds that lead to greenhouse-gas emissions from tailings ponds,[2,3] the dewatering and densification of tailings,[4] and the transformation of clay minerals,[5] which can affect the mobility of some trace metals in the porewater of tailings (unpublished data). The dewatering and densification of tailings can potentially reduce the volume of stored tailings and speed up the reclamation process.

The surface mining of oil sands ores followed by bitumen extraction using the modified Clark hot-water extraction process[6] generates enormous volumes of oil sands tailings that are retained indefinitely in tailings ponds. The current footprint of oil sands tailings ponds covers an area of 220 km^2, containing ~975 million m^3 of fluid fine tailings (http://osip.alberta.ca/map/), a colloidal slurry of water, silt, clay, unextracted bitumen (~5 wt %), and unrecovered solvent (<1 wt %) used in bitumen extraction. Tailings that have aged in situ and achieved ≥30% solids content are called mature fine tailings (MFT). The solvent used by most oil sands operators is naphtha, a hydrocarbon mixture composed of C_3–C_{14} paraffins (primarily *n*-, *iso*-, and *cyclo*-alkanes) and monoaromatics (BTEX; benzene, toluene, ethylbenzene, xylenes).[7] Although these components are volatile and have been detected above the oil sands area,[8] some of their potential evaporative loss from the ponds must be offset by the density of the tailings in situ because naphtha components are detected throughout depth profiles corresponding to >30 years of tailings deposition (K. Semple et al., unpublished data). Methane (CH_4) emissions[2] from Mildred Lake Settling Basin (MLSB, the oldest and largest tailings pond operated by Syncrude Canada Ltd., "Syncrude") are primarily due to microbial metabolism of these residual naphtha hydrocarbons. Our previous studies show that MLSB harbors well-developed indigenous microbial communities[9,10] that biodegrade *n*-alkanes and some BTEX components in naphtha to CH_4, whereas other components such as *iso*- and *cyclo*-alkanes remain recalcitrant to biodegradation during year-long incubation.[7,11]

In contrast to Syncrude's MLSB commissioned ~35 years ago, Shell Albian Sands ("Albian") began operations ~10 years

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ago with the Muskeg River Mine tailings pond by using a light paraffinic solvent (C_5–C_6, 85% of which is composed of *n*-alkanes and *iso*-alkanes) to aid bitumen extraction. The paraffinic solvent lacks the readily degradable longer-chain *n*-alkanes (C_7–C_{10}) and some BTEX compounds (toluene and *o*-xylene) present in Syncrude naphtha that are preferentially biodegraded in MLSB under methanogenic conditions.[7,11] However, C_5 and C_6 *iso*-alkanes constitute ~50% of Albian's paraffinic solvent and might represent a significant in situ carbon source if they were biodegradable. Methanogenic enrichment cultures established from Syncrude MFT recently were shown to utilize certain C_6, C_7, and C_8 *iso*-alkanes, whereas others resisted biodegradation.[12,13] However, the methanogenic biodegradation of other C_5 and C_6 *iso*-alkane components of the paraffinic solvent has not yet been demonstrated by either Syncrude or Albian MFT. For the long-term development of the Albian tailings ponds, it is important to understand the process, rate, and longevity of methanogenesis, a primary driver of biogeochemical processes in MFT, to help manage and reclaim the tailings and predict greenhouse-gas emissions. The findings can also assist in assessing reclamation strategies such as end-pit lakes, where sustained methanogenesis in MFT may influence the mobilization and transport of contaminants to the overlying cap water in the lake.

In this study, we assessed the methanogenic biodegradation of selected hydrocarbons comprising Albian paraffinic solvent, particularly highlighting the biodegradation of *iso*-alkanes. We wished to contrast the *iso*-alkane biodegradation potential of Albian and Syncrude tailings ponds due to their different solvent exposure and different deposition ages for the enrichment of competent anaerobic hydrocarbon-degrading communities in situ. This study also provided the opportunity to observe the response of an indigenous microbial community acclimatized to biodegrade a particular solvent when exposed to a different solvent. Rather than using the chemically complex authentic solvent as substrate, we formulated artificial mixtures of the dominant *iso*- and *n*-alkanes to simplify chemical analysis and added the mixtures to tailings individually at environmentally relevant concentrations. Furthermore, we incubated the cultures for extended times to allow the adaptation of the microbial communities to recalcitrant substrates. Hydrocarbon depletion and CH_4 production were monitored with intermittent analysis of the microbial communities by the use of pyrotag sequencing of 16s rRNA genes to infer the identity of key players in C_5 and C_6 *iso*-alkane biodegradation.

EXPERIMENTAL SECTION

Chemicals and Materials. 2-Methylbutane (2-MC_4; CAS no. 78-78-4, >99% purity), 2-methylpentane (2-MC_5; CAS no. 107-83-5, >99% purity) and 3-methylpentane (3-MC_5; CAS no. 96-14-0, >99% purity) were purchased from Sigma-Aldrich. *n*-Pentane (nC_5; CAS no. 109-66-0, >99% purity) was purchased from Caledon, Ontario, Canada, and *n*-hexane (nC_6; CAS no. 110-54-3, >95% purity) and methanol (CAS no. 67-56-1, >99% purity) were purchased from Fisher Scientific.

The Albian MFT was collected in bulk from the Muskeg River Mine tailings pond at a depth of 7 m below the surface (UTM coordinates, 0465371E 6342304N; solids, 25 wt %; bitumen, 0.87 wt %; pH 8.07; conductivity 3.1 dS m^{-1}) in June 2007 and used immediately. The Syncrude MFT was collected from the southern portion of MLSB at 7 m below the water surface in July 2005 (solids, 39.5 wt %; bitumen, 4.4 wt %; pH 7.8; conductivity 4.2 dS m^{-1} reported by Siddique et al.[11]) and stored in the dark at 4 °C for use in the experiments.

To select the appropriate *iso*-alkanes for the experiment, we analyzed the paraffinic solvent used by Albian by using a high-resolution procedure [method CAN/CGSB-3.0 no. 14.3, performed by Alberta Innovates–Technology Futures (Fuels & Lubricants), Edmonton, Alberta, Canada] developed for the determination of paraffins, olefins, naphthenes, aromatics, and unknown components (PONAU) in automotive gasolines. A total of five major hydrocarbons including nC_5 (24 wt %), nC_6 (11 wt %), 2-MC_4 (12 wt %), 2-MC_5 (24 wt %), and 3-MC_5 (13 wt %) constituted ~85% of the Albian paraffinic solvent. These hydrocarbons were added to Albian and Syncrude MFT as mixed substrates as described below to monitor biodegradation.

Experimental Setup for Hydrocarbon Biodegradation. To study the biodegradation of hydrocarbons by Albian MFT microbes, we prepared 158 mL anaerobic microcosms in sealed serum bottles as described previously.[11] Briefly, each microcosm received 50 mL each of Albian MFT and sterile methanogenic medium lacking organic carbon, with a headspace of 30% O_2-free CO_2 and balance N_2. An artificial mixture of *n*-alkanes was prepared from equal volumes of nC_5 and nC_6 and a mixture of *iso*-alkanes from equal volumes of 2-MC_4, 2-MC_5, and 3-MC_5. The mixtures were added individually to sealed triplicate microcosms to achieve environmentally relevant final concentrations of either ~0.1 or 0.15 wt % (~1000 or 1500 ppm total hydrocarbon), respectively, calculated based on the total volume of MFT plus medium.

To investigate the biodegradation of hydrocarbons in Syncrude MFT, we transferred 20 mL of a methanogenic naphtha-degrading culture (prepared from Syncrude MFT and substrate-free methanogenic medium as described above and incubated for ~125 d) to duplicate serum bottles containing 75 mL of methanogenic medium. After that, either the *n*-alkane or *iso*-alkane mixture was added to achieve final concentrations of either ~0.04 or 0.06 wt % (400 or 600 ppm total hydrocarbon), respectively.

Duplicate viable "baseline" control cultures consisting of MFT and medium without any additional hydrocarbons were prepared with respective MFT samples (Albian and Syncrude) and included in both experiments to account for the metabolism of endogenous substrates in the MFT. All cultures were incubated stationary in the dark at ~20 °C, which is similar to the in situ temperatures in the tailings ponds.[14] After the onset of CH_4 production, samples were periodically retrieved from the cultures for residual hydrocarbon measurement and 16S rRNA gene analyses.

Chemical Analyses. To determine CH_4 production in the sealed culture bottles, we removed 0.1 mL headspace using a sterile needle and syringe and injected it directly into a gas chromatograph (GC) with a flame ionization detector (FID).[15] The depletion of hydrocarbons during methanogenesis was quantified by GC-FID (Hewlett-Packard 6890) equipped with a purge and trap system (GC–PT) using a previously described protocol.[11] During the long incubation, residual volatile alkanes were also analyzed periodically by directly injecting 20 μL of headspace into an Agilent 5673 GC–mass spectrometry (GC–MS) system with a DB5-MS capillary GC column. The column was held at 35 °C for 7 min, and then temperature was increased at 10 °C min^{-1} to 100 °C. Helium was used as a carrier gas with a flow rate of 1.1 mL min^{-1} under splitless conditions. The relative peak areas of individual compounds

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were used to qualitatively assess the degradation of volatile alkanes compared with 1,1,3-trimethylcyclohexane that is endogenous to the MFT and was used as a conserved internal standard.[16]

The concentrations of the degraded hydrocarbons under methanogenic conditions were used to calculate the theoretical maximum CH_4 production using stoichiometric equations derived from the Symons and Buswell equation:[17]

$$2\text{-}MC_4 \text{ or } nC_5: C_5H_{12} + 2H_2O \rightarrow CO_2 + 4CH_4 \quad (1)$$

$$2\text{-}MC_5, 3\text{-}MC_5, \text{ or } nC_6: C_6H_{14} + 2.5H_2O \rightarrow 1.25CO_2 + 4.75CH_4 \quad (2)$$

Characterization of Microbial Communities. The microbial community structure in the microcosms was studied at intervals during methanogenic hydrocarbon biodegradation by pyrosequencing 16S rRNA genes using the protocol previously described.[5] Briefly, total genomic DNA was extracted from triplicate 300 μL culture subsamples using bead-beating, as in previously described reagents and conditions.[18] Extracted and then precipitated DNA was pooled for each culture and PCR-amplified in triplicate 25 μL reactions using previously validated universal primers targeting bacteria and archaea.[5,12,13,19] The touchdown PCR program consisted of one initial 5 min denaturation period at 95 °C; 10 cycles of [30 s at 95 °C, 30 s at 60 °C decreasing by 0.5 °C/cycle, and 30 s at 72 °C]; 30 cycles of [30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C]; and one final 5 min extension period at 72 °C. Negative controls consisting of only extraction reagents or only PCR reagents were included with every set of samples for quality assurance and quality control during DNA extraction and PCR amplification. PCR products were examined for quality control by electrophoresis on an agarose gel, purified using a QIAquick PCR purification kit (Qiagen), and pooled for sequencing. Purified PCR products were sent to Genome Quebec Innovation Centre (Montreal, Quebec, Canada) at McGill University for pyrosequencing using a GS FLX Titanium Series XLR70 kit (Roche Diagnostics Corporation). Raw pyrosequencing data were analyzed using Phoenix 2, a SSU rRNA data analysis pipeline providing quality control and chimera detection, following the procedure described by Soh et al.[20] Quality-verified sequences were compared against the SILVA 102 database (http://www.arb-silva.de) and clustered into Operational Taxonomic Units (OTUs) at ≤5% distance. Quality-controlled pyrotag sequences have been submitted to the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under BioProject PRJNA181037 with accession numbers SRR090195, SRR090199, SRR090200, SRR090201, SRR090204, SRR090205, SRR617773, SRR617774, SRR618982, SRR621639 - SRR621643, and SRR2179364–SRR2179370.

RESULTS

CH_4 Production during Metabolism of *iso*- and *n*-Alkanes. Methanogenic biodegradation of *iso*- and *n*-alkanes in Albian and Syncrude MFT was monitored for >6 years by measuring CH_4 production (Figure 1) and hydrocarbon depletion (Figure 2). The cumulative mass of CH_4 produced by Albian MFT incubated for ~2400 d with a mixture of either three *iso*-alkanes (2-MC_4, 2-MC_5, and 3-MC_5) or two *n*-alkanes (nC_5 and nC_6) is shown in Figure 1A. Although the three cultures for each substrate were carefully prepared as replicates,

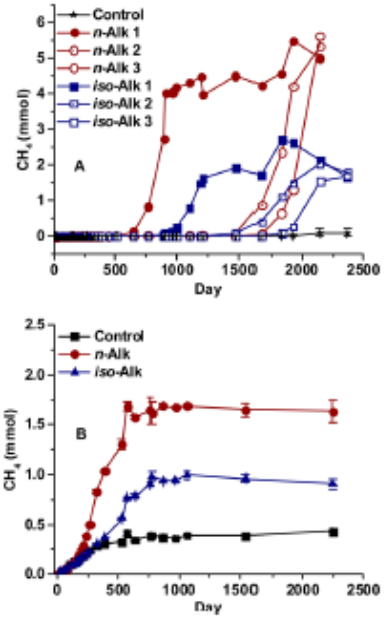

Figure 1. Cumulative methane (CH_4) production by (A) Albian MFT incubated with a mixture of three *iso*-alkanes [2-methylbutane (2-MC_4), 2-methylpentane (2-MC_5), and 3-methylpentane (3-MC_5)] or a mixture of two *n*-alkanes [pentane (nC_5) and hexane (nC_6)]. A total of three parallel cultures were established for each substrate mixture. (B) Syncrude MFT incubated with the same *iso*- and *n*-alkane mixtures. Data points are the mean of duplicate cultures, and error bars, where visible, represent one standard deviation.

they did not behave in this fashion: each culture exhibited a markedly different lag time before the onset of CH_4 production (~900–1800 d for *iso*-alkane and ~650–1675 d for *n*-alkane cultures). However, once methanogenesis began, the rates of CH_4 production were similar, and by 2150 days, the cumulative CH_4 production was comparable for all cultures in a set (1.6–2.1 mmol for *iso*-alkane and 5.0–5.6 mmol for *n*-alkane cultures). The length of the lag phase was remarkably long, despite the MFT having been exposed to the same hydrocarbons in situ for several years prior to laboratory incubation.

Methane production from Syncrude MFT incubated for ~2300 days with either *iso*- or *n*-alkane mixtures is shown in Figure 1B. Here, the pairs of cultures behaved like replicates and exhibited shorter lag phases than Albian MFT cultures, likely due to acclimatization of the microbial community to degrading naphtha hydrocarbons in situ and subsequently in laboratory culture. By only ~170 days of incubation, CH_4 production by both duplicate *n*-alkane-amended cultures exceeded that of the live baseline control, whereas the *iso*-alkane-amended cultures exhibited a longer lag phase of ~280 days. The production of 0.4 ± 0.02 mmol CH_4 by the live baseline control is due to the metabolism of endogenous substrates carried over in the MFT culture inoculum, including naphtha hydrocarbons and possibly residual metabolites. The greatest cumulative CH_4 production from *n*-alkanes (1.68 ± 0.02 mmol) and *iso*-alkanes (0.98 ± 0.04 mmol) was measured at ~570 and ~775 d, respectively.

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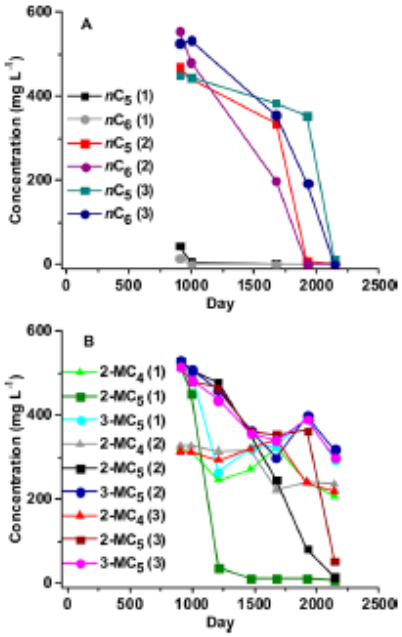

Figure 2. Concentrations of individual residual alkanes present in Albian MFT cultures incubated with either (A) *n*-alkane mixture or (B) *iso*-alkane mixture, corresponding to Figure 1. Numbers (1, 2, and 3) in legend parentheses represent Albian MFT cultures (*n*- or *iso*-Alk 1, 2, and 3, respectively).

Biodegradation of *iso*- and *n*-Alkanes. Because of the long lag period before the onset of CH_4 production by Albian cultures, the first sample for GC-PT analysis of residual hydrocarbons was collected at ~900 days of incubation, when only one culture (*n*-Alk 1) had begun to produce CH_4 (Figure 1A). At that time, little nC_5 (44 mg L^{-1}) and nC_6 (15 mg L^{-1}) remained in culture *n*-Alk 1 (Figure 2A), whereas the other two cultures (*n*-Alk 2 and *n*-Alk 3) still had residual nC_5 concentrations of 470 and 450 mg L^{-1}, respectively and nC_6 concentrations of 555 and 525 mg L^{-1}, respectively). Thereafter, the *n*-alkane concentrations in *n*-Alk 2 and *n*-Alk 3 decreased coincident with CH_4 accumulation (Figure 1A) until negligible concentrations remained by ~2150 and ~2370 d incubation, respectively. In addition to GC−PT analysis of residual hydrocarbons in culture solids and liquids, during the latter stages of incubation we also analyzed residual volatile hydrocarbons in culture-bottle headspace by using GC−MS analysis; results from the two analytical methods were congruent (Figures 2, S1, and S2).

GC−PT analyses of the three Albian *iso*-alkane cultures at ~900 d did not show any hydrocarbon depletion, with residual concentrations of 2-MC_4 (314−326 mg L^{-1}), 2-MC_5 (514−528 mg L^{-1}), and 3-MC_5 (514−529 mg L^{-1}) (Figure 2B) corresponding to a lack of CH_4 production greater than the baseline control (Figure 1A). In the first *iso*-alkane culture (*iso*-Alk 1) to produce CH_4, GC−PT analyses at ~1200 d showed an almost complete depletion of 2-MC_5 in that culture, followed at ~2150 days by complete 2-MC_5 depletion in the *iso*-Alk 2 and *iso*-Alk 3 cultures (Figure 2B) with concomitant CH_4 accumulation (Figure 1A). The other two *iso*-alkanes (2-MC_4 and 3-MC_5) were depleted by 30−34% and 40−44%, respectively, but only while 2-MC_5 was still present and actively being degraded in the cultures. After the complete depletion of 2-MC_5, no further depletion of 2-MC_4 and 3-MC_5 occurred, as determined by GC-headspace analysis of volatile hydrocarbons at ~2370 days (Figure S2).

Syncrude MFT cultures exhibited similar substrate depletion patterns, but biodegradation occurred much sooner than in Albian MFT cultures. Syncrude MFT had almost completely degraded nC_5, nC_6, and 2-MC_5 by the time of the first GC-PT analysis at ~650 d (Figure S3). 2-MC_4 and 3-MC_5 were partially depleted by this time (88% and 65% removal, respectively), after which no further decrease in concentration was measured even with extended incubation (~2250 days; Figure S4).

Bacterial Community Structure. Pyrosequencing of 16S rRNA genes was performed periodically to discern the enrichment of key taxa in cultures degrading *iso*- and *n*-alkanes in comparison to a live baseline control (unamended) culture. Bacterial reads dominated (61−75%) the prokaryotic sequences in Albian baseline (endogenous substrate) control cultures throughout incubation (Figure S5), comprising diverse members of Proteobacteria (26% of total DNA reads, with Betaproteobacteria being prominent), Chloroflexi (~15%), Actinobacteria (12%), and Firmicutes (3%) at the first sampling point (~900 days) (Figure 3A). The proportions of phyla were quite consistent during almost 2000 days (>5 years) of

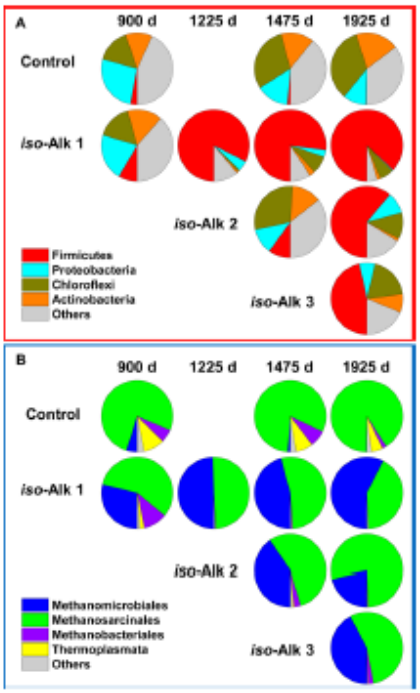

Figure 3. Community structures of (A) bacteria and (B) archaea in three parallel Albian MFT cultures (*iso*-Alk 1, 2, and 3) incubated with an *iso*-alkane mixture or without alkane addition (live baseline control), determined by analysis of 16S rRNA gene pyrosequences during a 1925 d incubation.

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incubation, providing a baseline against which to discern changes due to hydrocarbon amendment. The major family changes within these phyla were a decimal decrease in *Comamonadaceae* (Betaproteobacteria) from 22% to 2% and the doubling of the *Anaerolineaceae* from 15% to 34% abundance (Table S1). The proportion of rare taxa (OTUs present at <2% abundance at all time points; "Others") decreased only slightly from 43% to 35% of total reads during incubation, but diversity decreased from 212 to 84 OTUs (Figure 3A and Table S1)

Amendment with *iso*- or *n*-alkanes greatly increased the proportion of archaeal reads, exceeding >90% in some amended Albian MFT cultures (Figure S5). In *iso*-alkane-degrading Albian cultures, the bacterial community structure developed in parallel with the onset of methanogenesis and alkane degradation (Figure 3A and Table S2). In particular, sequences affiliated with *Peptococcaceae* (phylum Firmicutes, with genus *Desulfotomaculum* being the best-cultivated match in SILVA at ≤5% distance) came to dominate all three cultures by the end of incubation. *Peptococcaceae* sequences outnumbered all other prokaryotic taxa in culture *iso*-Alk 1, comprising 77% of the bacterial reads by 1475 d at a time when 2-MC_5 was completely depleted and the other two *iso*-alkanes were partially depleted, and further increasing to 87% abundance by ~1925 days (Figure 3A). The other two Albian *iso*-alkane cultures were also dominated by *Peptococcaceae* during the period of active *iso*-alkane degradation. The three parallel cultures had somewhat different proportions of major taxa at 1925 days, likely due to different lag times, although it is expected that *iso*-Alk 2 and *iso*-Alk 3 communities would eventually resemble *iso*-Alk 1. Similar results were observed in Albian MFT *n*-alkane cultures where *Peptococcaceae* constituted ~90% of the bacterial reads by the time of *n*-alkane depletion (Figure 4A; Table S3), and all three Albian *n*-alkane cultures, which had similar *n*-alkane degradation rates (but different lag times), also had similar community structures by the time of final sampling (1925 days) that were distinctly different from the endogenous substrate (baseline) control.

In the Syncrude live baseline control culture at 775 days, Proteobacteria constituted a greater proportion of the bacterial community than in the Albian control, representing 46% of total bacterial reads, at the expense of the combined rare OTUs ("Others", at 35% abundance) (Figure 5 and Table S4). The other three phyla in the control culture were present in proportions similar to those in the Albian MFT control: Chloroflexi (11%), Firmicutes (4%), and Actinobacteria (3%). However, as observed in the Albian cultures, significant changes in bacterial community composition were observed after alkane amendment, with *Peptococcaceae* (the *Desulfotomaculum* genus being the best SILVA match) again dominating the bacterial communities in the Syncrude *iso*-alkane and *n*-alkane cultures at 32–37% and 41–46%, respectively (Figure 5 and Table S4). Enrichment of these Firmicutes OTUs suggests that the same taxon is responsible for degrading both *n*- and *iso*-alkanes in both sources of MFT.

Archaeal Community Structure. All Albian and Syncrude cultures were dominated by only two orders of methanogenic Archaea (*Methanomicrobiales* and *Methanosarcinales*), although their relative proportions differed. The Albian baseline control at ~900 d was dominated (77% of archaeal reads) by *Methanosarcinales* (primarily genus *Methanosaeta*), which further increased to 91% abundance by ~1925 days (Figure 3B and Table S5). During active methanogenesis in both *n*- and

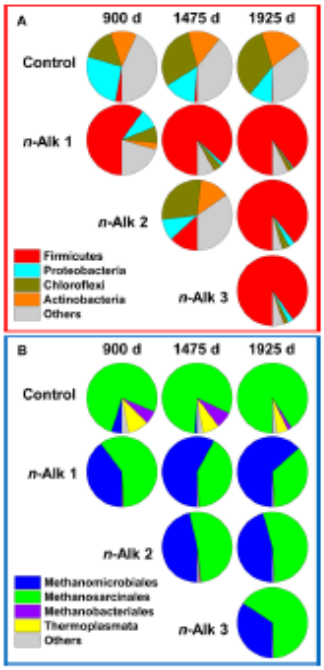

Figure 4. Community structures of (A) bacteria and (B) archaea in three parallel Albian MFT cultures (*n*-Alk 1, 2, and 3) incubated with an *n*-alkane mixture or without alkane addition (live baseline control), determined by analysis of 16S rRNA gene pyrosequences during a 1925 day incubation.

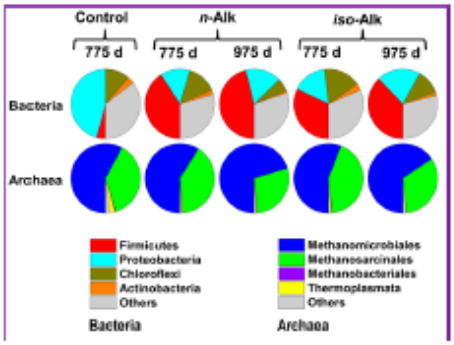

Figure 5. Community structures of bacteria and archaea in Syncrude MFT cultures incubated with an *n*-alkane or an *iso*-alkane mixture or without alkane addition (live baseline control), determined by analysis of 16S rRNA gene pyrosequences during a 975 day incubation.

iso-alkane-amended Albian cultures, *Methanosarcinales* OTUs decreased in relative abundance, and *Methanomicrobiales* increased with time. Additionally, within the order *Methanomicrobiales*, OTUs identified as genus *Methanolinea* were enriched during *iso*-alkane biodegradation to comprise 15–36% of archaeal reads along with candidatus *Methanoregula* at 5–25% of archaeal reads (Table S6). Conversely, candidatus *Methanoregula* dominated the *Methanomicrobiales* during *n*-alkane biodegradation and, depending on the stage of methanogenesis, increased from 34 to 52% of total archaeal

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which was not detected in control cultures), came to dominate the bacterial reads in Albian and Syncrude alkane-amended cultures, implying a major role in both *iso*- and *n*-alkane degradation. Similar results were reported by Abu Laban et al.[12] for Syncrude MFT cultures incubated with C_7–C_8 *iso*-alkanes under methanogenic conditions and by Tan et al.,[13] from which the dominant *Peptococcaceae* draft genome sequence was published using single-cell sorting.[29] The dominant phylotype of *Peptococcaceae*[12] had high sequence identity (99%) to a dominant cloned 16S rRNA gene sequence previously amplified from alkane- and naphtha-degrading primary cultures derived from Syncrude MFT.[10]

The low abundance of Proteobacteria (particularly the genera *Syntrophus* and *Smithella*) and Actinobacteria and the constantly low abundance of Chloroflexi in the alkane-degrading cultures versus the endogenous substrate control suggests that these taxa are not directly involved in the initial steps of *iso*- and *n*-alkane (C_5–C_6) degradation but rather are metabolizing pathway intermediates to H_2 and CO_2 or acetate for subsequent methanogenesis. It is interesting to note that in our previous studies on the methanogenic degradation of longer-chain *n*-alkanes (C_{14}–C_{18}) in Syncrude MFT, only *Syntrophus* dominated the bacterial population with no apparent contribution by Clostridia (i.e., *Peptococcaceae* or *Desulfotomaculum*) during degradation.[9] However, codominance of *Syntrophus* and *Desulfotomaculum* was observed during methanogenic degradation of C_6–C_{10} hydrocarbons such as *n*-alkanes, BTEX, and naphtha in Syncrude MFT.[10] In the present study with shorter *iso*- and *n*-alkanes (C_5–C_6), *Peptococcaceae* (ostensibly *Desulfotomaculum*) overwhelmingly dominated the bacterial populations with apparently little contribution from *Syntrophus*, showing preference of these bacterial taxa for certain ranges of hydrocarbons biodegraded in Syncrude MFT.

At all times and with all substrates, the archaeal sequences in Albian MFT were predominantly associated with acetoclastic *Methanosaeta* (75–84% of archaeal reads), some species of which recently have been shown to be subject to the quorum-sensing control of acetate metabolism, among other characteristics.[30] However, during *n*-alkane degradation, the proportion of hydrogenotrophic methanogens (particularly *Methanoregula*) increased, eventually codominating with *Methanosaeta* in Albian *n*-alkane cultures. Different results were observed in *iso*-alkane cultures, where *Methanolinea* dominated the hydrogentrophic methanogens with comparatively less contribution from *Methanoregula*. Both *Methanolinea* and *Methanoregula* codominated with *Methanosaeta* during biodegradation of *iso*-alkanes. The Syncrude MFT community was consistently codominated by *Methanosaeta* and *Methanoregula*, but because so few samples were analyzed, temporal shifts in Archaeal composition were not apparent during alkane degradation. The codominance of *Methanosaeta* and *Methanoregula* in alkane-amended Albian and Syncrude MFT suggests that both major methanogenic routes (acetoclastic and hydrogenotrophic) are active during *iso*- and *n*-alkane degradation.

In summary, we report here the biodegradation of short-chain *iso*-alkanes, a hydrocarbon fraction previously considered recalcitrant under methanogenic conditions. Complete and direct metabolism is inferred for biodegradation of 2-methylpentane, but incomplete cometabolic oxidation is inferred for 2-methylbutane and 3-methylpentane. For the Albian MFT cultures, degradation required several years to initiate and complete under laboratory conditions, suggesting that appropriate community members such as *Peptococcaceae* must be enriched or form functional syntrophic relationships to achieve *iso*-alkane biodegradation. This is important because it is the first microbial community pyrotag survey and detailed account of anaerobic hydrocarbon biodegradation capabilities in MFT from the only oil sands tailings pond (Albian) that is processed using paraffinic (C_5–C_6) diluent. It is also the only comparison to date between a well-established (Syncrude) and a recent (Albian) tailings pond, giving insight into the development of the indigenous microbial communities that have such fundamental contributions to the environmental impact of the enormous oil sands tailings ponds.

In addition to broadening the suite of hydrocarbons known to be degraded under methanogenic conditions, the results can be used to refine kinetic models predicting the onset and duration of greenhouse gas emissions from oil sands tailings ponds;[3] a refined model using data from the current report, among others, is in progress (J. King et al., in preparation). The current report can be used as a model with which to compare and monitor the success trajectories of reclamation approaches such as end-pit lakes,[31] in which MFT from tailings ponds will be placed in pits and capped with surface water to create viable lake ecosystems. Knowing the range of substrates susceptible to complete biodegradation and those apparently subject to co-metabolism will inform the prediction of end-pit lake biogeochemistry and greenhouse gas emissions. More broadly, these observations provide important insights into understanding methanogenesis in conventional oil reservoirs and devising strategies for the remediation of hydrocarbons in anoxic hydrocarbon-contaminated environments such as harbors and aquifers.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b04370.

Tables showing the relative abundance of bacterial 16S rRNA gene pyrosequences in the live Albian baseline, *iso*- and *n*-alkane-amended Albian MFT, baseline control and alkane-amended Syncrude MFT and archaeal 16S rRNA gene pyrosequences in the Albian baseline control MFT, *iso*-alkane-amended Albian MFT, *n*-alkane-amended Albian MFT, the baseline control and alane-amended Syncrude MFT. Figures showing methanogenic biodegradation of *n*C5 and *n*C6 in three parallel Albian MFT cultures and 2-MC_4, 2-MC_5, and 3-MC_5 in three parallel Albian MFT cultures; concentrations of residual *n*- (*n*C5 and *n*C6) and *iso*-alkanes (2-MC_4, 2-MC_5 and 3-MC_5) in Syncrude MFT cultures; methanogenic biodegradation of *n*- (*n*C5 and *n*C6) and *iso*-alkanes (2-MC_4, 2-MC_5 and 3-MC_5) by Syncrude MFT cultures; proportions of bacterial and archaeal quality-controlled 16S rRNA gene pyrosequencing reads; and proportions of bacterial and archaeal quality-controlled 16S rRNA gene pyrosequencing reads. PDF)

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Notes

The authors declare no competing financial interest.

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Supporting Information (SI) for

Long-term incubation reveals methanogenic biodegradation of C_5 and C_6 *iso*-alkanes in oil sands tailings

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The supporting information contains 8 tables (Table S1-S8) and 6 figures (Fig. S1-S6).

Table S1: Relative abundance (%) of bacterial 16S rRNA gene pyrosequences in the live Albian baseline (endogenous substrate) control MFT.

Taxa, assigned at <5% sequence difference by SILVA 102	**Day**		
	900	**1475**	**1925**
Firmicutes			
Clostridia; *Clostridiales; Peptococcaceae; Desulfotomaculum*	0.0	0.0	0.0
Erysipelotrichi; *Erysipelotrichales; Erysipelotrichaceae; Erysipelothrix*	3.1	1.2	0.2
Proteobacteria			
Deltaproteobacteria; *Syntrophobacterales; Syntrophaceae; Smithella*	1.5	2.2	4.2
Deltaproteobacteria; *Syntrophobacterales; Syntrophaceae; Syntrophus*	1.9	3.1	3.4
Betaproteobacteria; *Burkholderiales; Comamonadaceae; Rhodoferax*	21.9	7.9	1.9
Betaproteobacteria; *Hydrogenophilales; Hydrogenophilaceae; Thiobacillus*	1.0	1.5	1.2
Chloroflexi			
Anaerolineae; *Anaerolineales; Anaerolineaceae; uncultured*	11.6	21.3	24.5
Anaerolineae; *Anaerolineales; Anaerolineaceae; Leptolinea*	3.8	8.5	9.7
Actinobacteria			
Coriobacteridae; *Coriobacteriales; Coriobacteriaceae*	12.1	14.9	19.6
Others			
OTUs <2% [a]	43.1 (212)	39.2 (144)	35.3 (84)

Color highlights indicate abundance of total reads based on color gradient from lowest to highest percentages given in the table.

[a] Total OTUs present at <2% abundance in the control culture at each sample time; number of individual taxa shown in parentheses.

Table S2: Relative abundance (%) of bacterial 16S rRNA gene pyrosequences in the *iso*-alkane-amended Albian MFT.

Taxa, assigned at <5% sequence difference by SILVA 102	***iso*-Alk 1**				***iso*-Alk 2**		***iso*-Alk 3**
	Day						
	900	**1225**	**1475**	**1925**	**1475**	**1925**	**1925**
Firmicutes							
Clostridia; *Clostridiales; Peptococcaceae; Desulfotomaculum*	5.1	82.1	76.8	87.1	8.9	60.4	46.2
Erysipelotrichi; *Erysipelotrichales; Erysipelotrichaceae; Erysipelothrix*	3.4	0.3	0.3	0.0	1.1	0.7	0.4
Proteobacteria							
Deltaproteobacteria; *Syntrophobacterales; Syntrophaceae; Smithella*	1.3	1.6	1.4	0.0	3.7	9.0	4.1
Deltaproteobacteria; *Syntrophobacterales; Syntrophaceae; Syntrophus*	0.9	0.3	0.6	0.0	1.1	0.7	1.4
Betaproteobacteria; *Burkholderiales; Comamonadaceae; Rhodoferax*	18.5	2.6	1.1	0.4	5.6	0.0	0.8
Betaproteobacteria; *Hydrogenophilales; Hydrogenophilaceae; Thiobacillus*	0.7	0.2	0.0	0.0	1.2	0.0	0.6
Chloroflexi							
Anaerolineae; *Anaerolineales; Anaerolineaceae*; uncultured	11.7	0.3	5.6	4.3	20.9	4.9	12.2
Anaerolineae; *Anaerolineales; Anaerolineaceae; Leptolinea*	4.2	0.5	2.3	2.0	8.7	6.9	7.0
Actinobacteria							
Coriobacteridae; *Coriobacteriales; Coriobacteriaceae*	15.9	1.0	2.6	1.6	13.4	1.4	8.3
Others							
<2% OTUs [a]	38.4 (171)	11.1 (41)	9.2 (49)	4.7 (9)	35.6 (117)	16.0 (8)	18.8 (39)

Color highlights indicate abundance of total reads based on color gradient from lowest to highest percentages given in the table.

[a] Total OTUs present at <2% abundance in each culture at each sample time; number of individual taxa shown in parentheses.

Table S3: Relative abundance (%) of bacterial 16S rRNA gene pyrosequences in the *n*-alkane-amended Albian MFT.

Taxa, assigned at <5% sequence difference by SILVA 102	***n*-Alk 1**			***n*-Alk 2**		***n*-Alk 3**
	Day					
	900	**1475**	**1925**	**1475**	**1925**	**1925**
Firmicutes						
Clostridia; *Clostridiales; Peptococcaceae; Desulfotomaculum*	59.1	86.6	89.7	12.2	89.3	89.1
Erysipelotrichi; *Erysipelotrichales; Erysipelotrichaceae; Erysipelothrix*	1.0	0.1	0.0	1.0	0.0	0.9
Proteobacteria						
Deltaproteobacteria; *Syntrophobacterales; Syntrophaceae; Smithella*	2.7	0.6	0.0	2.8	0.6	1.4
Deltaproteobacteria; *Syntrophobacterales; Syntrophaceae; Syntrophus*	1.5	0.6	0.5	1.4	2.3	0.9
Betaproteobacteria; *Burkholderiales; Comamonadaceae; Rhodoferax*	4.4	0.1	0.0	4.0	0.0	0.0
Betaproteobacteria; *Hydrogenophilales; Hydrogenophilaceae; Thiobacillus*	0.4	0.1	0.0	1.7	0.0	0.0
Chloroflexi						
Anaerolineae; *Anaerolineales; Anaerolineaceae;* uncultured	5.0	1.9	1.3	19.5	0.0	1.9
Anaerolineae; *Anaerolineales; Anaerolineaceae; Leptolinea*	2.5	1.5	0.5	9.3	3.4	0.0
Actinobacteria						
Coriobacteridae; *Coriobacteriales; Coriobacteriaceae*	3.0	0.5	0.5	13.5	0.0	0.5
Others						
<2% OTUs [a]	20.5 (36)	8.0 (40)	7.4 (16)	34.7 (114)	4.5 (8)	5.2 (11)

Color highlights indicate abundance of total reads based on color gradient from lowest to highest percentages given in the table.

[a] Total OTUs present at <2% abundance in each culture at each sample time; number of individual taxa shown in parentheses.

Table S4: Relative abundance (%) of bacterial 16S rRNA gene pyrosequences in the baseline control and alkane-amended Syncrude MFT.

Taxa, assigned at <5% sequence difference by SILVA 102	**Control**	***n*-Alk**		***iso*-Alk**	
		Day			
	775	**775**	**975**	**775**	**975**
Firmicutes					
Clostridia; *Clostridiales; Peptococcaceae; Desulfotomaculum*	3.6	40.5	46.0	31.7	37.3
Erysipelotrichi; *Erysipelotrichales; Erysipelotrichaceae; Erysipelothrix*	0.5	0.3	0.0	0.4	0.5
Proteobacteria					
Deltaproteobacteria; *Syntrophobacterales; Syntrophaceae; Smithella*	4.8	3.0	4.7	2.1	6.8
Deltaproteobacteria; *Syntrophobacterales; Syntrophaceae; Syntrophus*	3.3	3.3	5.5	2.9	5.4
Betaproteobacteria; *Burkholderiales; Comamonadaceae; Rhodoferax*	30.0	5.2	5.6	9.4	5.8
Betaproteobacteria; *Hydrogenophilales; Hydrogenophilaceae; Thiobacillus*	7.8	2.1	1.0	1.5	2.2
Chloroflexi					
Anaerolineae; *Anaerolineales; Anaerolineaceae*; uncultured	7.1	10.2	4.3	10.9	8.9
Anaerolineae; *Anaerolineales; Anaerolineaceae; Leptolinea*	4.3	3.7	1.8	6.5	2.6
Actinobacteria					
Coriobacteridae; *Coriobacteriales; Coriobacteriaceae*	3.3	2.2	1.0	3.1	1.6
Others					
<2% OTUs [a]	35.4 (129)	29.3 (64)	30.1 (69)	31.5 (45)	28.8 (98)

Color highlights indicate abundance of total reads based on color gradient from lowest to highest percentages given in the table.

[a] Total OTUs present at <2% abundance in all cultures at each sample time; number of individual taxa shown in parentheses.

Table S5: Relative abundance (%) of archaeal 16S rRNA gene pyrosequences in the Albian baseline control MFT.

Taxa, assigned at <5% sequence difference by SILVA 102	**Day**		
	900	**1475**	**1925**
Methanomicrobiales			
Methanomicrobia; *Methanomicrobiales;* Candidatus *Methanoregula*	4.8	1.2	0.0
Methanomicrobia; *Methanomicrobiales; Methanolinea*	0.0	0.0	0.2
Methanomicrobia; *Methanomicrobiales; Methanomicrobiaceae*	0.0	0.0	0.0
Methanosarcinales			
Methanomicrobia; *Methanosarcinales; Methanosaetaceae; Methanosaeta*	70.7	76.3	83.7
Methanomicrobia; *Methanosarcinales; Methanosarcinaceae; Methanosarcina*	5.8	4.6	7.1
Methanobacteriales			
Methanobacteria; *Methanobacteriales; Methanobacteriaceae; Methanobacterium*	6.0	7.5	1.9
Thermoplasmata			
Thermoplasmata; WCHA1-57	9.5	7.7	5.2
Others			
<2% OTUs [a]	3.2 (14)	2.7 (6)	2.0 (6)

Color highlights indicate abundance of total reads based on color gradient from lowest to highest percentages given in the table.

[a] Total OTUs present at <2% abundance in control culture at each sample time; number of individual taxa shown in parentheses.

Table S6: Relative abundance (%) of archaeal 16S rRNA gene pyrosequences in the *iso*-alkane-amended Albian MFT.

Taxa, assigned at <5% sequence difference by SILVA 102	***iso*-Alk 1**				***iso*-Alk 2**		***iso*-Alk 3**
	Day						
	900	**1225**	**1475**	**1925**	**1475**	**1925**	**1925**
Methanomicrobiales							
Methanomicrobia; *Methanomicrobiales;* Candidatus *Methanoregula*	13.8	21.3	17.9	25.5	5.3	5.0	6.4
Methanomicrobia; *Methanomicrobiales; Methanolinea*	15.1	28.2	27.9	32.0	35.1	16.3	35.9
Methanomicrobia; *Methanomicrobiales; Methanomicrobiaceae*	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Methanosarcinales							
Methanomicrobia; *Methanosarcinales; Methanosaetaceae; Methanosaeta*	55.3	49.7	53.0	41.6	53.7	78.1	54.5
Methanomicrobia; *Methanosarcinales; Methanosarcinaceae; Methanosarcina*	1.8	0.0	0.0	0.1	1.1	0.1	0.2
Methanobacteriales							
Methanobacteria; *Methanobacteriales; Methanobacteriaceae; Methanobacterium*	10.7	0.2	0.4	0.2	3.0	0.3	2.5
Thermoplasmata							
Thermoplasmata; WCHA1-57	2.1	0.0	0.1	0.0	0.8	0.2	0.2
Others							
<2% OTUs [a]	1.2 (9)	0.7 (4)	0.6 (4)	0.5 (2)	1.0 (7)	0.1 (2)	0.2 (3)

Color highlights indicate abundance of total reads based on color gradient from lowest to highest percentages given in the table.

[a] Total OTUs present at <2% abundance in each culture at each sample time; number of individual taxa shown in parentheses.

Table S7: Relative abundance (%) of archaeal 16S rRNA gene pyrosequences in the *n*-alkane-amended Albian MFT.

Taxa, assigned at <5% sequence difference by SILVA 102	***n*-Alk 1**			***n*-Alk 2**		***n*-Alk 3**
	Day					
	900	**1475**	**1925**	**1475**	**1925**	**1925**
Methanomicrobiales						
Methanomicrobia; *Methanomicrobiales*; Candidatus *Methanoregula*	34.5	47.7	52.2	45.1	45.6	34.1
Methanomicrobia; *Methanomicrobiales; Methanolinea*	5.2	10.1	11.1	1.4	0.1	0.1
Methanomicrobia; *Methanomicrobiales; Methanomicrobiaceae*	0.0	0.0	0.1	0.0	0.0	0.0
Methanosarcinales						
Methanomicrobia; *Methanosarcinales; Methanosaetaceae; Methanosaeta*	59.5	41.2	35.6	50.9	53.5	65.2
Methanomicrobia; *Methanosarcinales; Methanosarcinaceae; Methanosarcina*	0.1	0.0	0.0	0.6	0.0	0.1
Methanobacteriales						
Methanobacteria; *Methanobacteriales; Methanobacteriaceae; Methanobacterium*	0.3	0.1	0.1	0.6	0.1	0.0
Thermoplasmata						
Thermoplasmata; WCHA1-57	0.1	0.0	0.2	0.5	0.1	0.0
Others						
<2% OTUs [a]	0.3 (3)	0.7 (5)	0.7 (3)	0.8 (7)	0.7 (2)	0.4 (3)

Color highlights indicate abundance of total reads based on color gradient from lowest to highest percentages given in the table.

[a] Total OTUs present at <2% abundance in each culture at each sample time; number of individual taxa shown in parentheses.

Table S8: Relative abundance (%) of archaeal 16S rRNA gene pyrosequences in the baseline control and alkane-amended Syncrude MFT.

Taxa, assigned at <5% sequence difference by SILVA 102	**Control**	***n*-Alk**		***iso*-Alk**	
		Day			
	775	**775**	**975**	**775**	**975**
Methanomicrobiales					
Methanomicrobia; Methanomicrobiales; Candidatus *Methanoregula*	53.1	49.9	50.4	45.4	45.4
Methanomicrobia; *Methanomicrobiales; Methanolinea*	4.0	4.5	5.2	7.6	7.6
Methanomicrobia; *Methanomicrobiales; Methanomicrobiaceae*	0.8	4.5	14.8	2.8	12.7
Methanosarcinales					
Methanomicrobia; *Methanosarcinales; Methanosaetaceae; Methanosaeta*	37.3	39.7	28.3	42.5	32.7
Methanomicrobia; *Methanosarcinales; Methanosarcinaceae; Methanosarcina*	0.0	0.0	0.0	0.0	0.0
Methanobacteriales					
Methanobacteria; *Methanobacteriales; Methanobacteriaceae; Methanobacterium*	0.4	0.1	0.1	0.2	0.1
Thermoplasmata					
Thermoplasmata; WCHA1-57	2.5	0.6	0.4	0.9	0.5
Others					
<2% OTUs [a]	2.0 (10)	0.7 (7)	0.8 (9)	0.5 (6)	1.0 (11)

Color highlights indicate abundance of total reads based on color gradient from lowest to highest percentages given in the table.

[a] Total OTUs present at <2% abundance in each culture at each sample time; number of individual taxa shown in parentheses.

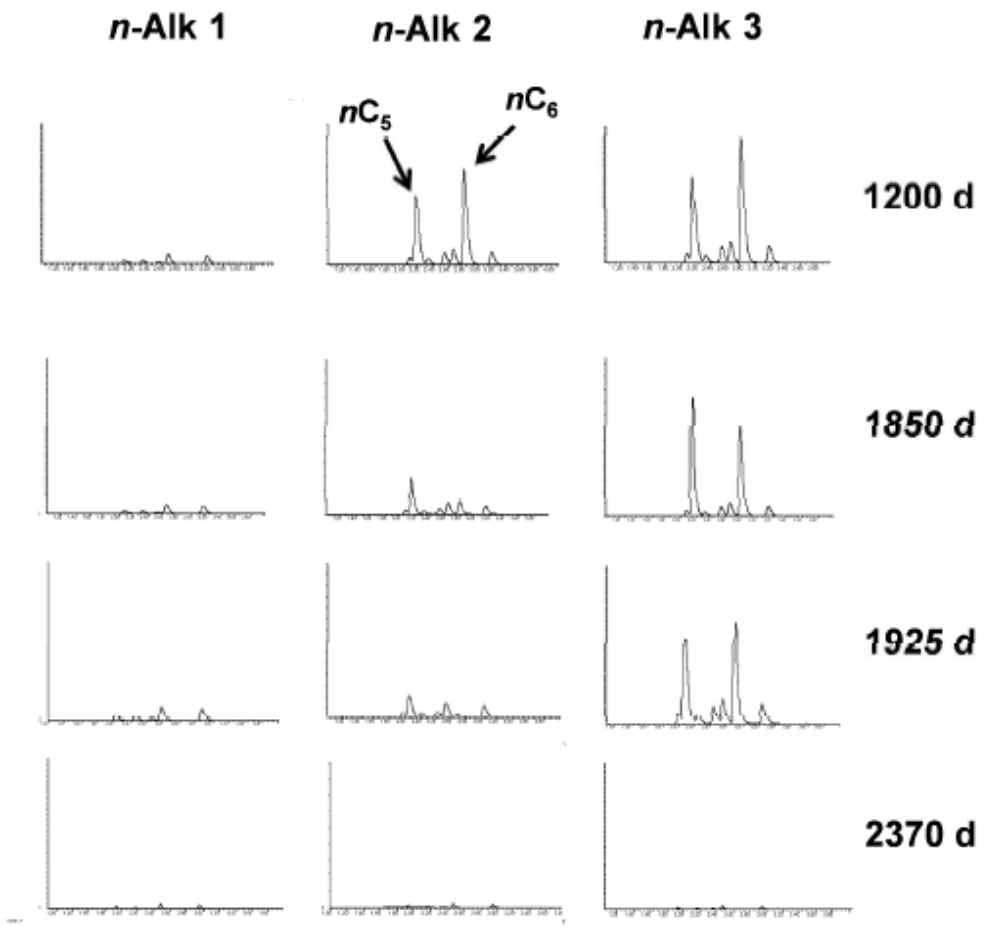

Fig. S1. Methanogenic biodegradation of nC_5 and nC_6 in three parallel Albian MFT cultures (*n*-Alk 1, -2 and -3) incubated with an *n*-alkane mixture for ~2400 d. Degradation was monitored by analyzing headspace gas using GC-MS, in addition to the GC-PT data presented in Figure 2 in the main text.

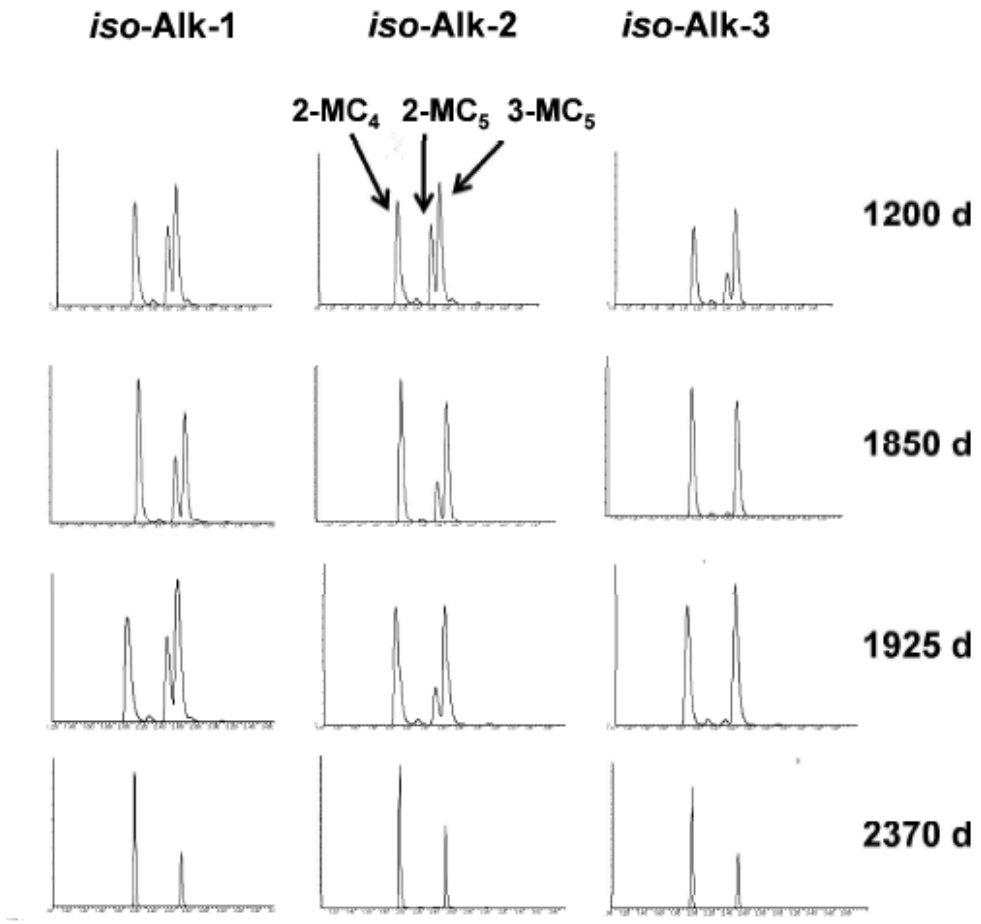

Fig. S2: Methanogenic biodegradation of 2-MC_4, 2-MC_5 and 3-MC_5 in three parallel Albian MFT cultures (*iso*-Alk 1, -2 and -3) incubated with an *iso*-alkane mixture for ~2400 d. Degradation was monitored by analyzing headspace gas using GC-MS, in addition to the GC-PT data presented in Figure 2 in the main text.

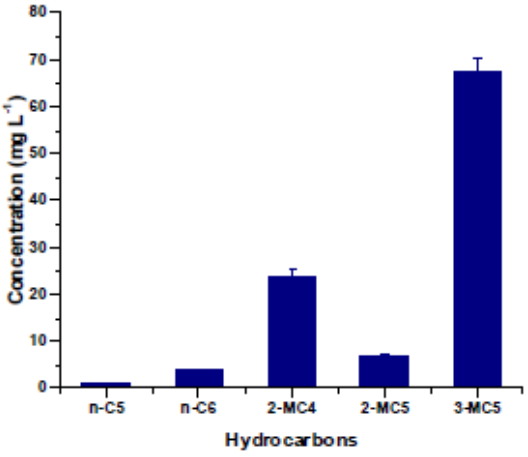

Fig. S3: Concentrations of residual *n*- (nC_5 and nC_6) and *iso*-alkanes (2-MC_4, 2-MC_5 and 3-MC_5) in Syncrude MFT cultures incubated with *n*- or *iso*-alkane mixtures for ~650 d. Degradation was monitored by using GC-PT. Data are the mean of duplicates; error bars (where visible) are the standard error.

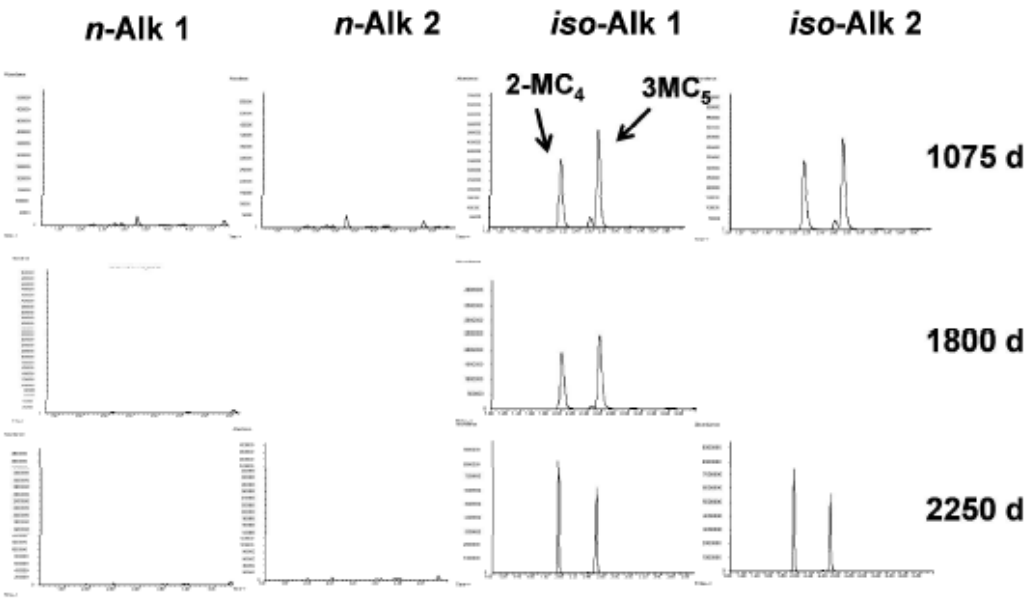

Fig. S4: Methanogenic biodegradation of *n*- (nC_5 and nC_6) and *iso*-alkanes (2-MC_4, 2-MC_5 and 3-MC_5) by Syncrude MFT cultures incubated with *n*- or *iso*-alkane mixtures for ~2250 d. Degradation was monitored by analyzing headspace gas using GC-MS, in addition to GC-PT data presented in Fig. S3.

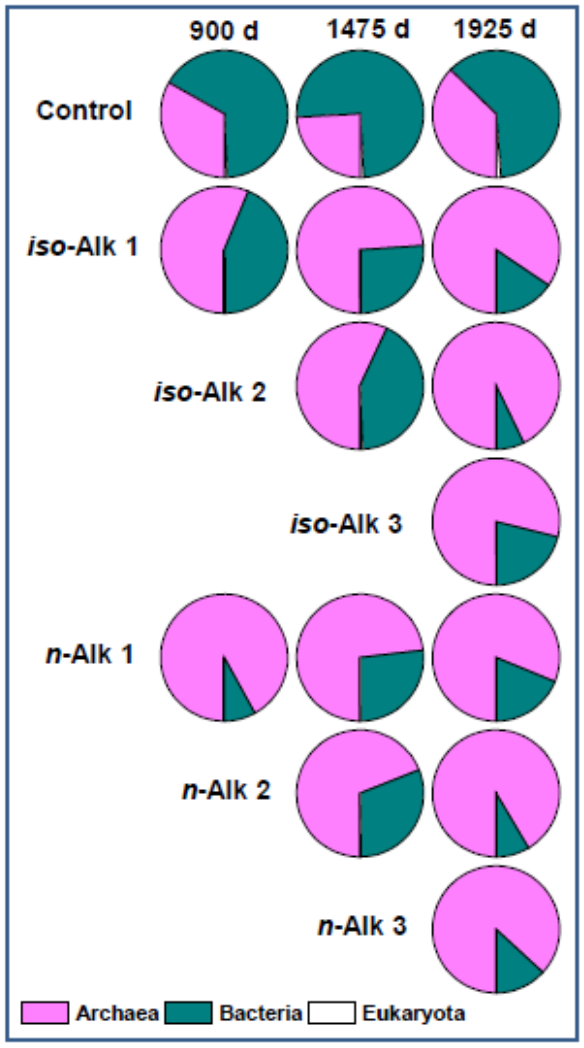

Fig. S5: Proportions (%) of bacterial and archaeal quality-controlled 16S rRNA gene pyrosequencing reads in baseline control and in *n*-alkane- and *iso*-alkane-amended Albian MFT cultures during incubation.

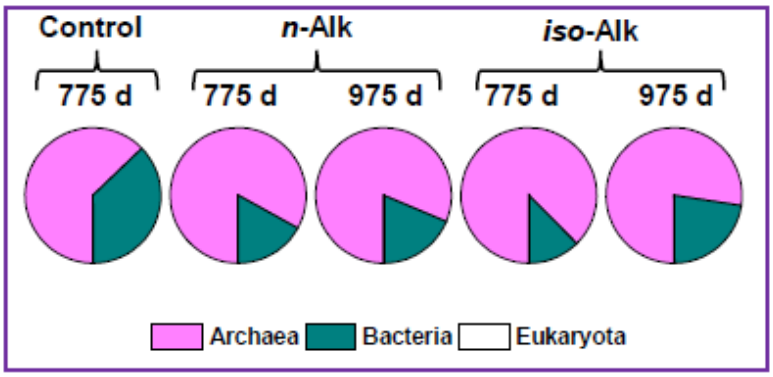

Fig. S6: Proportions (%) of bacterial and archaeal quality-controlled 16S rRNA gene pyrosequencing reads in baseline control and in *n*-alkane- and *iso*-alkane-amended Syncrude MFT cultures during incubation.