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Methanogenesis in oil sands tailings: An analysis of the microbial community involved and its effects on tailings densification

by

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Abstract

Densification of tailings slurries to mature fine tailings (MFT) is important in the oil sands industry for tailings inventory reduction, pore water recovery and tailings reclamation. The cause of methane release from the tailings pond of Shell Albian Sands (Albian) and the effects this process has on densification of Albian tailings was investigated. Citrate, added to tailings with polyacrylamide and hydrocarbon-diluent, was identified as the methanogenic substrate. Bacterial and Archaeal 16S rRNA gene sequences in Albian MFT were dominated by matches to *Rhodoferax*, some Clostridia and sulfate-reducing bacteria, and acetoclastic methanogens. Citrate-, diluent-, and polyacrylamide-amendments to Albian MFT did not cause a microbial shift over a 10-month laboratory incubation period. A potential pathway for microbial methane production in Albian MFT is proposed. Methane production and release from citrate-amended Albian MFT correlated to accelerated densification. Though diluent and polyacrylamide did not affect methanogenesis, they potentially affect gas bubble formation and release.

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List of Abbreviations

°C	degrees Celsius
AOM	Anaerobic oxidation of methane
BSA	bovine serum albumin
BTEX	benzene, toluene, ethylbenzene, xylenes
DGGE	denaturing gradient gel electrophoresis
dNTP	dideoxy nucleotide triphosphate
FeRB	iron-reducing bacteria
HPLC	high performance liquid chromatography
g	gram
GC-MS	gas chromatography-mass spectrometry
km	kilometer
L	liter
m	meter
MFT	mature fine tailings
mg	milligram
mL	milliliter
MLSB	Mildred Lake Settling Basin
MPN	most probable number
NCBI	National Center for Biotechnology Information
NREF	Natural Resources Engineering Facility
OSTRF	Oil Sands Tailings Research Facility
OUT	operational taxonomic unit
PCR	polymerase chain reaction
ppm	parts per million
RDP	Ribosomal Database Project
RFLP	restriction fragment length polymorphism
rRNA	ribosomal ribonucleic acid
SIP	stable isotope probing
SRB	sulfate-reducing bacteria
Vol%	volume percent

1 INTRODUCTION

1.1 OIL SANDS TAILINGS

1.1.1 Oil sands industry

Alberta is one of the world's main producers of oil. As one of the world's largest oil sands deposits, Alberta's Athabasca oil sands are estimated to hold at least 1.7 trillion barrels of bitumen (MacLean 1998). Oil sands are a mixture of bitumen, clay and water surrounding particles of sand. The bitumen, which is a viscous oil material, must first be removed from the sand and clay particles and then is upgraded to synthetic crude oil which can be further refined into jet fuels, gasoline, home heating fuels and petroleum products. Bitumen is recovered from oil sands using different methods depending on where the oil sands are located. If the oil sand deposit is shallow, it is surface mined, a process termed open-pit mining. If deposits are located deep below ground surface, *in situ* recovery methods are used, including cyclic steam stimulation and steam-assisted gravity drainage. In the Athabasca oil sands deposits where reserves are shallow, open-pit mining is used.

The oil sands material collected from the ground is crushed and broken into smaller pieces. In traditional processing, caustic hot water is then added to the oil sands to cause the asphaltic acids in bitumen, which are partly aromatic and contain phenolic, carboxylic and sulphonic functional groups, to become water-soluble and act as surfactants. This reduces surface and interfacial tensions, allowing the bitumen to be removed from the sand and clays (Chalaturnyk et al. 2002). A slurry forms from this process and is transported to a primary separation vessel where the components of this slurry separate. Aeration promotes the flotation of bitumen to the top of the slurry as froth (Chalaturnyk et al. 2002). The bitumen froth is skimmed and pumped to an extraction plant. Middlings, a mixture of clay, water and a small amount of bitumen, are treated again in a secondary separation vessel to generate more bitumen froth. Solid matter, including sand and clay, settles to the bottom of each vessel. The majority of the collected sand is used to form dykes for tailings ponds. The remainder of the bottom fraction is pumped to the tailings pond as fine tailings.

1.1.2 Tailings management

Fine tailings consist predominantly of clay particles with a small amount of sand and residual bitumen mixed with water, from the bottom and middle layers of the separation vessels, and are stored in tailings ponds on site of oil sands mining operations. At the current rate of oil production, staggering volumes of tailings are being produced and stored. There are currently more than 130 km^2 of tailings ponds in the Alberta oil sands region alone (Alberta Energy, 2009). As fine tailings settle, sand quickly falls to the bottom of the pond followed by clays. Tailings become denser while water is released to the surface of the pond. This water can be reused for the extraction of bitumen from freshly-mined oil sands. Settled tailings containing at least 30% solids by weight are termed mature fine tailings (MFT) (Chalaturnyk et al. 2002). When MFT reaches an appropriate density, in theory the tailings pond can be reclaimed or the MFT used in dry reclamation landscapes.

Two main methods of reclamation have been proposed for oil sands tailings ponds. The first is a "wet landscape approach" and involves covering MFT basins with a water cap to create a viable lake system. A natural freshwater ecosystem will eventually establish in or around the pond (List and Lord 1997). The second method is a "dry landscape approach" where soft fluid tailings are mixed with solid deposits and stored in a basin. Consolidated tailings, which are MFT treated with gypsum ($CaSO_4 \cdot 2H_2O$) and sand to create a non-segregating clay matrix, were designed for dry reclamation landscapes (Mikula et al. 1998). Dry tailings should eventually revegetate as a natural ecosystem (List and Lord 1997).

Densification of MFT in tailings ponds is therefore important for managing tailings inventory, water recovery, and reclamation. However, fine clay particles suspended in water settle very slowly because of their colloidal nature. As a result, densification of MFT for reclamation is predicted to take over a hundred years (Eckert et al. 1996).

1.1.3 Methane production and release from tailings

The extremely slow settling process of oil sands fine tailings was addressed by Syncrude Canada Ltd. (hereafter referred to as Syncrude) in the mid-1990s through the use of gypsum in their tailings pond (List and Lord 1997; Tara Penner, Syncrude Canada Ltd., personal communication). Gypsum causes a rapid release of water from tailings, which accelerates their densification and reduces their volumes. However, gypsum also causes a high concentration of sulfate in the released water from tailings. Foght et al. (1985) surveyed the microbial community in the MFT of Mildred Lake Settling Basin (MLSB), one of the main tailings ponds of Syncrude. Both aerobic and anaerobic microbes were detected, including sulfate-reducing bacteria (SRB). Methane production from MLSB MFT was observed when samples were amended with acetate or glucose and incubated at 37°C, indicating that methanogens were also present, but in numbers below detection limits. SRB and methanogens are microbial groups involved in producing methane in anaerobic environments. Tailings ponds are highly anaerobic environments due to their depth as well as the properties of tailings material, which prevent light and oxygen from entering into the ponds.

By the early 1990s, MLSB began to release methane. Methane contributes strongly to the greenhouse gas effect and it was thought at the time that release of methane bubbles would hinder tailings densification by disrupting the settling process. Holowenko et al. (2000) enumerated the methanogens and SRB in MLSB MFT and also incubated MLSB MFT with sodium acetate, the substrate acetoclastic methanogens use to produce methane, and sodium sulfate. Methanogens were now present in numbers above detection limits. Methanogens were generally present in higher numbers when sulfate concentrations were low and methanogenesis was inhibited by the addition of sulfate. This latter observation suggested that the addition of gypsum to MFT could inhibit methanogenesis in MFT.

Research moved towards identifying the substrates available in MFT for microbial methane production. Holowenko (2000) tested a number of organic compounds under anaerobic conditions, including bitumen and naphthenic acids, a component of oil sands, for the development of methane when incubated with Syncrude MFT. However,

no methane production was observed from any tested compound and mixtures of naphthenic acids were found to be toxic to the microbes. Research in understanding methanogenesis in MFT made significant progress when researchers were informed that during the process of bitumen recovery from oil sands, a diluent is added to help separate bitumen from solid particles. Syncrude uses a diluent called naphtha, a mixture of aliphatic and aromatic hydrocarbon compounds such as *n*-alkanes (C_6-C_{10}) and BTEX compounds (benzene, toluene, ethylbenzene, and xylene isomers). Siddique et al. (2006; 2007) were able to correlate the anaerobic degradation of *n*-alkanes (C_6, C_7, C_8, and C_{10}) and BTEX compounds by the microbial consortium in Syncrude MFT to methane production from Syncrude MFT.

Fedorak et al. (2003) reported the accelerated densification rate of methanogenic Syncrude MFT compared to nonmethanogenic MFT. This observation has large implications for the oil sands industry in regards to tailings storage and management, water recovery, and reclamation. If methane production causes tailings densification to occur at a faster rate, free water from the settling process will also be generated more quickly. This will provide more water to re-use for bitumen extraction and require less water from freshwater sources like the Athabasca River. Furthermore, the reduction of tailings volumes at a faster rate means less storage space is required for the immense volumes of tailings generated from bitumen extraction, delaying the need for new tailings basins and allowing tailings ponds to be reclaimed sooner.

If organic compounds are supporting methane gas production from MLSB MFT, then methane emissions could potentially be reduced by the addition of substrates that stimulate alternative anaerobic pathways or, conversely, increase methane emissions to cause tailings to undergo rapid densification. Shell Albian Sands (hereafter referred to as Albian) is a recently established oil sands company in the Athabasca oil sands region. Albian has observed a relatively rapid development of methane emissions from its tailings pond. Whereas Syncrude experienced a considerable lag, about 15 years of operation, before methane emissions were observed from MLSB, the tailings ponds at Albian began emitting methane after only about 5 years of operation. There are several possible substrates for the microbial population in Albian tailings. Albian uses a different diluent during bitumen extraction than Syncrude. The diluent used by Albian is a mixture

of linear, branched and cyclic short-chain aliphatics (C_5 and C_6) as opposed to the longer aliphatics and BTEX compounds in the naphtha diluent used by Syncrude. Albian also differs from Syncrude because Albian adds trisodium citrate to its tailings as a water-softening agent and polyacrylamide as a chemical flocculant.

The cause(s) and effects of methane production from Albian MFT are currently unclear. A better understanding of the process of methanogenesis in oil sands tailings is therefore required. The information available on anaerobic degradation of organic compounds in other natural environments may provide insight on the details of this process in oil sands tailings.

1.2 ANAEROBIC METHANE PRODUCTION

1.2.1 Microbial communities

The biodegradation of complex organic compounds to methane and carbon dioxide is a widespread process in anoxic environments including freshwater sediment, waterlogged solids such as rice paddies, sewage treatment plants, and the intestinal tracts of ruminants, humans and insects such as termites (Schink 1997). Research on anaerobic hydrocarbon degradation has been of interest because of the prevalence of hydrocarbons in the environment. Hydrocarbons occur in the environment naturally and anthropogenically; they are produced by living organisms and are also major constituents of petroleum products. The main hydrocarbon compounds in petroleum products such as gasoline or crude oil are aromatics and saturated alkanes. The degradation of these hydrocarbons has been observed frequently by mixed consortia found in anoxic environments, including oil-contaminated sediments (Massias et al. 2003; Miralles et al. 2007), fuel-contaminated groundwater (Cunningham et al. 2000), and gas condensate-contaminated aquifers (Gieg et al. 1999). The degradation of alkane and aromatic substrates has also been demonstrated in the laboratory using microbes from contaminated sites and enriched under iron-reducing (Botton and Parsons 2006), nitrate-reducing (An et al. 2004), and sulfate-reducing conditions (Coates et al. 1997; Edwards et al. 1992; Gieg et al. 1999; Elshahed et al. 2001).

Complex interactions among several different groups of microbes are required for the anaerobic breakdown of hydrocarbons into methane (Figure 1.1). The microbes involved are from Domains Bacteria and Archaea. Of the Bacteria, three main metabolic types are responsible for the degradation of complex, organic matter into simple substrates that the methanogens, belonging to the Archaea, can use to produce methane. The first metabolic group of Bacteria initiates attack on the organic compound and includes sulfate-, iron- and nitrate-reducing bacteria. Hydrocarbon compounds are often relatively stable under anaerobic conditions and these microbes are required to activate the hydrocarbons for further degradation. Activation of a hydrocarbon can occur through several mechanisms (Callaghan et al. 2006; Elshahed et al. 2001), discussed in Section 1.2.1.1. Following activation, the hydrocarbon is degraded by fermentative bacteria, which includes some sulfate- and iron-reducing bacteria, into simpler compounds like small organic acids and alcohols. The last group of bacteria, the acetogens, degrades the intermediate compounds further into simple substrates, such as acetate or hydrogen and carbon dioxide. Two main types of methanogens, acetoclastic and hydrogenotrophic, then produce methane from either acetate or hydrogen plus carbon dioxide, respectively.

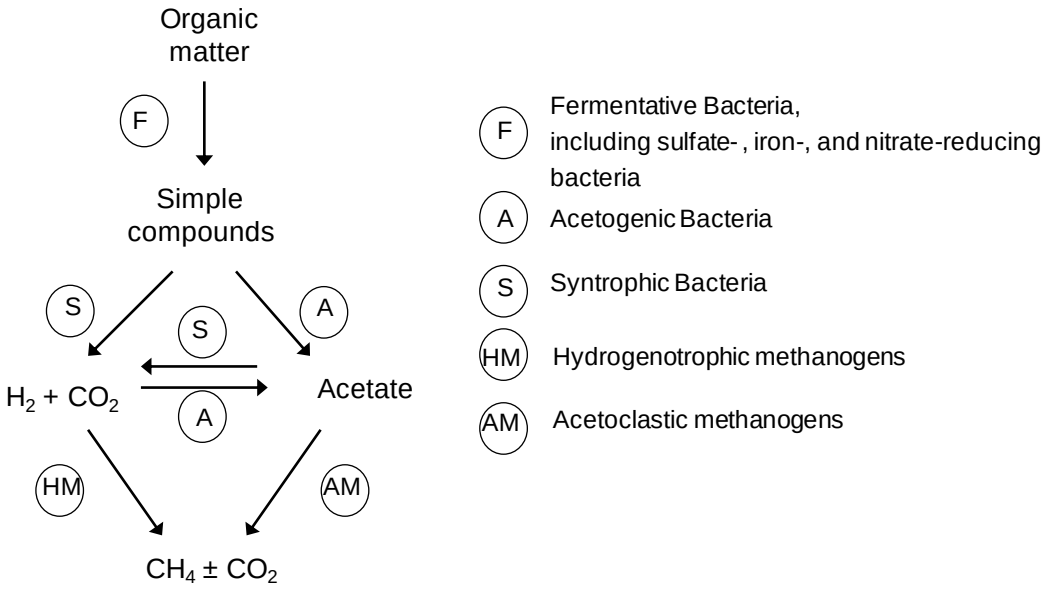

Figure 1.1. Schematic representation of the degradation pathway of organic matter under anaerobic conditions. Adapted from Lay et al. (1998).

The anaerobic degradation of hydrocarbons under methanogenic conditions has attracted the least attention; degradation of hydrocarbon compounds with carbon dioxide as a terminal electron acceptor has not been reported as often as with sulfate, nitrate, or iron as terminal electron acceptors. Reinhard et al. (2005) were able to demonstrate the degradation of BTEX into methane *in situ* at a gasoline-contaminated site under methanogenic conditions. Zengler et al. (1999) were the first group to demonstrate the full conversion of long-chain saturated alkanes into methane under strictly anoxic conditions, using incubations of ditch sediment and ^{13}C-labeled hexadecane. Using restriction fragment length polymorphism (RFLP) analysis of 16S rRNA genes, they were able to show the presence of bacteria affiliated with delta-Proteobacteria, the genera *Syntrophus* and *Desulfovibrio,* a hydrogen-utilizing sulfate-reducing bacterium, and archaeal species that affiliated with acetoclastic and hydrogenotrophic methanogens. The rapid degradation of hexadecane under methanogenic conditions was again shown by Anderson and Lovley (2000) who incubated oil-bearing sediments with ^{14}C-labeled hexadecane and observed the production of ^{14}C- methane.

The network of associations between the microbial species involved in degrading organic compounds is important for the growth of all groups of microbes. Cooperation among microbes is particularly important in methanogenic environments where the breakdown of substrates provides a very small amount of energy compared to oxidation of substrates with nitrate-, iron-, or sulfate-reduction (Schink 1997). Some substrates cannot be degraded unless coupled with the reaction of the downstream group. For example, anaerobic oxidation of some organic compounds like propionate and butyrate by acetogenic bacteria, including syntrophic bacteria, is thermodynamically unfavorable and requires interspecies hydrogen transfer through the efficient removal of the products by methanogens. The endergonic reaction becomes exergonic for acetogens or syntrophs through the low hydrogen partial pressure created by methanogens. Interactions between syntrophic bacteria and methanogenic species have been observed in numerous studies of anaerobic environments (references within de Bok et al. 2004). It is clear that each group of microbes in hydrocarbon-degrading consortia is crucial for the successful oxidation of an organic substrate to produce methane. Species within each microbial group have

successfully been isolated and identified, providing a more detailed understanding of their function, as described below.

1.2.1.1 Initiating anaerobic hydrocarbon degradation

Aromatic hydrocarbons and *n*-alkanes are the most readily available hydrocarbon substrates for use by anaerobic bacteria, particularly in environments such as aquifers, marine sediments, and landfill plumes contaminated by gasoline spillage, crude oil spills, and landfill leachate. Gasoline, crude oil, and other petroleum products contain numerous hydrocarbon compounds, but among these, aromatics and saturated alkanes are the most prominent and readily biodegradable. The majority of research in anaerobic hydrocarbon degradation has provided evidence for microbial species capable of anaerobically degrading aromatic and *n*-alkane hydrocarbon compounds.

In the cascade of interactions among microbial species, the first set of bacteria responsible for the activation of hydrocarbons for further degradation play an essential role. Aromatic and aliphatic hydrocarbons are relatively stable under anaerobic conditions. Without these bacteria, the degradation of aromatic and aliphatic hydrocarbon compounds would not be initiated and the microbes that act downstream to break down oxidized hydrocarbons into methane would not have substrates to use for energy or growth. The mechanisms of the initiating attack on aromatic and saturated alkanes appear to share similar characteristics.

Bacteria capable of initiating the degradation of aliphatic and aromatic hydrocarbons are mainly sulfate-reducing bacteria and fermenters. Fermenters are also involved in the degradation of activated hydrocarbons into smaller compounds. The BTEX compounds tend to be the most prominent aromatic hydrocarbons available in the aqueous phase of oil- or petroleum- contaminated environments because the solubility of hydrocarbons in water decreases as their molar volumes increase (Eganhouse et al. 1996); some aromatics are also hazardous to human health (López et al. 2008). Thus, there has been much focus on *in situ* microbial biodegradation of these compounds. The isolation of aromatic-degrading bacteria has proven to be difficult, particularly under methanogenic conditions. Interactions among syntrophic partners during anaerobic

growth on hydrocarbon substrates appear to be crucial for the survival of many hydrocarbon-degrading bacterial species (de Bok et al. 1994).

Despite these challenges, however, pure cultures of known species and newly isolated strains of Bacteria have been reported to degrade BTEX compounds in the absence of oxygen. The first microbial mixed culture to demonstrate anaerobic aromatic hydrocarbon degradation degraded benzene under methanogenic conditions (Vogel and Grbić-Galić 1986). However, the majority of aromatic hydrocarbon-degrading bacteria identified since this discovery degrade toluene and are denitrifying bacteria, such as *Azoarcus* species (Dolfing et al. 1990; Zhou et al. 1995, Fries et al. 1994; Rabus and Widdel 1995b) or *Thauera aromatica* strains (Anders et al. 1995; Evans et al. 1991) or sulfate-reducers, such as *Geobacter* species (Lovley and Lonergan 1990; Coates et al. 2001). Several pure strains of denitrifying bacteria that degrade more BTEX compounds than toluene have been successfully isolated and grow on toluene, *m*-xylene, ethylbenzene, and *n*-propylbenzene (Rabus and Widdle 1995b; Hess et al. 1997). These strains also matched most closely to *Thauera* and *Azoarcus* species (Zhou et al. 1995). There is also evidence of *o*-xylene, *m*-xylene (Harms et al. 1999) and ethylbenzene (Kniemeyer et al. 2003) degradation by sulfate-reducing isolates, which matched most closely to *Desulfosarcina* and *Desulfococcus*.

Anaerobic biodegradation of BTEX compounds occurs in different ways (reviewed by Foght 2008). Toluene (Biegert et al. 1996) and the xylene isomers (Beller and Spormann 1997; Krieger et al. 1999; Morasch and Meckenstock 2005) degrade via a fumarate addition reaction, forming benzylsuccinate or methylbenzylsuccinate, respectively. These intermediates are converted to benzoyl-CoA or benzoate, for toluene and xylene respectively, which can be degraded to produce carbon dioxide. Benzene has been proposed to degrade through three mechanisms (Heider et al. 1999): 1) benzene is hydroxylated to produce phenol (Vogel and Grbić-Galić 1986; Grbić-Galić and Vogel 1987) which is then either reduced to form cyclohexanone (Grbić-Galić and Vogel 1987) or carboxylated to form 4-hydroxybenzoate (Caldwell and Suflita 2000); 2) benzene is methylated to produce toluene (Coates et al. 2002) which then undergoes fumarate addition; 3) benzene is carboxylated to produce benzoate (Coates et al. 2002). These three benzene activation steps could all result in the formation of the central metabolite

benzoyl-CoA that is further oxidized to produce carbon dioxide (Chakraborty and Coates 2004). Ethylbenzene biodegradation has been reported to occur by two different mechanisms: 1) fumarate addition resulting in 1-phenylethylsuccinate, analogous to that of toluene (Kniemeyer et al. 2003), and 2) hydroxylation to form 1-phenylethanol (Rabus and Widdel 1995a; Heider et al. 1999) for carbon dioxide production.

Although aromatic hydrocarbon-degrading bacteria have not been isolated from methanogenic conditions, the biodegradation of aromatic compounds has been observed under methanogenic conditions (Gieg et al. 1999; Reinhard et al. 2005). Aromatic degradation has also been observed in denitrifying (Phelps and Young 1999; Cunningham et al. 2000), iron-reducing (Villatoro-Monzón et al. 2003; Jahn et al. 2005), and sulfate-reducing (Gieg et al. 1999; Elshahed et al. 2001) conditions in a variety of environments. Analysis of BTEX biodegradation suggests that both the available electron acceptors and environmental characteristics of a setting, including the pollution history of an environment, geochemical conditions, and temperature, can influence the degree of BTEX biodegradation that occurs (Phelps and Young 1999; Roychoudhury et al. 2006; Cunningham et al. 2000).

Aliphatic hydrocarbon biodegradation under anaerobic conditions has been studied considerably less than aromatic hydrocarbon biodegradation. There has been particularly little research focused on short-chain aliphatics. A few SRB have been isolated and demonstrated to use aliphatic compounds found in gas and oil (Rueter et al. 1994; Widdel and Bak 1992; Aeckersberg et al. 1991; 1998; So et al. 1999b; Kniemeyer et al. 2007). These strains grouped with Desulfobacteriaceae including *Desulfosarcina* and *Desulfococcus*, and with Desulfovibrionaceae according to 16S rRNA gene sequence similarity. *Desulfotomaculum*-like species detected in an enrichment culture from a gas condensate-contaminated aquifer were suggested to degrade alicyclic hydrocarbons under sulfate-reducing conditions (Rios-Hernandez et al. 2003). Even fewer denitrifying bacteria have been isolated that degrade *n*-alkanes under anaerobic conditions (Ehrenreich et al. 2000). This is due in part to limitations in cultivation techniques and a lack of knowledge of the microbial processes and species involved with anaerobic hydrocarbon degradation.

Thus far, using these isolated strains of alkane-degrading bacteria, two different mechanisms of alkane activation have been suggested (Kropp et al. 2000). A carboxylation reaction, although rarely cited (So et al. 2003), has been proposed to occur at C-3 of the alkane followed by the loss of two carbons to generate a fatty acid that either undergoes beta-oxidation to form acetate and ultimately carbon dioxide or transforms through chain elongation or methylation (Callaghan et al. 2006). A fumarate addition reaction analogous to that of toluene and xylene activation has been reported more often (Aeckersberg et al. 1998; Wilkes et al. 2002; Cravo-Laureau et al. 2005; Kropp et al. 2000; Rios-Hernandez et al. 2003; Davidova et al. 2005) and has been proposed to first produce a fatty acyl succinic acid that is then rearranged and decarboxylated to produce a fatty acid that is degraded through beta-oxidation to carbon dioxide or undergoes chain elongation and desaturation (So et al. 1999a; Wilkes et al. 2003). The activation reactions of *n*-alkanes have mainly been elucidated using isolates from sulfate- (Callaghan et al. 2006; Cravo-Laureau et al. 2005; Kropp et al. 2000; Davidova et al. 2005) and nitrate- reducing conditions (Rabus et al. 2001; Wilkes et al. 2003). Evidence of these reactions has not been collected under methanogenic conditions.

1.2.1.2 Anaerobic polyacrylamide degradation

Polyacrylamide is a general category for polymers containing acrylamide as a major constituent. These polymers may have various charges (anionic, cationic, neutral), charge densities and molecular weights (Lipp and Kozakiewicz 1991). Polyacrylamides are often used as flocculants in wastewater treatment and are used in the Albian tailings pond.

Polyacrylamides are relatively resistant to biodegradation (Kay-Shoemake et al. 1998a; b); this is likely due to their high molecular weights as well as cross-linking, making them inaccessible to cytoplasmic enzymes. Under aerobic conditions there is evidence of polyacrylamide biodegradation, though in these cases, polyacrylamide did not serve as a carbon source. Kay-Shoemake et al. (1998a; b) showed that polyacrylamide could be used as a nitrogen source. Aerobic microorganisms are able to deaminate polyacrylamide to form ammonia using extracellular amidases. Several studies

have demonstrated microbial growth on polyacrylamide as a sole nitrogen source (Kay-Shoemake et al. 1998a; b; Soponkanaporn and Gehr 1989; Nakamiya and Kinoshita 1995; Grula et al. 1994).

Under anaerobic conditions, there is little reported evidence that polyacrylamide is biodegraded. Chang et al. (2001) observed the partial degradation of polyacrylamide under anaerobic conditions. The presence of polyacrylamide significantly enhanced gas production from an anaerobic culture. However, this gas was not analyzed so its methane content is unknown. They observed that the compound resulting from microbial activity on the polyacrylamide was trimethylamine, a substrate for some methanogens. The ability of polyacrylamide to serve as a nitrogen source under anaerobic conditions was confirmed in various wastewater samples, including oil sands tailings samples (Haveroen et al. 2005). Polyacrylamide stimulated methanogenesis from the microbial consortia of oil sands tailings samples when amended as the sole nitrogen source in the presence of abundant carbon, amended as acetate or benzoate. Polyacrylamide has also been suggested as an electron source for SRB, although no direct evidence has been reported (Grula et al. 1994).

1.2.1.3 Anaerobic citrate degradation

Trisodium citrate ($Na_3C_6H_5O_7$) is added to the Albian tailings pond as a water softener. Citrate chelates divalent cations such as calcium and magnesium ions from the pore water of the MFT. However, the sodium concentrations of the pore water increase as a result of trisodium citrate-treatment.

As in the case of hydrocarbon degradation, citrate degradation under anaerobic conditions has been studied less than under aerobic conditions. Citrate is ubiquitously found in nature and occurs in all living cells. A large variety of microbial species are capable of using citrate as a carbon source for growth. In the presence of oxygen, citrate is typically metabolized by the tricarboxylic acid cycle; however, anaerobes have developed different metabolic pathways, generally termed the “citrate fermentation pathway” (Antranikian and Giffhorn 1987). Citrate fermentation has been studied extensively in lactic acid bacteria and in Enterococci due to the occurrence of citrate in

many natural foods, such as dairy products, vegetables, and fruits. Several species of *Clostridium* are also known to be capable of using citrate (Antranikian et al. 1984; Liou et al. 2005). The enzymes and mechanisms involved in citrate fermentation in these groups of bacteria have been elucidated (Antranikian and Giffhorn 1987). Different microbial species produce different end products from fermentation of citrate but a common product is acetate.

Citrate has not been considered a preferred substrate for sulfate- , nitrate- or iron-reducing bacteria. However, recent evidence suggests that in anaerobic environments, citrate may play a more significant role in microbial communities than previously thought. Coates et al. (1999) isolated an iron-reducing bacterium capable of fermenting citrate in the absence of other electron acceptors and the primary products were acetate and succinate. *Desulfovibrio oxamicus*, a nitrate- and sulfate-reducing bacterium, has been shown to oxidize citrate with the reduction of nitrate (López-Cortés et al. 2006). The degradation of citrate by microbial consortia under anaerobic conditions has rarely been examined. Stams et al. (2009) analyzed sulfate-reduction by the microbial consortium in a biological treatment plant in the presence of citrate-containing wastewater. Citrate was fermented to mainly acetate and formate and these acted as electron donors for the reduction of sulfate. The two isolated bacterial species responsible for these fermentation products matched most closely to *Trichococcus* and *Veillonella* by 16S rRNA gene sequences. Whereas several *Trichococcus* species are capable of fermenting citrate, only a single species of *Veillonella* is reported to ferment citrate (De Vries et al. 1977), although citrate has rarely been tested as a growth substrate for isolates of this genus. To date, there is a lack of research on citrate degradation under methanogenic conditions. Perhaps the only study of citrate degradation by a methanogenic microbial consortium was done by Gámez et al. (2009) who observed the fermentation of citrate by the microbial consortium of methanogenic granular sludge. Acetate, carbon dioxide, and hydrogen were the main products of this fermentation and acetate was then degraded completely to produce methane. Acetate concentrations were noticeably higher when citrate fermentation by the methanogenic consortium occurred in the presence of bicarbonate medium; this was attributed to the activity of acetogenic bacteria.

1.2.1.4 Acetogens

There are numerous genera of acetogens, including *Acetobacterium*, *Clostridium*, *Syntrophococcus*, and *Treponema* (Drake et al. 2006), which are widely dispersed throughout the domain Bacteria, according to 16S rRNA gene sequence. Acetogens are defined by their hydrogen-dependent reduction of carbon dioxide to synthesize acetate, specifically by the acetyl-CoA pathway (Drake 1994). Though identification of acetogens in hydrocarbon-degrading communities is generally lacking in the literature, acetogens have been isolated from a very wide range of habitats, including gastrointestinal tracts, anoxic sediments and aquifers, and even aerated soils, where they are exposed to substrates such as those they would encounter as part of a hydrocarbon-degrading consortium (Drake and Küsel 2003).

It has been suggested that the main role of acetogens is not to use hydrogen, but to convert available organic matter into acetate (Lay et al. 1998). Within the collection of trophic links that are required for the anaerobic degradation of organic matter into methane, acetogens participate in several different ways. Acetogens use simple compounds formed from the fermentation of an organic substrate, including single-carbon compounds as well as hydrogen plus carbon dioxide, to produce acetate. Some acetogens are capable of producing end products other than acetate by using terminal electron acceptors besides carbon dioxide (Drake et al. 2006). One example is *Clostridium carboxidivorans* sp. nov., which demonstrated growth on a large variety of carbohydrates, alcohols, amino acids, and organic acids with the production of acetate, ethanol, butyrate and butanol (Liou et al. 2005). Drake et al. (2006) outlined the many end products of organisms that use the acetyl-CoA pathway.

A few acetogens are also capable of consuming acetate to produce hydrogen and carbon dioxide; however, growth on acetate is affected by the presence of a hydrogen-consuming methanogen (Hattori et al. 2000). The use of acetate, hydrogen and carbon dioxide by acetogens plays a significant role in the overall cascade of reactions in the generation of methane because these substrates are also used by acetoclastic and hydrogenotrophic methanogens in the generation of methane.

Acetogens compete with fermenters for simple compounds and with sulfate-, nitrate-, iron-reducers, and hydrogenotrophic methanogens for hydrogen. The competition for hydrogen between acetogens and methanogens has been frequently observed. The outcome of this competition is affected by different factors of the surrounding environment, including temperature and pH, which is reflected in the activity and abundance of these two groups of microbes in an environment. Acetogens are superior competitors for hydrogen at low pH values (i.e. pH <7; Phelps and Zeikus 1984) and at low temperatures (<15°C) when hydrogen is not a limited substrate (Kotsyurbenko et al. 2001). Under these environmental conditions, hydrogen and carbon primarily flow through acetate to methane. However, low hydrogen partial pressure has been observed to be disadvantageous for acetogenic bacteria (Kotsyurbenko et al. 2001). There has been conflicting evidence for the role of bacterial density in determining which group of microbes has the competitive advantage for hydrogen. High bacterial density has been reported to both promote and inhibit acetogenic bacteria. According to Lay et al. (1998), despite the theoretically more favorable methanogenic reaction, at high bacterial density acetogens can outcompete hydrogenotrophic methanogens because they are capable of using a variety of other substrates for growth besides hydrogen and carbon dioxide. However, acetogens have also been observed as the stronger competitor in low density microbial population and in the presence of an excess of substrate (Kotsyurbenko et al. 1996). This may be due to differences in other factors such as temperature and available growth substrates.

Acetogens thus play a number of roles in the anaerobic degradation of organic substrates. They are capable of degrading simple compounds into acetate, hydrogen and carbon dioxide, as well as catalyzing the forward and reverse conversion of hydrogen and carbon dioxide into acetate. Finally, acetogens also participate in a fine balance of hydrogen and acetate consumption with hydrogenotrophic and acetoclastic methanogens.

1.2.1.5 Methanogens

Methanogens are a unique group of obligate anaerobes of the kingdom Euryarchaeota that are characterized by their ability to produce methane. There are three

main classes of methanogens: Methanobacteria, Methanococci, and Methanomicrobia (Demirel and Scherer 2008). The growth substrates of methanogens are relatively restricted and include hydrogen and carbon dioxide, formate, acetate, methanol, and methylamines. Methanogens inhabit a variety of environments, ranging from gastrointestinal tracts, hydrothermal vents, ride paddy soils, to anaerobic sediments, sewage sludge and Antarctic lakes (Chaban et al. 2006). In order to survive in these diverse environments, methanogens participate in microbial communities where complex organic matter is degraded by bacteria to produce simpler compounds that are within their limited range of growth substrates. Two types of methanogens, acetoclastic and hydrogenotrophic methanogens, are the most prevalent methanogens among methane-producing anaerobic sediments and have been identified within oil sands tailings (Penner 2006).

In the degradation of organic matter, acetoclastic methanogens convert acetate, generated by the acetogens or fermenters, into methane whereas hydrogenotrophic methanogens use hydrogen to reduce carbon dioxide, producing methane. Zengler et al. (1999) were the first to show the anaerobic microbiological conversion of long-chain alkanes to methane, and through 16S rRNA gene analysis, identified archaeal species that associated with acetoclastic and hydrogenotrophic methanogens. Acetoclastic and hydrogenotrophic methanogens were also identified in a high-temperature, sulfur-rich oil reservoir by molecular and culture-based methods (Orphan et al. 2000). Among the thermophilic methanogens detected were species closely related to Methanomicrobiales, hydrogenotrophic methanogens, and Methanosarcinales, which includes acetoclastic and hydrogenotrophic methanogens. This was the first report of Methanosarcinales in petroleum reservoirs. Few studies have since been conducted on the activity of acetoclastic or hydrogenotrophic methanogens in the conversion of hydrocarbon substrates into methane. *Methanosaeta* spp., obligate acetoclasts, have been identified in a hydrocarbon-contaminated aquifer (Dojka et al. 1998); Methanomicrobiaceae, hydrogenotrophs, and *Methanosaeta* were found in a petroleum-hydrocarbon-contaminated aquifer (Kleikemper et al. 2005); and Methanosaetaceae dominated the Archaeal species in the groundwater of a gas condensate-contaminated aquifer (Struchtemeyer et al. 2005).

Smith and Mah (1978) analyzed a strain of *Methanosarcina* capable of growing on acetate as well as hydrogen plus carbon dioxide. They provided evidence suggesting that acetate is not the preferred substrate for methane generation in the presence of hydrogen and carbon dioxide. This is in agreement with the theoretical energy that methanogenesis through acetoclastic and hydrogenotrophic reactions provide. Acetoclastic methanogenesis (Equation 1.1) provides considerably less energy than hydrogenotrophic methanogenesis (Equation 1.2).

$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$ ($\Delta G°' = -36.0$ kJ/mol CH_4) (Eq. 1.1)

$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ ($\Delta G°' = -131.0$ kJ/mol CH_4) (Eq. 1.2)

However, beyond energetic considerations, there are a number of environmental factors that affect the balance between acetoclastic and hydrogenotrophic methanogenic activity, including acetate concentrations, pH, and temperature (Drake and Küsel 2003). It has been reported that at low temperatures (~5°C), acetoclastic methanogens dominate and at high temperatures (~50°C), hydrogenotrophic methanogens dominate (Nozhevnikova et al. 2007; Glissman et al. 2004). Under mesophilic conditions (~30°C), both hydrogenotrophic and acetoclastic methanogenesis were observed.

1.3 MOLECULAR ANALYSIS OF ENVIRONMENTAL SAMPLES

Relatively little research has focused on the identification of the microbial species involved in anaerobic hydrocarbon degradation in their natural environments and even less has centered on the isolation and characterization of these species. The major limiting factor in expanding our understanding of microbial methane production from organic compounds has been the availability of informative analytical techniques. Culture-based methods involve isolating and growing individual microbial strains to analyze their physiology and metabolic capabilities. However, the preferred growth conditions of species are often unknown or laboratory conditions are inadequate to supply them (Amann et al. 1995). The *in situ* environment of a microbial consortium provides very specific conditions, including temperature, salinity, pH, growth substrates,

availability of electron acceptors, moisture level, and exposure to light. A change in any one of these conditions could prevent growth or cause biased growth of the isolated species. Molecular-based analyses have become the predominant method for studying environmental samples because they do not require cultivation. Some techniques for analysis of microbial consortia and their capabilities and limitations for application to environmental samples are discussed here.

1.3.1 DNA extraction

Molecular analysis of environmental samples requires first obtaining total genomic DNA. Numerous extraction techniques have been developed for the lysis of microbial cells, removal of cellular debris, and purification of DNA. However, these techniques range in their ability to produce high quality and high yield of DNA; the type of environmental sample being analyzed can also affect quality and yield due to contaminating or interfering substances, such as humic acids in soil samples. Microbial cells can be disrupted by mechanical and chemical means. Common DNA extraction methods include using detergents like sodium dodecyl sulfate or enzymes like proteinase K and include mechanical methods like bead-beating, freeze-thaw cycles, or sonication. In most cases, a combination of chemical and mechanical techniques is used (Rajendhran and Gunasekaran 2008). Mechanical lysis (e.g. bead-beating and sonication) produces sheared DNA that is suitable for subsequent amplification, whereas chemical methods (e.g. detergents and enzymatic digestion) produce high-molecular weight DNA suitable for cloning without amplification.

1.3.2 Polymerase chain reaction (PCR)-based analyses

Molecular-based analyses often involve polymerase chain reaction (PCR) for the amplification of an identifying sequence of DNA from the total extracted genomic DNA. For analysis of a microbial community with unknown diversity, universal primers for PCR amplification of an identifying gene common to all species, such as the 16S rRNA gene, are typically used. Primers can also be chosen to target an identifying gene of a

specific group of Bacteria or Archaea in an environmental sample. For example, the methyl coenzyme-M reductase (*mcrA*) can be used to identify methanogens (Luton et al. 2002). Another factor in selecting primers is the length of the amplicons they produce. Longer amplicons are more informative for identification based on sequence similarity to database collections, such as the National Center for Biotechnology Information (NCBI).

The amplicons can be analyzed in a variety of ways to obtain information of the microbial community of a sample. Microbial community analysis techniques involving PCR include RFLP analysis of 16S rRNA gene sequences, denaturing gradient gel electrophoresis (DGGE), microarrays, and stable-isotope probing. The use of PCR in studying microbial communities has increased the efficiency and sensitivity of analysis. PCR amplifies very small amounts of DNA which increases the likelihood that rare organisms will be detected. PCR also significantly reduces the volume of sample required for analysis (von Wintzingerode et al. 1997). However, PCR can produce biased results and universal primers can fail to detect the entire microbial community (Sipos et al. 2007). The results generated from PCR-based analyses must be considered carefully. This thesis project involved two DNA-based techniques which are discussed in greater detail below.

1.3.2.1 Clone library construction and restriction fragment length polymorphism (RFLP) analysis

RFLP analysis of the microbial species within an environmental sample first requires cloning the PCR products from the amplification of Bacterial and Archaeal 16S rRNA genes from total extracted genomic DNA. A sufficient number of clones are selected for restriction enzyme digestion to generate digested fragment patterns. Because each species of Bacteria or Archaea has slightly different 16S rRNA gene sequences, different species should generate different restriction fragment patterns. Typically tetrabase recognition restriction enzymes, such as *HaeIII*, *HhaI*, and *AluI*, are chosen to generate digestion patterns that can differentiate among species. If an enzyme does not cut often and produces large DNA fragments, some of the community diversity will be missed. Several enzymes should be screened on database 16S rRNA gene sequences to

determine the optimal ones to use on an environmental sample (Heyndrickx et al. 1996; Engebretson and Moyer 2003). Similar restriction patterns are grouped and a representative clone from each pattern is sequenced for identification of the species. The 16S rRNA gene sequences can be used for phylogenetic analyses to infer potential metabolic functions of the species. The general steps of clone library preparation and RFLP analysis of 16S rRNA gene sequences are outlined in Figure 1.2.

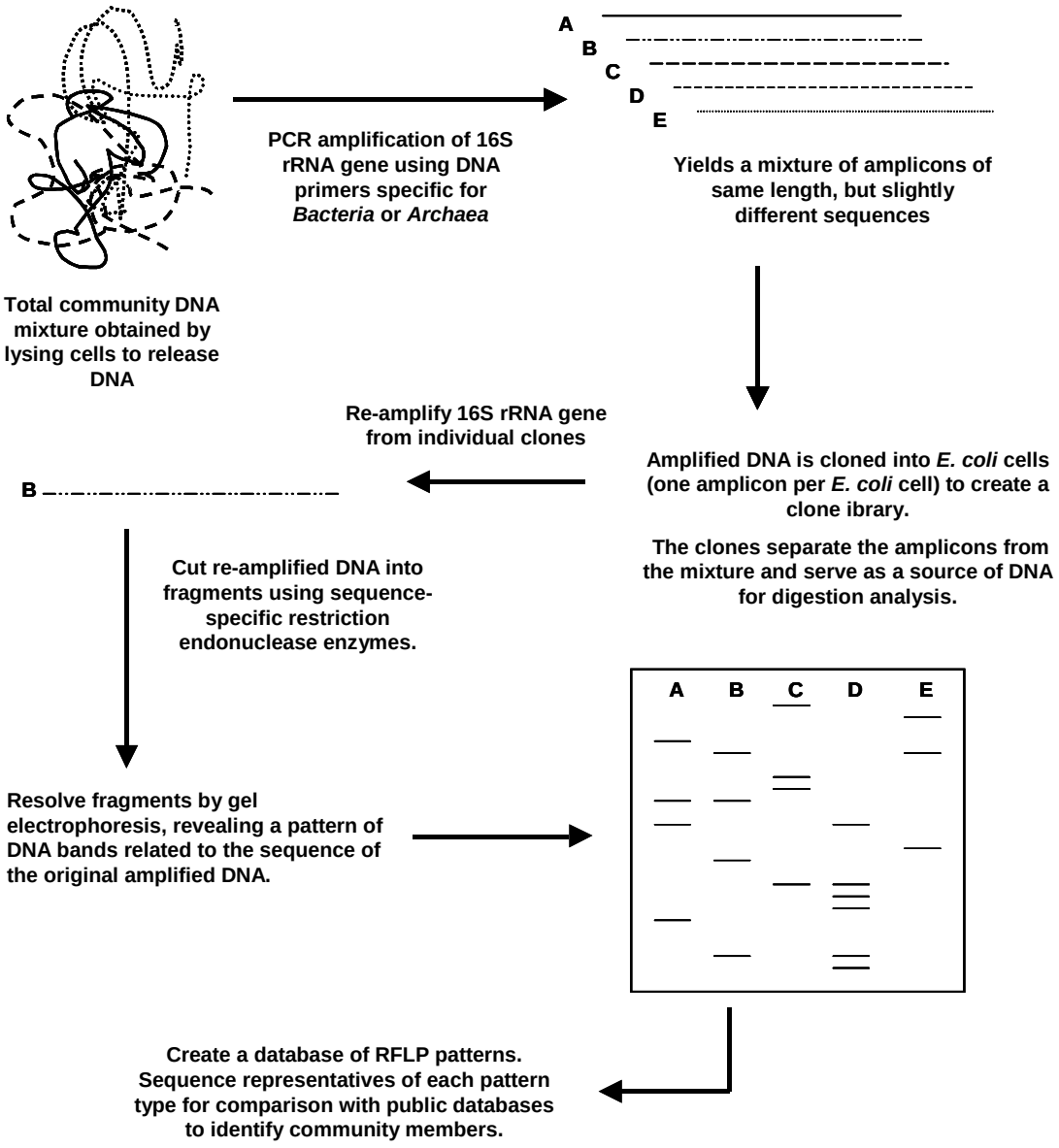

Figure 1.2. Schematic representation of clone library construction and RFLP analysis

Clone library construction and RFLP analysis allow for the presumptive identification of the microbial species within a community. Although clone library and RFLP analysis is highly time-consuming, it offers valuable information. Theoretically, clone libraries should provide a rough estimate of the proportion of each presumptive species as well as an indication of the dominant and rare species within a community. However, this technique cannot compensate for the possibility that new and unknown sequences are not represented in public databases. In this event, clone libraries are able to suggest the metabolic capabilities of a community of microbes based on their closest known relatives.

1.3.2.2 Denaturing gradient gel electrophoresis (DGGE) analysis

Denaturing gradient gel electrophoresis (DGGE) analysis, like clone libraries and RFLP analysis, offers the potential of identifying the microbial species of a community within a sample. However, DGGE analysis is a relatively rapid and efficient technique in comparison to clone libraries and RFLP analysis. DGGE can quickly offer information on the diversity of a sample and compare the diversity of different samples. Like RFLP analysis, DGGE analysis exploits the fact that 16S rRNA gene sequences differ between species. Unlike clone library and RFLP analysis, DGGE analysis operates on the fact that different DNA sequences have different melting points. The sequence of a DNA fragment determines its melting point, the temperature at which the double-stranded DNA fragment denatures into single strands. Thus, when a mixture of 16S rRNA genes amplified from a microbial community is run on a denaturing gel, the total amplicons from a microbial community will separate and cease to migrate at sequence-specific positions in the gel. Therefore, each band in a DGGE gel lane theoretically represents a different microbial species. The general steps of DGGE analysis are outlined in Figure 1.3.

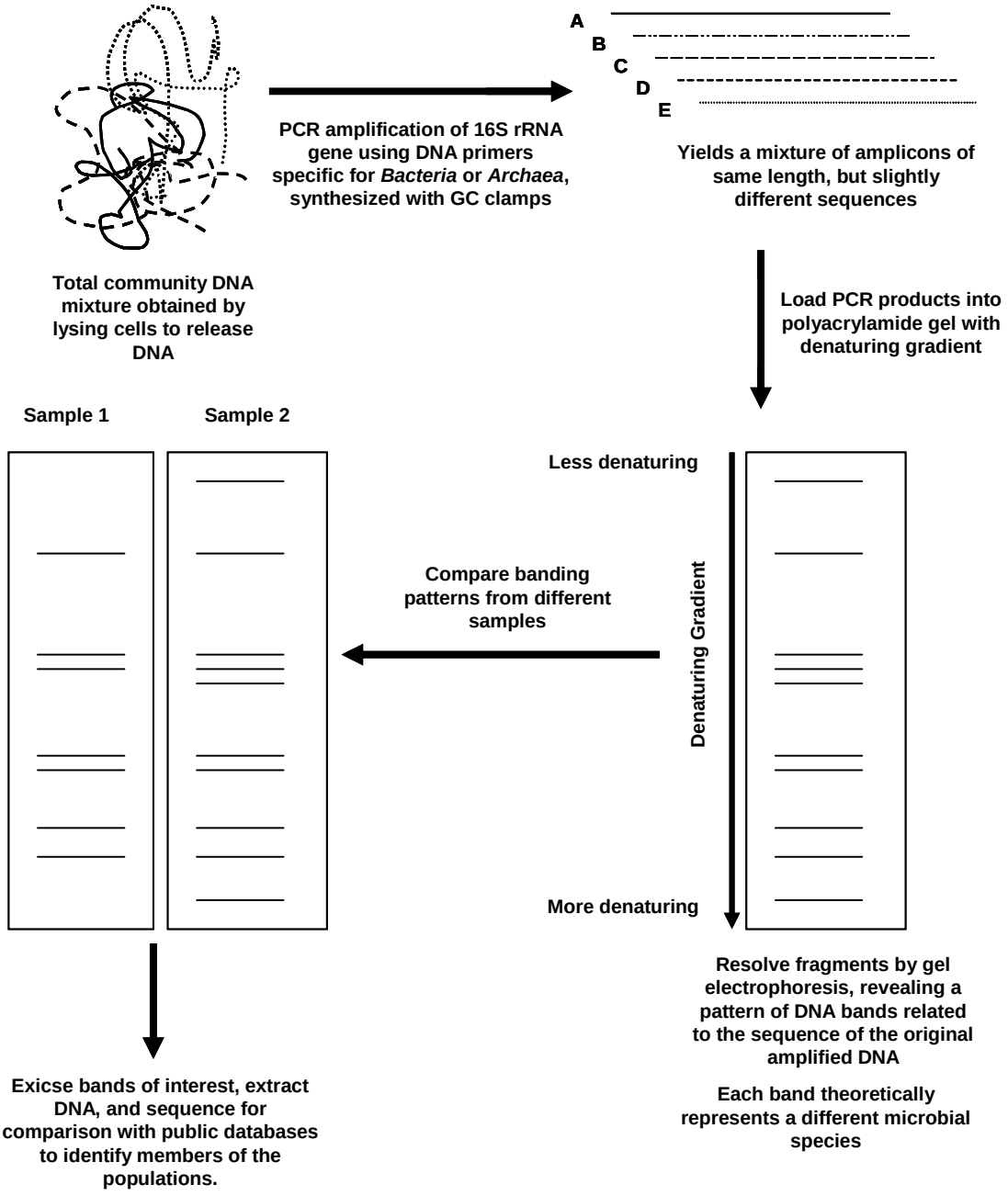

Figure 1.3. Schematic representation of denaturing gradient gel electrophoresis (DGGE) analysis.

1.3.2.3 Biases and misrepresentation

The greatest concern in using molecular techniques for the analysis of environmental samples is whether or not an accurate representation of the *in situ* microbial community is obtained. Biases inherent to the techniques of analysis can lead to misrepresentation of the microbial diversity of a sample. Limitations of the chosen technique must be taken into consideration when analyzing the results generated by each technique.

The first step of extracting total genomic DNA from an environmental sample can be the main limiting factor in an analysis. Microbial cells may not lyse with equal efficiency (Moré et al. 1994) or inhibiting factors may prevent the complete collection of genomic DNA from a sample. Sagova-Mareckova et al. (2008) found that DNA yields varied among soil samples with different pH levels and clay content. Also, some extraction techniques can be severe, such as bead-beating, and cause shearing of the genomic DNA. Small fragments of DNA can lead to chimaeras which will influence the apparent diversity of the microbial species (von Wintzingerode et al. 1997).

A major concern with techniques involving PCR is whether amplification can occur. Substances in environmental samples, such as humic acids, are often coextracted with nucleic acids and prevent amplification of template DNA (Tebbe and Vahjen 1993). There are also inherent problems in PCR amplification that can lead to an inaccurate depiction of the microbial population *in situ*. Templates with higher-order structures or with high GC content are less likely to dissociate, resulting in selective priming (Reysenbach et al. 1992). Annealing temperatures have also been found to heavily skew the amplification of different templates. Ishii and Fukui (2001) found that lower annealing temperatures were better than higher temperatures for the amplification of high quantities of DNA from both pure cultures and environmental samples of compost. A bias in PCR amplification of rRNA genes was also observed to be caused by the primers and the number of replication cycles used (Suzuki and Giovannoni 1996). Furthermore, rare species may remain undetected in diverse populations and the amplification of contaminating DNA can also lead to ambiguous results.

In addition to the biases of genomic DNA extraction and PCR amplification, there are also potential problems in RFLP analysis of clone libraries. The rRNA gene sequences of different species may have different cloning efficiencies and different digestion efficiencies, resulting in misrepresentative proportions of each species. The choice of restriction enzymes affects the observed digest patterns and can cause biased results; an enzyme that generates large digest fragments may miss variation among DNA sequences and an enzyme that cuts too often will not differentiate different microorganisms with very similar DNA sequences. In both cases, a lower diversity is observed which is not reflective of *in situ* microbial diversity. Wright and Pimm (2003) observed that *HaeIII* was the best restriction enzyme to use for differentiating species of methanogens and that different restriction enzymes could be used to specifically differentiate between two species of methanogens. Therefore, the restriction enzymes used for RFLP analysis can influence the observed diversity of a sample. Dojka et al. (1998) asserted that quantitative assessments of a community are better made with whole-cell *in situ* rRNA gene probes or hybridization to extracted RNA. Chimaeric sequences are also not apparent in RFLP analysis; the digestion of chimaeric sequences generates an increased variety of digestion patterns, giving an inaccurate depiction of the microbial diversity *in situ*.

DGGE analysis is subject to the inherent limitations of genomic DNA extraction and PCR amplification. In addition, it has been reported that the bacterial primers commonly used in DGGE analysis of communities are a perfect match for only a fraction of the available bacterial sequences and that the intensity of bands from DGGE gels are not accurate representations of the quantities of microbial species in the original sample (Ishii and Fukui 2001). DGGE analysis is also less informative than RFLP analysis due to the short sequences that are amplified. RFLP analysis involves cloning almost the entire 16S rRNA gene in Bacteria and Archaea, about 1500 and 900 nucleotides respectively; DGGE amplifies a fragment of each gene that is ~200-500 nucleotides long.

1.3.2.4 Analysis of microbial communities in oil sands MFT

Despite the numerous available methods of microbial community analysis, to date minimal work has been done on the microbial consortia of oil sands MFT. There are many difficulties in performing molecular analysis on MFT. Culture methods and even some DNA-based analyses such as fluorescent *in situ* hybridization and microarrays are not feasible because of the lack of biological information on MFT. RFLP and DGGE analyses are more practical for MFT analysis, but still present complexities. The extraction of genomic DNA from MFT material is problematic. Like some soils, MFT has a high clay content which can interfere with the extraction of genomic DNA. Released DNA from lysed cells is likely to be adsorbed to the surfaces of clay particles due to the negative charge of the phosphate backbone of DNA molecules (Cai et al. 2006). MFT can also contain humic acids and other inhibitors that prevent proper PCR amplification from template genomic DNA. These contaminants can lead to poor amplification combined with biased results. Feinstein et al. (2009) have suggested that pooling three successive extractions from a single sample helps to reduce extraction biases. In addition, the 16S rRNA genes of the microorganisms successfully analyzed and sequenced may not have been previously identified or cultured. Therefore, metabolic functions of these unknown tailings microorganisms can only at best be inferred from their most closely-related species, which in some cases are not well-characterized.

1.4 CURRENT STATE OF KNOWLEDGE

1.4.1 Syncrude Canada

Penner (2006) provided the first analysis of the microbial consortia of oil sands tailings using Bacterial and Archaeal 16S rRNA genes in Syncrude MLSB MFT. Penner (2006) optimized genomic DNA extraction, PCR amplification, cloning, and RFLP analysis for Syncrude MLSB MFT and putatively identified numerous species of the microbial community. The Bacteria that Penner (2006) identified in MLSB MFT were composed primarily of Proteobacteria. Clones related to each subgroup, *alpha-*, *beta-*, *gamma-*, *delta-*, and *epsilon-*, were found; however, the majority of clones were related to

beta-proteobacteria, particularly *Rhodoferax ferrireducens*, and the genus *Thiobacillus*. Clones matching to *Syntrophus* species and SRB such as *Desulfobacterium* species were detected as well, although at lower frequency. A considerable number of clones related to species within the class Clostridia were also identified. Clostridia are general fermenters of organic matter and some are acetogens. The remainder of the 16S rRNA gene sequences were most closely related to uncultured clones. The Archaea in MLSB MFT consisted of clones matching to hydrogenotrophic methanogens, such as *Methanocalculus* and *Methanoculleus*, and to acetoclastic methanogens such as *Methanosaeta*. Methanogens closely related to *Methanosarcina* were also detected; these methanogens are able to use hydrogen plus carbon dioxide or acetate as substrates for methane production.

Based on the identified species from Syncrude MLSB MFT, Penner (2006) constructed a potential scheme of the anaerobic degradation of hydrocarbon compounds found in naphtha to methane (Penner 2006). The naphtha hydrocarbons are first degraded by SRB, such as *Desulfocapsa* and *Desulfobacterium*, to generate aromatic and aliphatic acids and alcohols. The activity of Clostridia species such as *Tissierella, Soehngenia,* and *Clostridium* generate simple compounds, including acetate and propionate, which are then used by either syntrophic bacteria or acetogens to produce hydrogen plus carbon dioxide or acetate, respectively. Finally, hydrogen plus carbon dioxide are used by hydrogenotrophic methanogens and acetate is used by acetoclastic methanogens, to produce methane. The microbial analysis of MLSB MFT (Penner 2006) laid the groundwork for future studies on microbial communities in oil sands tailings.

The recent discovery that short-chain alkanes and BTEX compounds are causing and sustaining methanogenesis from the consortium in Syncrude MLSB (Siddique et al. 2006; 2007) combined with the work of Penner (2006) has presented many options for further research, including isolating bacteria capable of activating short-chain hydrocarbons for degradation and determining the mechanism(s) they use. In terms of research on oil sands tailings, the task at hand is to broaden our understanding of anaerobic biodegradation of organic matter and its effects in tailings ponds beyond Syncrude MLSB.

Methane release from tailings ponds can either be prevented, in environmental interests, or stimulated to quicken the densification of tailings, in industrial interests for tailings management, water recovery, and reclamation. However, before a decision can be made, knowledge of the microbial consortia in oil sands tailings and their activities is required. Actions towards inhibiting or stimulating methane release from tailings ponds cannot be implemented without first understanding the metabolic capabilities of the microbial species within tailings. Potential reactions of the microbial consortium to methane-enhancing or -inhibiting procedures must be known in order to prevent unwanted outcomes like the formation of equally or more environmentally dangerous effects, such as production of hydrogen sulfide.

1.4.2 Shell Albian Sands

The tailings pond at Albian became methanogenic like Syncrude MLSB in the absence of naphtha diluent. The diluent used by Albian provides only aliphatic (C_5-C_6) compounds to the MFT. Albian also adds trisodium citrate and polyacrylamide to its MFT. Studying and comparing methanogenesis in Albian MFT to Syncrude MLSB MFT will broaden our knowledge of this process and its effects in oil sands tailings ponds. It will also provide insight into the effects of different substrates on methane production in MFT and may explain the rapid development of methane in the Albian tailings pond compared to Syncrude MLSB.

The main questions surrounding methane production in the Albian tailings pond include what microbial species are in this MFT and what substrate(s) the microbes use to produce methane. By answering these questions, it may be possible to determine if the rapid methane emissions from this tailings pond are due to different microbial consortia and/or different substrates than those of MLSB. Examining tailings densification in Shell Albian tailings could also provide further insight into the several mechanisms of accelerated tailings densification by methane production and release.

1.5 STATEMENT OF OBJECTIVES

This thesis comprised a portion of a larger research project which aimed 1) to determine the methanogenic substrate(s) in Albian tailings pond, 2) to identify the microbial species in Albian MFT, and 3) to determine the effects of methane production on the densification of Albian MFT. The first objective was addressed by Dr. Tariq Siddique through microcosm analyses. The latter two objectives were addressed through the work done for this thesis.

The specific goals of this thesis project were:

1. To enumerate fermenters, SRB, and methanogens in Albian MFT;
2. To identify the Bacterial and Archaeal species in Albian MFT samples through clone library construction and RFLP analysis;
3. To identify the effects of substrate amendment by comparing Albian diluent-, citrate- and/or polyacrylamide-amendments on the microbial community in Albian MFT through DGGE analysis of mid- (7 L) and large-scale (300 L) studies;
4. To observe the effects of substrate amendment on methane release from Albian MFT through mid-scale settling columns;
5. To observe the effects of gas production and release on densification of Albian MFT through small- (0.15 L), mid-, and large-scale studies.

This is the first microbiological study of Albian MFT. The data collected from this work revealed a likely pathway for methane generation in the Albian tailings pond, including the substrate being degraded and the microbes involved. This work has also identified key differences between the microbial communities of Syncrude MLSB MFT and Albian MFT. The effects of gas production on tailings densification in Albian MFT were also determined and these observations complemented work on Syncrude MLSB MFT, strengthening the importance of understanding methanogenesis in oil sands tailings and its potential in accelerating tailings densification.

2 MATERIALS AND METHODS

2.1 MFT SAMPLES RECEIVED

The Albian Sands MFT received are listed in Table 2.1 with the corresponding date the sample was received, the location and depth from the tailings pond where the sample was collected, and the approximate volume of each sample received. These samples were used in various studies described below. For the sake of brevity, samples subsequently will be referred to by the abbreviations in Table 2.1. The locations in the tailings pond of Albian Sands from which M1, M2, and M6 MFT samples were taken is depicted in Figure 2.1. The specific location (UTM coordinates) of M4 and M5 MFT samples is unclear and was omitted from the figure.

Table 2.1. Inventory of Albian MFT samples received. Assigned names, dates of sampling, site coordinates, and depth of sampling are indicated.

MFT sample	Date sampled	UTM coordinates		Depth (mbs[a])	Approximate volume received (L)
M1	June, 2007	0465371E	6342304N	7	40
M2	June, 2007	0465769E	6342185N	22.5	20
M4	August, 2007	0465737E	634025N	5	20
M5	August, 2007	0465737E	634025N	9	20
M6	August, 2007	0465426E	6343397N	9	40
Bulk sample	June, 2008	0465371E	6342304N	7	2000

[a] meters below surface

2.2 MICROBIAL AND MOLECULAR TECHNIQUES

The different studies in this project largely used the same analytical techniques: most probable number (MPN) enumeration, clone library construction and restriction fragment length polymorphism analysis, DNA sequencing and denaturing gradient gel electrophoresis analysis. For this reason, these techniques are first described in this section and details specific to each part of the project are provided in their respective sections.

Figure 2.1. Sample locations within the tailings pond of Shell Albian Sands for M1, M2, and M6 MFT samples. Locations are marked with triangles. M4 and M5 MFT samples have been omitted because their exact sample location is unclear. The background is a false natural color composite of Landsat 5 Thematic Mapper satellite imagery of Path 42 Row 24, taken on September 14, 2009. Map was obtained from and sample locations were marked by Charlene Nielsen, Geographic Information Systems in Biological Sciences, University of Alberta.

2.2.1 MPN analyses

A 3-tube MPN method with selective media designed to support each of the metabolic groups of interest was used. Four different metabolic groups were analyzed: fermenters, SRB, iron-reducing bacteria (FeRB), and methanogens. Media were aliquoted in 9 mL portions per Hungate tube.

Fermenter medium contained (per liter) 30 g BBL Trypticase Soy Broth (BDL), 0.5 g cysteine, and resazurin dye (1 mg/L). Media were dispensed into Hungate tubes under nitrogen gas and all tubes were autoclaved before inoculation.

Two different sulfate-reducing media were made. SRB medium #1, used for MPN analyses of M1, M2, M4, M5, and M6 MFT samples (Section 2.4.1), was prepared as described for freshwater medium (Collins and Widdel 1986), with the addition of six carbon sources (per liter): sodium lactate (1.2 g), sodium acetate (0.4 g), sodium benzoate (0.1 g), sodium propionate (0.3 mL), ethanol (0.35 mL), and decanoic acid (0.1 g). Tubes contained one iron finishing nail, which was rinsed with dichloromethane (Fisher Scientific) then water prior to use, and were autoclaved after dispensing the medium. One-tenth milliliter of a sterile vitamin solution (ATCC 1992) was then added to each tube.

SRB medium #2, used for MPN analyses of megacosm (Section 2.7.5) and settling column (Section 2.8.6) MFT samples, was a modified API-RST medium (Tanner 1989), with the addition of (per liter) pyruvic acid (1 g) and sodium acetate (2 g) as extra carbon sources. Medium was dispensed into each tube containing one iron finishing nail and autoclaved. Each nail was rinsed with dichloromethane (Fisher Scientific) then water before use. Before inoculation, 0.1 mL of sterile vitamin solution (ATCC 1992) was added to each tube.

Iron-reducing medium was prepared as described by Salloum et al. (2002) and contained (per liter) 10 mL mineral solution (ATCC 1992) and 30 mL of $Fe(OH)_3$ slurry (Lovley and Phillips 1986) with sodium acetate as the carbon source. After dispensing, tubes were flushed with O_2-free 30% CO_2 balance N_2 gas, sealed and autoclaved. Prior to inoculation, 0.1 mL of sterile, anoxic 250 mM $FeCl_2$ and 0.1 mL of sterile vitamin solution (ATCC 1992) were added.

Methanogenic medium was prepared as described by Holowenko et al. (2000) with bicarbonate-buffered medium and Mineral Solutions I and II (Fedorak and Hrudey 1984). The pH was adjusted to 7.2 to 7.4 by sparging with O_2-free 30% CO_2 balance N_2. After sterilization by autoclaving, each tube received 0.1 mL sterile vitamin B solution (Fedorak and Hrudey 1984) and 0.25% (w/v) sterile sodium sulfide nonahydrate, prior to inoculation.

Samples were first diluted 10-fold up to 10^{-8} (for SRB, FeRB, and methanogen media) or 10^{-9} (for fermenter medium) in serum bottles containing sterile phosphate buffer. Phosphate buffer contained (per liter) 0.406 g KH_2PO_4 and 1.219 g K_2HPO_4; pH was 7.2. MPN tubes were inoculated with 1 mL of each 10-fold dilution and then incubated in the dark at room temperature (~22°C) with biweekly analysis.

Positive growth was indicated by turbidity in fermenter medium and black precipitate in SRB medium. Tubes containing at least 400 mg/L more Fe(II) than the uninoculated medium (as assayed by the ferrozine method described by Phillips and Lovley 1987) were scored positive for FeRB. Methane in the headspace gas was measured using a Hewlett Packard 5700A gas chromatograph and a flame ionization detector with a 2 m x 0.3 cm column packed with TenaxGC (60/80 mesh). The carrier gas used was N_2 at 50mL/min. H_2 and air were set at 50 mL/min and 200 nL/min, respectively. Injector, oven and detector temperatures were set at 30, 30 and 200°C, respectively. Chromatograms and peak areas were determined using a Hewlett Packard 3380A integrator (Holowenko et al. 2000). Tubes containing >0.1% (v/v) methane were scored positive for methanogenesis. Using the statistical table (APHA 1985), the numbers of each microbial type in each MFT sample were estimated. Significant differences between pairs of MPN values were determined using the statistical method of Cochran (1950).

2.2.2 DNA extraction by bead-beating

Typically, 300 µL MFT sample was suspended in equal volume of 100 mM phosphate buffer (pH 8), 10% SDS lysis buffer (10% SDS, 0.5M Tris-HCl (pH 8), 0.1 M NaCl) and a 24:1 mixture of chloroform:isoamyl alcohol, plus 1.0 g zirconia/silica beads

(Biospec Products Inc., Bartleville OK, USA; equal weights of 2.5 mm and 0.1 mm beads), as outlined by Penner (2006). Extraction was done in 2-mL screw cap unskirted conical bottom tubes with O-ring seals (Fisher Scientific). Samples were shaken in a FastPrep^{TM} FP120 Homogenizer and Isolation System (Bio 101, Thermo Fisher Scientific Inc.) at a setting of 5.0 m/s for 45 s. Ammonium acetate (7 M) was used at a final concentration of 2.5 M to precipitate protein and DNA was precipitated in isopropanol (0.54 volumes) overnight at 4°C and collected by centrifugation. DNA was redissolved in sterile nuclease-free water (Promega, Madison WI, USA) and stored at -20°C. Unless otherwise stated, DNA extractions of each sample for clone library analysis and DGGE analysis consisted of three subsamples which were each extracted twice. Extractions were analyzed by agarose gel electrophoresis and successful amplification reactions using primers PB36 and PB38 (described below) were pooled.

Numerous precautions were taken to ensure DNA extractions from tailings samples were not contaminated. Sterile stocks of extraction solutions, including sterile distilled water, were dedicated solely for DNA extraction from Albian MFT samples and only sterile materials were used throughout each extraction step. MFT samples were transferred to sterile bead-beating tubes by sterile syringes (Becton Dickinson, NJ, USA) and filtered-pipette tips (Molecular BioProducts, Inc., CA, USA) were used for transferring supernatants at each step of DNA extraction into sterile microcentrifuge tubes (Axygen Scientific, Inc., CA, USA). An extraction negative control containing only the extraction solutions was included with every set of extractions and was verified as negative by PCR using Bacterial and Archaeal primers and agarose gel electrophoresis as outlined in Section 2.2.3.

2.2.3 Clone library and RFLP analysis

All primers for PCR gene amplification (for clone library and RFLP analysis and DGGE analysis) were synthesized by Integrated DNA Technologies (IA, USA). Bacterial 16S rRNA genes were amplified using primers PB36 (5'-AGR GTT TGA TCM TGG CTC AG-3') and PB38 (5'-GKT ACC TTG TTA CGA CTT-3') (Saul et al. 2005), corresponding to *Escherichia coli* positions 8-27 and 1492-1509, respectively. PCR

reactions were made to a final volume of 25 µL and contained 1X PCR buffer (Finnzymes, Inc., MA, USA), 200 µM each dNTP (Fermentas Life Sciences, ON, Canada), 1 µM of each primer, 3% dimethyl sulfoxide (DMSO), 2.5 U Phusion DNA polymerase (Finnzymes, Inc., MA, USA), and ~7.5 ng of extracted genomic DNA. The PCR conditions consisted of initial denaturation at 98°C for 3 min, followed by 30 cycles of 98°C for 30 s, 54°C for 30 s and 72°C for 1 min, and a final extension step of 72°C for 10 min.

Archaeal 16S rRNA genes were amplified using primers 21F (5'-TTC CGG TTC ATC CYG CCG A-3') and 958R (5'-YCC GGC CTT GAM TCC AAT T-3') (DeLong 1992). Because Archaeal 16S rRNA genes would not amplify using the PCR reaction described for amplification of Bacterial 16S rRNA genes, a different Taq polymerase (Biological Sciences, University of Alberta), in combination with Pfu polymerase (Biological Sciences, University of Alberta), was used with a 1X PCR buffer (50 mM Tris-HCL, 1.5 mM $MgCl_2$, 0.4 mM β-mercaptoethanol, 0.1 mM bovine serum albumin (BSA), 10 mM $(NH_4)_2SO_4$, 200 µM each dNTP). PCR reactions were made to a final volume of 25 µL and contained 1X PCR buffer, 2.5 U *Taq* polymerase, 2.5 U *Pfu*, 1 µM of each primer, and 3% DMSO, and ~75 ng of extracted genomic DNA. The PCR program consisted of 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, 51°C for 30 s, and 72°C for 1.5 min, and a final extension step of 72°C for 10 min.

A negative control was included in each set of PCR reactions to ensure that contamination had not occurred in any of the PCR reagents or during setup of the reactions. The negative control contained all PCR reagents but lacked a sample of genomic DNA. Instead, sterile distilled water was used as a template and no amplification was expected. The negative control for PCR reactions was verified by agarose gel electrophoresis.

The amplified 16S rRNA gene fragments were separated by electrophoresis on a 1% agarose gel. The expected amplicon sizes were ~1500 bp for Bacteria and ~900 bp for Archaea. Gels were stained in ethidium bromide and visualized by UV illumination. DNA bands of the expected size were excised from the agarose gel using a sterile scalpel and the DNA was recovered from the agarose using the QIAquick Gel Extraction Kit (Qiagen Sciences, Maryland USA), following manufacturer's protocol.

The purified 16S rRNA gene PCR products were then ligated into a plasmid (CloneJET PCR Cloning Kit #K1221, Fermentas Life Sciences, ON, Canada) and transformed into *Escherichia coli* competent cells (New England BioLabs Ltd., ON, Canada). These cells were plated on Luria-Bertani (LB) agar plus ampicillin (100 mg/L). CloneJET PCR Cloning Kit uses a plasmid that, if recircularized in the absence of an insert, will express a lethal restriction enzyme in its host after transformation. Therefore, any colonies that successfully grew on LB + Amp^{100} were expected to contain an insert. After overnight growth, at least 96 *E. coli* colonies were randomly selected for each sample using sterile toothpicks and inoculated into 100 mL of LB and ampicillin in a 96-well round-bottom culture plate (Corning, Inc, NY, USA).The selected clones were screened by PCR for the correct insert size using vector-specific primers, pJETF (5'-GCCTGAACACCATATCCATCC-3') and pJETR (5'-GCAGCTGAGAATATTGTAGGAGATC-3') as described by the manufacturer. The PCR reaction consisted of 1X PCR buffer (50 mM Tris-HCL, 1.5 mM $MgCl_2$, 0.4 mM β-mercaptoethanol, 0.1 mM BSA, 10 mM $(NH_4)_2SO_4$, 200 µM each dNTP), 1 µM each of primers pJETF and pJETR, 2.5 U *Taq* DNA polymerase, and 1 µL of the liquid clone culture. The PCR amplification steps started with an initial denaturation of 95°C for 4 min, followed by 25 cycles of 95°C for 45 s, 58°C for 30 s, and 72°C for 1 min, and a final extension step of 72°C for 10 min. One microliter of each PCR product was run on a 1% agarose gel. The gel was stained in ethidium bromide and visualized by UV illumination to verify amplification of the insert.

Each PCR reaction that showed the correct insert size was digested by restriction enzymes *HaeIII* and *Cfo1* (New England BioLabs Ltd., ON, Canada). Each reaction (10 µL) contained 1X reaction buffer, 2.5 U of the restriction enzyme, 0.2 µg BSA/mL (Roche Diagnostics GmbH), and 3 µL of the PCR product. Reactions were incubated at 37°C for 2 h. The digested fragments were separated by electrophoresis on 2% agarose gels at 2.75 V/cm for 3 h in 1X Tris-acetate EDTA buffer. Gels were stained in ethidium bromide and visualized by UV illumination.

The resulting fragment patterns were visually analyzed and by a computer program, Gel-Pro Analyzer 4.5 (Media Cybernetics, Inc.). Clones carrying inserts that generated the same fragment pattern were grouped together and presumed to represent

the same operational taxonomic unit (OTU). One representative clone from each OTU represented by ≥2 clones was reamplified in preparation for sequencing by using pJET primers and the reaction described above (Fermentas Life Sciences, ON, Canada, kit #K1221). The amplified DNA product was purified using the High Pure PCR Purification Kit (Roche Diagnostics GmbH) according to manufacturer's protocol and eluted in 25 µL of elution buffer.

Each sequencing reaction (20 µL) contained 3 µL of BigDye Terminator v3.1 premix (PE Applied Biosystems, Foster City CA), 80 mM Tris-HCl, 2 mM $MgCl_2$, 4 pmol of pJETF primer, and 90-150 ng of purified DNA template. The PCR program was 96°C for 2 min, followed by 20 cycles of 96°C for 30 s, 55°C for 15 s, and 60°C for 2 min, and a final extension step of 60°C for 5 min. Sequence reaction products were cleaned by precipitating with 0.3 M sodium acetate, 25 mM EDTA and 95% ethanol, followed by a 70% ethanol wash. Sequencing was performed on an Applied Biosystems 373A automated DNA sequencer (Applied Biosystems Inc., USA) by the Molecular Biology Services Unit (University of Alberta) to give single reads of ~600 nt from one strand.

After checking for and excluding chimeric sequences using PinTail software (Ashelford, 2007; http://www.bioinformatics-toolkit.org/Pintail/index.html), BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search two public databases, the National Center for Biotechnology Information (NCBI) and the Ribosomal Database Project II (RDP-II), to identify the most closely related Bacterial or Archaeal species. RFLP patterns that appeared only once, termed singletons, were not analyzed because sequencing all single OTUs would be costly and time-consuming. As well, sequencing only well-represented OTUs operates on the premise that dominant Bacterial or Archaeal species (i.e. those that make up a higher proportion of the total population) are more likely to be metabolically dominant and thus more likely to play a role in methane production from substrates within the MFT.

2.2.4 DGGE analysis

Total community DNA was extracted by bead-beating as described in Section 2.2.2. Bacterial 16S rRNA genes were amplified using primer 341F with a GC clamp attached to its 5' end (5'- CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG- 3'; Muyzer et al. 1996) and primer 907R (5'- CCG TCA ATT CMT TTG AGT TT- 3'; Muyzer et al. 1998). PCR reactions contained 1X reaction buffer (Promega, Madison WI, USA), 1 µM of each primer 341F-GC and 907R, and ~15 ng of genomic DNA template. PCR conditions included an initial denaturation of 94°C for 5 min, followed by 30 cycles of 94°C for 45 s, 45°C for 1 min, and 72°C for 1 min, and a final extension step of 72°C for 10 min. Archaeal 16S rRNA genes were amplified using primer 340F with a GC clamp attached to its 5' end (5'- CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC CCT ACG GGG YGC ASC AG- 3'; Vetriani et al. 1999) and primer 934R (5'- GTG CTC CCC CGC CAA TTC CT -3'; DasSarma and Fleischmann 1995). PCR reactions contained the same reagents as described for Bacterial DGGE amplification, except for the primers used. The PCR conditions were as follows: 95°C for 2 min, followed by 30 cycles of 94°C for 30 s, 50°C for 1 min, and 72°C for 1 min, and a final extension step of 72°C for 10 min. Triplicate PCR amplifications for each sample were pooled before purification using the High Pure PCR Purification Kit (Roche Diagnostics GmbH), eluting into a final volume of 25 µL. As was stated in Section 2.2.3, a PCR negative control was included in each set of PCR reactions and verified by agarose gel electrophoresis.

The amplified PCR products were run on a 0% to 80% formamide and urea denaturing gradient gel: 0% denaturant solution contained (per 50 mL) 1 mL 50X TAE buffer (Bio-Rad Laboratories Inc., CA, USA) and 7.5 mL 40% 37.5:1 acrylamide/bis solution (Bio-Rad Laboratories, Inc., CA, USA); 80% denaturant solution contained (per 50 mL) 1 mL 50X TAE buffer, 7.5 mL 40% 37.5:1 acrylamide/bis solution, 16.8 g of molecular grade urea (Fisher Scientific, ON, Canada), and 16 mL formamide (Fisher Scientific, ON, Canada). Each solution was brought to 50 mL using MilliQ water, filtered through 0.4 µM syringe driven filter units (Millipore Corporation, Bedford, USA), and stored at 4°C in the dark for up to 3 weeks. Polyacrylamide gels consisted of 17.5 mL of

each 0% and 80% denaturant solution separately mixed with 90 µL of 10% APS (Fisher Scientific, ON, Canada) and 15 µL of TEMED (Bio-Rad Laboratories, Inc., CA, USA). The 80% denaturant solution was also mixed with 90 µL of 5X bromophenol blue loading dye. Gels were cast using the D-code Universal Mutation Detection System (Bio-Rad Laboratories, Inc., CA, USA) according to manufacturer's protocol and allowed to solidify for at least 2 h at room temperature. Wells were thoroughly flushed with distilled water prior to loading. The entire 25-µL purified PCR reaction was mixed with 5X bromophenol blue loading dye and loaded on the gel. After loading, gels were run at 130 V for 18 h and stained with 1X SYBRGold® (Invitrogen, ON, Canada). Stained gels were visualized using Image reader FLA-5000 (Fuji Medical Systems USA Inc., CT USA) with a 473 nm laser and an LPB filter to take a high-resolution fluorescence image (16 bit, 100 µm resolution). Gels were then left under fluorescent light in distilled water to destain and re-stained with ethidium bromide. The gel was visualized with UV illumination and dominant bands were excised using a sterile scalpel. Excised bands were stored in sterile microcentrifuge tubes in 10 µL of nuclease-free water (Promega, Madison WI, USA) at 4°C for at least 2 h to elute the DNA from the gel before long-term storage at -20°C.

Three microliters of eluted DNA from each gel band was reamplified by PCR using the same set of Bacterial (341F and 907R) or Archaeal (340F and 934R) DGGE primers, listed above, but lacking the GC clamp and the same corresponding PCR reaction mix and PCR program. Amplified DNA products were cleaned using either the High Pure PCR Purification Kit (Roche Diagnostics GmbH) or ExoSAP-IT (USB Corporation, OH, USA). Purified products were sequenced using BigDye Terminators v3.1 sequencing mix (Applied Biosystems), as described in Section 2.2.3, to give single reads of ~500 nt from one strand. The sequences were then compared to BLASTn of NCBI and RDP databases to determine the most closely related Bacterial or Archaeal species for presumptive identification.

2.2.5 Phylogenetic analysis

Phylogenetic trees were constructed using the 'Tree Builder' program of Ribosomal Database Project- Release 10 (http://rdp.cme.msu.edu/) which uses the weighted neighbor-joining (Weighbor) tree building algorithm (Cole et al. 2007; 2009). DNA sequences of named Bacterial species were selected from the NCBI database to include representative species from Bacterial groups often involved in anaerobic biodegradation of organic substrates to methane and some of which have been detected in Syncrude MFT (Penner 2006). These included alpha-, beta-, delta-, gamma-, and epsilon-proteobacteria, Clostridia, Chloroflexi, and Spirochaetes. DNA sequences of named Archaeal species were selected from the NCBI database to focus on methanogenic groups, including Euryarchaeota like Methanomicrobiales, Methanococcales, Methanobacteriales and Methanosarcinales. DNA sequences of Bacterial and Archaeal clones from Syncrude MFT deposited in the NCBI database were also included.

2.3 ANALYSIS OF INITIAL MFT SAMPLES – M1, M2, M4, M5, AND M6

2.3.1 MPN analyses

The 3-tube MPN technique was used to enumerate fermenters, SRB, and methanogens in MFT samples M1, M2, M4, M5, and M6. Media were prepared as described above (Section 2.2.1); SRB medium #1 was used in this analysis. Tubes were inoculated with M1, M2, M4, M5, or M6 tailings in 10-fold serial dilutions up to 10^{-8} for all media.

2.3.2 Clone library and RFLP analysis

DNA was extracted and clone libraries were constructed for Bacteria and Archaea in samples M1 and M2 (Table 1) as described above (Sections 2.2.2 and 2.2.3). DNA was extracted twice in triplicate from 300-µL subsamples of M1 and M2 MFT and the DNA was pooled for each. After amplification of 16S rRNA gene fragments in triplicate, the three DNA bands of the expected size, for each Bacterial and Archaeal PCR of M1 and

M2 MFT, were excised from the agarose gel as one piece and the DNA was eluted into 30 µL of elution buffer (QIAquick Gel Extraction Kit, Qiagen Sciences, Maryland USA). Clone library construction and RFLP analysis proceeded as described in Section 2.2.3.

2.3.3 Densification analysis

M1 MFT was chosen for this experiment because 1) we had sufficient quantities of M1 MFT, 2) microcosm analysis had shown M1 MFT to actively produce methane (data not shown), and 3) MPN analyses showed M1 MFT to have considerable quantities of fermenters, SRB and methanogens (described in Results, Section 3.2.1). Two-liter glass cylinders were filled with 1.4 L of M1 MFT amended with a constant volume (20 mL) of sterile distilled water (baseline control) or solutions of sodium acetate, trisodium citrate, methanol, citrate plus sodium 2-bromoethanesulfonate (BES), or citrate plus a concentrated nutrient medium. Each solution was added to achieve a final concentration of 5 mM, except for the methanogenic inhibitor BES (10 mM final concentration). Amendments were added to M1 MFT by first mixing the MFT in a sterile glass beaker with the appropriate sterile solution using a sterile glass pipette. Approximately 2 L of amended MFT was then poured into each glass cylinder, avoiding aeration as much as possible. Each glass cylinder was covered with an aluminum foil cap, incubated in the dark at 28°C and monitored biweekly for the first month of incubation. Densification of MFT was measured as the volume of pore water released and the volume of settled solids. MFT columns were again measured by Week 14 of incubation. Because the glass cylinders were covered with aluminum foil caps (i.e. they were not air tight), no effort was made to trap or measure produced gas from the amended tailings. We assumed that the high density of the tailings material and relatively small surface area exposed to air prevented O_2 diffusion below the first few millimeters of MFT. An incubation temperature of 28°C was used instead of 22°C as was done for microcosm analyses (Section 2.5) and MPN analyses (Section 2.2.1) because the slightly higher temperature should accelerate microbial activity to make densification occur more rapidly and there was sufficient space in a controlled temperature chamber where the columns would not be disturbed and would be dark.

In order to correlate gas production with densification of M1 MFT amended with each different solution, 100 mL of each amended MFT was placed into sterile serum bottles prior to filling each glass cylinder. These serum bottles were sealed with rubber septum stoppers (Bellco Glass, Inc. NJ, USA) and incubated in the dark at 28°C parallel to their corresponding glass MFT cylinders. The headspace of each serum bottle was sampled for methane and carbon dioxide at 2, 4, and 14 weeks of incubation by gas chromatography. Methane production was monitored using a Hewlett Packard 5700A gas chromatograph and a flame ionization detector, as described for methane analysis in MPN enumeration tubes (Section 2.2.1). To determine methane concentrations, a standard curve was constructed using methane standards of 0.16%, 4%, 8% and 15% methane. Carbon dioxide analysis was also performed by gas chromatography using a Hewlett-Packard 5890 Series II gas chromatograph equipped with a thermal conductivity detector. The column was 2 m × 0.3 mm diameter stainless steel tubing packed with 5% polyphenyl ether 6-ring (Chromatographic Specialties, Brockville, Ontario) coated Tenax-GC, 60/80 mesh (Alltech, Deerfield, IL). The carrier gas was helium at 52 mL/min. Injector, oven and detector temperatures were set at 35, 37 and 80°C, respectively. Chromatographs and peak areas were produced using a Hewlett Packard 3396 Series III integrator. To determine carbon dioxide concentrations, a standard curve was constructed using carbon dioxide standards of 0.16%, 4%, 8% and 15% (v/v) CO_2.

2.4 MICROCOSM ANALYSIS

Microcosm analysis of Albian MFT was performed by Dr. Tariq Siddique. A portion of his work has been included in this thesis as supporting data.

The contribution of diluent, citrate, and polyacrylamide to methane production in Albian MFT was studied through microcosm analysis using standard techniques described by Siddique et al. (2006; 2007). Microcosms containing M1, M2, M4, M5 and M6 MFT (50 mL of MFT per serum bottle), prepared as described in Section 2.4.3, were preincubated with methanogenic medium (50 mL) for 2 weeks at room temperature in the dark to allow acclimation of the microbial community to the methanogenic medium and consumption of endogenous substrates. Some microcosms were sterilized by autoclave to

serve as negative controls. The headspace of all microcosms was thoroughly flushed with O_2-free 30% CO_2 balance N_2 to remove any gas production from preincubation. The flushed microcosms containing M1, M2, M4, M5, or M6 MFT were then amended with 0.1% or 0.5 vol% Albian diluent. A positive control was included in this experiment consisting of M1 and M2 MFT amended with acetate (to a final concentration of 1000 ppm). In separate studies, flushed microcosms of M1 MFT were amended with trisodium citrate dihydrate for final concentrations of 100 or 200 ppm citrate in the presence or absence of 0.2% diluent and M1 MFT were amended with citrate (200 ppm) in the presence or absence of polyacrylamide (40 ppm, prehydrated in sterile distilled water; Hyperfloc® Af 246, Hychem, Inc. FL, USA). Concentrations of amendments reflect *in situ* concentrations.

Microcosms were incubated in the dark at room temperature. An additional control in each study was MFT with only methanogenic medium and no amendment (baseline control) in order to determine a baseline level of methane production from endogenous substrates remaining after preincubation. The headspace gas of each microcosm was sampled weekly and the concentrations of methane were measured by gas chromatography, as described in Section 2.2.1. Percent methane in headspace gas of each microcosm was converted into milliliters using the equations given by Nelson (1971) for gas concentrations in rigid containers, assuming methane gas is insoluble in the liquid medium of the microcosms (i.e. the estimated volume percent of methane in the headspace gas is equal to the volume of methane produced divided by the initial headspace volume plus the volume of produced methane). The volume of methane was then converted into moles of methane using the ideal gas equation.

2.5 MEGACOSM STANDING COLUMNS – MOLECULAR ANALYSIS AND DENSIFICATION ANALYSIS

The effects of citrate on the microbial community of Albian MFT and the effect of gas production on the densification rate of Albian MFT were examined in a large-scale study. Approximately 2000 L of M1 MFT and approximately 800 L of cap water from the same location were collected from the Albian Sands tailings pond and transferred in

three plastic tanks to the Oil Sands Tailings Research Facility (OSTRF) in Devon, Alberta in June, 2008 ("bulk sample", Table 2.1). Two 400-L columns located at the OSTRF facility were filled with approximately 300 L of M1 MFT (Figure 2.2). Each column was filled from a port at the bottom of the column; tailings were pumped into the column concurrently with a steady flow of ~10 L of either cap water for the baseline control column or trisodium citrate dihydrate solution for the experimental column (a final concentration of 200 ppm citrate). MFT was incubated in these columns at ambient temperature (~18°C) and were sampled through four of the eight ports located along the length of each column (Figure 2.2), effectively sampling tailings from different depths. Samples were taken from each of the four ports on each column at the time of set up (Time zero) and after 1, 3, 5, 8 and 10 months of incubation.

Sampling from ports on each column was done through a sampling device provided by the Department of Civil and Environmental Engineering at the University of Alberta. The sampling device consisted of a steel tube that was inserted into each port to reach the middle of the column (Figure 2.3). The steel tube was fitted with a valve that opened and closed to control the outflow of MFT from the column. The inside of the sampling tube was rinsed twice with a 5% hypochlorite solution followed by thorough rinsing with sterile distilled water. The outside of the sampling tube was washed with 70% ethanol. Samples were first collected from all four ports of the baseline control column and the first 300 mL of MFT that was collected from each port was discarded in order to "flush" the sampling tube in between each sample. Prior to sampling the citrate-amended column, the exterior of the sampling tube was wiped with 70% ethanol. The inside of the sampling tube was not cleaned because the first 300 mL of tailings from each port on the citrate-amended column were not collected. Two hundred and forty milliliters of tailings were collected into sterile PYREX 250-mL glass bottles from each sampling port to give a total of eight samples per sampling time. In total, 48 samples (250 mL each) were collected over the course of this experiment: one sample per port per column per time point (0, 1, 3, 5, 8, and 10 months). Ten-milliliter portions of each 240-mL sample were frozen at -80°C for molecular analysis to compare the microbial communities at different depths within the same column over time and also between the two columns over time. The remainder of each sample was stored in the dark at 4°C.

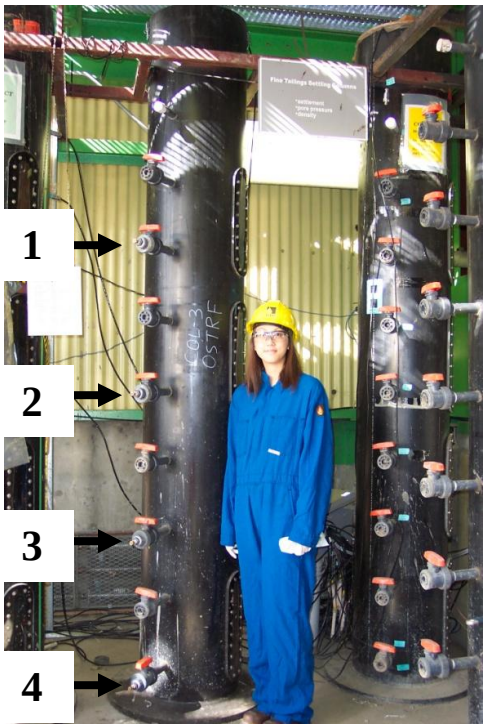

Figure 2.2. Megacosms filled with Albian MFT at the OSTRF facility in Devon, Alberta. Arrows and numbers indicate the ports from which samples were taken after 0, 1, 3, 5, 8, and 10 months of incubation.

Figure 2.3. Saidul Alam, Department of Civil and Environmental Engineering, University of Alberta, holding the sampling device which has been inserted into the column through one of the sampling ports.

2.5.1 Bicarbonate and pH analyses of megacosm samples

Megacosm samples were sent to the Department of Renewable Resources at the University of Alberta for bicarbonate and pH analyses. Forty-milliliter subsamples of MFT collected from Ports 1 and 4 (Figure 2.2) of the baseline control and citrate-amended columns at Time zero and 1, 3, 5, 8, and 10 months were centrifuged at 16,000 x g at 4°C for 30 min. The supernatant was collected (~ 24 mL each) and this was submitted for analysis. Bicarbonate concentrations were determined by acid-base titrations. Because only 40 mL of each tailings sample was collected for this specific analysis, the volume of supernatant collected could only allow for two replicate measurements of bicarbonate.

2.5.2 Percent solids analysis of megacosm samples

The solids content of all 48 samples from these megacosms were analyzed. The solids content is an indication of sample densities and was used to compare the densification of citrate-treated and baseline control MFT, as well as to compare the density of MFT at different depths within the same column. Approximately 1 g of each MFT sample in triplicate was dried at 100°C for at least 24 h to constant weight. The weight of the dry MFT sample was converted to a percentage using Equation 2.1.

$$\frac{\text{Weight (g) of dry tailings sample}}{\text{Weight (g) of wet tailings sample}} \times 100\% = \text{percent solids} \qquad \text{(Eq. 2.1)}$$

2.5.3 Densification measurements of megacosm samples

Concurrent with measuring the changes in percent solids of the MFT from each port of each column, MFT settling was measured by Saidul Alam, a graduate student in Civil and Environmental Engineering. Saidul monitored the settling behavior of Albian MFT by measuring the water-solids-interface height in these columns, with time. He also

collected samples from the megacosm columns to analyze the chemical composition of pore water.

2.5.4 DGGE analysis of megacosm samples

MFT samples collected from all ports of the baseline control and citrate-amended columns from Time zero and 5 and 10 months were analyzed by DGGE as described in Sections 2.2.2 and 2.2.4 to compare the microbial diversity in each column at different depths and over time. Bacterial diversity was examined for a total of 32 samples (4 ports on each of the two columns at 4 time points). The samples collected at each time point were analyzed on individual gels.

Some of the DNA eluted from excised bands could not be sequenced. The DNA was amplifiable but, upon sequencing, resulted in poor sequences with many unknown nucleotides. This happened with the Time zero and 5 month Bacterial DGGE analyses. In order to sequence the DNA from these excised bands, the eluted DNA was cloned using the CloneJET PCR cloning Kit and the method outlined in Section 2.2.3, including plating, selecting colonies by sterile toothpick, overnight growth in 96-well plates, and reamplification of the selected clones to verify that inserts were the expected size. Because each band was assumed to represent a single DNA sequence, only ten clones were selected for each sample of eluted band DNA. The primers used to reamplify the insert from the selected clones were pJETF1.2 (5'-CGA CTC ACT ATA GGG AGA GCG GC-3') and pJETR1.2 (5'-AAG AAC ATC GAT TTT CCA TGG CAG-3'). These primers differ from the first set of pJET primers described (Section 2.2.3) because Fermentas Life Sciences (ON, Canada) had changed their CloneJET kit to use a slightly different plasmid. Clones were not screened by restriction enzyme digestion because all selected clones per sample were expected to be the same. Instead, after verifying that the clones had the correct size insert, the PCR products for at least two clones for each gel band were sequenced to give single reads of ~400-500 nt, following the sequencing protocol described in Section 2.2.3.

For 10 month Bacterial DGGE analysis and Time zero Archaeal DGGE analysis, dominant bands could not be excised from the DGGE gel due to weak staining with

ethidium bromide. Instead, the purified DNA products intended for the DGGE gel were cloned using the CloneJET PCR cloning Kit with reamplification using primers pJETF1.2 and pJETR1.2. Typically, 96 colonies were chosen except in the case of Archaea (Time zero) where lower diversity was expected; in this case, 40 clones were selected. The remainder of this analysis was performed as described in Section 2.2.3. This technique of cloning DGGE PCR fragments was only used after verifying that all samples (8 samples per time point) showed the same microbial diversity (ie. number and placement of bands in the DGGE gel) and one representative sample of the baseline control MFT was cloned along with one representative sample of the citrate-amended MFT.

2.5.5 MPN analyses of megacosm samples

MPN analyses were done on Time zero and 10-month baseline control and citrate-amended megacosm MFT samples to enumerate fermenters, SRB, FeRB, and methanogens in order to monitor for changes over time and depth within each column and between the two columns. SRB medium #2 (Section 2.2.1) was used in this analysis. A total of eight samples from the megacosms were analyzed by MPN analyses. These samples represented Time zero tailings (which were archived at 4°C in the dark until used for MPN analyses) and tailings after 10 months of incubation (ie. beginning and end of column incubation) and were taken from the top and bottom of each column (Ports 1 and 4, Figure 2.2).

2.6 SETTLING COLUMNS (MESOSCOSMS)- METHANE PRODUCTION AND DENSIFICATION ANALYSIS

"Bulk sample" MFT, from the same location and depth as M1 MFT (Table 2.1), was used for filling 7-L acrylic settling columns. This was the same sample of MFT used to fill the 400-L megacosm columns (Section 2.7). Each column consisted of a cylindrical acrylic column (11.5 cm OD, 10.8 cm ID, 91 cm H; Johnston Industrial Plastics Ltd., Edmonton, AB, Canada) solvent-welded to an acrylic base plate (Fedorak et al. 2003). Columns were filled with 7 L of M1 MFT, amended as described below, under an N_2

headspace and sealed with an acrylic top plate fitted with a Teflon port and a 1-L Tedlar bag (SKC Inc., PA, USA) for collection of produced gas. Tape measures were attached along the lengths of the outside of each column in order to measure tailings height over time (Figure 2.4).

Each settling column was filled with 7 L of MFT amended with 233 mL of different combinations of three solutions prepared in Albian cap water: trisodium citrate dihydrate (to give 200 ppm final concentration of citrate), polyacrylamide (100 ppm final concentration, Hyperfloc® Af 246, Hychem, Inc. FL, USA), or Albian diluent (0.2% v/v final concentration). For baseline controls, the same volume (233 mL) of cap water (collected from the same site as M1 MFT) was added to the MFT. In each case, the amendment was carefully mixed into the MFT to avoid aeration, then poured down the side of the column (through a funnel) under a flowing stream of N_2. A summary of the treatments is shown in Table 2.2. Each treatment was prepared in duplicate to give a total of 16 settling columns.

The filled columns were covered in black plastic and incubated stationary at room temperature (~20°C) in Natural Resources Engineering Facility at the University of Alberta; MFT height measurements and gas sampling began after one week and continued biweekly.

2.6.1 Water release and solids settlement measurements

The rate of densification was monitored by measuring the height of the solids in each column and the height of the released pore water (water cap), using measuring tape on the exterior of each column. The initial height of the MFT was marked on the exterior of each acrylic column. Solids height readings were measured as a decrease in height and were taken from the marked initial height of MFT to the cap water-solids interface. Measurements of released water were taken from the cap water-solids interface to the surface of the cap water. The measured heights of released pore water and solids were converted into volume using Equation 2.2,

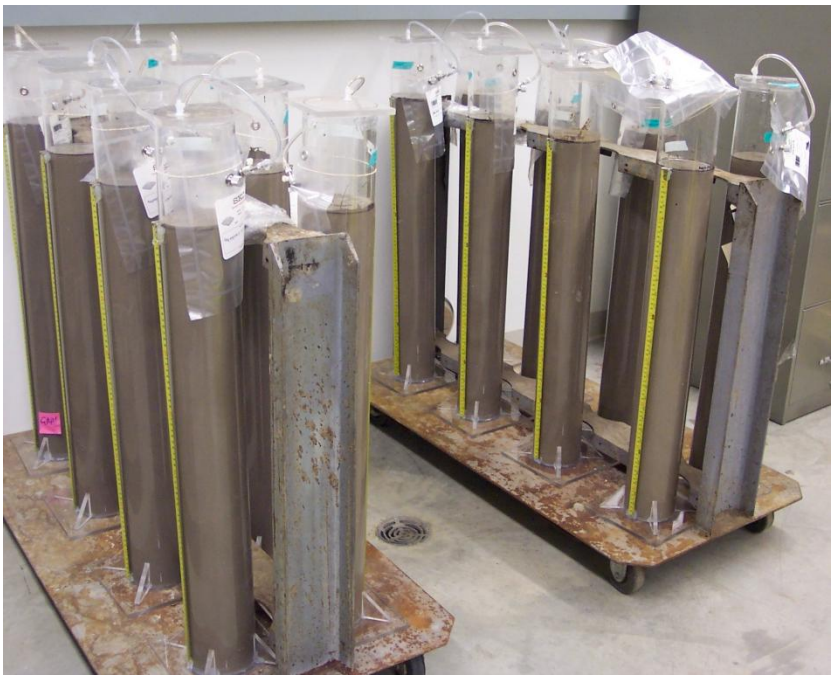

Figure 2.4. Settling columns filled with 7 L of Albian MFT, amended with citrate, diluent, polyacrylamide, or a combination of two or three of these, or with cap water (baseline control), sealed and fitted with 1 L Tedlar bags and tape measures for monitoring gas production and MFT densification.

Table 2.2. Experimental layout for settling columns of M1 Albian MFT. Amendments comprised: diluent (D) at final concentrations of 0 or 0.2 vol%; polyacrylamide (P) at final concentrations of 0 or 100 ppm; and citrate (C) at final concentrations of 0 or 200 ppm. The different combinations of amendments are indicated.

	Diluent (D) 0		Diluent (D) 0.2%	
	Citrate (C) 0 ppm	Citrate (C) 200 ppm	Citrate (C) 0 ppm	Citrate (C) 200 ppm
Polyacrylamide (P) 0 ppm	C0 D0 P0	C200 D0 P0	C0 D0.2 P0	C200 D0.2 P0
Polyacrylamide (P) 100 ppm	C0 D0 P100	C200 D0 P100	C0 D0.2 P100	C200 D0.2 P100

$$V = \Pi r^2 h \qquad \text{(Eq. 2.2)}$$

where ‘r’ = inner radius of the settling column (5.25 cm), and ‘h’ = measured height of the released pore water or the measured drop in solids height (cm).

2.6.2 Methane and carbon dioxide measurements

The methane and carbon dioxide composition of the released gas from each column was measured. Each 1-L Tedlar bag had a sampling port into which two sterile insulin syringes (Becton Dickinson, NJ, USA) were successively inserted and 100 µL of total gas was drawn into each syringe. The needles were immediately inserted into a rubber stopper and methane analysis and carbon dioxide were analyzed within 2 h of collection, as described previously (Section 2.4.3). In preliminary trials with known concentrations of methane, this method was shown to give accurate results up to several hours after sample collection (K. Londry, Edmonton Waste Management Centre of Excellence, personal communication).

2.6.3 Entrapped gas calculations

The total volume of gas trapped in the MFT solids material of each settling column was calculated using Equation 2.2, where the height was the difference in total height of the MFT (cap water plus solids) in each column between the end of experiment (after 10 months of incubation) and the beginning of the experiment (Time zero), as depicted in Equation 2.2a.

$$V = \Pi r^2 \left(h_{\text{Time 10 months}} - h_{\text{Time zero}}\right) \qquad \text{Eq. (2.2a)}$$

A positive value of entrapped gas therefore indicates an increase in MFT volume from Time zero to Time 10 months and a negative value of entrapped gas indicates a decrease in volume.

2.6.4 Settling column (mesocosm) deconstruction and sampling

After 10 months of incubation, six settling columns were decommissioned. One of each pair of replicate columns of the following treatments was deconstructed: baseline control; C200D0P0; C200D0.2P0; C200D0P100; C200D0.2P100; and C0D0.2P0 (Table 2.2). Before deconstruction of each column, two clamps were placed along the tubing connecting the Tedlar bag to the port of the lid. This prevented any gas leakage from the Tedlar bag after removal of the lid from each column. The lid to each column was removed by first drilling a hole in the column just below the lid and then using a jigsaw to cut around the circumference of the column. After the lid was removed, a 50-mL sample of cap water was removed using a sterile pipette and stored in a sterile container (Corning, Inc, NY, USA). The rest of the cap water was discarded. Samples of MFT were collected from each column using a glass tube connected by Tygon® tubing to a glass pipette. Samples were drawn up the tube by applying suction using a sterile 60 mL syringe (Becton Dickinson, NJ, USA) at the opposite end of the glass pipette. All of these components for sampling were first sterilized by autoclaving and each column was sampled using a different sampling device. Four 100-mL samples were collected from each deconstructed column at depths of 5 cm below the black precipitate that had formed in every column (discussed below), 25 cm below the water-solid interface (approximately 1/3 of the tailings height), 50 cm below the water-solid interface (approximately 2/3 of the tailings height), and from near the bottom of the column. Three milliliters of each collected sample was archived in microcentrifuge tubes (Axygen Scientific, Inc., CA, USA) at -80°C for molecular analysis (Section 2.8.5) and small aliquots of each were taken directly for percent solids analysis (Section 2.8.4). The remainder of each collected sample was stored in the dark at 4°C.

The development of a ring of black precipitate around the circumference slightly under the cap water-solids interface in every column (described in Section 3.4.4) prompted collection of MFT within this region, with as much of the black precipitate as possible, from the baseline control column, where this band was the most prominent (in width and intensity). This sampling was done in the same manner as described above, using a sterile glass pipette and sterile syringe. There was also development of an orange

precipitate on the surface of solids material in certain columns (described in Section 3.4.4) and an attempt was made to collect some precipitate from the settling column of MFT amended with citrate, diluent, and polyacrylamide. This was done first using a sterile glass pipette and sterile syringe, but this appeared to be ineffective as mostly MFT was being collected. Next, a metal spatula sterilized through thorough wiping with 95% ethanol was used but this method only allowed a very small amount of the precipitate to be collected.

2.6.5 Percent solids analysis of 7-L settling column MFT

The solids content of each of the four samples from each decommissioned settling column was measured in order to compare densification of the treated MFT to the baseline control MFT as well as to compare the 'density' of MFT at different depths within the same column. A total of 24 samples were analyzed. Measurements were performed as described previously (Section 2.5.2).

2.6.6 DGGE analysis of 7-L settling column MFT

To minimize the time and cost of this analysis, equal volumes of the four archived samples collected per deconstructed settling column were combined to make one composite sample per column. DNA was extracted from each composite MFT sample, amplified and analyzed by DGGE, as described in Sections 2.2.2 and 2.2.4. In most cases, DGGE bands were also sequenced as previously described; however, a few of the resulting gel bands required cloning after excision and eluting the DNA from the band (Section 2.5.4). Only Bacterial diversity was analyzed in these samples.

2.6.7 MPN analyses of 7-L settling column MFT

The four samples taken from each column were pooled in equal volumes to make a composite sample for MPN analyses, prior to storage of the MFT samples at 4°C, to give a total of 7 samples (five amended MFT samples, one baseline control MFT sample,

and one “black band” sample). Each composite MFT sample was then used for serial dilutions in sterile phosphate buffer and MPN analyses (Section 2.2.1) of fermenters, SRB (using SRB medium #2), FeRB, and methanogens (Section 2.2.1). MPN tubes were incubated in the dark at room temperature (~22°C) for 4 months with biweekly analysis.

3 RESULTS AND DISCUSSION

3.1 MICROCOSM ANALYSIS

Microcosms, containing 50 mL of MFT per serum bottle, were used to determine which substrates in Albian MFT can contribute to methanogenesis. This work was done entirely by Dr. Tariq Siddique. Though he did numerous microcosm analyses of Albian MFT, only the data from select experiments have been included here as supporting data that provide the necessary background for the focus of this thesis project.

To determine whether Albian diluent can support methanogenesis from Albian MFT, M1 and M2 MFT were amended with either 0.1 vol% or 0.5 vol% diluent (representative of *in situ* concentrations) and were incubated in sealed serum bottles in the dark at room temperature for 57 weeks. The headspace gas in each of these serum bottles was sampled biweekly for methane. Each amendment was done in triplicate. A baseline control of M1 MFT without diluent-amendment was included. Figure 3.1 presents representative methane measurements from these MFT.

The addition of 0.1 or 0.5 vol% diluent to M1 and M2 MFT did not result in significant methane production when compared to the baseline control over a period of 57 weeks (Figure 3.1). It does not appear that the lack of significant methane production is a result of a lag period as 57 weeks should be sufficient time for the microbial community to adapt to the methanogenic medium and amended substrates and there is no indication that methane production would increase significantly in the amended MFT over the baseline control. It is unlikely that the diluent is a methanogenic substrate for the microbial community in M1 and M2 MFT. Instead, residual endogenous substrate(s) likely supported the small amount of methane produced during the first few weeks of incubation. M4, M5, and M6 MFT were also tested for methane production after amendment of diluent (0.1 or 0.5 vol%) and similar results were obtained (data not shown).

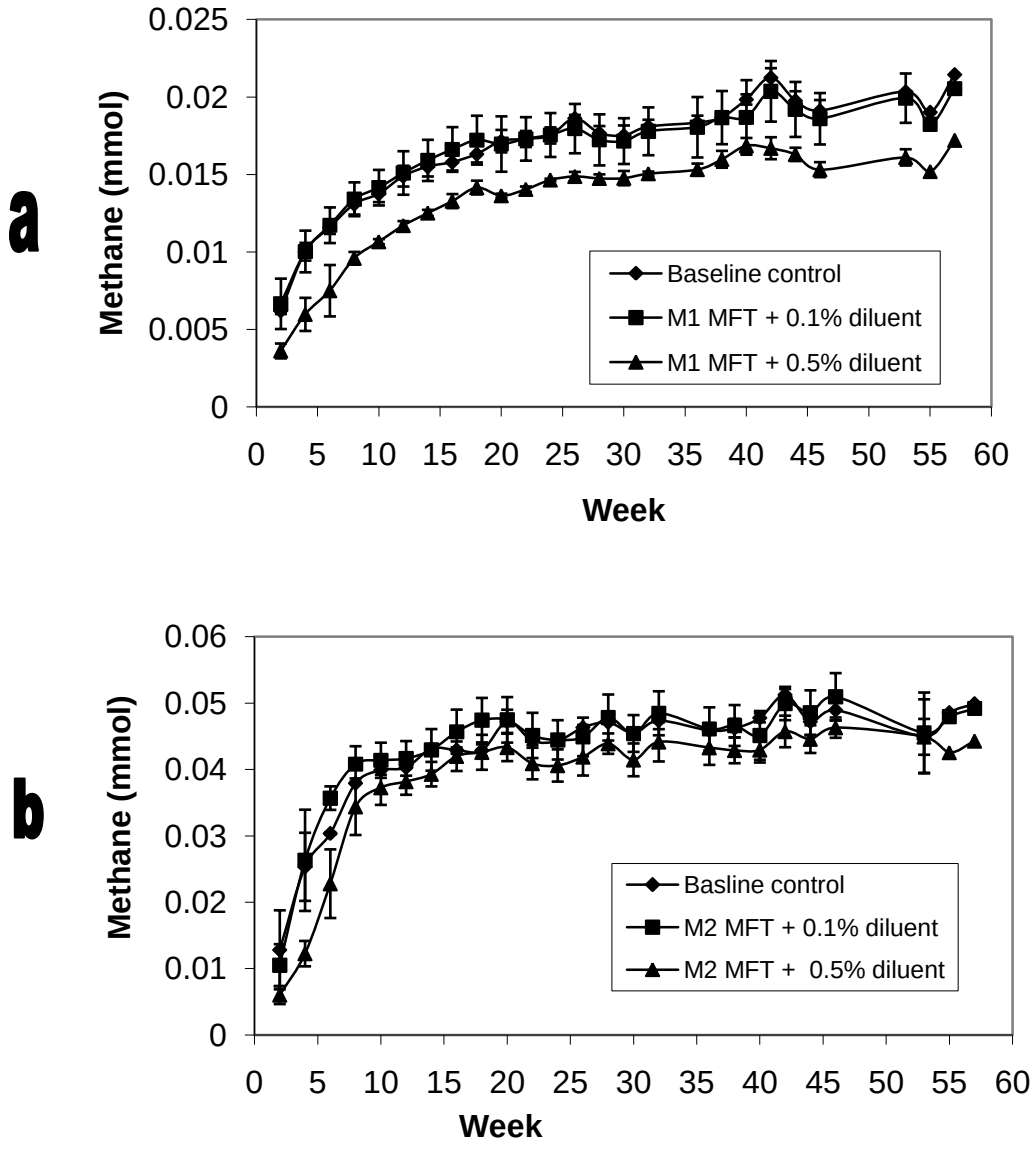

Figure 3.1. Methane production in microcosms by Albian a) M1 and b) M2 MFT amended with Albian diluent (0.1 or 0.5 vol%) or not amended with diluent (baseline controls) during 57 weeks of incubation in the dark at room temperature. Values represent the mean of triplicate microcosms ± 1 standard deviation, expressed as millimoles of methane detected in microcosm headspace gas. All measurements were taken by Dr. Tariq Siddique, Renewable Resources, University of Alberta.

Citrate was tested as a potential methanogenic substrate in Albian MFT. M1 MFT was amended with citrate at final concentrations of 100 or 200 ppm. Concentrations of citrate *in situ* are expected to be ~100 ppm (Brad Komishke, Shell Albian Sands, personal communication). Citrate was tested at 200 ppm concentrations to ensure its effects, if any, would be readily apparent. Amended MFT was incubated in sealed serum bottles in the dark at room temperature. A baseline M1 control was included. M1 MFT was also amended with a combination of citrate solution (100 ppm final concentration) plus diluent (0.2 vol%). Each amendment was done in triplicate. This experiment was incubated for 24 weeks with weekly sampling of headspace gas from each serum bottle for methane. The results of methane measurements from these MFT are summarized in Figure 3.2.

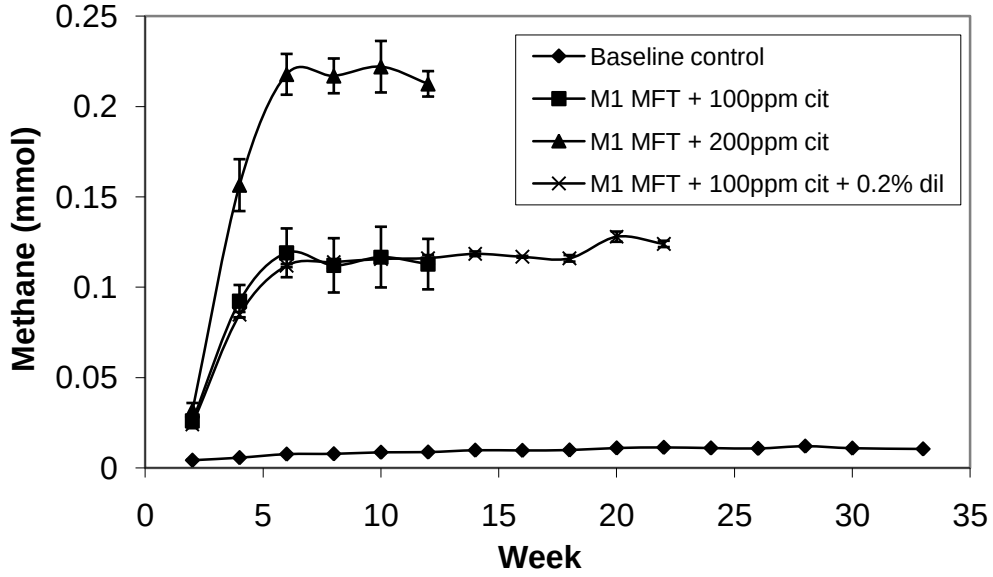

Figure 3.2. Methane production in microcosms of Albian M1 MFT amended with citrate (100 or 200 ppm cit) or with citrate (100 ppm) plus 0.2 vol% diluent (dil) or not amended with either (baseline control) measured during 24 weeks of incubation at room temperature in the dark. Values represent the mean of triplicate microcosms ± 1 standard deviation, expressed as millimoles of methane detected in headspace gas. All measurements were taken by Dr. Tariq Siddique, Renewable Resources, University of Alberta.

The addition of citrate to M1 MFT caused the production of significant amounts of methane, in comparison to the baseline control that was comparable to the previous experiment (Figure 3.1). Approximately 0.213 mmol of methane was produced from M1 MFT amended with 200 ppm citrate, a 95% conversion of citrate to methane based on theoretical yield calculated using Equation 3.1. M1 MFT amended with 100 ppm citrate produced approximately half the amount of methane (0.113 mmol methane) which is a 99% conversion of citrate to methane based on calculated theoretical yield (Equation 3.1).

$$4\ C_6H_8O_7 + 2\ H_2O \rightarrow 15\ CO_2 + 9\ CH_4 \qquad \text{(Eq. 3.1)}$$

Methane production began almost immediately and reached a plateau after 6 weeks of incubation. The addition of a combination of citrate (100 ppm final concentration) and 0.2 vol% diluent showed that the diluent neither inhibited nor contributed to methane production, because the amount of produced methane was the same for M1 MFT amended with 100 ppm citrate in the absence and presence of diluent. From these results, it was concluded that Albian MFT are methanogenically active if provided with a suitable substrate, and that citrate but not Albian diluent is a good methanogenic substrate for M1 MFT.

Polyacrylamide was amended to M1 MFT at a concentration of 40 ppm in the presence of citrate (representative of *in situ* concentrations) to test its effects on methane production from Albian MFT. A baseline control and M1 MFT amended with only citrate (200 ppm) were included. These microcosms were incubated in the dark and analyzed for methane production over a period of 16 weeks. The results of methane measurements from headspace gas of each serum bottle are summarized in Figure 3.3.

There is published evidence that polyacrylamide can act as a nitrogen source in MFT to enhance methane production and that it may stimulate microbial activity in anaerobic environments where a carbon source is available but a nitrogen source is not (Haveroen et al. 2005). Polyacrylamide was amended to M1 MFT in the presence of citrate to see the combined effect of the two treatments. Adding polyacrylamide to M1 MFT in the presence of citrate resulted in the same amount of methane production

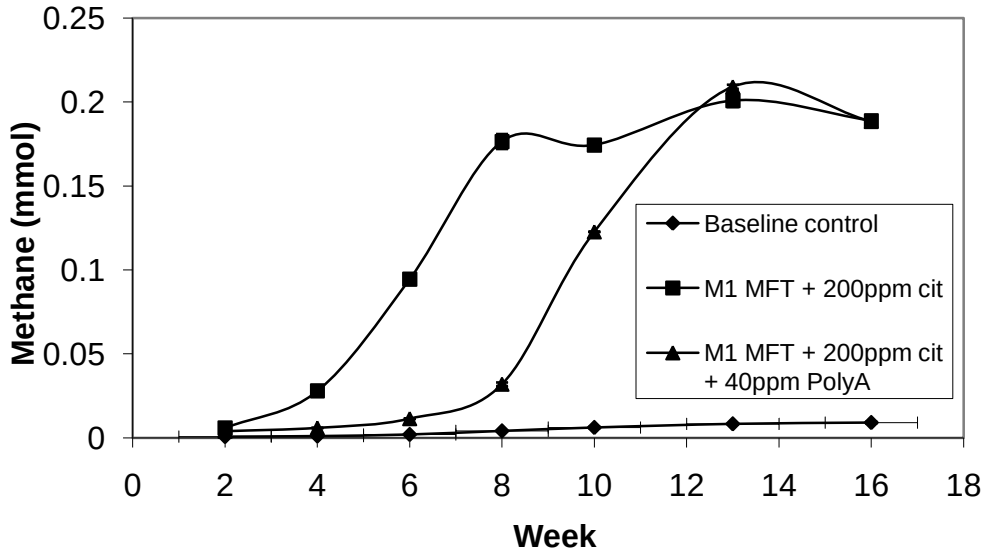

Figure 3.3. Methane production in microcosms of Albian M1 MFT amended with citrate (200 ppm cit) or with citrate (200 ppm) plus polyacrylamide (40 ppm PolyA) or amended with neither (baseline control) during 16 weeks of incubation at room temperature in the dark. Values represent the mean of triplicate microcosms ± 1 standard deviation, expressed as millimoles of methane detected in headspace gas. All measurements were taken by Dr. Tariq Siddique, Renewable Resources, University of Alberta.

from M1 MFT as only citrate-amendment. Polyacrylamide did not enhance methane production from citrate suggesting that there is sufficient fixed nitrogen for methanogenesis in Albian MFT. Haveroen et al. (2005) observed enhanced methane production from the addition of polyacrylamide to microcosms of Syncrude MFT and sewage sludge incubated with benzoate or acetate only after the MFT and sewage sludge samples had been serially diluted to reduce fixed nitrogen from the inocula to almost zero, making polyacrylamide the most abundant source of fixed nitrogen. Polyacrylamide amendment to M1 MFT caused a lag in methane production, as compared to M1 MFT amended with only citrate. The reason(s) for this lag is unclear. It is possible that polyacrylamide reduced the accessibility of citrate to microbes due to the flocculation effects of polyacrylamide on the MFT.

3.2 ANALYSIS OF INITIAL MFT SAMPLES

The following analyses using M1, M2, M4, M5, and M6 MFT, in combination with the microcosm experiments done by Dr. Tariq Siddique (Section 3.1) established the experimental setup for the 400-L megacosms and the 7-L settling column (mesocosm) experiments. These analyses laid the groundwork for further investigation into the microbial community, methane production, and densification of Albian MFT, and included MPN enumeration, clone library construction and RFLP analysis, and 2-L densification columns of MFT amended with various substrates.

3.2.1 MPN analyses of M1, M2, M4, M5, and M6 MFT

MPN analyses were performed on Albian MFT to confirm the presence of microbes potentially involved in the anaerobic degradation of organic matter into methane. MPN analyses also allowed for estimations of the quantities of each metabolic class of microbes in MFT samples which guided the decision of which MFT sample to use for further analysis (for megacosm and settling column (mesocosm) experiments). The results of MPN analyses of all received MFT samples, except for the bulk sample, enumerating fermenters, SRB, and methanogens are presented in Table 3.1. The bulk sample was collected from about approximately the same location and depth as M1 MFT and was not analyzed by MPN methods. Intermediate readings after 8 and 12 weeks of incubation are included to indicate growth rates.

The MPN results generally indicate an abundant population of fermenters, SRB, and methanogens in all MFT samples. There are differences, however, in which metabolic group dominates in each MFT sample. M4 and M6 MFT have significantly more SRB than fermenters or methanogens whereas M5 MFT has significantly more fermenters than SRB or methanogens (Cochran 1950; $p<0.05$). M1 and M2 MFT have significantly more methanogens than fermenters or sulfate-reducers ($p<0.05$).

Table 3.1. Summary of MPN analyses for fermenters, SRB, and methanogens in five Albian MFT samples (M1, M2, M4, M5, and M6) after 8, 12, and 24 weeks of incubation. By Week 24, all readings had stabilized.

MFT sample	Metabolic Type	MPN/mL by Week 8	MPN/mL by Week 12	MPN/mL by Week 24[a]
M1	Fermenters	2.3×10^6	2.3×10^6	2.3×10^6 ($4 \times 10^5 – 1.2 \times 10^7$)
	Sulfate-reducers	2.3×10^5	2.3×10^6	1.2×10^7 ($3 \times 10^6 – 3.8 \times 10^7$)
	Methanogens	2.3×10^4	3.9×10^5	1.1×10^8 ($1.5 \times 10^7 – 4.8 \times 10^8$)
M2	Fermenters	4.3×10^5	4.3×10^5	4.3×10^5 ($7 \times 10^4 – 2.1 \times 10^6$)
	Sulfate-reducers	1.5×10^5	1.5×10^5	1.5×10^5 ($3 \times 10^4 – 4.4 \times 10^5$)
	Methanogens	2.3×10^5	9.3×10^5	7.5×10^6 ($1.4 \times 10^6 – 2.3 \times 10^7$)
M4	Fermenters	7.5×10^5	7.5×10^5	7.5×10^5 ($1.4 \times 10^5 – 2.3 \times 10^6$)
	Sulfate-reducers	1.5×10^4	1.5×10^7	1.5×10^8 ($3 \times 10^7 – 4.4 \times 10^8$)
	Methanogens	1.5×10^4	4.3×10^4	2.3×10^6 ($4 \times 10^5 – 1.2 \times 10^7$)
M5	Fermenters	$\geq 2.4 \times 10^8$	$\geq 2.4 \times 10^8$	$\geq 2.4 \times 10^8$ -[b]
	Sulfate-reducers	7.5×10^4	2.1×10^5	1.5×10^6 ($3 \times 10^5 – 4.4 \times 10^6$)
	Methanogens	2.1×10^4	7.5×10^5	2.4×10^7 ($3.6 \times 10^6 – 1.3 \times 10^8$)
M6	Fermenters	1.2×10^6	1.2×10^6	1.5×10^7 ($3 \times 10^6 – 4.4 \times 10^7$)
	Sulfate-reducers	3.9×10^4	1.5×10^5	$\geq 2.4 \times 10^8$ -[b]
	Methanogens	2.3×10^4	3.9×10^4	9.3×10^6 ($1.5 \times 10^6 – 3.8 \times 10^7$)

[a] 95% confidence interval in parentheses.
[b] confidence intervals cannot be calculated for these values

SRB are a versatile group of microbes and most are capable of fermentative growth, depending on their environment. Because of this, some sulfate-reducers in these MFT samples may have been enumerated in both the fermenter and sulfate-reducer MPNs. Regardless, the presence of fermenters and SRB in substantial quantities in Albian MFT suggests the potential for methane production from hydrocarbon and/or

citrate degradation because these are the metabolic key players required for production of hydrogen, carbon dioxide, and acetate required for methane generation by methanogens. The differences in quantities of each group of microbes between the MFT samples may reflect heterogeneity in the Albian MFT pond, as each sample was taken from a different location in the pond or from a different depth (Table 2.1). This heterogeneity may also reflect an uneven distribution of biodegradable substrates in the pond.

Based on the relatively high numbers of fermenters, SRB, and methanogens in M1 MFT, this sample was chosen as the MFT with highest potential for methane production and densification. This was also supported by microcosm analyses of M1 MFT amended with acetate, a preferred substrate for methanogenesis, which showed rapid methane production (data not shown).

3.2.2 Clone libraries and RFLP analysis of M1 and M2 MFT

Because of the time-demanding nature of clone library construction and RFLP analysis, only M1 and M2 MFT were analyzed using this technique. In total, four libraries were constructed: a Bacterial and Archaeal library for each sample. The results of these analyses are described in Sections 3.2.2.1 and 3.2.2.2.

3.2.2.1 Bacterial species in M1 and M2 MFT

The Bacterial species in M1 and M2 MFT were determined through clone library and RFLP analysis. Pictures of RFLP patterns generated from *HaeIII* and *HhaI* digests of Bacterial 16S rRNA genes are given in Appendix A. Representative clones of 11 RFLP patterns that each occurred at least twice in M1 and M2 Bacterial clone libraries were sequenced for putative identification. The closest sequence matches to these clones from NCBI and RDP databases are summarized in Table 3.2. In the interests of time and cost, clones of RFLP patterns that occurred only once in M1 and M2 Bacterial clone libraries were not sequenced.

Table 3.2. Results of RFLP analysis of Bacterial DNA clones from M1 and M2 MFT. Clones representing 22 unique RFLP patterns are listed with the closest sequence match (most closely related) and closest named match (most closely related named taxon) in NCBI GenBank, determined by the BLASTn program and confirmed by the RDP database.

RFLP Pattern	# of clones	Representative clone name	1. Closest related sequence in GenBank 2. Closest related named taxon in GenBank	Accession # in GenBank	% Sequence Similarity
			M1		
CA	10	11H	1. Uncultured *Acidovorax* sp. clone Ctrl2-2H from oil sands tailings	EU522644.1	97
			2. *Acidovorax defluvii* strain R-31649	AM943035.1	97
AA	8	8H	1. Uncultured bacterium clone ZZ9C12 from benzene-contaminated groundwater	AY214177.1	97
			2. *Rhodoferax ferrireducens* T118	CP000267.1	93
HD	2	E9C	1. Uncultured Desulfuromonadales bacterium clone BTEX1-2A from oil sands tailings	EU522640.1	95
			2. *Geoalkalibacter subterraneus* strain Red1	EU182247.1	91
NG[a]	1	12D	1. Uncultured bacterium clone Rap2_24C from sediments of an artificial lake	EF192905	95
			2. Uncultured Clostridiales bacterium clone D10_02 from a tar oil contaminant plume	EU266777.1	80
13X	2	10C	1. Uncultured bacterium clone:LS4-260 from polychlorinated dioxin-contaminated sediments	AB234256	97
			2. Uncultured Chloroflexi bacterium clone HS07Ba03 from a coal gasification site	EU016411.1	97
AP	2	E6G	1. Uncultured *Rhodoferax* sp. clone Ctrl1-7 from oil sands tailings	EU522643.1	95
			2. *Rhodoferax ferrireducens* T118	CP000267.1	95
BA	2	4D	1. Beta-proteobacterium PB7 from diesel-contaminated borehole water	AY686732	96
			2. *Rhodoferax ferrireducens* T118	CP000267.1	94
6A	2	6A	1. Bacterium ROMEm4sh242 from continental deep subsurface shale-sandstone	AY998130	95
			2. *Geobacter psychrophilus* strain P39	AY653548.1	90
25A	2	E9E	1. Beta-proteobacterium PB7 from diesel contaminated borehole water	AY686732	98
			2. *Rhodoferax ferrireducens* T118	CP000267.1	94
26 29	2	3B	1. Uncultured bacterium clone Er-LLAYS-23 from sediment slurries amended with polychlorinated biphenyls	EU542498.1	86
			2. *Clostridium thermocellum* ATCC 27405	CP000568.1	86
28R	2	9C	1. Uncultured *Acidovorax* sp. clone Ctrl2-2H from oil sands tailings	EU522644.1	95
			2. *Acidovorax defluvii* strain R-31649	AM943035.1	95

Continued on next page

Table 3.2. continued

RFLP Pattern	# of clones	Representative clone name	1. Closest related sequence in GenBank 2. Closest related named taxon in GenBank	Accession # in GenBank	% Sequence Similarity
			M2		
CA	13	10E	1. Uncultured *Acidovorax* sp. clone Ctrl2-2H from oil sands tailings 2. *Acidovorax defluvii* strain R-31649	EU522644.1 AM943035.1	94 94
AA	9	2C	1. Uncultured bacterium clone ZZ9C12 from benzene-contaminated groundwater 2. *Rhodoferax ferrireducens* T118	AY214177.1 CP000267.1	98 94
HD	2	10B	1. Uncultured Desulfuromonadales bacterium clone BTEX1-2A from oil sands tailings 2. *Pelobacter acetylenicus*	EU522640.1 X70955.1	95 90
NG[a]	2	6A	1. Uncultured bacterium clone Rap2_24C from sediments of an artificial lake 2. Uncultured Acidobacteriaceae bacterium clone Amb_16S_1379 from soil cores	EF192905 EF018888.1	92 82
BA	6	6F	1. Beta-proteobacterium PB7 from diesel-contaminated borehole water 2. *Rhodoferax ferrireducens* clone EB01	AY686732 AM265401.1	97 95
DD	2	2D	1. Uncultured Desulfuromonadaceae bacterium clone Ctrl2-8D from oil sands tailings 2. *Pelobacter acetylenicus*	EU522641.1 X70955.1	95 89
DP	2	6B	1. Uncultured bacterium gene clone: YWB15 from deep coal seam groundwater 2. *Desulfuromonas palmitatis*	AB294284 U28172.1	94 88
EF	3	5C	1. Uncultured Chloroflexi bacterium clone Alk1-2G from oil sands tailings 2. uncultured Chloroflexi bacterium; MLSB_20m_12A_B from oil sands tailings	EU522645.1 EF420218	94 93
OC	3	E2A	1. Uncultured delta-proteobacterium MLSB_10m_14E_B from oil sands tailings 2. Uncultured Desulfobacterales bacterium clone SIMO-2387 from a salt marsh	EF420214 AY711753.1	99 90
GB	2	1G	1. Uncultured bacterium isolate cMM319-24 from mine drainage water 2. *Hydrogenophaga taeniospiralis* clone SE57	AJ536813 AY771764.1	93 93
XK	2	11D	1. Uncultured bacterium clone anNF05 from Arctic permafrost soil 2. Uncultured Clostridiaceae bacterium clone A839 from a laboratory model of early earth conditions	EF034735 EU283554.1	98 92

[a]Note that RFLP pattern "NG" occurred only once in the M1 Bacterial library. However, RFLP pattern "NG" occurred twice in the M2 Bacterial library and so clones from both libraries were sequenced for comparison.

The Bacterial clone libraries for MFT samples M1 and M2 comprised a total of 62 and 56 distinct RFLP patterns, respectively, from HaeIII and HhaI digests suggesting that there are potentially at least 62 and 56 different Bacterial species or OTUs in each MFT sample. This diversity is lower than the diversity observed in Syncrude MFT (Penner, 2006) and may be low due to insufficient coverage (discussed below). It is also possible that this relatively low diversity reflects the effects of the different diluent Albian uses than Syncrude in its extraction process as well as the use of citrate in its tailings pond, or the relatively young age of the MFT.

When the sequences of representative clones of each RFLP pattern were searched for similarity to sequences in NCBI and RDP databases, all of the sequences matched most closely to unnamed, uncultured bacterial sequences. This is one of the difficulties in analyzing unique environments such as tailings ponds; many of the microbes identified here have never been isolated or described before and the closest match to the sequenced clones provides little or no information about the metabolic capabilities of the microbes in Albian MFT. As suggested by the relatively low percent sequence similarities (Table 3.2), even matches to named species may not be an accurate indication of the roles the Bacteria play in producing methane from Albian MFT. However, the closest genus match can allow some inference into the metabolic capabilities of the unknown clones. The sequenced clones were aligned with sequences of more distantly related Bacterial species and aligned with one another in a phylogenetic analysis (Figure 3.4).

The majority of Albian clones show a high degree of relatedness to *Acidovorax* and *Rhodoferax* species. Both of these genera have been reported to degrade complex organic compounds (Eriksson et al. 2003; Lin et al., 2007). *Acidovorax* species have been reported as part of the microbial community degrading polycyclic aromatic hydrocarbons with the reduction of nitrate in enrichment cultures of soils (Eriksson et al. 2003) and as part of the microbial population in sludge from a wastewater treatment system to degrade acetate under denitrifying conditions (Osaka et al. 2006). *Rhodoferax antarcticus* and *Rhodoferax fermentans* have been reported to grow photoheterotrophically on citrate (Madigan et al. 2000; Hiraishi et al. 1991), whereas *R. ferrireducens* can grow on propylbenzene under iron-reducing conditions (Eriksson et al. 2005) and oxidizes acetate with iron reduction (Finneran et al. 2003). *R. ferrireducens* is the only member of this

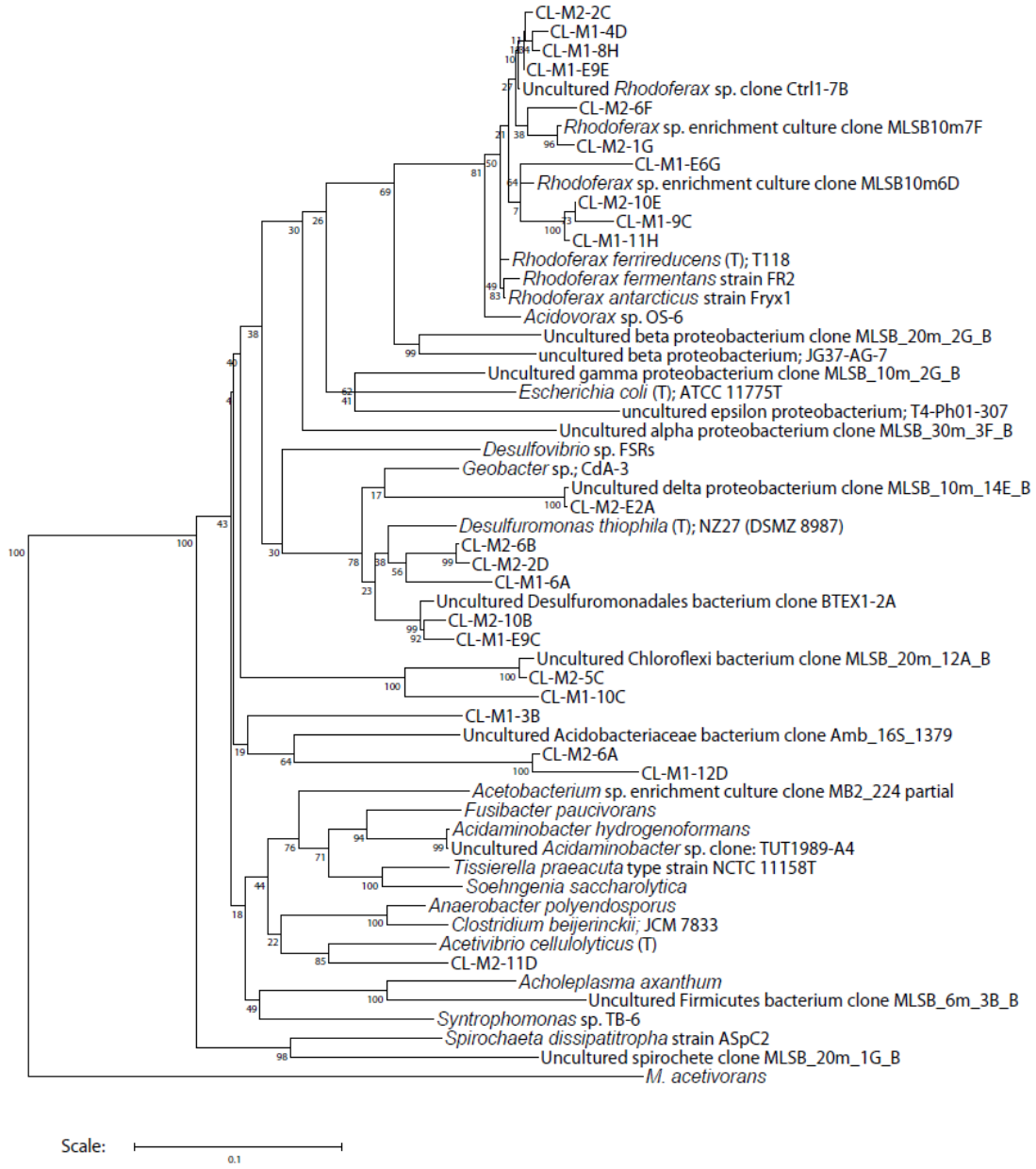

Figure 3.4. Phylogenetic tree comprising sequenced representative Bacterial clones in clone libraries constructed from M1 and M2 MFT samples and named species or clones from GenBank. Clones from this study are labeled with the prefix "CL-". Sequences in GenBank deposited from previous studies with Syncrude MFT (Penner 2006) have the prefix "MLSB_". Lengths of lines are proportional to the relatedness of the species. The scale bar represents 10% difference in DNA sequence.

genus that does not grow phototrophically and does not have photosynthetic pigments. The reclassification of *R. ferrireducens* to a new genus as *Albidoferax ferrireducens* has been proposed (Ramana and Sasikala 2009). However, because this name change has not been ratified, throughout this thesis, this species will be referred to as *R. ferrireducens.*

Though these findings cannot indicate which substrate(s) (i.e., citrate, Albian diluent, or both) are predominantly used for methanogenesis, they do suggest that the microbial consortium in Albian MFT should have the ability to produce methane from either or both substrates. Microcosm analysis (Section 3.1) has suggested that citrate is the likely methanogenic substrate; however, Albian diluent may have the potential of becoming a methanogenic substrate for the microbial community in Albian MFT eventually.

Several clones clustered with species identified in Syncrude MLSB MFT (Figure 3.4). CL-M1-E9C and CL-M2-10B clones grouped with a Desulfuromonadales bacterium clone from Syncrude MFT submitted to GenBank (EU522640). However, the sequence similarities for these matches were all quite low. One clone from M2 MFT grouped most closely with a delta-protebacterium clone from Syncrude MFT (EF420214). Clones CL-M2-5C and CL-M1-10C grouped with a Chloroflexi clone found in MLSB MFT (EF420218). The phylum Chloroflexi, also known as green non-sulfur bacteria, includes genera of filamentous gliding phototrophic bacteria and contains both thermophiles and mesophiles (Garrity and Holt 2001). This group of Bacteria has been identified in numerous anaerobic environments including as part of the core microorganisms involved in anaerobic sludge digestion (Rivière et al. 2009). In methanogenic sludge reactor vessels, they are thought to play a role in forming sludge granules on which biofilms can form (Yamada et al. 2005). Chloroflexi have also been found in hot springs (Lau et al. 2008), subsurface environments (Costello and Schmidt 2006), and marine environments (Inagaki et al. 2006) and have been found among the microbial communities involved in benzene-degradation (Herrmann et al. 2008; Kleinsteuber et al. 2008). Chloroflexi were recognized as a Bacterial group over 20 years ago (Woese 1987), but even now, few isolates have been described and their role in these environments remains unclear. This overlap suggests some similarity between the microbial communities of Albian MFT and Syncrude MFT.

The remainder of the M1 and M2 clones clustered with various genera. Some clones clustered with *Desulfuromonas*, but are more related to the cultured *Desulfuormonas thiophila* than to the Desulfuromonodales bacterium clone from Syncrude MFT, suggesting different species of SRB are present in Albian MFT than in Syncrude MFT. Similarly, Clone CL-M2-11D is more closely related to *Acetivibrio cellulolyticus* in the Clostridia than it is to the Firmicutes clone found in Syncrude MFT. Some clones clustered with *Acidobacteriaceae*. Little is known about this genus of bacteria; however, they have been found in hydrocarbon-contaminated sediments (LaMontagne et al. 2004). Their role in methane production in Albian MFT is unclear.

3.2.2.2 Archaeal species in M1 and M2 MFT

Archaeal clone libraries prepared from M1 and M2 MFT comprised 17 and 28 different RFLP patterns, respectively (Table 3.3; Appendix A), indicating less diversity among the Archaeal species than Bacterial species in Albian MFT. This is not unexpected because species of methanogens are likely to be less diverse than fermenters and sulfate-reducers, which have wider-ranging metabolic abilities. A lower diversity of Archaeal species than Bacterial species was also observed in Syncrude MLSB MFT (Penner 2006).

As observed with the Bacterial library, sequenced representatives for each OTU in the Archaeal library were most closely related to unnamed, uncultured species in the NCBI database, providing little information about their roles in methanogenesis in Albian MFT. The closest match, based on sequence similarity, to a named Archaeal taxon should be considered a rough indication of the potential metabolic capabilities. The percent similarities between the sequenced clones of the Archaea libraries and their highest matches from the NCBI and RDP databases were generally higher than the percent similarities for matches to the sequenced clones of the Bacteria clone libraries (Table 3.2). This also occurred for the sequenced clones from Syncrude MFT (Penner 2006). Greater sequence similarity is expected among Archaea based on the relatively low diversity among Archaea found in anaerobic methanogenic environments in comparison to Bacteria. The sequenced Archaeal clones were aligned to one another as well as to a range of other methanogenic Archaea (Figure 3.5).

Table 3.3. Results of RFLP analysis on Archaeal DNA clones from M1 and M2 MFT. Clones representing 14 unique RFLP patterns are listed with the closest sequence match (most closely related) and closest named match (most closely related named taxon) in NCBI GenBank, determined by the BLASTn program and confirmed by the RDP database.

RFLP Pattern	# of clones	Representative clone name	1. Closest related sequence in GenBank 2. Closest related named taxon in GenBank	Accession # in GenBank	% Sequence Similarity
			M1		
AA	50	8D	1. Uncultured archaeon clone P41 from low-temperature sludge	EU662673.1	98
			2. No matches to species; RDP gives Methanobacteriaceae	-	0.931[a]
AP	3	12F	1. Uncultured archaeon 39-2 from wastewater sludge	AF424769	98
			2. No matches to species; RDP gives Methanobacteriaceae	-	0.927 [a]
BA	12	E3C	1. Uncultured archaeon clone MHLsu47_B8F from minerotrophic peatland	EU155906.1	99
			2. Uncultured *Methanosaeta* sp. clone LH11 from Antarctic sediments	AY177805.1	98
BH	4	1F	1. Uncultured archaeon SJC-125a from a bed reactor for dechlorination of trichlorobenzene	AJ009509	96
			2. Uncultured *Methanosaeta* sp. clone KB-1 2 from a chlorinated ethane-degrading culture	AY780569.1	94
DK	7	5B	1. Unidentified archaeon, clone 123 from landfill leachate	AJ831143	93
			2. *Methanosarcina lacustris* strain MS	AY260431.1	93
IA	6	5E	1. Uncultured archaeon clone MHLsu47_6E from minerotrophic peatland	EU155905	99
			2. Uncultured *Methanosaeta* sp. clone LH11 from Antarctic sediments	AY177805.1	98
			M2		
AA	34	6A	1. Uncultured archaeon clone P41 from low-temperature sludge	EU662673.1	94
			2. No matches to species; RDP gives Methanobacteriaceae	-	0.845 [a]
AP	2	9G	1. Uncultured archaeon 39-2 from wastewater sludge	AF424769	86
			2. No matches to species; RDP gives Methanobacteriaceae	-	0.652 [a]
BA	25	8A	1. Uncultured archaeon clone MHLsu47_B8F from minerotrophic peatland	EU155906.1	99
			2. Uncultured *Methanosaeta* sp. clone LH11 from Antarctic sediments	AY177805.1	99
CD	3	7H	1. Uncultured archaeon isolate Str6_6/K4 from cold sulfidic marsh water	AM055705	98
			2. No matches to species; RDP gives Thermoprotei	-	0.982 [a]
EF	6	E3B	1. Uncultured archaeon SAGMA-4 gene from gold mine waters	AB050235	92
			2. Uncultured Methanospirillaceae archaeon from lake sediment, rice field soil, granular sludge	AB236096.1	96
BH	2	4E	1. Uncultured archaeon SJC-125a from a bed reactor for dechlorination of trichlorobenzene	AJ009509	98
			2. Uncultured *Methanosaeta* sp. clone KB-1 2 from a chlorinated ethane-degrading culture	AY780569.1	96
IA	2	E1F	1. Uncultured archaeon clone MHLsu47_6E from minerotrophic peatland	EU155905	99
			2. Uncultured *Methanosaeta* sp. clone LH11 from Antarctic sediments	AY177805.1	99

[a] similarity score from RDP

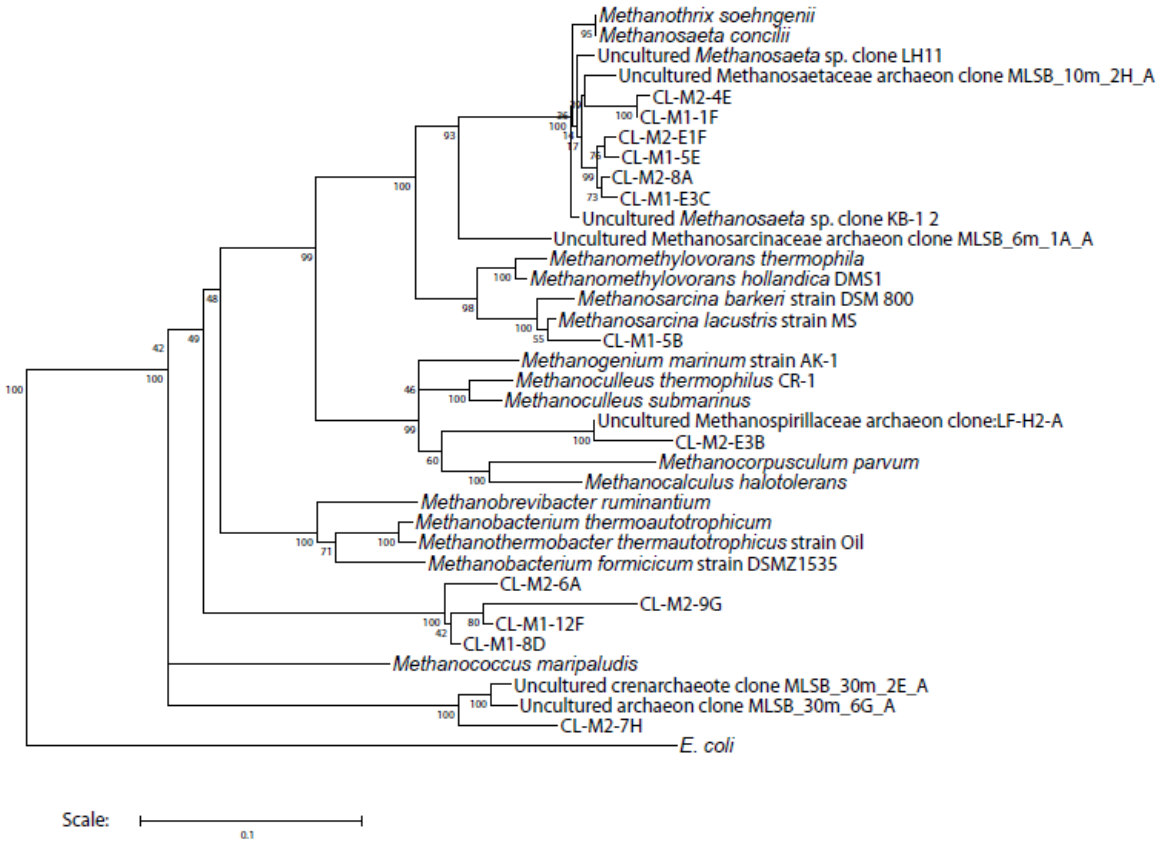

Figure 3.5. Phylogenetic tree comprising sequenced representative Archaeal clones in clone libraries constructed from Albian M1 and M2 MFT samples and named species or clones from GenBank. Clones from this study are labeled with the prefix "CL-". Sequences in GenBank deposited from previous studies with Syncrude MFT have the prefix "MLSB_". Lengths of lines are proportional to the relatedness of the species. The scale bar represents 10% difference in DNA sequence.

Only one representative clone clustered with the *Methanosarcina* and one clustered with a Methanospirillaceae clone. *Methanosarcina* prefer methylated compounds such as methanol, but can also use acetate or hydrogen plus carbon dioxide to produce methane (Smith and Ingram-Smith 2007). Methanospirillaceae include the genus *Methanospirillum*, which are able to use formate and hydrogen plus carbon dioxide to produce methane, but not acetate (Ferry et al. 1974). The majority of Archaeal clones grouped with the genus *Methanosaeta*, which comprises obligate acetoclasts. This is not unexpected because citrate, as described in Section 3.2.3, is expected to rapidly and directly degrade to acetate in Albian MFT. The relatively high quantities of

Methanosaeta clones versus other methanogen clones suggest that acetoclastic methanogenesis is the predominant pathway for methane production in Albian MFT. One group of clones appear to have little relatedness to any described methanogenic Archaeal species (CL-M1-8D, CL-M2-6A, CL-M1-12F, and CL-M2-9G from Figure 3.5). Their closest related matches, based on DNA sequences, were uncultured archaeal clone P41 (EU662673), which was sequenced during the analysis of microbes in low-temperature sludge samples, and uncultured archaeon 39-2 (AF424769), which was sequenced as part of a survey of the Archaeal diversity of wastewater sludge. Sludge and MFT are both anaerobic and dark environments where microbial communities can be exposed to substrates like organic acids and hydrocarbons. Methane production is commonly observed in anaerobic wastewater sludge (Gomec et al. 2009). Because only a few Archaea are capable of anaerobic methane production, it is not surprising to find overlap in the species detected from methane-producing anaerobic environments.

3.2.2.3 Coverage of Bacterial and Archaeal clone libraries of M1 and M2 MFT

The coverage of the Bacterial and Archaeal clone libraries constructed for M1 and M2 MFT was calculated using Good's equation (Good 1953); Equation 3.2. Coverage refers to how well a clone library represents the true diversity that exists in the original sample, which is affected by the number of clones that are sampled. Coverage values for the M1 and M2 clone libraries (both Bacterial and Archaeal) are listed in Table 3.4 where C is coverage, n is the number of OTUs that appear only once in a library, and N is the total number of clones in a library.

$$C = [1 - (n / N)] \times 100 \qquad \text{(Eq. 3.2)}$$

Table 3.4. Coverage of Bacterial and Archaeal libraries constructed from M1 and M2 MFT, calculated using Good's coverage equation (Good 1953).

Library	M1		M2	
	Bacteria	Archaea	Bacteria	Archaea
Coverage	40%	89%	52%	78%

According to Kemp and Aller (2004), a minimum Good's coverage value is between 70% and 95% for clone library analysis of Bacterial 16S rRNA genes in aquatic environments. The coverage values of the Bacterial clone libraries for M1 and M2 MFT do not meet this range, suggesting that more clones must be analyzed from these two libraries to more accurately represent the Bacterial diversity that exists in Albian MFT. Though the true Bacterial diversity remains unknown based on this clone library analysis, it has provided an initial indication of some of the Bacterial species we can expect to find in Albian MFT and likely some of the more dominant Bacterial species. The coverage values of the Archaeal libraries for M1 and M2 MFT do fall within the suggested range of 70 to 95 suggesting that sufficient numbers of clones have been selected to provide a reasonable representation of the Archaeal diversity that exists in Albian MFT.

3.2.3 Anaerobic citrate degradation

The degradation of citrate under methanogenic conditions has been recently investigated (Gámez et al. 2009). In their study, Gámez et al. (2009) analyzed citrate degradation by a methanogenic consortium from a bioreactor treating recycled paper effluent. They found that under methanogenic conditions, citrate was rapidly fermented with acetate as the main product. When methanogenesis was inhibited, acetate accumulated, accounting for 90-100% of the degraded citrate. In the absence of an inhibitor, acetate was degraded completely to methane. Acetate, carbon dioxide and hydrogen were the main products of citrate fermentation in a ratio of 2:2:1 per mole of citrate; however, in the presence of bicarbonate, the yield of acetate was increased due to acetogenesis. Based on their observations, Gámez et al. (2009) proposed the pathway for anaerobic citrate degradation under methanogenic conditions shown in Figure 3.6 (adapted from Gámez et al. 2009).

This pathway potentially occurs in oil sands MFT as well. Clone library and RFLP analysis of M1 and M2 MFT detected numerous *Rhodoferax*-like sequences, some Clostridia-like sequences as well as sequences similar to SRB. Among Archaeal species detected, numerous *Methanosaeta*-like sequences were found; *Methanosaeta* are obligate acetoclasts. Few hydrogenotrophic methanogens were identified, which agrees with the

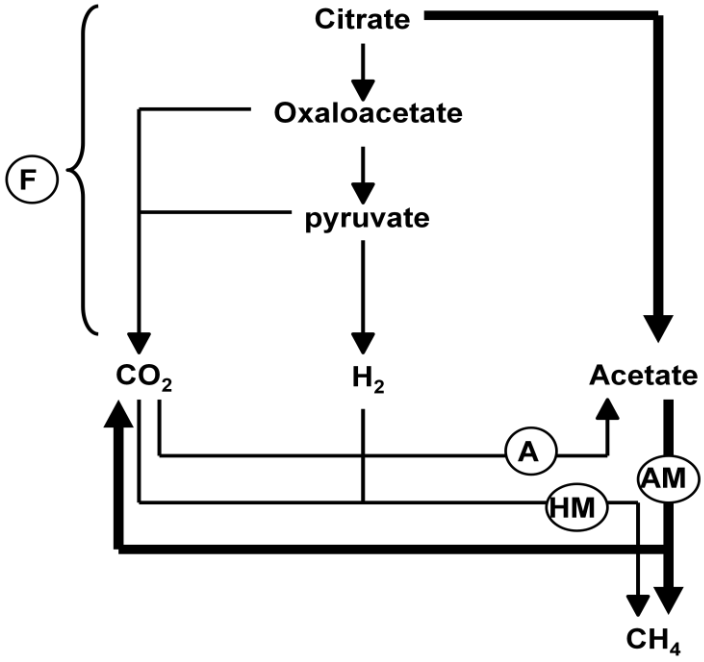

Figure 3.6. Proposed pathway of anaerobic citrate degradation to methane. Adapted from Gámez et al. (2009). F= citrate fermentation; A= acetogenesis using hydrogen; AM= acetoclastic methanogenesis; HM= hydrogenotrophic methanogenesis. Heavy lines indicate the expected dominance of acetoclastic methanogenesis.

proposed pathway for anaerobic citrate degradation. The pathway of hydrogenotrophic methanogenesis has been included in Figure 3.6 to indicate its possibility; however, the expected dominant pathway for methane production is acetoclastic methanogenesis. An additional pathway that may occur is acetogenesis from hydrogen and carbon dioxide; the class Clostridia includes some acetogens.

3.2.4 Densification analysis of M1 MFT

Microcosm analyses (Section 3.1) suggested a central role for citrate in methane production from Albian MFT. Before setting up the large (400-L) megacosms at the Oil Sands Tailings Research Facility in Devon, AB and the smaller (7-L) settling columns (Sections 3.3 and 3.4), citrate was first confirmed to be a major factor in Albian MFT densification by preparing small-scale (2-L) settling columns and monitoring water release and solids densification. Acetate was added to M1 MFT as the most readily

useable substrate for methane production by methanogens. This was done to test the methanogenic ability of these MFT and to serve as a positive control, as used previously with Syncrude MFT (J. Foght, University of Alberta, personal communication). Whereas acetate can be used by both fermenters and methanogens, methanol is a substrate solely for certain methanogens, such as *Methanosarcina* species (Müller et al. 1986), and so it was used as a test for the activity of the methylotrophic methanogens in M1 MFT. Citrate was the main experimental amendment. BES or nutrient solutions were added along with citrate to inhibit (Smith and Mah 1981) or promote methanogenesis, respectively. The addition of BES plus citrate addressed whether densification specifically required activity of methanogens producing methane plus carbon dioxide, or more generally of fermenters producing carbon dioxide. BES acts to inhibit methanogenesis, which is required for removing the end products of fermenters and SRB. Thus, BES may indirectly prevent fermenters and SRB from breaking down hydrocarbons and/or citrate in Albian MFT. The nutrient medium, which contained fixed nitrogen, trace metals, salts and vitamins, was added to promote as much microbial metabolic activity as possible.

In parallel to each amended MFT column, 100 mL of each baseline control or amended MFT sample was incubated in a sealed anaerobic serum bottle. Each microcosm headspace was monitored using gas chromatography for production of methane and carbon dioxide whenever the glass cylinders were measured for densification (2, 4, and 14 weeks of incubation). This was done to observe correlations between gas production and densification in Albian MFT. The results of densification and gas measurements are summarized in Table 3.5.

By week 14, the largest change in solids volume had occurred in Albian MFT amended with citrate, with a 9.3% solids volume decrease. This MFT also released the most porewater. MFT amended with citrate (citrate only or citrate plus nutrients) were the first to show a change in MFT volume (Table 3.5, week 2), as increased solids volume. This increase was the result of gas pockets dispersed throughout the amended MFT (Figure 3.7); over time, an overall decrease in solids volume was observed in MFT amended with citrate (Table 3.5, weeks 4 and 14) as degassing occurred near the surface. The large gas pockets often appeared to be ‘lined’ with bitumen, which may affect their stability. Interestingly, the addition of citrate alone caused slightly more MFT

Table 3.5. Results from 2-L densification columns after 0, 2, 4, and 14 weeks of incubation at 28°C. MFT was amended with acetate, citrate, methanol, citrate plus BES, citrate plus concentrated nutrient solution, or sterile water (baseline control).

Incubation time	Parameters	Treatment					
		Baseline control	Acetate (5 mM)	Citrate (5 mM)	Methanol (5 mM)	Citrate (5 mM) and BES (10 mM)	Citrate (5 mM) and nutrient solution
0 weeks	Water cap (mL)	0	0	0	0	0	0
	Solids (mL)	1420	1420	1400	1400	1420	1420
	% Volume change[a]	0	0	0	0	0	0
	% Methane[b]	0	0	0	0	0	0
	% Carbon dioxide[b]	0	0	0	0	0	0
2 weeks	Water cap (mL)	10	80	140	100	10	140
	Solids (mL)	1420	1420	1420	1400	1420	1440
	% Volume change[a]	0	0	+1.4	0	0	+1.4
	% Methane[b]	0.1	2.3	4.6	3.1	0	5.6
	% Carbon dioxide[b]	2.1	2.2	5.7	3.2	10.2	4.7
4 weeks	Water cap (mL)	20	110	210	120	20	200
	Solids (mL)	1400	1390	1310	1380	1400	1340
	% Volume change[a]	-1.4	-2.1	-6.4	-1.4	-1.4	-5.6
	% Methane[b]	0.3	6.4	18.2	7.7	0.4	16.0
	% Carbon dioxide[b]	2.0	2.3	7.4	3.0	0[c]	25.2
14 weeks	Water cap (mL)	70	90	210	150	50	210
	Solids (mL)	1330	1330	1270	1330	1350	1310
	% Volume change[a]	-6.3	-6.3	-9.3	-4.9	-4.9	-7.7
	% Methane[b]	0.8	7.2	20.7	18.5	0.4	18.1
	% Carbon dioxide[b]	2.2	2.5	5.7	2.8	9.1	14.1

[a] percent change where '-' means a decrease in total solids volume due to densification, and '+' means an increase in total solids volume, due to gas pockets in the MFT, compared to the original volume.

[b] measured in headspace of parallel serum bottles.

[c] believe to be an anomalous reading.

densification and methane production than the addition of citrate plus nutrients. However, MFT amended with citrate plus nutrients produced a much greater amount of carbon dioxide. These results suggest that fermentative bacteria that are initiating attack on citrate in Albian MFT may be limited in nutrients whereas the methanogens are limited by other factors.

BES appeared to reduce methane production and MFT densification as well as water release as indicated by the lower % volume change and lower % methane from MFT amended with citrate and BES than from baseline control MFT (Table 3.5). The highest concentration of methane at each time point was produced from citrate-amended MFT whereas the most carbon dioxide was generated from MFT amended with citrate solution plus nutrients. The addition of nutrients like fixed nitrogen, metals, salts, and vitamins, may stimulate alternative pathways of citrate and acetate degradation to methane (Figure 3.6), resulting in an increase in carbon dioxide production. BES acts to prevent the metabolic activity of methanogens and so it was expected that MFT amended with BES would produce little methane. Gámez et al. (2009) observed that in the presence of a methanogen-inhibitor like BES, methane production but not acetate production from citrate fermentation was hindered. The increased carbon dioxide concentration in BES-amended MFT was likely a result of BES inhibiting the consumption of hydrogen plus carbon dioxide by hydrogenotrophic methanogens. The absence of carbon dioxide at week 4 may be due to carbon dioxide gas dissolving into the MFT as bicarbonate; however, because this trend is not observed in the other columns of amended MFT, it is more likely an anomalous gas chromatography reading. The addition of methanol to M1 MFT caused a smaller decrease in solids volume than in MFT amended with acetate. This suggests that methane production by methanogens alone is not sufficient to cause MFT densification and that the concerted metabolic actions of the entire microbial community in Albian MFT are much more effective. Overall, Albian MFT showed a high methanogenic potential with citrate as a carbon source, and methane production in Albian MFT caused considerable MFT densification over the short period of 14 weeks.

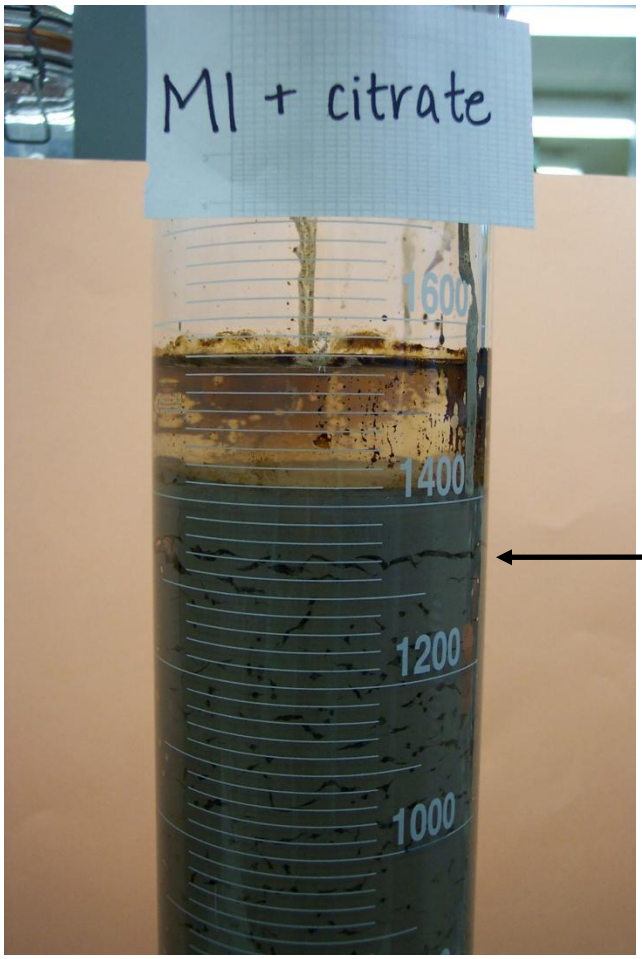

Figure 3.7. Densification of M1 MFT amended with citrate solution (final concentration of 1000 ppm) after 2 weeks of incubation at 28°C. Total volume (1580 mL) has surpassed the original volume of 1420 mL (because of gas pocket formation) although MFT solids volume has remained the same.

3.2.5 Summary of initial analyses

MPN analyses showed that M1 MFT was one of two MFT samples that had the greatest number of methanogens among the MFT samples analyzed (M1, M2, M4, M5, and M6) and also had a great number of fermenters and SRB, suggesting it had a strong potential for methane production. For this reason, M1 MFT was selected for microcosm (Section 3.1), megacosm (Section 3.3) and meso-settling column (Section 3.4) experiments. Clone library and RFLP analysis of the Bacterial species in M1 and M2

MFT suggested a predominance of clones similar to *Rhodoferax* and *Acidovorax* species. Clones similar to Clostridia and Chloroflexi were also identified. Bacterial species in Albian MFT were similar to the bacterial species in Syncrude MFT (Penner 2006), including clones that matched to Clostridia, Chloroflexi and *Rhodoferax*; however, the strong predominance of *Rhodoferax* species in Albian MFT is unique. Analysis of the Archaeal species in M1 and M2 MFT suggested predominance of an unknown archaeon that did not match closely to any cultured or described Archaeal species. *Methanosaeta*-like species were also dominant, particularly in M2 MFT. Clones resembling *Methanosarcina* were observed in low quantities. *Methanosaeta* and *Methanosarcina* were previously identified in Syncrude MFT (Penner 2006).

Densification analysis of M1 MFT indicated that stimulating methanogenesis in this MFT results in accelerated MFT densification. Citrate was an effective inducer of methane production and MFT densification. It was confirmed that both methane and carbon dioxide are produced in the presence of acetate and citrate. In the absence of methane production, MFT densification was not accelerated; however, methane production alone did not stimulate MFT densification as much as the activity of the total microbial community, including carbon dioxide production.

3.3 MEGACOSMS: MICROBIAL COMMUNITY ANALYSIS OVER TIME

Because citrate had been determined to be the primary methanogenic substrate by Dr. Tariq Siddique using microcosm studies (Section 3.1), a large-scale study was set up to analyze the effects of citrate on the microbial community and densification of Albian MFT. Two 400-L columns were filled with Albian MFT, one with MFT amended with cap water (baseline control) and the other with citrate-amended MFT (200 ppm). During incubation at ambient temperature in the dark for over 10 months, samples were collected from four different ports of each column. Analyses included bicarbonate concentration, pH values, solids content (weight %), and DGGE analysis of microbial profiles and MPN enumeration.

3.3.1 Bicarbonate and pH analyses of megacosm MFT during incubation

3.3.1.1 Bicarbonate concentrations

If citrate is the main substrate contributing to methane production in Albian MFT, the most likely pathway of degradation to occur would be through fermentation to acetate (Figure 3.6). In this case, acetate concentrations should be high in citrate-amended MFT, and is expected to promote acetoclastic methanogenesis which would result in the production of carbon dioxide as well as methane. It was postulated that as carbon dioxide was produced it would dissolve in the MFT pore water as bicarbonate. Therefore, MFT from each column was analyzed to compare bicarbonate as an indirect measurement of microbial activity. Bicarbonate in samples from the top and bottom of each column (Figure 2.2; ports 1 and 4 respectively) were compared to determine the effect(s) of hydrostatic pressure, with the expectation that higher pressure would increase the amount of carbon dioxide dissolved into pore water (Al-Anezi et al. 2008). Bicarbonate concentrations of MFT from each column were tracked over time and compared within the same column and between each column. The summary of bicarbonate measurements of MFT collected from the top and bottom ports of each column (Ports 1 and 4) from 0, 1, 3, 5, 8, and 10 months of incubation is given in Figure 3.8. The corresponding pH values of these MFT samples have been included in this figure for ease of comparison; pH values are discussed further below.

A limited amount of each MFT sample was available for bicarbonate analysis. Though each megacosm column contained ~300 L of MFT, only small (250 mL) samples were taken from each port at each sampling time in order to minimize disturbance of the columns, particularly because they were being monitored for densification (Section 3.3.3). As a result, the values shown (Figure 3.8) are means of two bicarbonate measurements for each sample. Bicarbonate concentrations in the porewater of these MFT samples were measured by acid-base titration. As a result of this analytical method, other acids in the porewater, including citrate, could potentially cause an overestimation of bicarbonate concentration. Because citrate concentrations were not measured in these samples, the degree to which the bicarbonate concentrations have been affected is

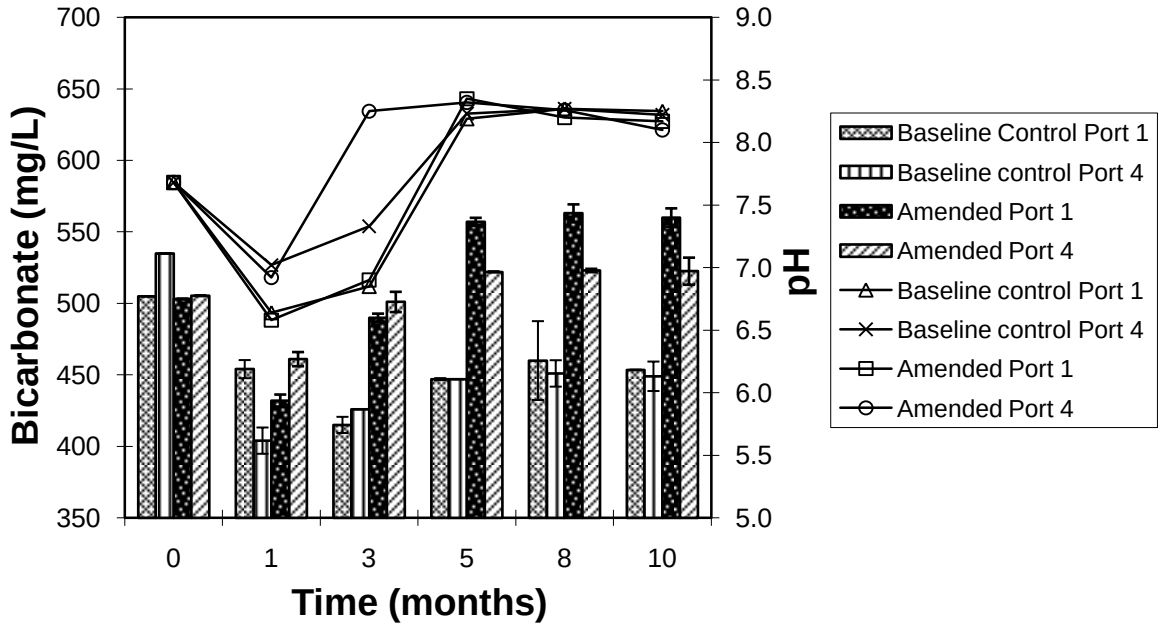

Figure 3.8. Bicarbonate concentrations (bars) and pH values (lines) of MFT samples collected from the top (port 1) and bottom (port 4) of baseline control and citrate-amended megacosm columns at 0, 1, 3, 5, 8, and 10 months of incubation. Bicarbonate values at Time zero represent single measurements; error bars for all other samples indicate the range of two measurements.

unknown. Still, with these considerations in mind, these measurements suggest some general trends of bicarbonate concentrations in Albian MFT amended with cap water (baseline control) and Albian MFT amended with citrate.

Bicarbonate concentrations were expected to be similar among all samples taken at Time zero since columns had just been filled with the MFT and no activity had takenplace yet. MFT sampled from all ports except Port 4 of the baseline control column had very similar bicarbonate concentrations; note that all Time zero measurements were made on single samples so differences between samples cannot be assessed statistically.

Bicarbonate concentrations first decreased in samples from both columns and both ports as incubation began (after 1 month) but then slowly increased again (after 3 months of incubation and onwards). Bicarbonate is used as an electron acceptor by acetogens during hydrogen oxidation (Doré and Bryant 1990). Bicarbonate

concentrations would decrease in the first few months of incubation (Months 1 and 3) as acetogens used the bicarbonate to generate acetate. There is reported evidence of increased acetate production from citrate fermentation in the presence of bicarbonate due to acetogenesis (Gámez et al. 2009). Acetate would be used by acetoclastic methanogens to produce methane as well as carbon dioxide, which would dissolve in pore water to elevate bicarbonate concentrations. Carbon dioxide can also be reduced to methane by hydrogenotrophic methanogens; however, few sequences similar to hydrogenotrophic methanogens were detected during clone library and RFLP analysis of M1 and M2 MFT (Section 3.2.2.2).

In the baseline control MFT from both ports, bicarbonate concentrations remained low after the initial drop in concentration at 1 month of incubation and never reached the same concentration as at Time zero. Bicarbonate concentrations likely first decreased due to reduction of bicarbonate by acetogens to produce acetate, which the acetoclastic methanogens used to produce methane. However, because citrate is lacking, there is no source for additional acetate for production of methane and carbon dioxide. As a result, bicarbonate concentrations do not significantly increase between Time 1 month and Time 10 months samples from the top (port 1) or bottom (port 4) of the baseline control MFT column (two-sample t-test, $p<0.05$). Bicarbonate concentrations in citrate-amended MFT increased to surpass original concentrations because of carbon dioxide production from citrate fermentation and acetate production from citrate which would be used to produce methane and carbon dioxide. As a result, there were significant increases in bicarbonate concentrations between Time 1 month and Time 10 months in citrate-amended MFT from the top of the column and from the bottom of the column (two-sample t-test, $p<0.05$).

Bicarbonate concentrations reached a plateau in citrate-amended MFT after 5 months of incubation suggesting the completion of citrate degradation. In the microcosm study of methane production from citrate (Figure 3.2), methane concentrations reached a plateau in citrate-amended MFT after approximately 1.5 months of incubation. In microcosm studies, MFT was supplemented with methanogenic medium, which improved the conditions for citrate degradation. The MFT in these megacosm columns were not supplemented with medium.

By 5 months of incubation, in amended MFT, bicarbonate concentrations were significantly higher (two sample t-test, $p<0.05$) in samples from the top of the column than from the bottom of the column, which was not expected. Carbon dioxide dissolution was expected to be greater at lower depths due to increased hydrostatic pressure. This same effect is not observed for MFT in the baseline control column where bicarbonate concentrations remained similar between Port 1 and Port 4 samples throughout incubation. Bicarbonate concentrations were significantly different at 3 months of incubation onwards between MFT sampled from the baseline control at Port 1 and from the citrate-amended column at Port 1 (two-sample t-test, $p<0.05$). It is possible that insufficient carbon dioxide was produced in baseline control MFT; concentrations of bicarbonate never surpassed the initial amount of bicarbonate. Baseline control MFT also settled less and released less water which is perhaps why bicarbonate concentrations remained similar between baseline control MFT from Ports 1 and 4. Overall, an effect of pressure on carbon dioxide dissolution in MFT pore water was not observed.

3.3.1.2 pH values

As carbon dioxide dissolves in the MFT as bicarbonate, it is likely to decrease the pH of the MFT; therefore pH values were also monitored. Changes in pH could potentially affect the microbial community of Albian MFT and also the densification rate. Carbon dioxide dissolves in water to form carbonic acid which dissociates to form bicarbonate and a single hydrogen ion, as shown in Equation 3.3.

$$CO_{2(aq)} + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \qquad \text{(Eq. 3.3)}$$

The pH of MFT at Time zero was measured as a collective sample immediately prior to filling the columns and was about 7.7. One month after incubation began, the pH value of MFT had decreased, with baseline control and citrate-amended MFT at Port 1 reaching a pH of about 6.6 and baseline control and citrate-amended MFT at Port 4 reaching a pH value of about 6.9-7. The drop in pH does not correspond to the initial decrease in bicarbonate that was observed for these MFT (between Time zero and 3

months of incubation, Figure 3.8). As bicarbonate concentrations in all MFT samples increased again after 3 months and remained steady by 5 months of incubation, the pH values surprisingly followed the same trend. The pH values of baseline control MFT were very nearly the same as the pH values of citrate-amended MFT after 3 months of incubation despite having lower bicarbonate concentrations. Furthermore, differences in bicarbonate concentrations are not consistently reflected in pH values. For example, in amended MFT, samples from Port 1 and Port 4 taken from 5 months onwards show a difference of ~25 mg/L but no difference in pH values. However, the difference in bicarbonate concentrations of amended MFT from Port 4 between 3 and 5 months (~50 mg/L) does correlate with a difference in pH values. Although it is possible that <50 mg/L bicarbonate does not affect the pH of these MFT samples, the same trend is not observed in baseline control MFT. These observations suggest that other factors besides bicarbonate concentrations are affecting the pH of the column MFT, such as other dissolved ions. Saidul Alam measured the pore water chemistry of baseline control and citrate-amended MFT and saw a decrease in sulfate concentrations in both MFT during the first 3 months of incubation, as discussed below (Section 3.3.4).

In summary, bicarbonate concentrations decreased within the first month of incubation in both baseline control and citrate-amended MFT. This was likely due to acetogenic bacteria using bicarbonate for acetate production. In amended MFT, bicarbonate concentrations increased again to surpass initial bicarbonate concentrations, likely because acetoclastic methanogens produced methane and carbon dioxide using acetate from the degradation of citrate and because carbon dioxide was produced directly from citrate fermentation. There was no observed effect of hydrostatic pressure on bicarbonate concentrations in either baseline control or citrate-amended MFT. The pH values of baseline control and citrate-amended MFT did not correspond to changes in bicarbonate concentrations, suggesting that other factors in the columns are affecting pH, such as other dissolved ions.

3.3.2 Solids content analysis of megacosm MFT

The solids content of MFT in each column was measured, based on weight, as an indication of density. Samples were taken from all four ports of each column at selected 0, 1, 3, 5, 8, and 10 months of incubation to compare the solids content of MFT over time at each location and between treatments. These measurements are summarized in Figure 3.9.

There was a considerable difference in percent solids of the MFT material between each column and within the same column at Time zero. This was unexpected because MFT were expected to be about the same at the beginning of the experiment. Prior to filling the columns, the bulk sample of MFT was mixed but not thoroughly homogenized to prevent extensive aeration of the MFT. The variation in percent solids between Time zero megacosm MFT samples could be due to the lack of complete mixing.

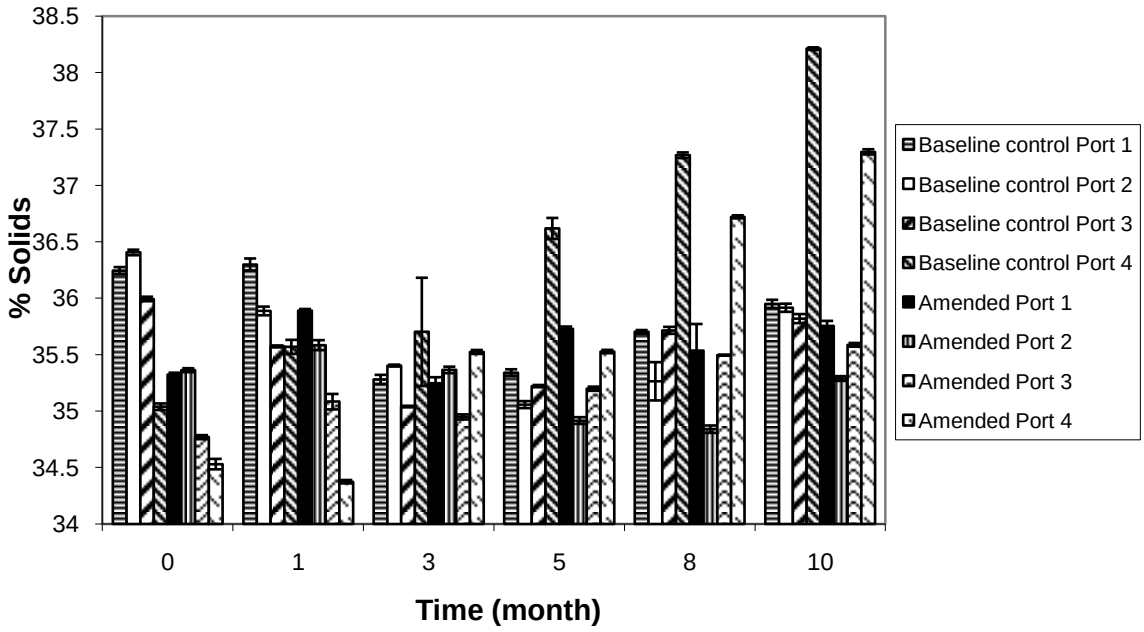

Figure 3.9. Percent solids of MFT samples from four ports of baseline control and citrate-amended megacosm columns at 0, 1, 3, 5, 8, and 10 months of incubation. Values represent the mean of triplicate subsamples ± 1 standard deviation.

After 10 months of incubation, there was a definite distinction between upper and lower MFT (Ports 1 and 4, respectively) in each column; about 2.5% difference in solids content for the baseline control column and about 1.5% difference in solids content for the citrate-amended column. There was also a general decrease in solids content in MFT sampled from Ports 1 and 2 of each column between and after 10 months of incubation and a general increase in solids content in MFT sampled from Ports 3 and 4. An increase of ~3.2% solids was observed in the MFT from Port 4 of the baseline control column and an increase of ~2.8% was observed in MFT from Port 4 of the citrate-amended column. However, the solids content measurements for the MFT at the ports in between (Ports 2 and 3) did not increase correspondingly with depth. An increase in solids content was expected to be observed over time in both baseline control and citrate-amended MFT, with a larger increase in the citrate-amended MFT. Citrate was observed to accelerate MFT densification in 2-L columns (Section 3.2.4) and the same was expected in these megacosm columns. Instead, baseline control MFT showed a slightly greater increase in solids content over time than the citrate-amended MFT did.

The percent solids in baseline control MFT sampled from Ports 1, 2, and 3 first showed a decrease in solids content (from Time zero to 3 months of incubation) and then a gradual increase over the remaining months. There are slight fluctuations in solids measurements from one time point to the next for the citrate-amended MFT. For example, MFT from Port 1 of the citrate-amended column increase then decrease in solids content from Time zero to 1 month of incubation and this pattern repeats for the remainder of the time points sampled. MFT from port 2 of the citrate-amended column did not follow a trend either and fluctuated across the 10-month incubation period.

The irregularity of the percent solids measurements in megacosm MFT samples within and between baseline control and citrate-amended columns over time may be due to the lack of proper homogenization of the bulk MFT sample prior to filling each megacosm column. There may also be more factors affecting the change in percent solids in megacosm MFT, including microbial activity and the production of gas bubbles, pore water chemistry, or other unknown aspects of the MFT environment.

There is potentially a relationship between bicarbonate concentrations and pH values to percent solids in Albian MFT. Percent solids in amended MFT, particularly

from the bottom of the column (Port 4), first decreased after 1 month of incubation and then increased again by 3 months and continued to increase until the end of incubation. This is the same trend observed for bicarbonate concentrations and pH values in Albian MFT (Figure 3.9) suggesting that bicarbonate concentrations and/or pH are related to solids content of this MFT. However, baseline control MFT, particularly from Port 4 of this column, also increased in percent solids over the 10-month incubation period and increased by a greater amount between Time zero and Time 10 months than amended MFT did. Bicarbonate concentrations were steadily low in baseline control MFT samples (Figure 3.8) and this is not reflected in the percent solids of these MFT samples. The pH values of amended MFT remained at a constant value of ~8.2 despite an observed increase in dissolution of carbon dioxide, as evidenced by bicarbonate concentrations, from 5 months of incubation and onwards. It is likely that other unknown factors in the MFT, such as other dissolved ions, are preventing the decrease in pH we expect from the dissolution of carbon dioxide and may also be preventing an increase in the percent solids of the amended MFT by affecting the interactions between clay particles. These factors are probably due to citrate-amendment since baseline control MFT show a continual increase in percent solids without a change in bicarbonate concentration or pH value. However, this is speculation and with the experiments done here, it is difficult to conclude the relationship between bicarbonate, pH, and percent solids in Albian MFT.

3.3.3 Measuring solids settling of megacosm MFT

Densification and pore water chemistry analyses of MFT in these two columns were done as part of an independent project by another student, Saidul Alam, from Civil and Environmental Engineering, University of Alberta. As part of his PhD project, Saidul monitored the water-solids interface of the MFT in the baseline control and citrate-amended MFT columns over a period of 10 months (Figure 3.10).

Baseline control and citrate-amended megacosm MFT started with different interface depths, indicating that the MFT were filled to slightly different heights in each column. However, within approximately 1 week, the interface depth of the citrate-amended MFT column reached about the same interface depth as the baseline control

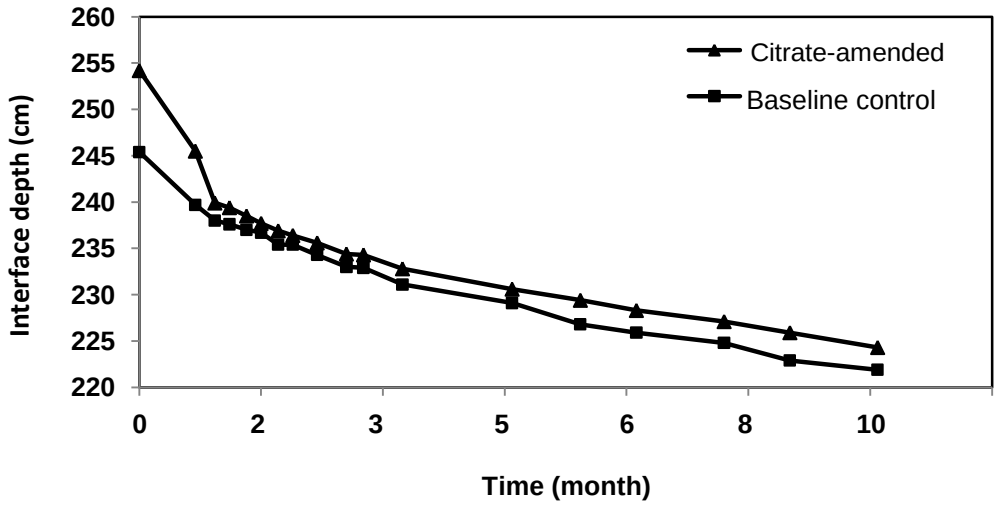

Figure 3.10. Changes in the water-solids interface of baseline control and citrate-amended MFT incubated in megacosm columns in the dark at ambient temperature over 10 months. Measurements were taken by Saidul Alam, Civil and Environmental Engineering, University of Alberta.

MFT column. The more rapid densification rate of the citrate-amended MFT within the first month is potentially due to microbial degradation of the citrate to methane. Methane production from citrate amendment to Albian MFT was complete by 6 weeks of incubation in microcosm experiments (Section 3.1) and was observed to accelerate MFT densification in as little as 4 weeks (Section 3.2.4; Table 3.5).

The effects of citrate on densification of MFT in megacosm columns were expected to occur over a longer period of time than was observed in 2-L column experiments because of the increased pressure of the megacosm columns which would decrease the mobility of gas bubbles. This is reflected in the bicarbonate concentrations of citrate-amended MFT samples taken from the megacosm column which took approximately 5 months to reach a plateau (Figure 3.8). It is more likely that the lack of thorough homogenization of the MFT prior to filling each column resulted in the rapid settling of the citrate-amended MFT within the first month of incubation. The MFT used to fill the citrate-amended column may have had higher water content, as indicated by the lower solids content in citrate-amended MFT than in baseline control MFT (Figure 3.9).

The bulk sample of MFT used for filling megacosm columns was stored in two containers of approximately 600 L each. To homogenize these MFT would have required an electric mixer which had the potential to aerate the MFT. For these reasons, thorough homogenization of the MFT was not done.

After about 1 month of incubation, the interface readings of MFT in both columns were approximately the same and they remained similar until the end of the experiment (9 months later). No substantial differences in interface depths were observed between the citrate-amended MFT column and baseline control MFT column. Because citrate has been observed to accelerate Albian MFT settling and water release (Table 3.5), the interface depth of the citrate-amended MFT was expected to be considerably lower than the interface depth of baseline control MFT. Instead, the interface depth of citrate-amended MFT is slightly higher than that of the baseline control MFT. This may be caused, in part, by the production of gas in the citrate-amended MFT, which would increase the overall volume of solids material in the MFT, thereby increasing the reading of the interface depth.

Percent solids and solids settling measurements were expected to correlate. As solids settle towards the bottom of each column, the percent solids of MFT at the bottom of each column should increase. Because citrate was observed to accelerate settling in 2-L column studies (Table 3.5), citrate-amended MFT in the megacosm column was expected to show considerably greater settling and higher percent solids than the baseline control MFT. Over the 10-month incubation period, there was a general increase in percent solids of both baseline control and citrate-amended MFT with the greatest increase of percent solids in MFT located at the bottom of each column. However, a greater increase in percent solids was observed in baseline control MFT than citrate-amended MFT. Settling of the solids in megacosm columns was greater initially in citrate-amended MFT than in baseline control MFT. After about 1.5 months of incubation, settling rates between citrate-amended and baseline control MFT were similar and remained similar until the end of the experiment.

Based on the measurements done here, there appears to be an effect of citrate in accelerating solids settling in Albian MFT but not on increasing percent solids of the MFT. It is possible that citrate-amendment prevents an increase in percent solids in MFT

due to constant gas production. Gas pockets have been observed to develop throughout MFT during incubation in small-scale studies (Figure 3.7) and as the gas moves through the MFT to the surface, it may disrupt packing of the fines. Gas movement through MFT may also form channels for pore water to reach the surface of the MFT, resulting in overall accelerated settling of the MFT. However, the MFT was poorly homogenized prior to filling each column and it is likely this affected the observed results.

3.3.4 Changes in ion concentrations in megacosm MFT

Over the 10-month incubation period, Saidul Alam also tracked the concentration of sodium, potassium, magnesium, calcium, ammonium, fluoride, chloride, and sulfate ions in the pore water of MFT collected from each port of both columns. All ion concentrations, except for sulfate, in MFT between columns and between ports did not show substantial differences over time (S. Alam, personal communication). Because of this, only sulfate concentrations of MFT in both columns from all ports have been reported here (Figure 3.11).

Within the first 3 months of incubation, sulfate concentrations dropped to near zero in baseline control and citrate-amended MFT sampled from all four ports of each column. By the fifth month, sulfate concentrations in all samples had risen very slightly, but then decreased again and remained low for the remainder of the incubation time. This decrease in sulfate concentrations suggests the activity of SRB. SRB have been reported to use acetate as the electron donor for reduction of sulfate to sulfide during anaerobic citrate degradation (Gámez et al. 2009).

Unexpectedly, there was a large variability in sulfate concentrations among the eight samples of MFT at the beginning of the experiment (Time zero). All MFT samples from the baseline control and citrate-amended columns were handled, stored and prepared in the same manner before being analyzed for sulfate concentrations (Saidul Alam, University of Alberta, personal communication). A possible cause of the differences in Time zero sulfate concentrations among the MFT samples is the incomplete homogenization of MFT prior to filling the columns (as mentioned in Section 3.3.3). Microbes in the MFT may be unevenly dispersed and their localized activity could

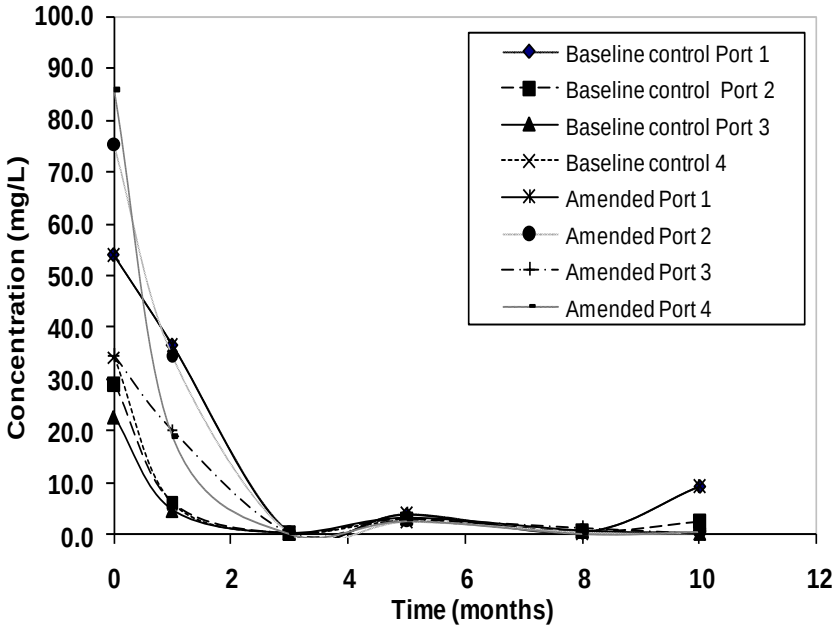

Figure 3.11. Sulfate concentrations in pore water of baseline control and citrate-amended MFT from four ports of each megacosm column during incubation in the dark at ambient temperature for 10 months. Measurements were taken by Saidul Alam, Civil and Environmental Engineering, University of Alberta.

cause localized changes in sulfate concentrations within the MFT material, which would result in heterogeneous sulfate concentrations.

3.3.5 DGGE analysis of Megacosm column MFT

DGGE analysis was used to monitor shifts in the microbial populations of Albian MFT in these columns as a result of citrate amendment. Several difficulties were encountered during DGGE analysis of these megacosm MFT samples. Bacterial DGGE analysis of baseline control and citrate-amended MFT samples from three time points have been included here: Time zero, 5 months, and 10 months. Amplification of Bacterial 16S rRNA gene fragments was consistently very weak and did not improve with commonly used strategies for enhancing PCR product, such as changing the annealing temperature, altering template or primer concentrations, or adding 100% DMSO. A considerable amount of time was spent testing different extraction protocols. Sodium pyrophosphate was used to remove humic materials and metal-organic compounds

(Hayes et al. 1975; Keefer et al. 1984) and skim milk was tested as a clay-adsorption competitor (Hoshino and Matsumoto 2004). Different extractions kits were also tested, such as FastDNA® SPIN Kit for Soil (Qbiogene, Inc. CA, USA), and PowerSoil® DNA (Mo Bio Laboratories, Inc. CA, USA), which was previously tested in the lab (T. Siddique, University of Alberta, personal communication). Mechanical disruption by frozen mortar and pestle (Zhou et al. 1996) was also tried. Classical phenol-chloroform extractions were tested as well; however, ultimately, the standard bead-beating protocol standardized by Penner (2006) for DNA extraction from MFT was the best technique in quality (as measured through PCR amplification) and quantity of recovered DNA.

Amplification of Archaeal 16S rRNA gene fragments consistently failed unless two rounds of PCR amplification were done (i.e. nested PCR). Nested PCR gave visible PCR product when checked on an agarose gel; however, when these visibly clean PCR products were run on the denaturing gradient polyacrylamide gel, gel lanes showed a very high background with smears such that the DNA bands were barely visible. When PCR products were purified in an attempt to remove the smears and background, DNA gel bands became very weak using ethidium bromide staining and were barely visible by the naked eye, causing excision of gel bands to be impossible. For this reason, only the DGGE analysis of Archaea in baseline control and citrate-amended Time zero MFT has been included in the Results and Discussion section.

3.3.5.1 Time Zero

Bacteria

The Bacterial species in baseline control and citrate-amended MFT taken at Time zero from all four ports of each column were analyzed by DGGE. Figure 3.12 is the gel image obtained after staining with SYBR®Gold nucleic acid stain. Bands were excised as labeled.

No discernible difference in bacterial diversity, represented through the number of bands in each gel lane, was observed between baseline control and citrate-amended MFT samples. Samples taken from the same column also showed the same bacterial diversity

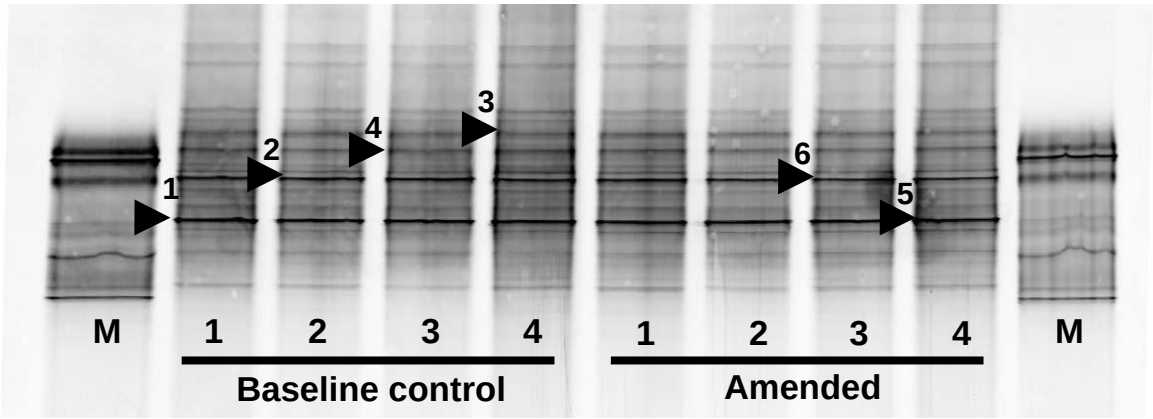

Figure 3.12. DGGE gel image of Bacterial species in baseline control and citrate-amended megacosm MFT sampled from four ports (Ports 1 to 4 as labeled) on each column at Time zero. Each gel lane is labeled with its respective Port (1-4) sample from the baseline control or citrate-amended column, marker lanes are indicated by 'M' and bands that were subsequently excised for sequencing are labeled on their left side with their corresponding number.

compared to one another. Since these MFT were taken at the start of the experiment, it was expected that all MFT samples would display the same diversity as citrate amendment would not have caused an effect yet. Because many of the observed bands stained weakly even with SYBR®Gold, only a few of them were visible after ethidium bromide staining. These bands were considered to be the dominant bands of the sample and were excised for sequencing, as labeled in Figure 3.12. Sequencing these bands proved difficult. As a result, of the six excised bands, four were successfully sequenced through subcloning the DNA eluted from the excised bands. Band 1 and Band 2 were sequenced to represent Bands 1 and 5 and Bands 2 and 6, respectively. The results of comparing the band sequences to NCBI and RDP databases are summarized in Table 3.6.

Generally, there were few dominant bands available for excision. This is likely a reflection of: 1) limitations in the DGGE technique (including DNA extraction and PCR amplification) and the relatively low sensitivity of ethidium bromide staining, and 2) a relatively low bacterial diversity in Albian MFT, as was observed through clone library and RFLP analysis. DNA sequences matched to *Rhodoferax* and very weakly to

Table 3.6. Identities of sequenced DGGE bands of Bacterial species in baseline control and citrate-amended Time zero megacosm samples of Albian MFT. Sequence length was typically ~500 nt. The closest sequence matches (most closely related) and closest named match (most closely related named taxon) in NCBI GenBank, determined by the BLASTn program and confirmed by the RDP database, are indicated. Clones that had no matches to named taxon in NCBI are indicated with taxon information from the RDP database.

Band #	Sample	1. Closest related sequence in GenBank 2. Closest related known taxon in GenBank	Accession # in GenBank	% Sequence Similarity
1 (and 5)[a]	Baseline control Port 1	1.Uncultured *Rhodoferax* sp. clone Ctrl1-7B from oil sands tailings	EU522643.1	97
		2.Uncultured beta-proteobacterium from mountain lakes	AM946181.1	97
2 (and 6)[a]	Baseline control Port 2	1.Uncultured bacterium clone Rap2_24C from sediments of an artificial lake	EF192905.1	98
		2.Uncultured Acidobacteria bacterium clone GASP-WC2W1_G05 from agricultural soils	EF075212.1	82
3	Baseline control Port 4	1. Uncultured bacterium clone 27A from a MTBE-degrading microbial community	FN396939.1	97
		2. No matches to species; RDP gives genus *Erysipelothrix*	-	0.806[b]
4	Baseline control Port 3	1. Denitrifying bacterium enrichment culture clone NOB_2_E12 from river sediment	FJ802273.1	97
		2. No matches to species; RDP gives Caldilineacea	-	0.739 [b]

[a] band numbers in parentheses did not yield sequences but have similar mobility, therefore are presumed to represent the same sequence identity.
[b] similarity scored from RDP.

Acidobacteria species through DGGE analysis. *Rhodoferax* species were present in considerable proportions in M1 and M2 MFT based on clone library and RFLP analysis (Section 3.2.2). Intensity of gel bands in DGGE analysis is not an appropriate way to estimate proportions of microbial species. DGGE analysis shows only the presence or absence of microbial species. Greater intensity of gel bands can be caused by numerous factors including biases in DNA extraction and PCR amplification. However, having said that, it is reassuring to see that the high proportion of *Rhodoferax* in clone libraries of M1 and M2 Albian MFT correlates to the high intensity of bands 1 and 5. Sequenced DNA from one of the excised bands (Band 4) matched to Chloroflexi which was also previously observed in clone libraries of M1 and M2 MFT. Band 3 matched to an uncultured bacterium clone with no genus or species information.

Archaea

Amplification of Archaeal 16S rRNA gene fragments from baseline control and citrate-amended Time zero MFT samples proved to be extremely difficult, probably due to a combination of the presence of inhibitors that are not removed during DNA extraction, a low concentration of Archaeal genomic DNA relative to Bacterial genomic DNA, and suboptimal PCR conditions. As described earlier, attempts to refine DNA extraction and PCR conditions failed to improve the yield of PCR products. Nested PCR was attempted and gave strong PCR product; however, upon running these products on DGGE gels, only smears without distinct bands were repeatedly observed. DGGE analysis of baseline control and citrate-amended Time zero MFT samples was successfully accomplished by using a much lower annealing temperature in PCR amplification and without the use of nested PCR; however, the quality of the PCR products and the resulting DGGE gel are quite low (Figure 3.13).

Four main bands were observed in each sample of MFT and profiles across all samples were the same between baseline control and citrate-amended MFT. Archaeal profiles were also the same in MFT samples taken from different ports in the same column. As was stated for Bacterial DGGE analysis, the profiles of all samples taken from Time zero were expected to be the same.

Though a picture of the DGGE gel was obtained through SYBR® GOLD staining, as stated before, excising the bands based upon ethidium bromide staining was difficult; therefore, the DGGE PCR products were cloned instead and separated through RFLP analysis for sequencing OTUs. Generally, a low diversity of Archaeal species was observed. This has been seen in numerous other environmental studies of anaerobic microbial methane production (Kotelnikova 2002), and also in the clone library analysis of M1 and M2 Albian MFT (Section 3.2.2.2). The results of the sequenced Archaeal DGGE sequences from baseline control and citrate-amended Time zero MFT is summarized in Table 3.7.

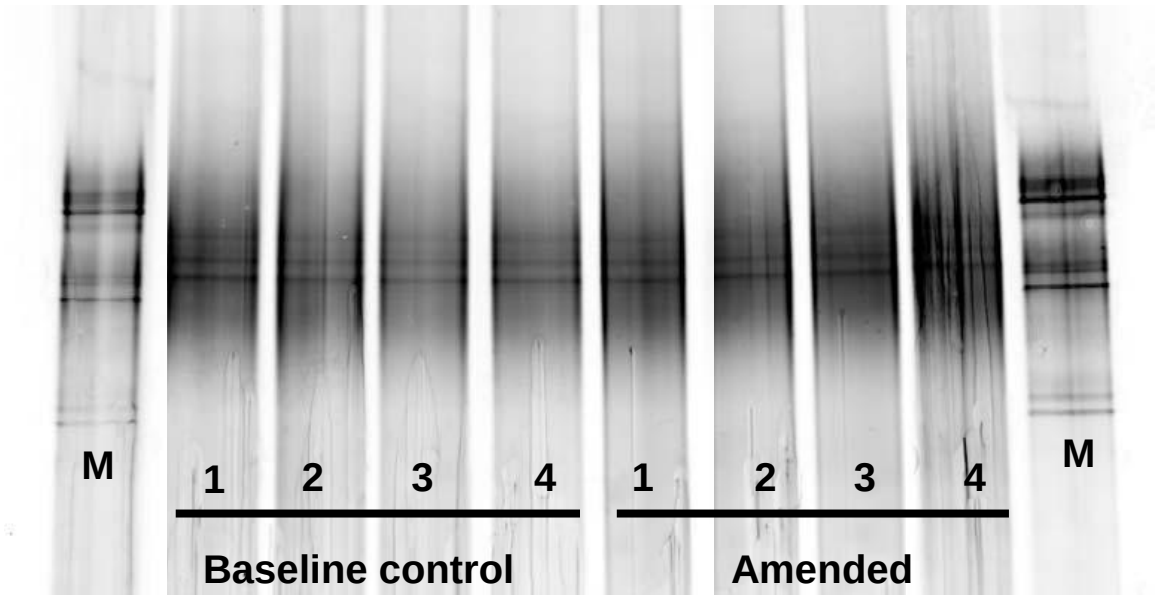

Figure 3.13. DGGE gel image of Archaeal species in baseline control and citrate-amended megacosm MFT sampled from four ports (Ports 1 to 4 as labeled) on each column. Each gel lane is labeled with its respective Port (1-4) sample from the baseline control or citrate-amended column and marker lanes are indicated by 'M'. No bands could be excised from this gel.

In total, six clones representing different RFLP patterns were sequenced out of 10 total different RFLP patterns for the baseline control MFT and seven clones representing different RFLP patterns were sequenced out of a total of 11 different RFLP patterns for the citrate-amended MFT. These RFLP patterns comprised two or more clones and were therefore sequenced. All other RFLP patterns were singletons and clones of these patterns were not sequenced.

Sequenced representatives matched most closely with *Methanosaeta* and *Methanosarcina* species, which was also observed in clone library analysis of Archaeal species in M1 and M2 MFT. The presence of sequences that match to these two genera is not surprising as they have been commonly found in anaerobic methanogenic environments, such as oil fields, aquifers, and marine sediments (Kotelnikova 2002). The remainder of the sequenced representatives matched most closely to uncultured species with no genus or species information. Some information can be inferred from the environment in which these uncultured species were identified. For example, uncultured

Table 3.7. Identities of cloned and sequenced DGGE PCR products of Archaeal species in baseline control and citrate-amended Time zero megacosm samples of Albian MFT. Sequence length was typically ~500 nt. The closest sequence match (most closely related) and closest named match (most closely related named taxon) in NCBI GenBank, determined by the BLASTn program and confirmed by the RDP database, are indicated. Clones that had no matches to named taxon in NCBI are indicated with taxon information from the RDP database.

RFLP Pattern	# of clones	Representative clone name	1. Closest related sequence in GenBank 2. Closest related named taxon in GenBank	Accession # in GenBank	% Sequence Similarity
			Baseline control		
AA	23	2A	1. Uncultured archaeon clone LT-SA-A4 from lake sediment	FJ755706.1	94
			2. Uncultured *Methanosaeta* sp. clone KB-1 2 from a chlorinated ethene-degrading culture	AY780569.1	94
BA	4	1F	1. Uncultured archaeon clone PL-10D12 from production waters of an oil reservoir	AY570673.1	91
			2. No matches to species; RDP gives Methanobacteriaceae	-	0.985[a]
DB	1	2D	1. Uncultured Methanomicrobiaceae archaeon clone YBCAr12 from rice paddy soil	FM165668.1	99
			2. No matches to species; RDP gives Methanomicrobiaceae	-	0.891[a]
CA	1	1E	1. Uncultured Methanosarcinales archaeon from oil-contaminated groundwater	AB077213.1	97
			2. Uncultured *Methanosaeta* sp. clone KB-1 2 from a chlorinated ethane-degrading culture	AY780569.1	96
EB	1	5G	1. Uncultured Methanosarcinaceae archaeon from rice rhizosphere	AJ879021.1	99
			2. *Methanosarcina* sp. HC-2	AB288264.2	99
GA	1	3C	1. Uncultured methanogenic archaeon clone 2R1A01 from an anaerobic bioreactor treating wastewater	EF592658.1	92
			2. No matches to species; RDP gives Methanomicrobiaceae	-	0.710[a]
			Citrate-amended		
AA	14	7C	1. Uncultured archaeon clone LT-SA-A4 from lake sediment	FJ755706.1	99
			2. Uncultured *Methanosaeta* sp. clone KB-1 2 from a chlorinated ethane-degrading culture	AY780569.1	99
BA	4	10A	1. Uncultured archaeon clone PL-10D12 from production waters of an oil reservoir	AY570673.1	98
			2. No matches to species; RDP gives Methanobacteriaceae	-	0.872[a]
GA	3	6E	1. Uncultured archaeon clone PL-10D12 from production waters of an oil reservoir	AY570673.1	99
			2. No matches to species; RDP gives Methanobacteriaceae	-	0.923[a]
CA	3	6H	1. Anaerobic methanogenic archaeon E15-4 from a cellulose-degrading microbial culture	AJ244290.1	99
			2. Uncultured *Methanosaeta* sp. clone KB-1 2 from a chlorinated ethane-degrading culture	AY780569.1	99
EB	2	10C	1. Uncultured archaeon WCHD3-33 from a hydrocarbon-contaminated aquifer	AF050619.1	98
			2. Uncultured Methanosarcinaceae archaeon in rice rhizosphere	AJ879026.1	95
JA	2	9H	1. Uncultured archaeon clone LT-SA-A4 from lake sediment	FJ755706.1	100
			2. Uncultured *Methanosaeta* sp. clone KB-1 2 from a chlorinated ethane-degrading culture	AY780569.1	100
DB	1	7F	1. Uncultured Methanomicrobiaceae archaeon clone YBSAr06 from rice paddy soil	FM165673.1	95
			2. No matches to species; RDP gives Methanobacteriaceae	-	0.833[a]

[a] Similarity score from RDP.

archaeon clone PL-10D12 was sequenced from an analysis of the microbial diversity in production waters of a biodegraded oil reservoir (Grabowski et al. 2005). Uncultured Methanomicrobiaceae archaeon clone YBCAr12 and uncultured Methanomicrobiaceae archaeon clone YBSAr06 were recovered as part of a survey of the methanogenic Archaeal community in rice paddy soil (FM165668.1 and FM165673.1, respectively). Uncultured methanogenic archaeon clone 2R1A01 was discovered among the methanogen populations of an anaerobic biofilm reactor used to treat wastewater (EF592658.1). These environments are similar to MFT in that they are anaerobic and have methanogenic potential, therefore, finding sequences in Time zero baseline control and citrate-amended Albian MFT is not surprising.

The sequences of the Time zero baseline control and citrate-amended Archaeal DGGE clones were aligned with each other as well as with a selection of Archaeal species (Figure 3.14). There is general agreement between the Archaeal species identified by DGGE analysis in Time zero Albian MFT and those identified by clone library and RFLP analysis in M1 and M2 MFT. Again, the majority of representative clone sequences matched to *Methanosaeta* and only one matched to *Methanosarcina*. Clone 10C of the citrate-amended MFT sample (CL-0A-10C, Figure 3.14) matched to *Methanosarcinaceae* on NCBI and RDP databases but at only 95% similarity, explaining its isolation in the phylogenetic tree. There is also a clade of clones that are isolated from the remainder of the tree, clones CL-0U-1F, CL-0A-10A, and CL-0A-6E, which was observed also in the phylogenetic analysis of clones of M1 and M2 MFT (Figure 3.5). An additional clade consisting of clones CL-0A-7F, CL-0U-3C, and CL-0U-2D, also does not group closely with any other methanogenic Archaea on the phylogenetic tree. This was also observed in clone library analysis of M1 and M2 MFT (Figure 3.5). These DGGE results further validate the results of the clone library and RFLP analysis of M1 and M2 MFT.

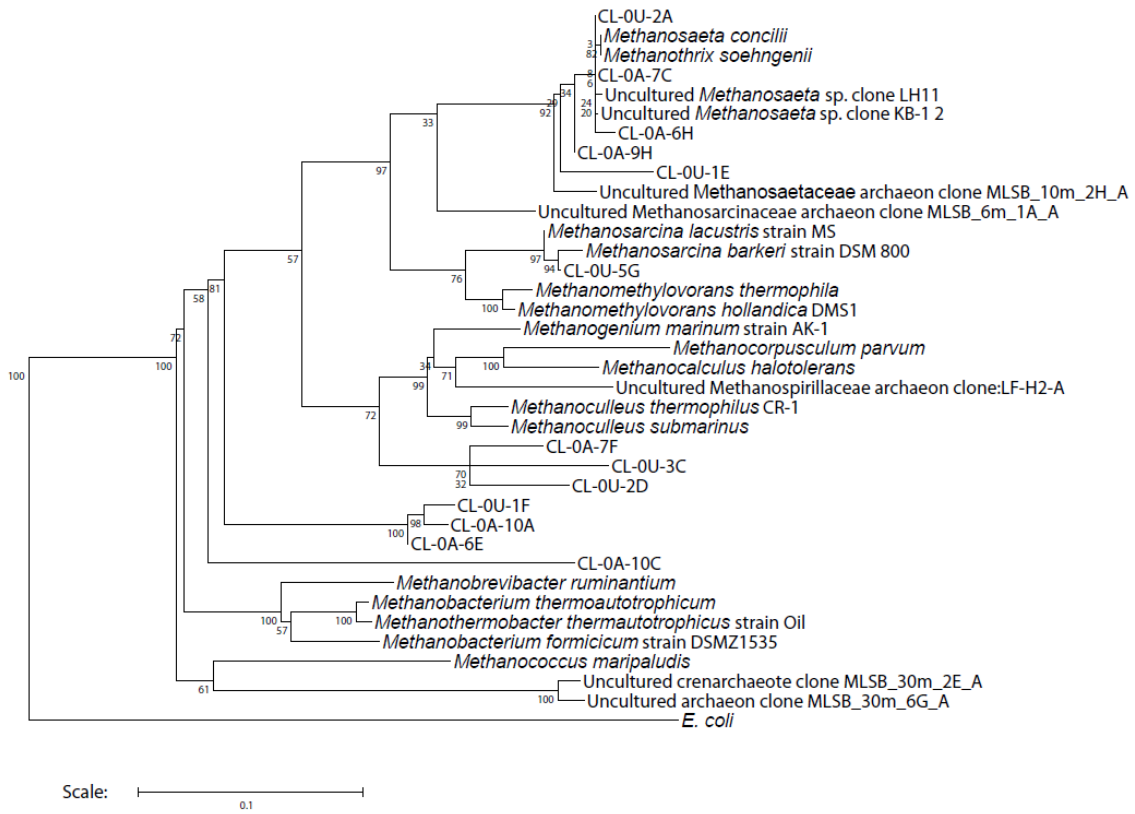

Figure 3.14. Phylogenetic tree of sequenced Archaeal clones of DGGE PCR products obtained from Time zero megacosm baseline control and citrate-amended Albian MFT and named species from NCBI GenBank. Clones from DGGE analysis of Time zero samples are labeled with the prefix "CL-0-". Sequences in GenBank deposited from previous studies with Syncrude MFT have the prefix "MLSB_". Length of the lines are proportional to the relatedness between species and the scale bar represents 10% difference in DNA sequence.

3.3.5.2 Time 5 months

Bacterial species were analyzed in MFT collected from all four ports of each baseline control and citrate-amended MFT column after 5 months of incubation using DGGE analysis. The image of the SYBR®Gold-stained polyacrylamide gel is shown in Figure 3.15. In this analysis, bands were successfully excised from the gel and sequenced directly without any cloning.

The diversity of each sample after 5 months of incubation appeared to be the same between all MFT samples of baseline control and citrate-amended MFT, regardless of

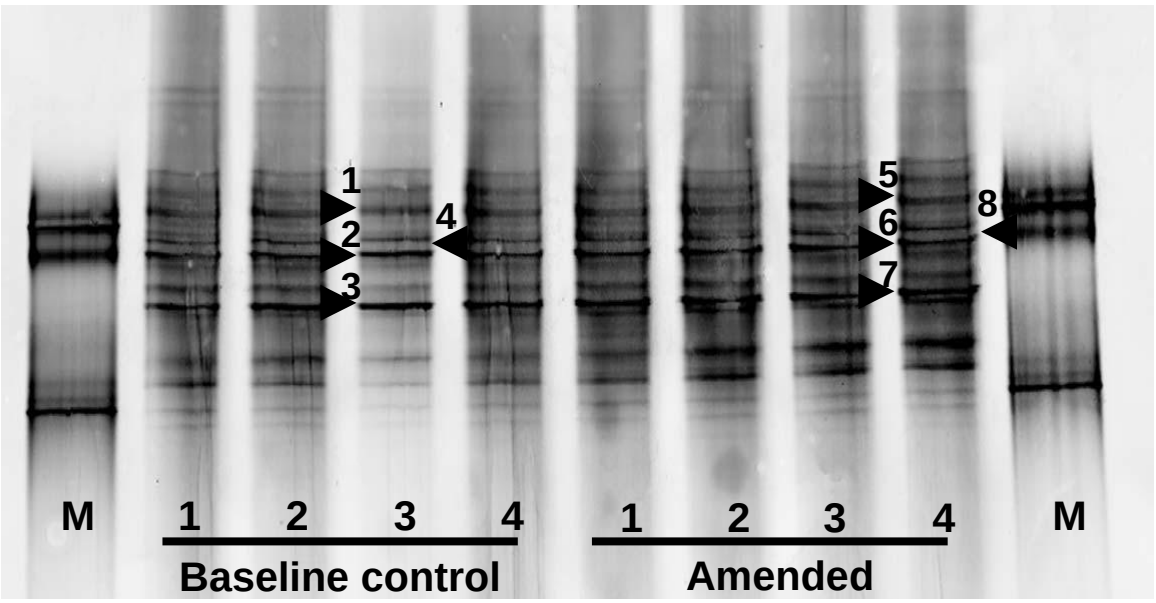

Figure 3.15. DGGE gel image of Bacterial species in baseline control and citrate-amended megacosm MFT sampled from four ports (Ports 1 to 4 as labeled) of each column after 5 months of incubation. Each gel lane is labeled with its respective Port (1-4) sample from the baseline control or citrate-amended column; marker lanes are indicated by 'M' and bands that were subsequently excised for sequencing are labeled with their corresponding number.

which port the MFT were taken from. Furthermore, the diversity observed in these 5 month samples highly resembles the diversity observed for Time zero samples, suggesting that no major shift in the microbial community occurred over 5 months in the presence of citrate. Citrate caused an increase in methane production in microcosms by the sixth week of incubation (Figure 3.2) and its effect on densification was observed in 4 weeks in 2-L densification columns (Table 3.5), suggesting that these changes take place without a change in the diversity of the microbial community in Albian MFT.

A total of eight bands were excised from this gel, as labeled in Figure 3.15. Attempts were made to sequence the DNA in each band despite having excised more than one band of the same mobility. In some cases, strong sequences were unobtainable from the DNA in gel bands, even after cloning each gel band separately and sequencing the resulting clones. This could be because the quality of the original PCR product that was

run on the polyacrylamide gel was very low, potentially because of inhibitors during PCR amplification. One excised band out of the pair for each targeted dominant band was successfully sequenced. The results of comparing these sequences to NCBI and RDP databases are summarized in Table 3.8.

Bacterial DNA sequences matched strongly to *Rhodoferax* species and Chloroflexi bacteria. There were two matches to uncultured bacteria (bands 2 and 4). Uncultured bacterium clone Rap2_24C was discovered in the sediments of an artificial lake (Dorador et al. 2007). Uncultured bacterium clone E42-966 was a part of the microbial community involved in anaerobic degradation of petroleum hydrocarbons ranging from C_{10} to C_{40} in boreal subsurface (unpublished submission to NCBI). Its closest match to a described bacterium is to an uncultured Spirochaetes. Spirochaetes are anaerobes perhaps best known by their association with the hindguts of termites and intestines of animals or with oral diseases. Although the majority of the cultivated species ferment carbohydrates (Canale-Parola 1977), there have been a few Spirochaetes isolates shown to perform acetogenesis (Leadbetter et al. 1999; Graber et al. 2004).

3.3.5.3 Time 10 months

The last samples of this experiment were collected after the columns had been incubating for 10 months. Samples were again taken from all four ports of the baseline control and citrate-amended MFT columns and were analyzed by DGGE analysis for Bacterial species. The image of the SYBR®Gold-stained polyacrylamide gel is given in Figure 3.16.

As was seen in the two previous DGGE analyses of Bacterial species in megacosm column MFT, after 10 months of incubation the bacterial diversity in all samples, whether from the baseline control or citrate-amended column and from all ports, appeared to be largely the same. Interestingly, the two most intense DNA bands in the DGGE gel image of Time zero samples (Bands 1 and 2, Figure 3.12) that remained quite prominent in the 5 month samples (Bands 3 and 2, Figure 3.15), do not show prominently in the 10 month samples. These two bands in the Time zero samples matched to

Table 3.8. Identities of cloned and sequenced DGGE bands of Bacterial species in baseline control and citrate-amended megacosm Albian MFT samples after 5 months of incubation. Sequence length was typically ~500 nt. The closest sequence match (most closely related) and closest named match (most closely related named taxon) in NCBI GenBank, determined by the BLASTn program and confirmed by the RDP database, are indicated. Clones that had no matches to named taxon in NCBI are indicated with taxon information from the RDP database.

Band #	Sample	1. Closest related sequence in GenBank 2. Closest related named taxon in GenBank	Accession # in GenBank	% Sequence Similarity
2 (& 6)[a]	Baseline control Port 3	1. Uncultured bacterium clone E42-966 from a petroleum hydrocarbon-degrading microbial community in boreal subsurface	DQ200741.1	98
		2. Uncultured Spirochaetes bacterium clone KS-419 from glacier soil	EU809520.1	98
3	Baseline control Port 3	1.Uncultured *Rhodoferax* sp. clone Ctrl1-7B from oil sands tailings	EU522643.1	98
		2.Uncultured bacterium clone ZZ12C5 from benzene-contaminated groundwater	AY214181.1	98
5 (& 1) [a]	Amended Port 4	1. Uncultured Chloroflexi bacterium clone Alk1-2G from oil sands tailings	EU522645.1	99
		2. No matches to species; RDP gives Caldilineacea	-	0.895[b]
7	Amended Port 4	1.Uncultured *Rhodoferax* sp. clone Ctrl1-7B from oil sands tailings	EU522643.1	96
		2.Uncultured bacterium clone ZZ12C5 from benzene-contaminated groundwater	AY214181.1	96
8 (& 4) [a]	Amended Port 4	1. Uncultured bacterium clone Rap2_24C from the sediment of an artificial lake	EF192905.1	98
		2. No matches to species; RDP gives unclassified bacterium	-	0.826[b]

[a] band numbers in parentheses did not yield sequences but have similar mobility, therefore are presumed to represent the same sequence identity

[b] Similarity score from RDP.

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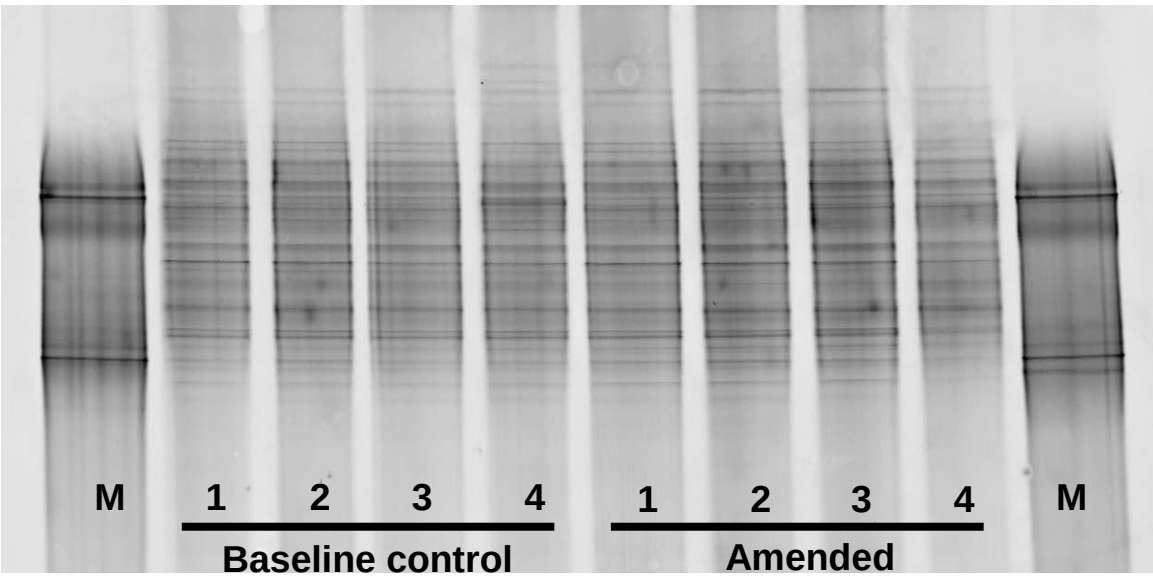

Figure 3.16. DGGE gel image of Bacterial species in baseline control and citrate-amended megacosm MFT sampled from four ports (Ports 1 to 4 as labeled) on each column after 10 months of incubation. Each gel lane is labeled with its respective Port (1-4) sample from the baseline control or citrate-amended column and marker lanes are indicated by 'M. No bands could be excised from this gel.

Rhodoferax and loosely to *Acidobacteria*, respectively, and in 5 month samples, they matched to *Rhodoferax* and Spirochaetes, respectively.

The decreased prominence of these bands could be due to biases in DNA extraction, PCR amplification and staining or it could be due to an actual change in the microbial population. It is possible that the species these prominent bands represent are no longer present in such high numbers as the concentration of citrate decreased due to degradation. However, as DGGE is not a quantitative technique, this is only speculation. By the naked eye, there appears to be more bands in each lane of the 10 month megacosm MFT DGGE analysis than was observed in the DGGE analyses of 5 month or Time zero MFT samples and a reduction of previously dominant bands. In order to determine if this is the case, the species present in the MFT had to be determined. Excising bands from this gel proved to be difficult and so instead DGGE PCR products were cloned and select clones were sequenced after screening by restriction enzyme digestions. The results of comparing the sequences of DNA in baseline control and citrate-amended MFT incubated for 10 months to NCBI and RDP databases is summarized in Table 3.9.

Table 3.9. Identities of cloned and sequenced DGGE PCR products of Bacterial species in baseline control and citrate-amended megacosm Albian MFT samples after 10 months of incubation. Sequence length was typically ~500 nt. The closest sequence match (most closely related) and closest named match (most closely related named taxon) in NCBI GenBank, determined by the BLASTn program and confirmed by the RDP database, are indicated.

RFLP Pattern	# of clones	Representative clone name	1. Closest related sequence in GenBank 2. Closest related named taxon in GenBank	Accession # in GenBank	% Sequence Similarity
			Baseline control		
BF	3	12C	1. Uncultured Peptococcaceae bacterium clone D25_37 from a tar oil contaminant plume 2. *Desulfotomaculum* sp. ECP-C5	EU266910.1 AF529223.1	99 99
CB	3	8A	1. Uncultured bacterium clone Rap2_24C from sediment of an artificial lake 2. Uncultured Acidobacteria bacterium clone GASP-WB1W1_B04 from agricultural soils	EF192905.1 EF073561	98 82
NJ	2	8D	1. Uncultured bacterium clone E42-966 from a petroleum hydrocarbon-degrading microbial community in boreal subsurface 2. Spirochetales bacterium enrichment culture clone D2CL_Bac_16S_Clone1 from a dechlorinating mixed culture	DQ200741.1 EU498367.1	99 94
KD	3	4A	1. Bacterium enrichment culture clone MA1 from an MTBE-degrading culture 2. Uncultured Actinobacteria bacterium clone from an anaerobic sludge digestor	FJ842566.1 CU924270.1	100 96
KN	6	5B	1. Uncultured *Rhodoferax* sp. clone Ctrl1-7B from oil sands tailings 2. *Rhodoferax ferrireducens* T118	EU522642.1 CP000267.1	99 97
MJ	4	4G	1. Uncultured eubacterium OCG8 from oil-contaminated groundwater 2. Uncultured Spirochaetales bacterium clone D15_39 from a tar oil contaminant plume	AB047119.1 EU266876.1	99 99
GJ	2	7E	1. Uncultured Bacteriodetes bacterium clone CS-42-11 from a submerged sinkhole 2. Uncultured Cytophagales bacterium clone TDNP_USbc97_186_1_46 from semiarid wetland	GQ406168.1 FJ516913.1	94 90
AK	2	1B	1. *Polaromonas* sp. RB76 2. Same as above	FJ898301.1	99
LI	2	5H	1. Uncultured Chloroflexi bacterium clone Alk2-10G from oil sands tailings 2. No matches to species; RDP gives *Levilinea*	EU522651.1 -	95 0.642[a]
KK	2	4C	1. Uncultured Chloroflexi bacterium clone Alk1-2G from oil sands tailings 2.No matches to species; RDP gives Caldilineacea	EU522645.1 -	96 0.772[a]
DN	2	9A	1. Uncultured bacterium clone G-30 from an undescribed reservoir 2. Uncultured *Tepidimonas* sp. clone HB116 from aerobic activated sludge	FJ901017.1 EF648100.1	99 99
HK	3	10D	1. Uncultured Comamonadaceae bacterium clone SIB2 1B from Arctic glacier 2. No matches to species; RDP gives *Polaromonas*	DQ628934.1 -	93 0.733[a]

Table 3.9 continued

RFLP Pattern	# of clones	Representative clone name	1. Closest related sequence in GenBank 2. Closest related named taxon in GenBank	Accession # in GenBank	% Sequence Similarity
			Citrate-Amended		
AB	3	10C	1. Uncultured bacterium clone RA13C8 from monochlorobenzene contaminated groundwater	AF407407.1	96
			2. Uncultured Firmicutes bacterium clone Ctrl1-5E from oil sands tailings	EU522659.1	96
BK	3	5B	1. Uncultured bacterium clone AV5-17 from lake sediment	AM181893.1	99
			2. No matches to species; RDP gives *Rhodoferax*	-	0.806 [a]
DJ	11	2B	1. Uncultured bacterium gene, clone: LCFA-B05 from long-chain fatty acid-degrading methanogenic microbial consortium	AB244312.1	99
			2. Uncultured Spirochaetales bacterium clone D15_39 from a tar oil contaminant plume	EU266876.1	95
ZH	2	9G	1. Uncultured bacterium clone D12 from penicillin G production wastewater	EU234285.1	99
			2. *Acidaminobacter hydrogenoformans*	AF016691.1	98
BD	2	10A	1. Uncultured Firmicutes bacterium isolate DGGE gel band 278 from gold mine tailings	FJ493562.1	95
			2. Uncultured Peptococcaceae bacterium clone D25_37 from a tar oil contaminant plume	EU266910.1	95
BB	2	9B	1. Uncultured bacterium SHD-245from a microbial consortium that dechlorinates 1,2-dichloropropane in an anaerobic bioreactor	AJ278174.1	95
			2. Uncultured Chloroflexi bacterium from an anaerobic sludge digestor	CU922167.1	95
LK	2	3G	1. Uncultured bacterium clone WC1_b40 from a simulated waste site	GQ263800.1	96
			2. Uncultured Chloroflexi bacterium from hydrocarbon-contaminated soil	AM935614.1	96
EJ	2	4G	1. Uncultured bacterium clone McSIPG07 from cave water	FJ604737.1	96
			2. Uncultured Spirochaetes bacterium from an anaerobic sludge digestor	CU925696.1	95
EZ	2	12G	1. Uncultured bacterium clone E42-966 from a petroleum hydrocarbon-degrading microbial community in boreal subsurface	DQ200741.1	99
			2. Uncultured Spirochaetes bacterium clone KS-419 from glacier sediment	EU809520.1	98

[a] Similarity score from RDP.

Almost all the sequenced clones matched to uncultured bacteria of which the large majority had no genus or species information. As the gel image suggested, there is a lack of dominance of any one type of RFLP pattern in the baseline control MFT. The highest number of clones belonging to a single RFLP pattern was 6 out of 78 clones and these matched to the genus *Rhodoferax* which was observed before as a dominant species, particularly in clone library and RFLP analysis (M1 and M2 MFT, Section 3.2.2). In citrate-amended MFT, a total of 15 clones grouped to one RFLP pattern out of 75 clones and these matched most closely to a Spirochaetales bacterium. The remainder of the sequenced representative clones grouped to the phylum Firmicutes, which include Clostridia, and to Chloroflexi bacteria.

DGGE analysis of 10 month MFT was meant to screen for differences between samples, either from different columns or different depths. The gel indicated that there are no substantial differences between any of the MFT samples. With a Good's coverage (Good 1953; Equation 3.1) of 42% for the baseline control MFT and 40% for the amended MFT of this 10 month MFT DGGE analysis, more clones needed to be selected to indicate the true diversity of these samples. Many of the singletons would also have to be cloned.

However, even with the clones selected and sequenced in this analysis, it appears that there are small differences in the Bacterial diversity between baseline control and citrate-amended 10 month MFT (Table 3.9). No clones matched to the genus *Rhodoferax* in the citrate-amended 10 month MFT despite the predominance of clones matching to *Rhodoferax* in clone library analysis of M1 and M2 MFT and in Time zero and 5 month MFT. Instead, there was an increase in clones matching to uncultured bacteria that loosely matched to Spirochaetales species in the citrate-amended MFT. Also, it is interesting to note that none of the dominant sequences from the baseline control 10 month MFT were found as dominant sequences in the citrate-amended 10 month MFT. It is difficult to say whether these differences are worth further investigation however, because ultimately each of these RFLP patterns only comprised of 2 or 3 clones out of 78 (baseline control) or 75 (citrate-amended) total clones. The similarity of these clones to Spirochaetales species was also generally low (95%). The relative increase in clones matching to uncultured bacteria and potentially Spirochaetales species in 10 month

citrate- amended MFT as compared to Time zero and 5 month MFT is possibly due to their growth on citrate or byproducts of citrate degradation leading to their increased numbers by 10 months. Spirochaetales-like sequence was detected by 5 months (Band 6, Table 3.8); however, these sequences were not dominant in Time zero MFT. This may suggest that Spirochaetales species increased over 5 months of incubation with citrate. However, since even the baseline control MFT used for these experiments had been previously exposed to citrate *in situ*, these species would be expected to be detected in Time zero MFT. An alternative explanation is that *Rhodoferax*-like clones decreased after citrate had been depleted at about 5 months of incubation and Spirochaetales-like species in the MFT were able to proliferate.

3.3.5.4 Summary of DGGE analyses of megacosm MFT samples

Phylogenetic analysis of the Bacterial sequences from baseline control and citrate-amended megacosm MFT samples at Time zero, 5 months, and 10 months was done to compare the sequences obtained by DGGE analysis from these samples to one another and to a range of Bacterial species (Figure 3.17). The Bacterial DGGE analyses of baseline control and citrate-amended MFT show numerous sequences from all three time points, Time zero, 5 months, and 10 months, that are highly related to *Rhodoferax*. Numerous sequences from the 10 month analysis, two sequences from the Time Zero analysis and one sequence from 5 month MFT analysis grouped to Chloroflexi. Chloroflexi were also found in the M1 and M2 MFT Bacterial analysis and were also found in Syncrude MFT (Penner 2006).

Two of the 10 month DGGE clones clustered with *Desulfotomaculum*, a group of SRB that are often found in anaerobic environments, such as sediments, ground water, and oil reservoirs. The genus *Desulfotomaculum* comprises species that are all capable of sulfate reduction, but some species are autotrophic and others grow by fermentation on organic substrates. Many of the sequences from the 10 month DGGE analysis clustered with *Spirochaeta* as did one band from the 5 month DGGE analysis. This reflects the increase in Spirochaetes observed in baseline control and citrate-amended MFT over time. Interestingly, they are most related to the Spirochaete clone from Syncrude MFT.

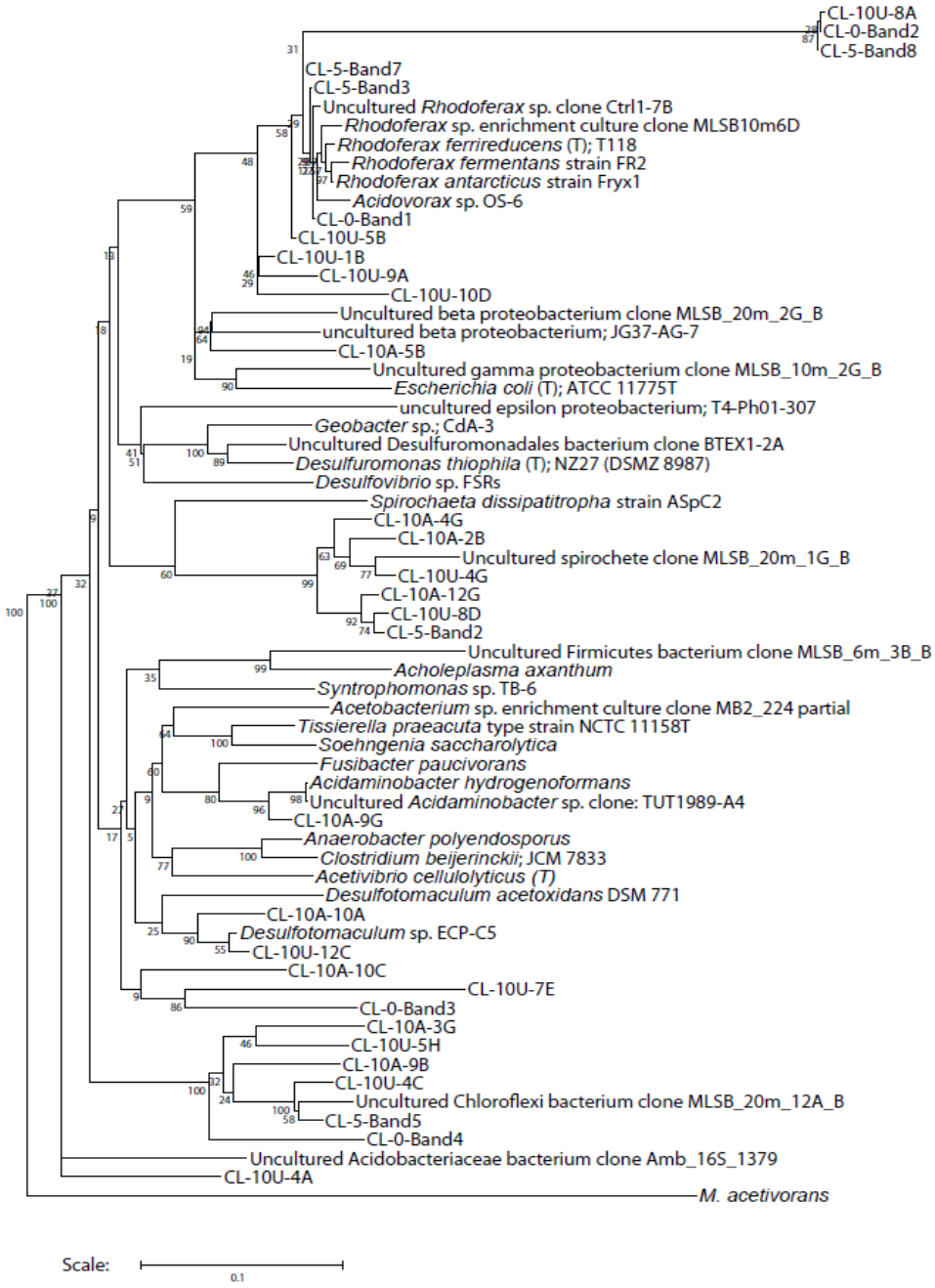

Figure 3.17. Phylogenetic tree of 16S rRNA gene sequences from DGGE gel bands amplified from Time zero and Time 5 months samples and sequenced clones of DGGE PCR products obtained from Time 10 months of baseline and citrate-amended Albian MFT and named species from NCBI GenBank. Sequences of Time zero, Time 5 month, and Time 10 month samples are labeled with the prefix "CL-0-", "CL-5-", and "CL-10-", respectively, followed by a "U" or "A" to indicate baseline control or citrate-amended, respectively. Sequences in GenBank deposited from previous studies with Syncrude MFT have the prefix "MLSB_". Length of the lines are proportional to the relatedness between species and the scale bar represents 10% difference in DNA sequence.

A few of the sequenced representatives of the 10 month bacterial DGGE analysis (CL-10U-1B, CL-10U-9A, and CL-10U-10D) matched closely with *Polaromonas* and *Tepidimonas*. These sequences form a clade separate from those sequences clustering with *Rhodoferax* but remain closely related, as *Polaromonas* and *Tepidimonas* are also part of the family Comamonadaceae. Though two species of *Polaromonas*, *P. vaculota* and *P. naphthalenivorans*, are capable of growth on citrate (Irgens et al. 1996; Jeon et al. 2004), neither grow under anaerobic conditions. *Tepidimonas* species are also capable of growth on citrate and a variety of organic acids (Chen et al. 2006; Moreira et al. 2000), but also do not grow anaerobically. Detecting DNA sequences that match to these genera in Albian MFT is suspicious. It is possible that contamination from lab strains occurred at some point during the analysis although negative controls were included at every step and remained negative. These genera were not detected in MLSB MFT (Penner 2006).

Some sequences did not match to any of the Bacterial species included in this phylogenetic analysis; for example, CL-10A-10C, CL-10U-7E, CL-0-Band 3 formed their own clade and CL-10A-3G and CL-10U-5H did as well. Three sequenced representatives, one from each time point likely representing the same sequence (clones CL-10U-8A, CL-0-Band 2, and CL-5-Band 8), clustered together and were not closely related to any other species included in the phylogenetic tree.

3.3.6 MPN analyses of megacosm MFT

MPN analyses were performed on megacosm MFT samples taken at the beginning of the experiment (Time zero) and at the end of the experiment (10 months of incubation) to compare the changes in numbers of fermenters, SRB, FeRB, and methanogens over time as well as throughout the depth of the column. MPN tubes were monitored regularly for 16 weeks. The results of these analyses are summarized in Table 3.10.

At the beginning of the experiment (Time zero) there were no significant differences in the number of fermenters, SRB, FeRB, or methanogens between each column and each location (port 1 or 4). Each type of microbe was present at the same order of magnitude between columns and between ports. After 10 months of incubation,

Table 3.10. Summary of MPN analyses for fermenters, SRB, FeRB and methanogens in Albian MFT sampled from the top (Port 1) and bottom (Port 4) of the baseline control (U) and citrate-amended (A) megacosm columns. MPNs were scored after 8, 12 and 16 weeks of incubation. All readings had stabilized by Week 16. Significant differences between Time zero and 10-month samples are in bold-face font.

Sampling Event		Time zero			10 months		
Port where MFT was sampled	Metabolic Type	MPN/mL by Week 8	MPN/mL by Week 12	MPN/mL by Week 16[a]	MPN/mL by Week 8	MPN/mL by Week 12	MPN/mL by Week 16[a]
U-1	Fermenters	9.3×10^5	9.3×10^5	**9.3×10^5** ($1.5 \times 10^5 – 3.8 \times 10^6$)	1.5×10^4	1.5×10^4	**1.5×10^4** ($3 \times 10^3 – 4.4 \times 10^4$)
	Sulfate-reducers	2.1×10^4	2.1×10^4	**2.1×10^4** ($3.5 \times 10^3 – 4.7 \times 10^4$)	9.3×10^2	9.3×10^2	**9.3×10^2** ($1.5 \times 10^2 – 3.8 \times 10^3$)
	Iron-reducers	9.3×10^2	1.5×10^3	1.5×10^3 ($3 \times 10^2 – 4.4 \times 10^3$)	1.5×10^3	1.5×10^3	1.5×10^3 ($3 \times 10^2 – 4.4 \times 10^3$)
	Methanogens	1.5×10^3	4.3×10^3	4.3×10^3 ($7 \times 10^2 – 2.1 \times 10^4$)	4.3×10^2	4.3×10^3	4.3×10^3 ($7 \times 10^2 – 2.1 \times 10^4$)
U-4	Fermenters	2.3×10^5	2.3×10^5	2.3×10^5 ($4 \times 10^4 – 1.2 \times 10^6$)	4.3×10^4	4.3×10^4	4.3×10^4 ($7 \times 10^3 – 2.1 \times 10^5$)
	Sulfate-reducers	9.3×10^4	9.3×10^4	**9.3×10^4** ($1.5 \times 10^4 – 3.8 \times 10^5$)	9.3×10^3	9.3×10^3	**9.3×10^3** ($1.5 \times 10^3 – 3.8 \times 10^4$)
	Iron-reducers	2.3×10^3	4.3×10^3	4.3×10^3 ($7 \times 10^2 – 2.1 \times 10^4$)	9.3×10^2	9.3×10^2	9.3×10^2 ($1.5 \times 10^2 – 3.8 \times 10^3$)
	Methanogens	2.3×10^3	4.3×10^3	4.3×10^3 ($7 \times 10^2 – 2.1 \times 10^4$)	4.3×10^2	9.3×10^2	2.3×10^3 ($4 \times 10^2 – 1.2 \times 10^4$)

Continued on next page

Table 3.10. continued

Sampling Event		Time zero			10 months		
Port where MFT was sampled	Metabolic Type	MPN/mL by Week 8	MPN/mL by Week 12	MPN/mL by Week 16[a]	MPN/mL by Week 8	MPN/mL by Week 12	MPN/mL by Week 16[a]
A-1	Fermenters	4.3×10^5	4.3×10^5	4.3×10^5 ($7 \times 10^3 – 2.1 \times 10^5$)	4.3×10^5	4.3×10^5	4.3×10^5 ($7 \times 10^3 – 2.1 \times 10^5$)
	Sulfate-reducers	1.5×10^4	1.5×10^4	**1.5×10^4** ($3 \times 10^3 – 4.4 \times 10^5$)	1.5×10^5	1.5×10^5	**1.5×10^5** ($3 \times 10^4 – 4.4 \times 10^6$)
	Iron-reducers	2.3×10^3	2.3×10^3	4.3×10^3 ($7 \times 10^2 – 2.1 \times 10^4$)	2.1×10^3	2.3×10^4	2.3×10^4 ($4 \times 10^3 – 1.2 \times 10^5$)
	Methanogens	7.5×10^1	2.3×10^3	2.3×10^3 ($4 \times 10^2 – 1.2 \times 10^4$)	4.3×10^3	4.3×10^3	4.3×10^3 ($7 \times 10^2 – 2.1 \times 10^4$)
A-4	Fermenters	2.1×10^5	2.1×10^5	2.1×10^5 ($3.5 \times 10^4 – 4.7 \times 10^5$)	7.5×10^4	1.5×10^5	1.5×10^5 ($3 \times 10^4 – 4.4 \times 10^5$)
	Sulfate-reducers	9.3×10^4	9.3×10^4	9.3×10^4 ($1.5 \times 10^4 – 3.8 \times 10^5$)	9.3×10^4	9.3×10^4	9.3×10^4 ($1.5 \times 10^4 – 3.8 \times 10^5$)
	Iron-reducers	1.2×10^3	1.2×10^3	9.3×10^3 ($1.5 \times 10^3 – 3.8 \times 10^4$)	2.3×10^2	9.3×10^2	4.3×10^3 ($7 \times 10^2 – 2.1 \times 10^4$)
	Methanogens	9.3×10^2	9.3×10^2	2.3×10^3 ($4 \times 10^2 – 1.2 \times 10^4$)	7.5×10^2	4.3×10^3	4.3×10^3 ($7 \times 10^2 – 2.1 \times 10^4$)

[a] 95% confidence interval in parentheses.

there were no significant differences in the numbers of fermenters in MFT from each column and each port, with the exception of MFT sampled from the baseline control column Port 1. Fermenters decreased significantly in number in this sample between Time zero and Time 10 months ($p<0.05$).

There were some changes in SRB abundance between the megacosm MFT samples. SRB decreased significantly in numbers between Time zero MFT and Time 10 months MFT taken from either port of the baseline control column ($p<0.05$). In the citrate-amended column, SRB increased significantly in MFT samples taken from Port 1, the upper port, and displayed no change in MFT sampled from Port 4, the bottom port ($p<0.05$). The decrease in SRB in the baseline control MFT may have occurred because of a lack of substrate. SRB were present in significantly greater quantities in citrate-amended MFT than in baseline control MFT after 10 months of incubation (in MFT from Ports 1 and 4; $p<0.05$). SRB include some fermenters, capable of using citrate. Some SRB also use acetate as an electron donor for the reduction of sulfate and production of sulfide (Gámez et al. 2009). Analysis of the Bacterial species in M1 and M2 MFT (Section 3.2.2.1) showed sequences matching to Desulfobacterales, which are SRB.

The number of methanogens in MFT collected from both ports of the citrate-amended column did not significantly change over 10 months. There was also no significant difference in the quantities of methanogens in MFT from either port of the baseline control column between Time zero and Time 10 months. Like the methanogens, numbers of FeRB did not significantly differ between MFT samples. After 10 months of incubation, FeRB abundance remained the same in baseline control MFT sampled from Port 1 and Port 4. The same trend was observed in the amended MFT column after 10 months of incubation.

3.3.7 Summary of microbial analyses of megacosm MFT

DGGE analysis of megacosm MFT samples at the start of the incubation period detected Bacterial species similar to the genus *Rhodoferax*. Of the Archaeal species, there was a predominance of *Methanosaeta* species and some *Methanosarcina* species. Several Archaeal sequences did not match closely to any methanogen. These results are in

general agreement with the Bacterial and Archaeal species detected in M1 Albian MFT using clone library construction and RFLP analysis (Section 3.2.2). There were no observable differences in the Bacterial or Archaeal species diversity between baseline control and citrate-amended MFT or between samples taken from different ports of each column.

After 5 and 10 months of incubation, there were still no major changes in the Bacterial diversity of each sample and no observable differences between baseline control and citrate-amended MFT samples. At 5 months, DNA sequences matched with *Rhodoferax*, Chloroflexi, which were found in clone library analysis of M1 MFT (Section 3.2.2), and Spirochaetes, which were not detected at Time zero. At 10 months, uncultured bacteria loosely matching to Spirochaetes instead of *Rhodoferax* species were dominant in citrate-amended MFT samples. It is possible that Spirochaete species were able to proliferate as *Rhodoferax* species decreased.

MPN enumeration showed that citrate amendment to Albian MFT and incubation for 10 months did not cause an increase in the quantities of fermenters, FeRB, or methanogens. SRB was the only group of microbes to show a significant increase in numbers between the baseline control column and citrate-amended column and this occurred in MFT samples from Port 1. Significant decreases in fermenters and SRB were observed in MFT samples from Ports 1 and 4 of the baseline control, likely caused by depletion of useable substrate. Overall, there were no drastic shifts in the Bacterial population, in either diversity or quantity, of Albian MFT as a result of citrate-amendment. These observations support the findings of microcosm experiments (Section 3.1) which indicate that citrate is a methanogenic substrate in Albian MFT. The lack of change in the microbial community in response to citrate-amendment, as observed in these megacosm columns, suggests that the microbial community is readily capable of degrading citrate. It is possible that the concentration of citrate that was tested (200 ppm) is not high enough to cause an increase in the microbial quantities of Albian MFT.

3.4 SETTLING COLUMNS (MESOCOSMS): GAS RELEASE AND DENSIFICATION ANALYSIS

Sixteen 7-L settling columns were filled in duplicate with Albian "bulk sample" MFT amended with one of citrate, diluent, polyacrylamide, or a combination of two or three of these substrates prepared in cap water, or with cap water only (baseline control). These columns were sealed and incubated in the dark at 18-22°C. Measurements of water release, settling of solids, and gas production were taken biweekly.

3.4.1 Water release and solids measurements in settling columns (mesocosms)

3.4.1.1 Water release

Amendments of citrate, diluent, and polyacrylamide were tested for their effects on water release from Albian MFT. Water release was measured as the height of the water caps that formed on the surface of the solids material in each column and then converted into volume (mL). Measurements over 31 weeks are summarized in Figure 3.18. The error bars of this figure, and several of the figures below (Figures 3.19, 3.20, and 3.23) indicate the standard deviations of duplicates.

Citrate treatments and diluent treatments had an effect of increasing water release whereas polyacrylamide did not appear to increase water release when applied alone to MFT. Water release from Albian MFT was most prominent when MFT was amended with citrate as part of its treatment. Whether alone or in combination with polyacrylamide and/or diluent, MFT amended with citrate released more water as a group than MFT without citrate.

It is difficult to interpret the effects of polyacrylamide or diluent on water release in relation to citrate. It must also be taken into consideration that this experiment was done only in duplicate. These trends are based on the mean values of duplicate columns and in cases of MFT treated with all three amendments, citrate and diluent, and citrate only, the error bars of change in water height of each treatment overlap. This suggests that these three treatments have the potential to reach the same amount of water release.

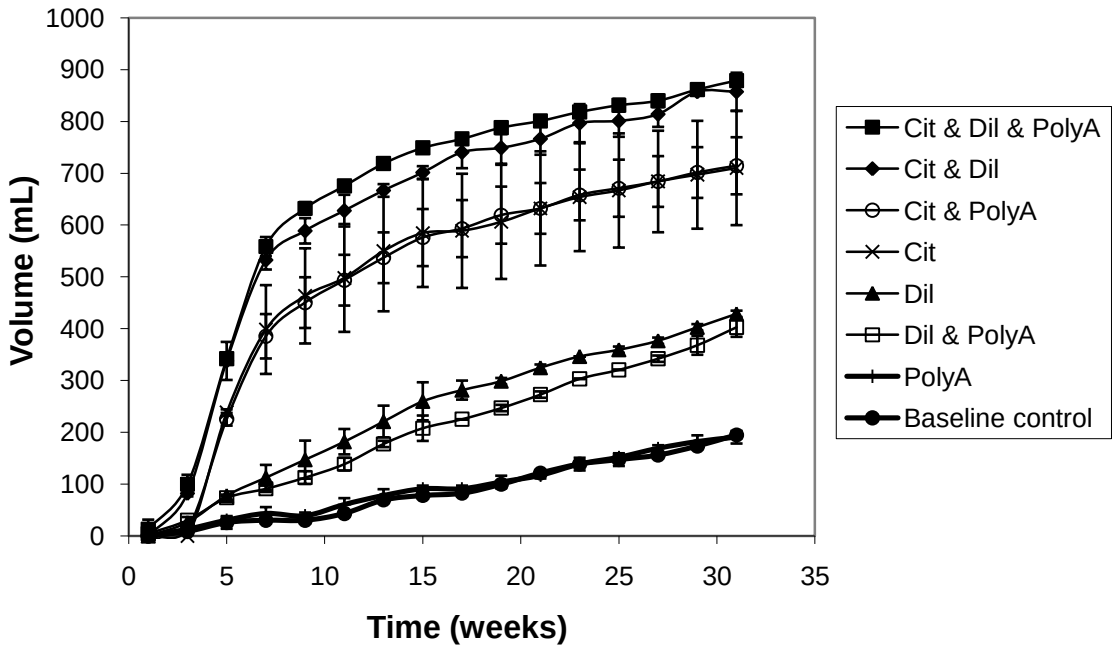

Figure 3.18. Volume of water released during 31 weeks of incubation from Albian MFT amended with cap water (baseline control), or citrate (200 ppm), diluent (0.2 vol%), polyacrylamide (100 ppm) or a combination of two or three of these prepared in cap water. Values represent the mean of duplicate settling columns ± standard deviation.

A third replicate of each column might have indicated which columns were significantly different from one another and allowed stronger conclusions to be made about the effect of polyacrylamide and diluent on water release from these MFT.

The variability between two seemingly identical columns should be noted. Although every effort was made to ensure the MFT were homogenous in each column, pairs of columns holding MFT with the same treatment showed noticeable differences. For example, the pair of columns with citrate-amended MFT had substantially different volumes of water released (at Week 31, 788 mL and 632 mL). The high degree of variability between the pairs of settling columns analyzed here suggest that there are numerous factors affecting water release from Albian MFT besides the amendments tested, including chemical and physical factors.

Based on duplicate column measurements, it remains clear that citrate and diluent, in combination and alone, have an effect on water release from Albian MFT. The

addition of citrate was shown in microcosm experiments to cause increased microbial methane production (Section 3.1; Dr. T. Siddique). Because methane production has already been shown to accelerate MFT densification in Syncrude MFT (Fedorak et al. 2003), methane production was hypothesized to cause accelerated settling in Albian MFT. The increased water release in citrate-amended MFT is speculated to be the result of 1) the formation and subsequent release of methane gas from MFT, which would disrupt the stacking of the solids in MFT and/or 2) the production of carbon dioxide, which is expected to dissolve in pore water as bicarbonate, decreasing the pH and allowing for cation exchange between the surfaces of clay particles. This would affect the stacking of the clays and facilitate settling. These hypotheses are being examined in other studies (J. Foght, University of Alberta, personal communication). The mechanism of diluent enhancing water release from MFT may be a physical effect because microcosm experiments showed that diluent did not cause increased methane production (Figure 3.1). The exact mechanism of how diluent aids water release from MFT is unknown. The combination of diluent and citrate is not additive, suggesting that unknown factors interact to cause increased water release in MFT amended with both, compared to MFT amended with only citrate or only diluent. Polyacrylamide is used in treating Albian MFT as a flocculant, causing the clay particles of MFT to aggregate, which facilitates settling. The results obtained here suggest that polyacrylamide alone cannot cause improved water release from MFT.

3.4.1.2 Solids settling

Amendments of citrate, diluent, and polyacrylamide were also tested for their effects on solids settling in Albian MFT over the incubation period of 31 weeks. Measurements were taken from the original height of the MFT at the start of the experiment to the water-solids surface interface, and these values were then converted into volume (mL). The results of solids settlement measurements are summarized in Figure 3.19.

In the first few weeks of incubation, the volume of MFT in columns increased (Figure 3.19) due to the production and entrapment of gas. When the solid material in

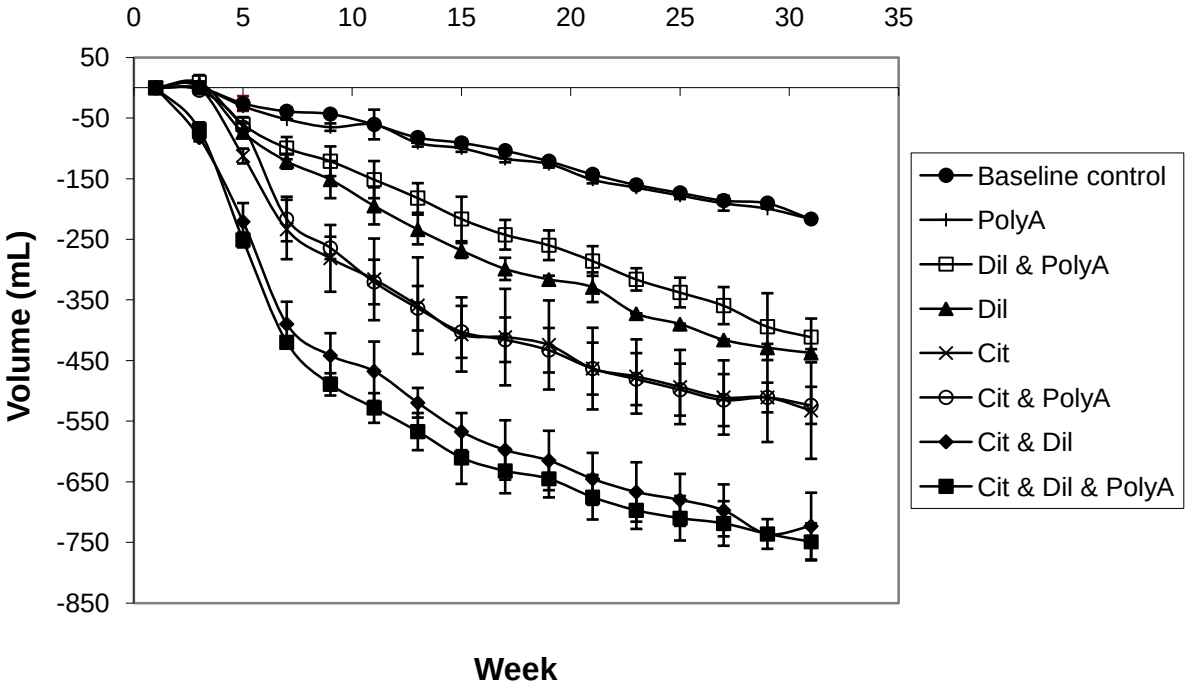

Figure 3.19. Change in solids volume during 31 weeks of incubation in Albian MFT amended with cap water (baseline control), or citrate (200 ppm), diluent (0.2 vol%), polyacrylamide (100 ppm) or a combination of two or three of these prepared in cap water. Values represent the mean of duplicate columns ± standard deviation.

each column began to settle, the same trend was observed for solids settling in these MFT as for water release. Polyacrylamide amendment resulted in the same amount of settling as in the baseline control whereas diluent caused more settling than the baseline control with or without polyacrylamide. MFT with citrate as part of their treatment settled the most. MFT treated with citrate and citrate plus diluent showed a greater distinction from MFT treated with citrate and citrate plus polyacrylamide in settling than in water release.

These results suggest that diluent alone has an effect on settling but also has an effect in the presence of citrate, further enhancing the settling caused by citrate alone. The mechanisms of how citrate and diluent accelerate solids settling are unknown. Citrate promotes microbial methane production but it is unclear whether this solely is responsible for accelerated settling or if citrate also operates on another mechanism. Diluent has an effect on settling but when amended in combination with citrate, the interactions, if any,

between diluent and citrate in causing accelerated settling are unknown. Polyacrylamide was expected to accelerate settling of the MFT. MFT visibly thickened upon the addition of polyacrylamide and yet minimal settling occurred over 31 weeks. This suggests that the aggregation of clay material in MFT alone does not result in accelerated settling.

3.4.2 Gas production and release in settling columns (mesocosms)

3.4.2.1 Released gas

The effect of citrate, diluent, and polyacrylamide on gas production and release from Albian MFT was tested concurrently with their effects on water release and solids settling to see whether there was a relationship among these three aspects of MFT densification. As gas was released from the MFT in each column, it was collected in a Tedlar bag attached to the lid of each column. Tedlar bags were sampled and the methane gas measured by gas chromatography. Because these columns were static, as compared to methane analyses in microcosms where bottles were thoroughly shaken before sampling, methane measurements were an analysis of released methane and not produced methane. The results of the biweekly percent methane gas analysis from each column are summarized in Figure 3.20. Methane percentages were not converted to absolute methane values because the volume of gas trapped in each Tedlar bag could not be confirmed.

Methane release from these columns, for the first 13 weeks of observation, followed the same trend as was observed for water release and solids settling, strongly suggesting that methane release in Albian MFT accelerates MFT densification as it does in Syncrude MFT (Fedorak et al. 2003). MFT amended with citrate resulted in the most methane release. MFT treated with diluent or polyacrylamide or the combination of the two released the same amount of methane as the baseline control which was minimal.

After about 13 weeks of incubation, released methane measurements began to decrease. Initially, this was attributed to leakage from the Tedlar bags. However, because these measurements were of methane percentage, it seemed unlikely that methane would leak more rapidly than other gases in the Tedlar bags to disproportionately affect the percent methane. Columns continued to incubate with continued sampling for methane to

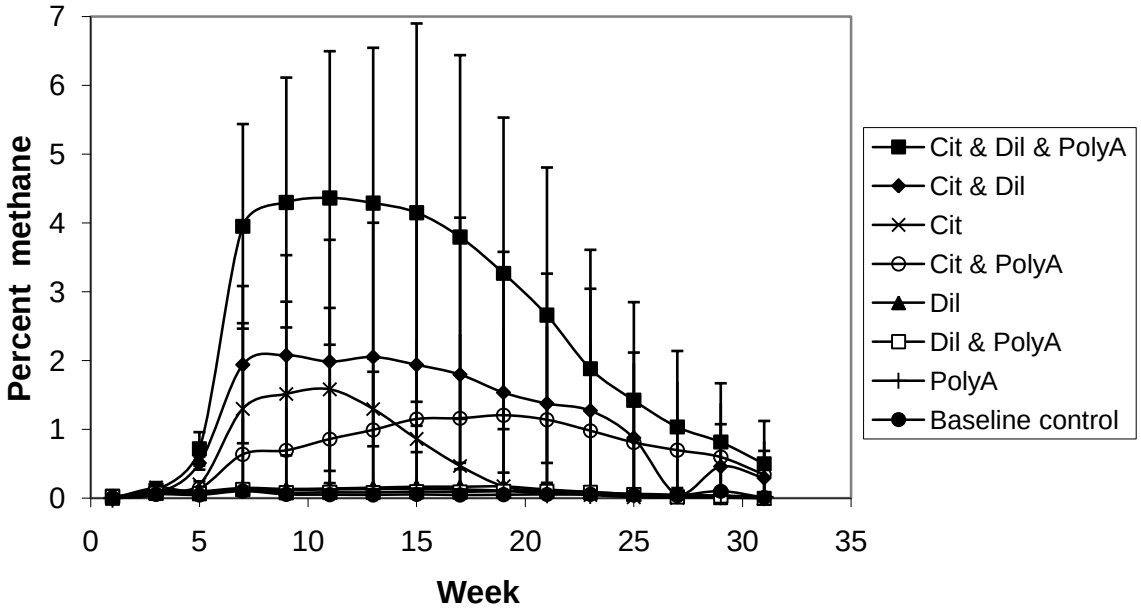

Figure 3.20. Methane content (%) of total released gas during 31 weeks of incubation from Albian MFT amended with cap water (baseline control), or citrate (200ppm), diluent (0.2 vol%), polyacrylamide (100 ppm) or a combination of two or three of these prepared in cap water. Values represent the mean of duplicate columns ± standard deviation.

see if methane proportion would increase again; however, methane percentages remained low. By Week 31, methane readings from all samples had reached zero or were near zero.

One possible explanation for the change in composition is that methane production had ceased and another gas had begun to be produced or was being produced at a greater rate than before. Carbon dioxide is also produced during citrate fermentation and methanogenesis and so this gas was measured from samples of the collected gas in the Tedlar bags. Carbon dioxide measurements, however, were low and did not follow a clear trend (data not included). Percent carbon dioxide did not steadily increase or decrease over time, but fluctuated slightly from week to week. Carbon dioxide production did not compensate for the decrease in methane from the columns.

It is still unclear why released methane measurements dropped to nearly zero in all columns. Leakage from Tedlar bags or tubing seems unlikely as this should not change the percentage of methane release, unless there was preferential leakage of methane and not other gases. Another possible explanation for the decrease in methane

from MFT is that the methane was consumed or reabsorbed into the MFT. Methanotrophs have been identified in anaerobic environments such as rice paddy soils and rice roots (Takeda et al. 2008), groundwater aquifers (Lindner et al. 2007), and compost heaps where the large majority of produced methane was oxidized (Jäckel et al. 2005). Few methanotrophs have been successfully isolated and relatively little is known about them. One subgroup of methanotrophic archaea are anaerobic methane oxidizers (AOM) which are thought to participate in a syntrophic relationship with SRB to carry out the reaction depicted in Equation 3.4 (Valentine 2002; Thauer and Shima 2008).

$$CH_4 + SO_4^{2-} + H^+ \rightarrow CO_2 + HS^- + 2H_2O \qquad (\Delta G^{\circ\prime} = -21 \text{ kJ/mol } CH_4) \qquad \text{(Eq. 3.4)}$$

Methane is oxidized by the reduction of sulfate to produce carbon dioxide and sulfide. This could potentially account for the severe drop in sulfate observed in megacosm MFT, as measured by Saidul Alam (Figure 3.11), and also the black precipitate observed in settling column MFT (Section 3.4.4) if the sulfide precipitated with metals in the MFT. Methanotrophs have a slow doubling time, estimated to be between weeks and months (Strous and Jetten 2004), which may explain the decrease in percent methane in settling columns by Week 17 (Figure 3.20). However, no anaerobic methanotroph-like clones were found during clone library analysis of M1 and M2 MFT (Section 3.2.2), or in DGGE analyses of megacosm (Section 3.3.4) and settling column (Section 3.4.6) MFT samples. 16S rRNA gene sequencing of AOM involved in the oxidation of methane using sulfate (Equation 3.4) has suggested that these species are closely related to Methanosarcinales and Methanomicrobiales. The SRB involved in methane oxidation fall within the genera *Desulfosarcina* and *Desulfococcus*, as well as *Desulfobulbus* (Valentine 2002; Knittel and Boetius 2009). Microorganisms of these genera were not detected in Albian MFT.

3.4.2.2 Entrapped gas

Observations of the MFT within each column showed a variety of gas bubble sizes and shapes entrapped in the MFT material and differing clarity of water caps. The

largest gas bubbles were observed in MFT amended with citrate plus polyacrylamide and citrate plus diluent plus polyacrylamide (Figure 3.21 a and c). These bubbles are better described as large gas pockets. MFT amended with only polyacrylamide or only diluent showed few to no gas bubbles (Figure 3.22 c and d). MFT amended only with citrate varied; one column had MFT with many small bubbles (Figure 3.21 d) and the other column had MFT with fewer bubbles that are better described as thin cracks (Figures B-1a and B-1b in Appendix B). The same was observed for MFT amended with citrate plus polyacrylamide; one column showed numerous small bubbles and the other had large cracks and larger bubbles (Figures B-2a and B-2b in Appendix B). The size and shape of gas bubbles as a result of citrate amendment appear to be affected by diluent and polyacrylamide. Gas release may potentially be indirectly affected by diluent and polyacrylamide; larger gas bubbles are more likely to successfully migrate through the MFT material to erupt through the MFT surface.

Generally, settling columns of MFT amended with diluent and/or polyacrylamide as well as the baseline control had the clearest water caps (Figure 3.22 a, c, and d). MFT amended with citrate, alone or in combination with diluent and/or polyacrylamide, had water caps that varied in turbidity (Figure 3.22 b). MFT amended with citrate plus polyacrylamide showed variation between columns, where one column had a very clear water cap and the other column showed very turbid water (Figures B-3a and B-3b in Appendix B). The turbidity of cap water is likely related to the amount of gas release that occurs in the MFT column, as well as the size of the gas bubbles being released. MFT that showed minimal to no methane release (baseline control MFT, MFT amended with diluent or polyacrylamide) had very clear water caps (Figure 3.22 a, c, and d). As gas bubbles move upward to break through the surface of the MFT, they are likely to perturb the settled fines and push some upwards into the water cap. Polyacrylamide acts as a flocculant and may hold the MFT matrix more strongly, preventing the movement of gas bubbles to the MFT surface until a very large gas bubble develops. As a result, cap water remains quite clear until a large gas bubble erupts through the MFT surface to cause turbidity. A selection of images of settling column MFT are shown in Figures 3.21 and 3.22; additional images of settling column MFT can be found in Appendix B.

Figure 3.21. Gas bubbles in 7-L settling column MFT: a) very large gas pockets dispersed through the solids material of MFT amended with citrate plus diluent plus polyacrylamide; b) some small and mid-sized gas bubbles dispersed through the solids material of MFT amended with citrate plus diluent; c) mid-sized gas pockets formed through solids material of MFT amended with citrate plus polyacrylamide; d) numerous small gas bubbles dispersed in MFT amended with citrate.

Figure 3.22. Water caps in 7-L settling column MFT: a) clear water released from baseline control MFT with few small gas bubbles in solids material; b) the slightly turbid water cap on the surface of solids material of MFT amended with citrate plus diluent plus polyacrylamide; c) clear water released from MFT amended with diluent; d) clear water released from MFT amended with polyacrylamide. Note the absence of gas bubbles in MFT of settling columns pictured in 3.22c and 3.22d.

To further look into the relationship between citrate, diluent, and polyacrylamide amendments on gas production and release in Albian MFT, the total volume of entrapped gas in each column was calculated (Section 2.6.3). The results of these calculations are summarized in Figure 3.23. Negative volumes of gas indicate MFT that have a lower volume at the end of the experiment than at the beginning of the experiment. This does not necessarily mean there was no gas entrapped in the MFT material; gas release may have surpassed gas entrapment to cause the total volume of the MFT to decrease. There was also a small amount of evaporation of the released water during incubation, evidenced by condensation collected on the lids of each column. This would minimally decrease the overall volume of the MFT. The degree to which the evaporation would affect these calculations is unknown because there was no method to measure the amount of evaporation and volume of condensate.

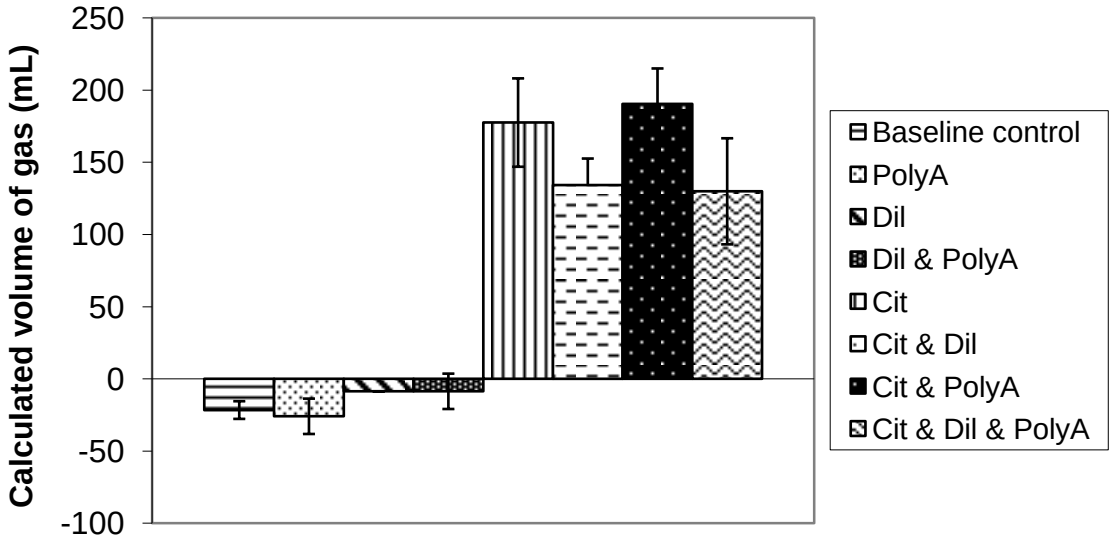

Figure 3.23. Calculated volumes of entrapped gas in settling column MFT after 31 weeks of incubation. MFT were amended with cap water (baseline control), diluent (0.2 vol%), polyacrylamide (100 ppm), citrate (200 ppm), or a combination of two or three of these substrates prepared in cap water. Values represent the mean of duplicate columns ± standard deviation.

The settling columns of MFT that released little to no methane gas also showed little settling and released small amounts of water compared to the baseline control MFT columns. These columns also had minimal gas entrapment compared to the baseline control MFT. MFT amended with diluent, or polyacrylamide, or a combination of these two had negative volumes of gas entrapment (Figure 3.23). The volume of the MFT by Week 31 of incubation was less than the volume of the MFT at the beginning of the experiment. According to methane measurements and observations of the MFT within the columns, gas release and entrapment were both minimal.

MFT amended with citrate and citrate plus polyacrylamide had the greatest amount of entrapped gas and MFT amended with citrate plus diluent and citrate plus diluent plus polyacrylamide had less. This corresponds with measurements of released methane where the opposite trend was observed. If citrate is the sole methanogenic substrate in these MFT, it follows that if more gas is released, less gas will be entrapped in the MFT. However, this also assumes that methane proportions in the headspace gas are a reliable indicator of the total amount of gas released.

3.4.3 Summary of water release, solids settling, and gas release in settling columns (mesocosms)

Citrate stimulated methane production from Albian MFT, as evidenced through methane release and entrapped gas, and increased the rate of water release as well as the rate of solids settling. Diluent amendment caused a small increase in the rates of water release and solids settling, but did not contribute to methane release. Polyacrylamide did not stimulate water release, solids settling, or methane production from MFT. Methane release strongly correlated to accelerated water release and solids settling, as observed in MFT amended with citrate, either alone or in combination with diluent and/or polyacrylamide.

There is evidence that factors beyond microbiological methane production, like physical and chemical factors, play a role in MFT densification. MFT amended with diluent alone showed greater water release and solids settling than baseline control MFT, despite a lack of methane production. Different sizes and shapes of gas bubbles were

observed in columns of MFT treated with different amendments suggesting further that physical factors affect MFT behavior. The largest gas bubbles, better termed gas pockets, were observed in MFT amended with citrate plus diluent plus polyacrylamide. MFT amended with citrate only or citrate plus polyacrylamide had the most trapped gas; these results suggest that diluent facilitates gas release from MFT. However, the mechanisms of citrate, diluent, and polyacrylamide in accelerating gas release and MFT densification are currently unknown.

3.4.4 Deconstructing the settling columns (mesocosms) for sampling

Six columns were deconstructed for sampling to analyze solids content and microbial diversity and population, through DGGE and MPN analyses: one of each pair of columns amended with citrate plus diluent plus polyacrylamide, citrate plus diluent, citrate plus polyacrylamide, citrate alone, diluent alone, and the baseline control. These were chosen on the basis that citrate is a methanogenic substrate in Albian MFT, as demonstrated by Dr. Tariq Siddique's microcosm experiments (Section 3.1) and also by its measured effect on water release, solids settling, and methane production in the settling columns. Therefore, all columns containing citrate as part of the MFT treatment were considered. MFT treated with diluent alone was also analyzed to see any potential effects on the microbial community of Albian MFT. Columns were each sampled at four heights of the solids material to give 24 total samples.

There were several peculiar observations of settling columns during their incubation, including the formation of a dark grey-to-black-colored band of MFT near the solids-water interface and the development of a flaky orange precipitate that settled on the solids surface. Black precipitation occurred in all 16 columns (Figure 3.24a) and suggested the possible activity of SRB. Sulfate concentrations in megacosm column MFT were also observed to decrease to zero (Figure 3.11; Saidul Alam), suggesting sulfate-reducing activity. The orange precipitate was observed in all columns (Figure 3.24b) except for the baseline control, MFT amended with solely polyacrylamide, and one of the two columns of MFT amended with citrate plus diluent. Orange precipitate suggested that a reaction involving iron may be occurring, possibly indicating the presence of Fe(III);

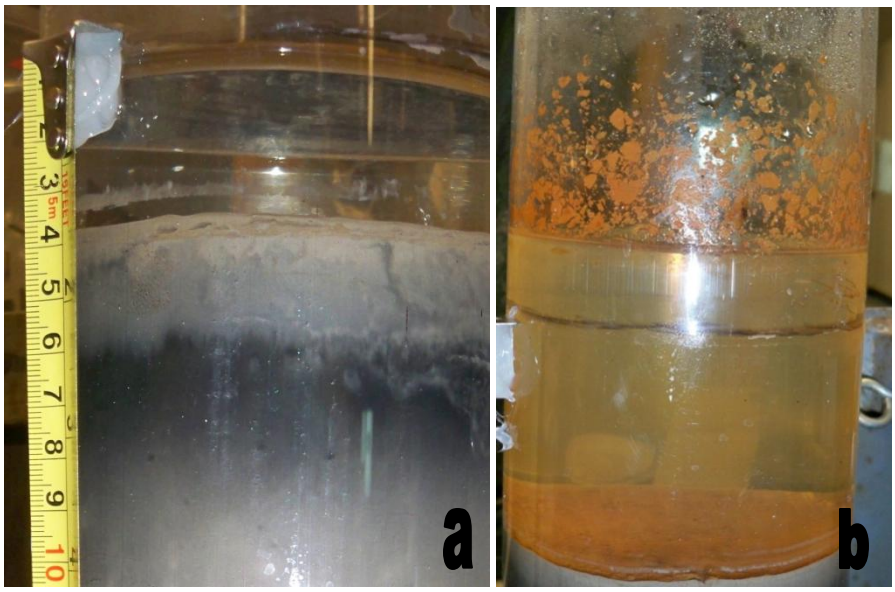

Figure 3.24. Band of black precipitate and orange precipitate in 7-L settling column MFT: a) black precipitate in the formation of a wide band around the circumference of the column of baseline control MFT; band is located several centimeters below solids-water interface; b) flaky orange precipitate observed in column of MFT amended with citrate and polyacrylamide; precipitate is observed on the surface of solids material as well as on the interior of the column wall above the water surface.

for example, $Fe(OH)_3$. Samples of the black precipitate and the orange precipitate were collected from a baseline control MFT column and a column of MFT amended with citrate and polyacrylamide, respectively. However, because only a minimal amount of the orange precipitate could be collected, DGGE and MPN analyses were not performed on it (Sections 3.4.6 and 3.4.7). All other samples were analyzed for percent solids, microbial diversity (DGGE analysis), and microbial counts of fermenters, SRB, FeRB, and methanogens (MPN analyses).

3.4.5 Percent solids analysis of settling column (mesocosm) MFT

The solids content of MFT samples taken from four different locations within each column were measured, by weight, to track densities of MFT with different amendments but also to track densities of MFT within the same column with increasing depth. The solids content of MFT samples when incubation began were not measured and

so changes in percent solids could not be compared over time. However, changes in percent solids as the result of amendments could be made in comparison to the baseline control. The percent solids measurements of settling column MFT samples are summarized in Figure 3.25.

All samples showed an increase in solids content with depth in the column. This is expected because MFT was left to incubate for 31 weeks and settling of solids material should have occured within this time, even without any treatment. Interestingly, the difference in percent solids with depth (between the top, position 1, and bottom, position 4) was greater in baseline control MFT than it was in the amended MFT, except for MFT treated with citrate. This trend was also observed in the percent solids measurements of megacosm MFT (Section 3.3.2). This is unexpected because treatments of citrate and diluent, in combination or alone, accelerated solids settling during the incubation of these columns (Figure 3.19), compared to the baseline control which showed little solids settling and water release. However, this may be because the percent solids of the

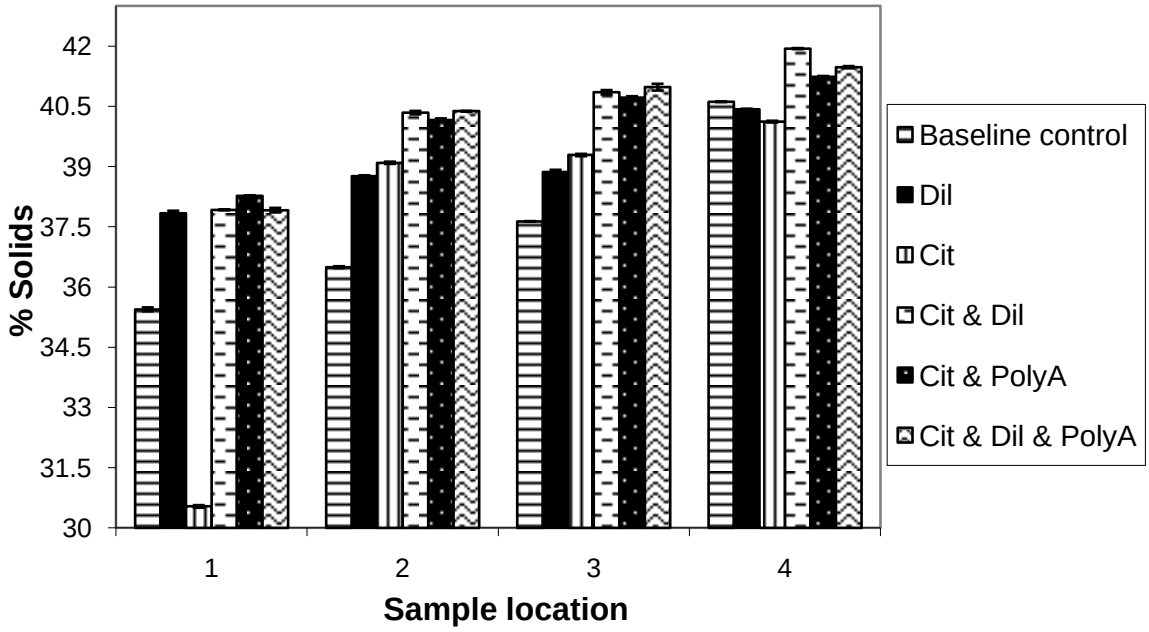

Figure 3.25. Percent solids of MFT samples from four locations of each of six settling columns after 31 weeks of incubation. Sample location are numbered in increasing depth, where 1= near the solids-water interface and 4= near the bottom of the column. Values represent the mean of triplicate subsamples ± 1 standard deviation.

amended MFT, except for citrate-amended MFT, at the most shallow depth tested (sample location 1) was already much higher than that of the baseline control. The effect of these treatments on Albian MFT was an increase of the percent solids throughout the column, at all locations sampled.

The percent solids measurement of MFT treated with citrate sampled from Location 1 was unusually low as compared to the percent solids measurements of MFT with other treatments or the baseline control at the same location. This sample was retested several times with the same result. The percent solids measurement of citrate-amended MFT at the next sampled location was much higher and was more consistent with the remaining measurements at Locations 3 and 4. A likely explanation for the low solids content at Location 1 of the citrate-amended MFT is that during sampling, a considerable amount of cap water was taken along with the MFT. It was likely not a representative sample of the MFT at this height of the column.

By measuring solids content, the role of polyacrylamide and diluent is made more obvious than by only measuring the change in water release and solids settling. MFT treated with citrate or citrate plus diluent had a lower solids content at every sampled location than MFT treated with citrate and polyacrylamide. This suggests that polyacrylamide was indeed acting as a flocculant and causing the aggregation of solids material; however, after aggregation, it appears that the solids failed to express water. Interestingly, MFT treated with diluent showed equal if not greater percent solids readings at each sample location than MFT treated with citrate. Solids settling data indicate diluent-amended MFT showed less solids settling than citrate-amended MFT (Figure 3.19). Diluent appears to affect the aggregation of solids particles in the MFT but cannot accelerate the settling of these aggregates to the same degree that citrate does. When citrate and diluent are combined as a treatment, the percent solids of such-treated MFT increases to be greater than MFT treated with either citrate or diluent alone. At all depths, MFT treated with citrate plus diluent had nearly, if not the same, solids content as MFT treated with citrate plus diluent plus polyacrylamide. This trend agrees with the water release and solids settling data (Figures 3.18 and 3.19).

Citrate likely affects settling through the production of gas from its biodegradation which suggests that gas production and carbon dioxide dissolution has a

greater effect on the solids settling in MFT than the aggregation of solids particles does. In other words, it appears that the effects of citrate allow fines to pack more tightly together and exclude water as opposed to flocculated fines that retain porewater. Another potentially influencing factor is that citrate was added to MFT as trisodium citrate. An increase in the concentration of sodium ions in MFT porewater may influence the interaction of clay particles. In their study of the effects of background water chemistry on sediment transport in turbidity currents, Packman and Jerolmack (2004) observed that the surface charge of kaolinite particles became less negative and settling velocities of kaolinite aggregates increased with increasing sodium ion concentrations. Kaolinite is one of the several minerals found in oil sands ore (Kaminsky et al. 2008). It is possible that sodium ions contributed to the observed increase in percent solids (Figure 3.25) and in solids settling (Figure 3.19) of sodium citrate-amended MFT. However, there remain many unknown factors that may affect the interactions between clay particles.

Ultimately, it appears that Albian MFT undergoes the greatest densification in terms of percent solids increase and solids settling with water release when more than one amendment is applied, and possibly when all three amendments are used. These three amendments appear to have a combined biological, physical, and potentially chemical effect on accelerating MFT densification. However, it remains unclear how these amendments interact with one another and the MFT.

3.4.6 Bacterial DGGE analysis of settling column (mesocosm) MFT

The Bacterial diversity in each of these columns was compared through DGGE analysis to see the potential effects of citrate, diluent, and/or polyacrylamide on the Bacterial species of Albian MFT. Figure 3.26 shows the gel image after staining with SYBR® Gold.

The DGGE profiles of Bacterial diversity in each sample were almost identical, including the baseline control MFT. A few bands varied in intensity between samples; however, as DGGE analysis is not quantitative, differences between samples can only be judged by the presence or absence of DNA bands between profiles. Based on what we

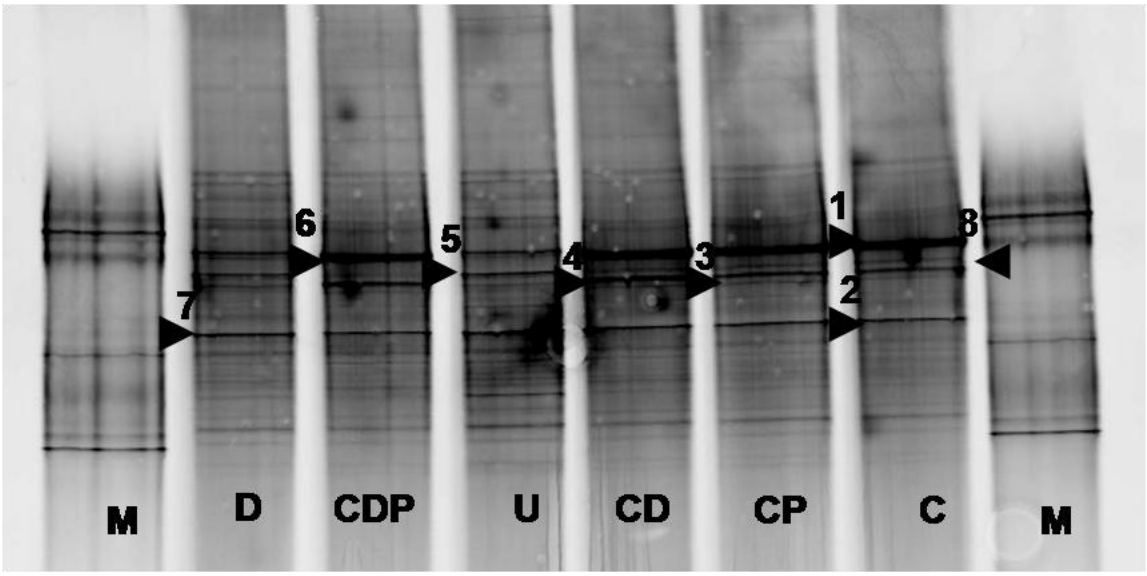

Figure 3.26. DGGE gel image of Bacterial species in settling column MFT amended with citrate, diluent, polyacrylamide or a combination of two or three of these, or with cap water (baseline control). Each gel lane is labeled with its respective settling column sample, where D= diluent, C= citrate, P= polyacrylamide, and U= baseline control. Marker lanes are indicated by 'M' and bands that were excised for sequencing are labeled with their corresponding number.

can observe in this gel, there are no major shifts in the Bacterial community in response to any of the tested amendments.

Gel bands were excised as labeled and sequenced. For certain DNA bands, two representative bands were excised for sequencing (Figure 3.26; Bands 2 and 7, 3 and 4, and 5 and 8 are pairs of excised bands theoretically representing three different DNA sequences). Some bands were particularly difficult to sequence possibly due to inhibitory substances from the process of excising and eluting DNA from the DGGE gel. In order to sequence these, the DNA eluted from each band was subcloned and sequenced from the plasmid. As a result, in most cases where pairs of bands representing the same DNA sequence were excised, only one of the two bands was sequenced. The results of comparing the obtained sequences with NCBI and RDP databases are summarized in Table 3.11.

Table 3.11. Identities of cloned and sequenced DGGE PCR products of Bacterial species in settling column Albian MFT amended with citrate (C), diluent (D), polyacrylamide (P), or a combination of two or three of these, or with cap water (baseline control). Typically ~500 nt was sequenced. The closest match (most closely related sequence) and closest named match (most closely related named taxon) in NCBI GenBank, determined by the BLASTn program and confirmed by the RDP database, are indicated.

Band # [a]	Sample	1. Closest related sequence in GenBank 2. Closest related named taxon in GenBank	Accession # in GenBank	% Sequence Similarity
1	C	1.Uncultured bacterium clone D12 from penicillin G production wastewater	EU234285.1	98
		2. *Acidaminobacter hydrogenoformans*	AF01669.1	97
2	C	1. Uncultured *Rhodoferax* sp. clone Ctrl1-7B from oil sands tailings	EU522643.1	98
		2. Same as above		
4 (and 3)	CD	1. Uncultured bacterium isolate DGGE gel band BLMA_25 from Antarctic sediment	GQ336963.1	100
		2. Uncultured *Rhodoferax* sp. clone RUGL6-339 from glacier sediment	GQ366604.1	99
6	CDP	1. Uncultured bacterium clone D12 from penicillin G production wastewater	EU234285.1	98
		2. *Acidaminobacter hydrogenoformans*	AF01669.1	97
7	D	1. Uncultured *Rhodoferax* sp. clone Ctrl1-7B from oil sands tailings	EU522643.1	98
		2. Same as above		
8 (and 5)	C	1. Uncultured bacterium clone D12 from penicillin G production wastewater	EU234285.1	99
		2. *Acidaminobacter hydrogenoformans*	AF016691.1	98

[a] band numbers in parentheses did not yield sequences but have similar mobility, therefore are presumed to represent the same sequence identity.

Once again DNA sequences matching to genus *Rhodoferax* were found. Other sequenced bands matched to *Acidaminobacter hydrogenoformans*. This species was the closest match to a named species of a sequenced clone in megacosm column MFT incubated with citrate after 10 months of incubation, with 98% similarity (Table 3.9). *Acidaminobacter hydrogenoformans* was first discovered in black mud as an obligate anaerobe capable of growing on glutamate to produce acetate, carbon dioxide, formate and hydrogen, as well as propionate (Stams and Hansen 1984). When grown in the presence of SRB, such as *Desulfovibrio*, or methanogens, such as *Methanospirillum*, fermentation of glutamate occurred more quickly. *Acidaminobacter hydrogenoformans* was also found to be capable of growth on citrate. It is not unusual to detect this species in Albian MFT; however, this species was not among the dominant sequenced bands of

DGGE analysis of megacosm column MFT at Time zero and Time 5 months (Tables 3.6 and 3.8). If time had permitted a more thorough analysis of Time zero and 5 month MFT samples from the megacosm columns by clone library and RFLP analysis, it would be clearer whether *A. hydrogenoformans* increased in proportion over time to be within detection limits or if technical biases are at fault.

Bands 1 and 6, and 8 (and, by inference, 5) matched most closely to *A. hydrogenoformans* which suggests they are a dominant group, possibly playing an important role in the activity of MFT. However, as noted before, DGGE analysis does not provide a complete analysis of the microbial diversity of a sample, due to limitations in the technique and analysis. Many of the bands observed in DGGE gels could not be excised because they were not visible with ethidium bromide staining. Constructing clone libraries for the baseline control and amended setting column MFT would permit more thorough analysis of the dominance of certain Bacterial species. DGGE analysis has, however, offered an initial screen that suggests addition of diluent, citrate, and polyacrylamide do not cause major changes in the Bacterial diversity of incubated Albian MFT, likely because these amendments are also present in the Albian tailings ponds.

The species detected by DGGE from these incubated settling columns were among those detected from Albian MFT in the initial clone library and RFLP analysis, as well as those found in the megacosm MFT. The sequences of the bands in Table 3.11 were aligned with the sequences of clones and DGGE bands from the megacosm MFT in a phylogenetic analysis (Figure 3.27). All these sequences were aligned with a range of described Bacterial species.

The sequenced bands of the DGGE analysis of settling column samples matched most closely to the genera *Rhodoferax* and *Acidaminobacter* and this is reflected in the phylogenetic tree (Figure 3.27). The sequenced bands from the settling column samples are also very highly related to the sequenced bands from analysis of 5 month megacosm samples and the sequenced clones of the 10 month megacosm DGGE analysis, particularly for the *Rhodoferax* species. This suggests that the sequences obtained from the settling column MFT samples and the megacosm MFT samples are the same sequence, and verifies the finding of both studies.

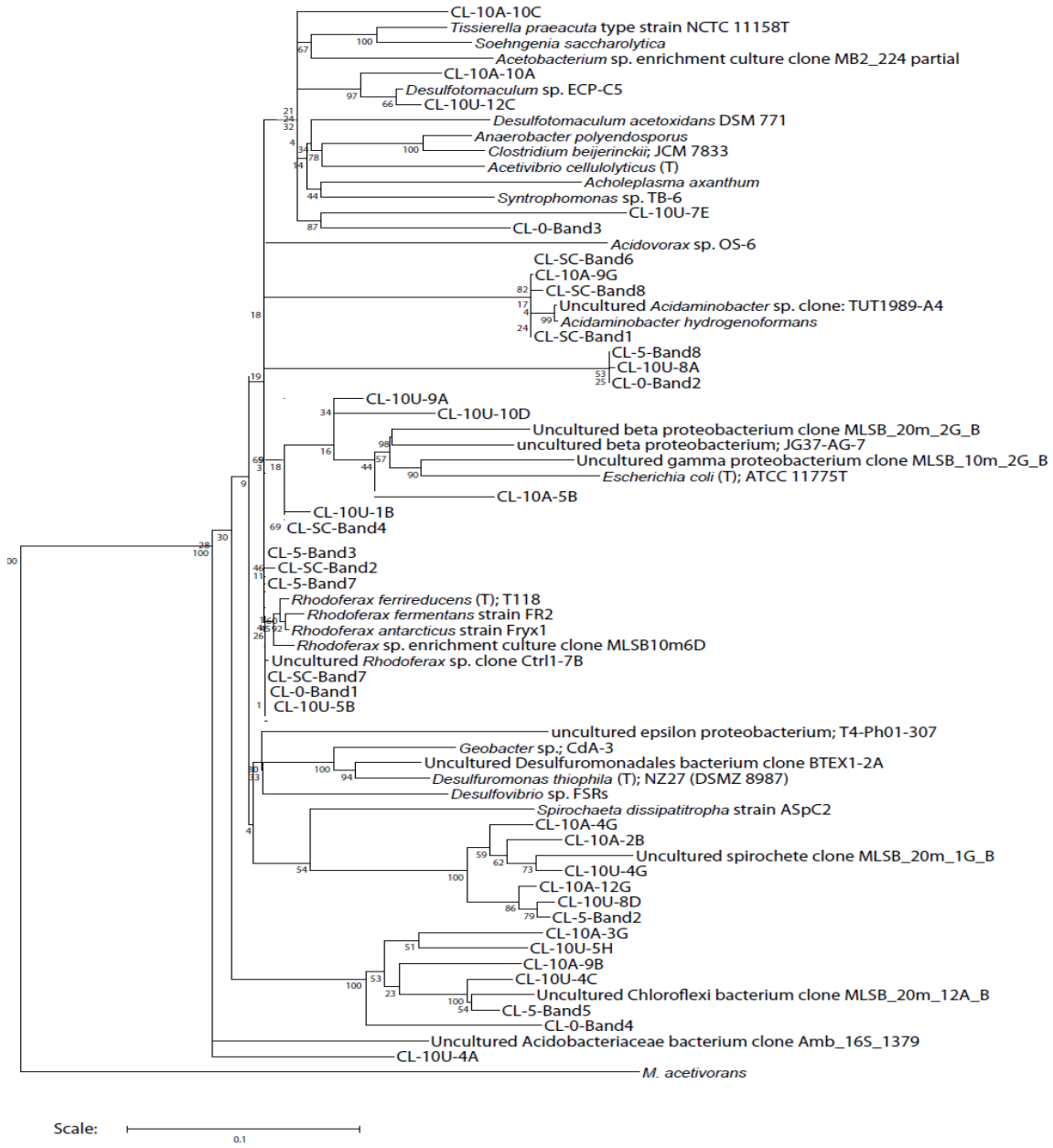

Figure 3.27. Phylogenetic tree of 16S rRNA gene sequences from DGGE gel bands amplified from settling column samples and Time zero and Time 5 month megacosm samples and sequenced clones of DGGE PCR products obtained from Time 10 month samples of baseline and citrate-amended Albian MFT and named species from NCBI GenBank. Sequences from settling column MFT are labeled with the prefix "CL-SC-". Sequences of Time zero, Time 5 month, and Time 10 month samples from megacosms are labeled with the prefix "CL-0-", "CL-5-", and "CL-10-", respectively, followed by a "U" or "A" to indicate baseline control or citrate-amended, respectively. Sequences in GenBank deposited from previous studies with Syncrude MFT have the prefix "MLSB_". Length of lines are proportional to the relatedness between species and the scale bar represents 10% difference in DNA sequence.

Overall, DGGE analysis of settling column MFT did not reveal any different species than DGGE analysis of the megacosm MFT. This is not surprising based on the lack of changes observed over time in megacosm MFT (Section 3.4.4) and also because the DGGE PCR products of the 10 month megacosm MFT samples were cloned and screened, which has shown to be a much more thorough examination of the microbial diversity of a sample of Albian MFT than DGGE analysis.

3.4.7 MPN analyses of settling column (mesocosm) MFT

The different treatments applied to the settling column MFT resulted in different amounts of gas release, solids setting, and water release. When DGGE analysis of the Bacterial diversity of these MFT did not show any substantial differences in the Bacterial species present, fermenters, SRB, FeRB, and methanogens were enumerated to see if the changes observed in the MFT after incubation with each amendment correlated to the numbers of each metabolic type of microbe. Samples from each deconstructed column were incubated in MPN media for four months with regular scoring. The black band of MFT that was observed in all settling columns was sampled from the baseline control column and included in the MPN analyses. The results of these analyses have been summarized in Table 3.12 and have been plotted as a log graph for easier comparison (Figure 3.28).

Fermenters were significantly higher in numbers in MFT amended with citrate, whether with diluent, with polyacrylamide, or with both ($p<0.05$) as compared to the baseline control MFT. Within the group of citrate-amended MFT samples, fermenters were significantly greater in MFT amended with only citrate than in MFT amended with citrate plus diluent ($p<0.05$). There were no significant differences in the number of fermenters between MFT amended with only citrate and MFT amended with either citrate plus polyacrylamide or citrate plus polyacrylamide plus diluent. These data suggest that there is potentially an effect of diluent on reducing the growth of fermenters and that this inhibition is relieved by polyacrylamide. However, in microcosm experiments (Section 3.1; Dr. T. Siddique), adding citrate in combination with diluent did not decrease the amount of methane produced as compared to Albian MFT treated with citrate alone

Table 3.12. Summary of MPN analyses for fermenters, SRB, FeRB and methanogens in Albian MFT samples from deconstructed settling columns, scored after 8, 12, and 16 weeks of incubation. All readings had stabilized by Week 16.

MFT sample	Metabolic Type	MPN/mL by Week 8	MPN/mL by Week 12	MPN/mL by Week 16[a]
Black Band from Baseline control	Fermenters	1.5×10^5	1.5×10^5	1.5×10^5 ($3 \times 10^4 – 4.4 \times 10^5$)
	Sulfate-reducers	7.5×10^4	7.5×10^4	7.5×10^4 ($1.4 \times 10^4 – 2.3 \times 10^5$)
	Iron-reducers	3.9×10^2	7.5×10^2	9.3×10^3 ($1.5 \times 10^3 – 3.8 \times 10^4$)
	Methanogens	2.3×10^3	2.3×10^3	4.3×10^3 ($7 \times 10^2 – 2.1 \times 10^4$)
Baseline control	Fermenters	9.3×10^4	9.3×10^4	9.3×10^4 ($1.5 \times 10^4 – 3.8 \times 10^5$)
	Sulfate-reducers	4.3×10^4	4.3×10^4	4.3×10^4 ($7 \times 10^3 – 2.1 \times 10^5$)
	Iron-reducers	7.5×10^1	7.5×10^1	2.1×10^3 ($3.5 \times 10^2 – 4.7 \times 10^3$)
	Methanogens	4.3×10^3	9.3×10^3	2.3×10^4 ($4 \times 10^3 – 1.2 \times 10^5$)
Diluent	Fermenters	7.5×10^4	7.5×10^4	7.5×10^4 ($1.4 \times 10^4 – 2.3 \times 10^5$)
	Sulfate-reducers	7.5×10^4	7.5×10^4	7.5×10^4 ($1.4 \times 10^4 – 2.3 \times 10^5$)
	Iron-reducers	9.3×10^1	9.3×10^1	2.3×10^3 ($4 \times 10^2 – 1.2 \times 10^4$)
	Methanogens	9.3×10^4	2.3×10^4	4.3×10^4 ($7 \times 10^3 – 2.1 \times 10^5$)
Citrate	Fermenters	2.3×10^7	2.3×10^7	2.3×10^7 ($4 \times 10^6 – 1.2 \times 10^8$)
	Sulfate-reducers	9.3×10^4	9.3×10^4	9.3×10^4 ($1.5 \times 10^4 – 3.8 \times 10^5$)
	Iron-reducers	9.3×10^2	2.3×10^3	2.3×10^3 ($4 \times 10^2 – 1.2 \times 10^4$)
	Methanogens	1.5×10^4	1.5×10^4	9.3×10^4 ($1.5 \times 10^4 – 3.8 \times 10^5$)
Citrate + Diluent	Fermenters	1.5×10^6	1.5×10^6	1.5×10^6 ($3 \times 10^5 – 4.4 \times 10^6$)
	Sulfate-reducers	7.5×10^4	7.5×10^4	7.5×10^4 ($1.4 \times 10^4 – 2.3 \times 10^5$)
	Iron-reducers	4.6×10^2	4.6×10^2	2.3×10^3 ($4 \times 10^2 – 1.2 \times 10^4$)
	Methanogens	7.5×10^3	1.5×10^4	1.5×10^4 ($3 \times 10^3 – 4.4 \times 10^4$)

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Table 3.12. continued

MFT sample	Metabolic Type	MPN/mL by Week 8	MPN/mL by Week 12	MPN/mL by Week 16[a]
Citrate + Polyacrylamide	Fermenters	3.9×10^6	3.9×10^6	3.9×10^6 ($7 \times 10^5 – 1.3 \times 10^7$)
	Sulfate-reducers	2.1×10^5	2.1×10^5	2.1×10^5 ($3.5 \times 10^4 – 4.7 \times 10^5$)
	Iron-reducers	2.4×10^2	2.4×10^2	3.9×10^2 ($7 \times 10^1 – 1.3 \times 10^3$)
	Methanogens	9.3×10^4	9.3×10^4	9.3×10^4 ($1.5 \times 10^4 – 3.8 \times 10^5$)
Citrate + Diluent + Polyacrylamide	Fermenters	9.3×10^6	9.3×10^6	9.3×10^6 ($1.5 \times 10^5 – 3.8 \times 10^7$)
	Sulfate-reducers	2.3×10^4	2.3×10^4	2.3×10^4 ($4 \times 10^3 – 1.2 \times 10^5$)
	Iron-reducers	2.4×10^2	4.3×10^2	9.3×10^2 ($1.5 \times 10^2 – 3.8 \times 10^3$)
	Methanogens	9.3×10^3	4.3×10^4	9.3×10^4 ($1.5 \times 10^4 – 3.8 \times 10^5$)

[a] 95% confidence interval in parentheses.

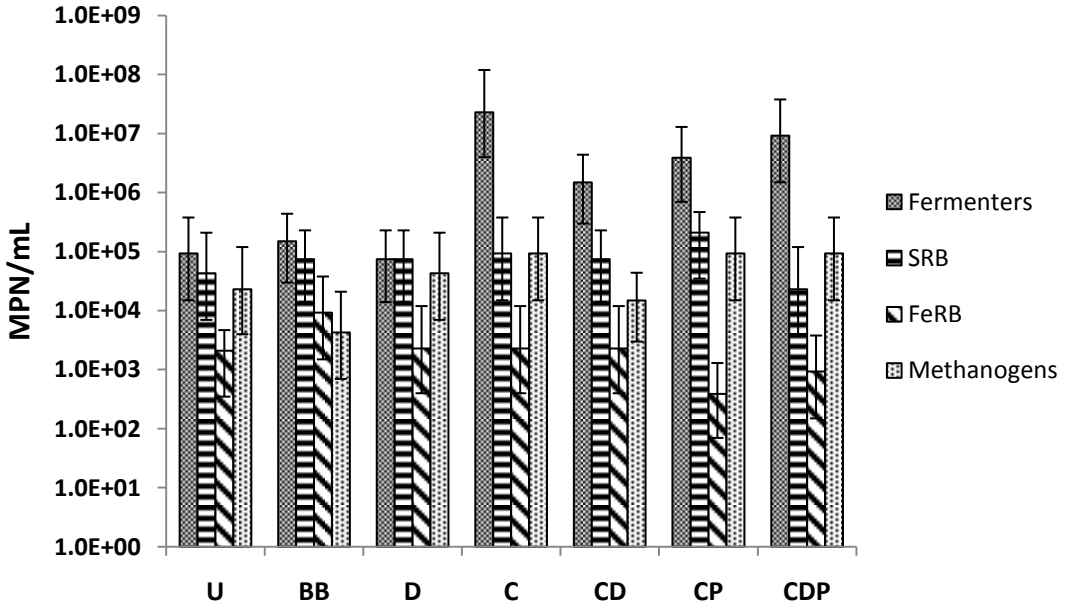

Figure 3.28. Summary of MPN analyses for fermenters, SRB, FeRB and methanogens in settling column MFT, scored after 16 weeks of incubation. U= baseline control; BB= black band; D= diluent; C=citrate; CD= citrate plus diluent; CP= citrate plus polyacrylamide; CDP= citrate plus diluent plus polyacrylamide. Error bars indicate 95% confidence intervals.

(Figure 3.2). Also, MFT amended with only diluent did not show significant differences in fermenters compared to the baseline control MFT (Table 3.12).

Because microcosm experiments (Section 3.1; Dr. T. Siddique) demonstrated that Albian diluent does not serve as a methanogenic substrate whereas citrate does, the trend of fermenter numbers is not surprising. Fermentative bacteria participate in the initial degradation of larger organic molecules and if diluent is not a suitable substrate, then no growth or increase in numbers of fermenters as compared to the baseline control would be expected. The number of fermenters may actually decrease instead because of a lack of useable substrate, which was observed in the megacosm MFT (Table 3.11). The significant increase in numbers in response to citrate amendment suggests that fermenters in Albian MFT use citrate as a substrate.

The number of methanogens did not differ significantly between the different amended MFT samples or the baseline control, suggesting that citrate, diluent, and polyacrylamide do not stimulate or inhibit their growth. FeRB followed a similar trend. FeRB were enumerated by MPN analyses for these settling columns with the particular interest of isolating *Rhodoferax* species; particularly *Rhodoferax ferrireducens*, which matched numerous sequences in clone library analysis of M1 and M2 MFT (Table 3.1) and in DGGE analyses (megacosm and settling column samples; Tables 3.6, 3.8, 3.9, and 3.11). In addition, the development of orange precipitate in several of the settling column MFT suggested the activity of FeRB. FeRB were relatively low in quantity and proportion in all settling column MFT samples. FeRB numbers did not differ significantly between MFT samples. These results suggest that citrate, diluent, and polyacrylamide amendments have little effect on FeRB quantities in Albian MFT. However, growth of FeRB in MPN culture tubes was also very slow. Perhaps with longer incubation periods, a greater difference would be observed. An effort was made to identify the microbes growing in FeRB MPN tubes through DNA extraction and DGGE analysis. However, all attempts to amplify DNA were met with difficulty, likely due to inhibitory substances in the FeRB MPN medium that were not fully removed during DNA extraction.

The numbers of SRB also did not differ significantly between each amended MFT sample or the baseline control with the exception of a significantly greater number of

SRB in MFT amended with citrate plus polyacrylamide compared to MFT amended with citrate plus polyacrylamide plus diluent ($p<0.05$). Though this suggests that diluent may inhibit the growth of SRB, there are no significant differences in SRB numbers between MFT amended with citrate and MFT amended with citrate plus diluent and between baseline control MFT and MFT amended with diluent. This difference is likely due to heterogeneity of the MFT and not caused by the difference in amendments.

Gas release measurements from settling column MFT were significantly different from one another between treatments (Figure 3.20) as were volumes of produced gas (Figure 3.23), even though the numbers of SRB, FeRB and methanogens do not significantly differ. It is possible that other unknown factors including substrate and nutrient availability are limiting the growth of the anaerobic respirers. Instead of increasing the abundance of microbes, these amendments appear to stimulate the activity of the microbes in Albian MFT.

An interest was taken in the development of a dark grey-to-black band of MFT around the circumference of each column, including the baseline control. Some columns had a darker band than others but after 31 weeks of incubation, this black band had faded to a faint grey in the MFT of all columns except the baseline control columns. Black precipitate was thought to be caused by the activity of SRB producing sulfide and so MPN analyses were done on MFT collected from the baseline control column in the area of the black precipitate. Black precipitate was also scraped from the sides of the column. There were no significant differences in fermenters or SRB in this black precipitate compared to the different amended MFT samples and the baseline control MFT. There were significantly less methanogens in this black precipitate compared to MFT amended with diluent, citrate, citrate plus polyacrylamide or citrate plus diluent plus polyacrylamide ($p<0.05$). There were also significantly more FeRB in the black precipitate compared to MFT amended with citrate plus polyacrylamide or citrate plus diluent plus polyacrylamide. However, it must be kept in mind that this black precipitate was collected from the baseline control column and the differences in methanogens and FeRB between the baseline control and the black precipitate were not significant. Therefore, the differences in methanogens and FeRB between the black precipitate and the amended MFT samples cannot be attributed to the amendments. The black precipitate

was collected close to the water-solids interface. It is possible that the location from which the MFT samples were taken has an effect, where FeRB decrease and methanogens increase in quantity with depth. However, this cannot be confirmed with the data presented here because each amended MFT sample, as well as the baseline control MFT sample, was tested as a single composite sample of subsamples taken from four different locations in each column (Sections 2.6.4 and 2.6.7). It is still unclear at this time what the black precipitate is. It may be a cause of microbial activity but it may also be a non-biological effect, such as a chemical reaction between a component in the MFT material and the acrylic column containing it. However, this has not been observed in previous experiments with Syncrude MFT incubated in similar acrylic columns (Eleisha Underwood, Civil and Environmental Engineering, University of Alberta, personal communication).

3.4.8 Summary of microbial analyses on settling column (mesocosm) MFT

There were no observed differences in the Bacterial diversity between the baseline control MFT and any of the amended MFT. Citrate and diluent amendments, alone or in combination with the other and/or polyacrylamide did not cause a detectable microbial shift in the community of Albian MFT. DGGE analysis found sequences matching to *Rhodoferax*-like species and also *Acidaminobacter* which was observed in low quantities in citrate-amended megacosm MFT after 10 months of incubation (Table 3.9). These findings are in agreement with microcosm experiments that suggest citrate, but not diluent, is a methanogenic substrate in Albian MFT (Section 3.1). If the microbial community used diluent as a methanogenic substrate, the DGGE profiles of diluent-amended MFT and citrate-amended MFT would be expected to be different.

MPN enumerations of fermenters, SRB, FeRB, and methanogens in settling column MFT samples showed that overall, the numbers of fermenters increased in response to citrate-amendment, SRB and methanogens numbers did not respond to any amendment, and FeRB were in relatively low quantities and were not affected by any of the amendments. It is possible that 200 ppm citrate is not a high enough concentration to cause an increase in microbial quantities in Albian MFT since SRB, FeRB, and

methanogen counts did not increase in response to citrate amendment. However, it is also possible that unknown factors in the MFT are restricting the growth of these microbial groups. These observations again support that citrate is the methanogenic substrate in Alian MFT because there was no major change in the microbial community of Albian MFT, either in quantity, with the exception of fermenters in citrate-amended MFT, or in diversity as a result of citrate, diluent, and/or poylacrylamide-amendments. The lack of change in abundance of these microbial groups in citrate-amended Albian MFT suggest that citrate stimulates their activity to cause significant differences in water release, solids settling, and methane release from citrate-amended MFT. The microbial analyses of megacosm MFT and settling column (mesocosm) MFT suggest that citrate is the methanogenic substrate of Albian MFT and is supporting a microbial community dominated by *Rhodoferax* species and acetoclastic methanogens.

4 CONCLUSIONS

4.1 ANAEROBIC MICROBIAL DEGRADATION OF CITRATE IN ALBIAN MFT

Microcosm studies with Albian MFT performed by Dr. Tariq Siddique have shown that citrate is the methanogenic substrate in Albian MFT. The microbial community in Albian MFT has been shown by the work in this thesis to consist predominantly of *Rhodoferax ferrireducens*-like species and acetoclastic methanogens, such as *Methanosaeta* species, with a few Clostridia and SRB as well. It is unclear what the role of *R. ferrireducens*-like species is regarding citrate metabolism; the anaerobic growth of this species on citrate has not been tested. The other two described *Rhodoferax* species, *R. fermentans* and *R. antarcticus*, have been reported to grow phototrophically on acetate and citrate (Hiraishi et al. 1991; Madigan et al. 2000). *R. fermentans* can ferment sugars and pyruvate, but has not been tested for fermentation of citrate or acetate. *R. ferrireducens* is the only species of this genus that does not grow phototrophically, lacks photosynthetic pigments, and has been reported to reduce Fe(III) by oxidizing acetate (Finneran et al. 2003). Because of these differences, the reclassification of *R. ferrireducens* to a new genus as *Albidoferax ferrireducens* has been proposed (Ramana and Sasikala 2009).

Because *in situ* phototrophic growth in MFT is unfeasible, *Rhodoferax* species in Albian MFT could potentially be acting as fermenters, like *R. fermentans*, or as iron-reducers, like *R. ferrireducens*. Based on the microorganisms putatively identified here and on the observations of Gámez et al. (2009) who reported mainly the production of acetate through fermentation of citrate by a methanogenic consortium, the following pathway of anaerobic citrate degradation in Albian MFT is proposed (Figure 4.1). Citrate is first fermented to produce acetate as well as hydrogen and carbon dioxide. Bacterial species including fermenters and sulfate- and iron-reducers from genera *Rhodoferax, Clostridium,* and *Desulfuromonas* may be involved. Acetate is then used for the production of methane plus carbon dioxide by acetoclastic methanogens from the genera *Methanosaeta* and *Methanosarcina*.

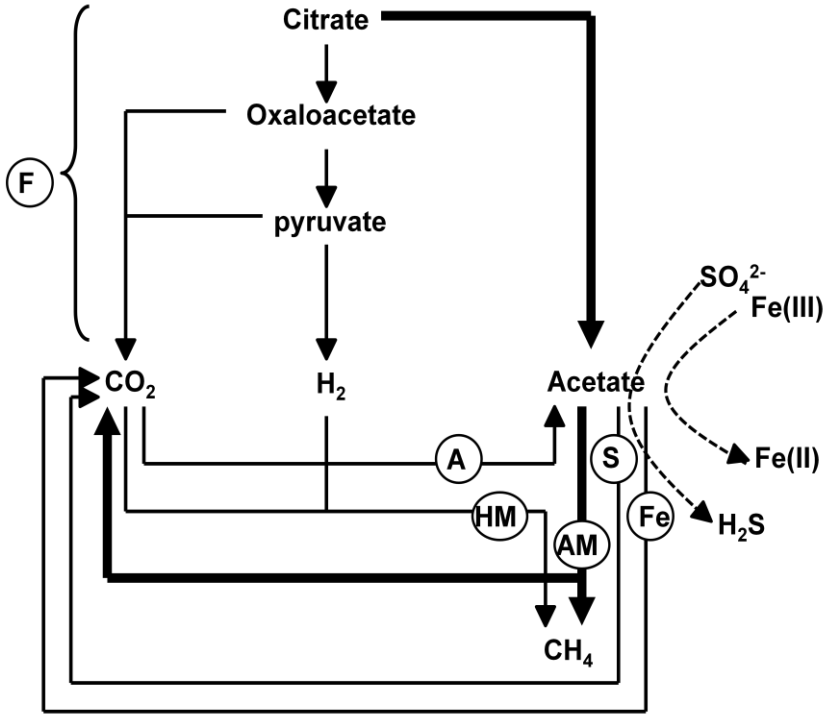

Figure 4.1. Proposed pathway of anaerobic citrate biodegradation to methane in Albian MFT. F= citrate fermentation; A= acetogenesis using hydrogen; AM= acetoclastic methanogenesis; HM= hydrogenotrophic methanogenesis; S= sulfate reduction; Fe= ferric iron reduction. The proposed dominant pathway of citrate degradation is indicated by thick lines; minor pathways are indicated by regular lines; potential side pathways are included in dotted lines. Adapted from Gámez et al. (2009).

Although acetoclastic methanogenesis is likely the dominant pathway of methane production from acetate, hydrogenotrophic methanogenesis is also a possible pathway. Few hydrogenotrophic methanogens were detected among the Archaeal species of Albian MFT. Hydrogen and carbon dioxide are produced during the fermentation of citrate to acetate and carbon dioxide is generated during the conversion of acetate to methane by acetoclastic methanogenesis. Hydrogenotrophic methanogens, however, likely have to compete with acetogenic bacteria for these substrates. Some *Clostridium* species, which are potential acetogens, were detected in Albian MFT. The group of microbes that is competitively advantageous is influenced by environmental factors such as temperature, hydrogen availability, and pH (Section 1.2.1.4). During the anaerobic degradation of citrate, Gámez et al. (2009) observed increased production of acetate at high

concentrations of carbon dioxide due to the activity of acetogens. Acetogenic production of acetate could potentially occur in Albian MFT as well.

Sulfate reduction has been included in Figure 4.1 to demonstrate that a small amount of sulfate reduction to sulfide may be occurring in Albian MFT. Sulfate concentrations in Albian MFT were observed to rapidly decline within 1 month of incubation with or without citrate (Figure 3.12) and a black precipitate was observed in Albian MFT settling columns, amended with cap water (baseline control) or amended with citrate (Figure 3.24). Acetate has been observed to stimulate sulfate reduction by a sulfate-reducing microbial consortium to produce sulfide (Gámez et al. 2009; Stams et al. 2009). In baseline control Albian MFT, it is possible that sulfate is being reduced using acetate from endogenous citrate. SRB typically outcompete methanogens for the same substrates; the endogenous acetate would be used towards sulfate-reduction and sulfide production rather than methane production. In citrate-amended MFT, acetate concentrations may be high enough that sulfate-reduction and methanogenesis both occur.

Iron-reduction has also been included as another potential pathway occurring in Albian MFT due to the observed predominance of *R. ferrireducens*-like species in the MFT (Figure 4.1) and also because of the development of an orange precipitate (presumptive iron oxides at the surface of settling columns) during incubation of Albian MFT with citrate (Figure 3.24). Acetate is a potential electron donor for the reduction of Fe(III) to Fe(II). The presence of Fe(III) has been observed to inhibit methanogenesis by enrichment cultures of the microbial community found in freshwater sediment by decreasing substrate availability (Lovley and Phillips 1986). The addition of more methanogenic substrate such as acetate relieved this inhibition. It is therefore possible that the elevated concentrations of acetate from citrate in citrate-amended MFT stimulated iron-reduction to occur. The source of ferric iron in Albian MFT is likely the clays. Kaminsky et al. (2008) observed iron sulfides, iron carbonates, and iron-titanium oxides in their scanning electron microscopy analysis of coarse heavy minerals in froth tailings. The form, concentration, and availability of ferric iron will affect its influence on methanogenesis by the microbial community in Albian MFT.

4.2 MICROBIAL SPECIES OF ALBIAN MFT AND MLSB MFT

The microbial species detected in Albian MFT in this study suggest that Albian MFT contains some Bacterial and Archaeal species similar to those detected in Syncrude MLSB MFT (Penner 2006). Both MFT contained Bacterial 16S rRNA gene sequences matching closely to genera *Rhodoferax, Acidovorax, Desulfotomaculum* and to species of the class Clostridia. Archaeal DNA from both MFT were largely dominated by *Methanosaeta*-like sequences, but also contained *Methanothermobacter* and *Methanosarcina* (Penner 2006). Syncrude MLSB MFT also contained Bacteria that matched closely to the genera *Thiobacillus, Thauera*, *Azoarcus*, *Pseudomonas*, and *Syntrophus* and Albian MFT did not. Other Archaeal species found in Syncrude MLSB MFT that matched to genera such as *Methanocalculus, Methanocorpusculum*, and *Methanoculleus* were not found in Albian MFT. Albian MFT has a less diverse microbial population than Syncrude MLSB MFT does. It should be kept in mind, however, that the survey of microbial species in Syncrude MLSB MFT was much more exhaustive (Penner 2006) than the work done here, which could in part explain the higher diversity observed in Syncrude MLSB MFT.

One of the main reasons why Albian MFT has a less diverse microbial community than Syncrude MLSB MFT may be because the Albian tailings pond is much younger. Whereas Syncrude MLSB has been operating for a few decades (~30 years), the Albian tailings pond began several years ago (~7 years). The microbial community in Albian MFT may not yet be fully established. Perhaps if given enough time, a more diverse population will be observed in Albian MFT and potentially, Albian diluent could serve as a methanogenic substrate. Microcosm experiments with Syncrude MLSB MFT incubated with Albian diluent showed small amounts of methane production after a lag period of over a year (Siddique, T.; personal communication), suggesting that an anaerobic microbial community well-acclimated to hydrocarbon substrates may be capable of using Albian diluent for methanogenesis.

Another explanation for the less diverse microbial community in Albian MFT is the availability of citrate as a methanogenic substrate. The anaerobic biodegradation of hydrocarbon substrates requires the concerted metabolic actions of several different

Bacterial and Archaeal groups for the activation and successive degradation of these relatively stable substrates and their intermediates into methane (Figure 1.1). In contrast, citrate is relatively labile. Fermentative bacteria are widespread and citrate is an amenable substrate. Acetoclastic methanogens use the acetate produced from the fermentation of citrate to produce methane. In this two-step process, less cooperation is required between the microorganisms than is required for the multi-step degradation pathway of hydrocarbons. Furthermore, the constant supply of citrate in Albian MFT removes the selective pressure on the microbial community to diversify. In the absence of readily available, easily degradable substrates such as citrate, it is possible that the microbial community of Albian MFT would shift over an extended period of time to be dominated by microbes capable of degrading hydrocarbon substrates, such as those present in Syncrude MFT.

In summary, Albian MFT has been shown to be quite different than Syncrude MLSB MFT. A different microbial community was found in Albian MFT which used a different methanogenic substrate than the diluent hydrocarbon compounds used by the microbial consortium in Syncrude MLSB MFT. It is now apparent that one tailings pond cannot serve as a representative for all tailings ponds and that each should be considered separately when it comes to strategies for tailings management and reclamation.

4.3 ACCELERATED TAILINGS DENSIFICATION BY MICROBIAL GAS PRODUCTION

One common factor between Albian MFT and Syncrude MLSB MFT that this thesis has confirmed is that anaerobic biological gas production accelerates densification and dewatering of MFT. Enhanced microbial production of methane stimulates MFT to settle and release water, suggesting a potential method for tailings management. However, the mechanism(s) of how gas production facilitates tailings densification remains unknown. In this thesis, some interesting observations were made that may provide insight into the mechanism(s). Gas production is not the only factor affecting the rate of tailings densification. There appeared to be non-biological effects of diluent-, and polyacrylamide-amendment to Albian MFT. Though neither diluent nor polyacrylamide

contributed to methane production from Albian MFT, gas bubbles in citrate-amended MFT varied in size and number depending on the presence or absence of diluent and/or polyacrylamide. Diluent alone caused a small amount of densification in Albian MFT and polyacrylamide did not affect the settling of the MFT solids but did increase the percent solids of the MFT. This suggests that diluent and polyacrylamide can affect the formation of gas bubbles and potentially play a role in whether the gas bubbles aggregate and release through the surface of the tailings. It appears that the other amendments in a tailings pond could potentially enhance the release of gas. The data collected in this thesis generally suggest that diluent and polyacrylamide in combination with citrate caused the most methane release from Albian MFT, compared to MFT amended with citrate alone or in combination with either diluent or polyacrylamide. Conversely, it is also possible that other amendments in a tailings pond could prevent gas release. This is a critical point to note. Although stimulating microbial gas production ultimately causes accelerated tailings densification, if the gas remains trapped in the MFT, the result will instead be an increase in the overall volume of the MFT.

Obviously, we need to elucidate the mechanism(s) of densification of tailings affected by microbial gas, while keeping in mind the effects of other factors *in situ*. Studying the mechanism(s) involved in tailings densification must encompass the biological, physical, and chemical effects of tailings amendments. Though both the Albian tailings pond and Syncrude MLSB show accelerated tailings densification from microbial gas production, the *in situ* environment of each tailings pond is likely different due to their different extraction methods, different diluents, and use of other tailings treatments, like citrate and polyacrylamide. This is more reason to be cautious in making generalizations on MFT behavior and to consider each tailings pond individually for management and reclamation.

5 FUTURE WORK

Since this project was the first microbiological analysis of Albian MFT, there remain unanswered questions regarding methane and densification of these tailings. Some of the questions addressed in this project could be further explored with different analytical techniques and some new questions need to be addressed as well. By doing so, methane production from tailings ponds can potentially be manipulated; in particular, stimulating gas production in MFT to accelerate densification, leading to options for improved tailings management, quicker recovery of water for reuse and earlier reclamation of tailings ponds.

Though the work done throughout this thesis project strongly suggests that citrate is the primary methanogenic substrate in Albian MFT, direct evidence of citrate degradation and conversion into methane was not obtained. Citrate concentrations in food products, such as juice (Igual et al. 2010) and wine (Kelebek et al. 2009), are often measured using high performance liquid chromatography (HPLC). HPLC analysis of citrate concentrations in Albian MFT pore water, based on the method outlined by Zhanguo and Jiuru (2002), was developed and performed by Jordan Hulecki (University of Alberta, personal communication, 2009). Unfortunately, this technique was unavailable during the megacosm (Section 3.3) and settling column (Section 3.4) experiments done in this thesis. As a result, megacosm and settling column MFT samples that had been archived at 4°C or -20°C for at least 6 months were analyzed and citrate was not detected in any of these samples. HPLC analysis of citrate-amended Albian MFT samples taken during incubation could correlate methane production to a decrease in citrate concentration over time to confirm it as a methanogenic substrate and allow calculation of stoichiometry. Along with citrate, acetate concentrations in incubating MFT samples could be analyzed to determine whether acetate is a key intermediate in the degradation pathway of citrate. Another method of measuring the degradation of citrate in Albian MFT is gas chromatography with mass spectrometry (GC-MS [Gray et al. 2005]). Albian MFT would be amended with ^{13}C- citrate and ^{12}C-diluent hydrocarbons and after incubation, the produced methane would be analyzed by GC-MS. Methane produced from the amended citrate would be ^{13}C-methane.

Though some of the dominant microbial species of Albian MFT were identified in this study, these data only suggest the participation of these species in methane production from citrate. The presence of a species does not signify its active contribution to a microbial community. Stable isotope probing (SIP) could confirm that the identified microbial species of Albian MFT are actively using citrate to produce methane. SIP is used to identify species that are performing metabolic processes in their environments (Dumont and Murrell 2005). This technique has been used to identify specific substrate-using microbial species in anaerobic environments (Schwarz et al. 2007; Lueders et al. 2004) and to determine substrate specificity of mixtures of uncultivated microbes (Singleton et al. 2007). SIP analysis of Albian MFT would involve the incubation of Albian MFT with ^{13}C-labeled citrate. The stable isotope is incorporated into cellular components, including the DNA, of microbial species actively using citrate and its subsequent byproducts for growth. Total genomic DNA is then extracted from the sample and subjected to density-gradient centrifugation for the separation of the "heavy" and the "light" DNA. The "heavy" DNA can be used for further analysis to identify which microbial species of the community actively used citrate early in the process.

With time and cost in mind, not every clone of the constructed libraries was sequenced; digest patterns that occurred only once in each library were not sequenced. The likely dominant species were putatively identified but the entire microbial community was not revealed. Furthermore, according to Good's coverage (Good 1953) the Bacterial clone library of Albian MFT was inadequate (Section 3.3.2.3), suggesting that substantially more sequences need to be cloned from 16S rRNA gene amplicons to uncover the true microbial diversity of Albian MFT. An alternate approach that would save on time and cost is to use high-throughput sequencing techniques, such as pyrosequencing. Pyrosequencing is a relatively new analytical technique with a wide range of applications and is becoming a more common technique for studying microbial diversity in environmental samples. Some examples of the work done in the past year using this method include: the correlation of bacterial communities to pH in a variety of different soils (Lauber et al. 2009), the characterization of the microbial community in a biogas plant (Kröber et al. 2009), and determining the bacterial community in alkaline

hot springs (Miller et al. 2009). Pyrosequencing has also been applied to study Archaeal diversity (Galand et al. 2009).

Pyrosequencing of the microbial community of Albian MFT would involve the extraction of total genomic DNA from an MFT sample followed by the amplification of partial 16S rRNA genes. All amplicons would then be sequenced using a synthesis-based method. Pyrosequencing involves the release of pyrophosphate during nucleotide-incorporation by a DNA polymerase. Through enzymatic reactions, the released pyrophosphates are converted to adenosine triphosphate which provides the energy for luciferase, an enzyme that oxidizes luciferin to generate light. Therefore, production of light is an indication of incorporated nucleotides and because each of the four nucleotides are added in succession, the sequence of the DNA can be determined (Ronaghi 2001). The obtained sequences would then be analyzed to determine the closest match to a cultured species by sequence similarity. High-throughput sequencing also offers the opportunity to study the microbial diversity in tailings samples from several locations within the same pond and from different depths of the pond. Because tailings ponds are heterogeneous, analyzing the microbial species in numerous locations of a tailings pond would provide a better indication of *in situ* microbial communities. There is also a potential to study other specific genes such as functional genes in tailings samples which offers a better idea of the metabolic capabilities of the microbial community compared to analyzing 16S rRNA genes. For example, genes encoding the dissimilatory sulfite reductase (*dsrAB*) that distinguishes SRB, the methyl-coenzyme M reductase (*mcrA*) that distinguishes methanogens (Wilms et al. 2006), or the ferric reductase enzymes (*ferA* and *ferB*) associated with Fe(III)-reduction (Magnuson et al. 2001), could be analyzed; however, the use of high-throughput techniques to study functional genes is still being developed (Cardenas and Tiedje 2008).

Functional genes can be analyzed by other techniques, such as quantitative real-time reverse-transcriptase PCR (qRT-PCR). This technique can be used to indirectly measure microbial activity by measuring gene expression. Citrate-, diluent-, and polyacrylamide-amendments did not affect the abundance of SRB, FeRB, and methanogens in Albian MFT and had no detectable effect on the diversity of the microbial community in Albian MFT. qRT-PCR could correlate differences in methane

production and MFT densification in amended MFT samples to differences in microbial activity. Total RNA from an MFT sample would be reverse-transcribed into its DNA complement by the enzyme *reverse transcriptase*. Primers specific to the functional gene of interest would then be used in quantitative real-time PCR to generate and quantify amplicons from the DNA. Within the same number of PCR cycles, a highly-expressed gene will result in more amplicons than a gene that is rarely expressed. The expression of different functional genes could be compared between Albian MFT samples amended with different substrates over time. Gene expression should increase in response to a useable substrate. For example, if acetate serves as an electron donor for Fe(III) reduction in Albian MFT, the microbial community in acetate-amended MFT would show a higher collective expression of *ferA* and *ferB* than the microbial community in MFT without amendment. Although this method does not necessarily identify the microbial species involved, it suggests the metabolic response of the community to different substrates which could indicate the resulting metabolic products of their activity.

There was a predominance of *Rhodoferax*-like sequences in Albian MFT and this genus was also a match to sequences in Syncrude MLSB MFT (Penner 2006). These bacteria play a currently unknown role in methane production from both tailings ponds. *Rhodoferax ferrireducens* was the closest match to numerous sequenced clones in Albian MFT; the *R. ferrireducens*-like clones could be participating as iron-reducers, as their most related cultured match suggests, or as fermenters like *R. fermentans* (Hiraishi et al. 1991). It is also possible that their large predominance in the Bacterial species of Albian MFT may be an artifact of DNA extraction and amplification bias, although this observation has not been reported in literature. These bacteria may be using a substrate that is common between the two tailings ponds, such as acetate, but it is also possible that they are using a wide range of substrates for growth. Isolation of the *Rhodoferax*-like species in Albian MFT would allow further study of their physiology and ability to grow on different substrates, including citrate, acetate, hydrocarbons and intermediates of hydrocarbon degradation. Determining the byproducts of their activity on different substrates would provide further insight into their role in methane generation from citrate or naphtha hydrocarbons in Albian and Syncrude MLSB MFT. The difficulty in isolating this species from Albian MFT is that its preferred growth conditions are unknown.

Attempts to isolate *Rhodoferax*-like species have begun using the traditional iron-reducing media as well as the medium and carbon substrates used to isolate *R. ferrireducens* (Finneran et al. 2003) to which sequences from Albian MFT matched with a similarity from 97% to 99% (Sections 3.2.2.1, 3.3.4, and 3.4.6).

The cause(s) of the black precipitate and orange precipitate in Albian MFT needs investigation. Thorough analysis of the chemical composition of the tailings pore water as well as the clays and fines material, in combination with analysis of the microbial community of Albian MFT, may help in identifying the cause(s) of their production. Tracking the development of black precipitate with sulfide concentrations in Albian MFT over time would help indicate whether the black precipitate is sulfide and, similarly, testing for ferric and ferrous iron would help indicate whether the orange precipitate is an iron precipitate. It would also be interesting to test for the production of these precipitates from other MFT, such as MLSB MFT, once their sources are identified. Understanding the cause of these precipitates is important for the goal of manipulating the MFT. Methods of enhancing methane production should be designed to hinder the formation of these precipitates especially when considering the great volumes of tailings to be treated.

Methane release has now been demonstrated to accelerate densification of Albian (this study) and Syncrude MLSB MFT (Fedorak et al. 2003); however, the mechanism of how this occurs remains unknown. Observations from this study on Albian MFT suggest that water release and solids settling correlate to the amount of methane produced and released from tailings. It is possible that gas production accelerates densification through the creation of channels in the tailings matrix as gas bubbles move to the surface; these channels would facilitate water release to the surface as well. Alternatively, gas production could potentially be altering the chemistry in the tailings matrix, affecting the interaction between the ions on clay surfaces and facilitating settling and packing of the solids material (references in Penner 2006). Some of the many things to test in regards to determining the mechanism of accelerated tailings densification include establishing how much of the accelerated densification is due to biological activity, whether other gases can have the same effect as methane and what the chemical and physical effects of diluent and polyacrylamide are. Dr. Julia Foght and colleagues (personal communication, 2009) are currently investigating some of these questions.

A detailed understanding of methane production in Albian MFT and its effects is necessary in order to modify this process. In the interest of accelerating tailings densification, the focus could be on stimulating microbial species that are currently involved in methane production or promoting non-active species to also contribute to methane production; this could involve the addition of substrates to MFT. However, we must understand the capability of the microbial community to degrade other substrates to prevent hazardous or unwanted effects from substrate-amendment. This could occur through production of unwanted byproducts or chemical reactions between intermediate compounds and compounds found in the MFT. Uncovering the details of gas production by the microbial community in MFT also offers the potential of manipulating MFT to produce harvestable methane. The idea of engineered MFT is currently being investigated by Dr. Julia Foght and colleagues (personal communication, 2009). By understanding how methane production accelerates tailings, it is also possible to look into non-biological approaches including chemical and physical alternatives that can mimic the effects of methane release. The work done in this thesis and the proposed future work aim to provide a better understanding of methane production in tailings ponds so that options for tailings management, pore water recovery, and reclamation can be better considered.

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7 APPENDICES

7.1 APPENDIX A- RFLP PATTERNS OF SEQUENCED CLONES FROM M1 AND M2 MFT

RFLP digest patterns of sequenced M1 and M2 MFT clones in Tables 3.2 and 3.3 were labeled first by the *HaeIII* digest pattern followed by the *HhaI* digest pattern. For example, RFLP pattern "CA" of the Bacterial clones of M1 and M2 MFT (Table 3.2) indicates a digest pattern of "C" with *HaeIII* and a digest pattern of "A" with *HhaI*. Images of the *HaeIII* and *HhaI* digest patterns of sequenced Bacterial and Archaeal clones of M1 and M2 MFT clones are shown below.

Bacteria

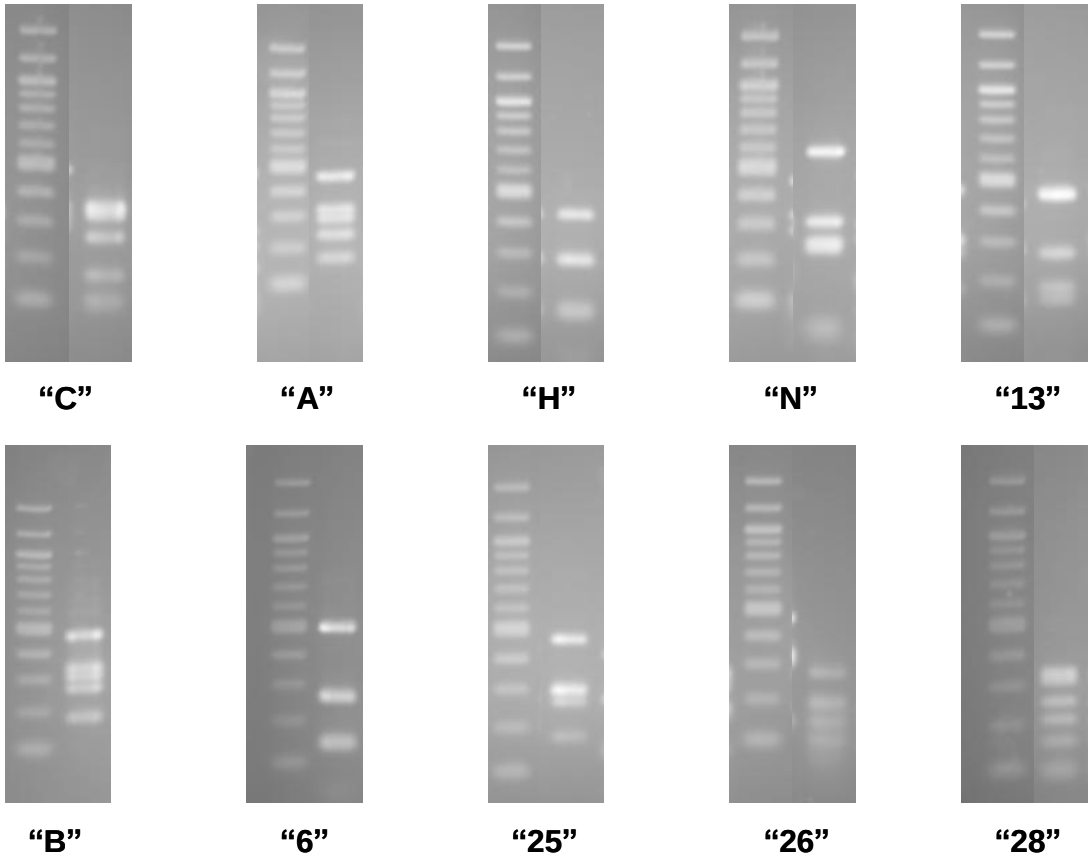


Figure A-1. RFLP digest patterns with *HaeIII* of sequenced M1 MFT Bacterial clones from Table 3.2. DNA ladders are shown on the left of each digest pattern.

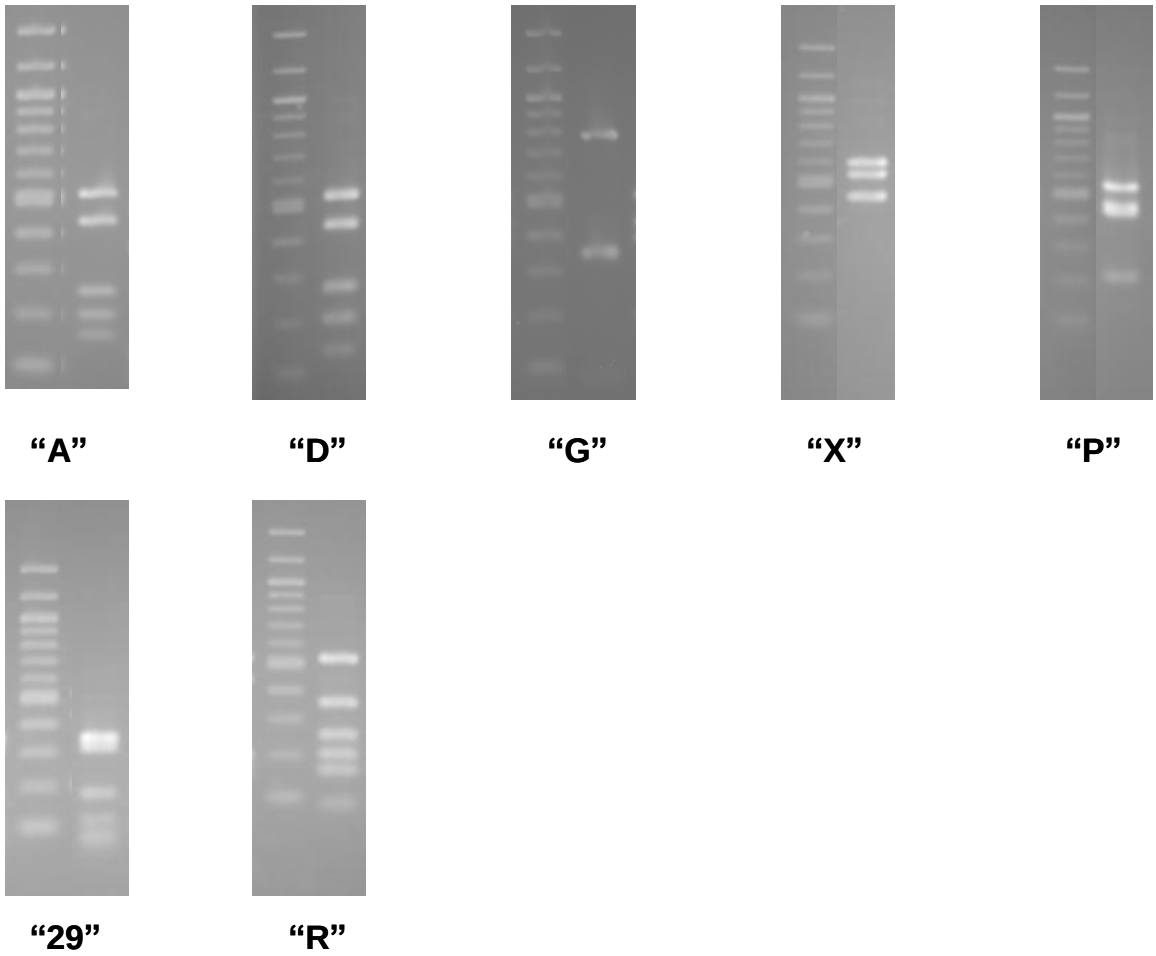


Figure A-2. RFLP digest patterns with *HhaI* of sequenced M1 MFT Bacterial clones from Table 3.2. DNA ladders are shown on the left of each digest pattern.

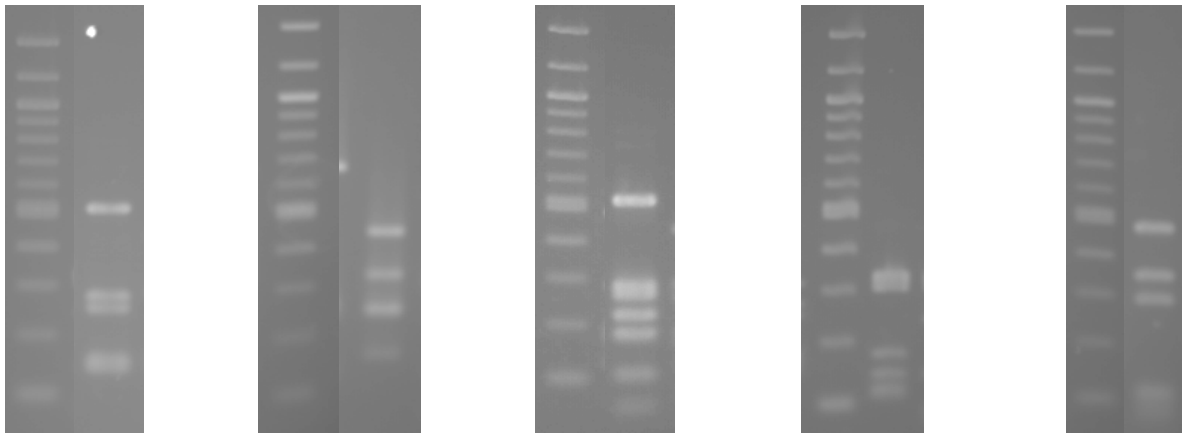


Figure A-3. RFLP digest patterns with *HaeIII* of sequenced M2 MFT Bacterial clones from Table 3.2. The *HaeIII* digest patterns of M2 MFT Bacterial clones that are the same as the *HaeIII* digest patterns of sequenced M1 MFT Bacterial clones have not been included. Refer to Figure A-1 to see those. DNA ladders are shown on the left of each digest pattern.

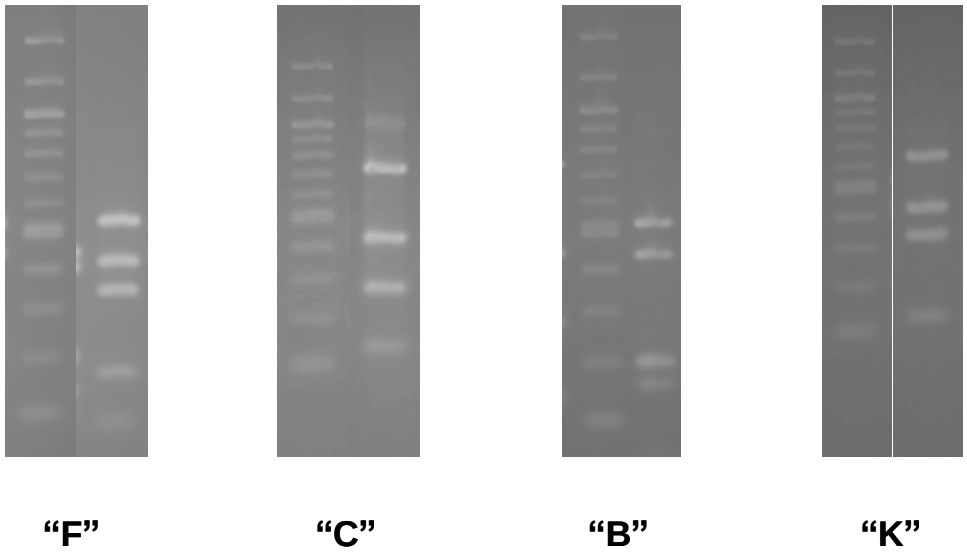


Figure A-4. RFLP digest patterns with *HhaI* of sequenced M2 MFT Bacterial clones from Table 3.2. The *HhaI* digest patterns of M2 MFT Bacterial clones that are the same as the *HhaI* digest patterns of sequenced M1 MFT Bacterial clones have not been included. Refer to Figure A-2 to see those. DNA ladders are shown on the left of each digest pattern.

Archaea:

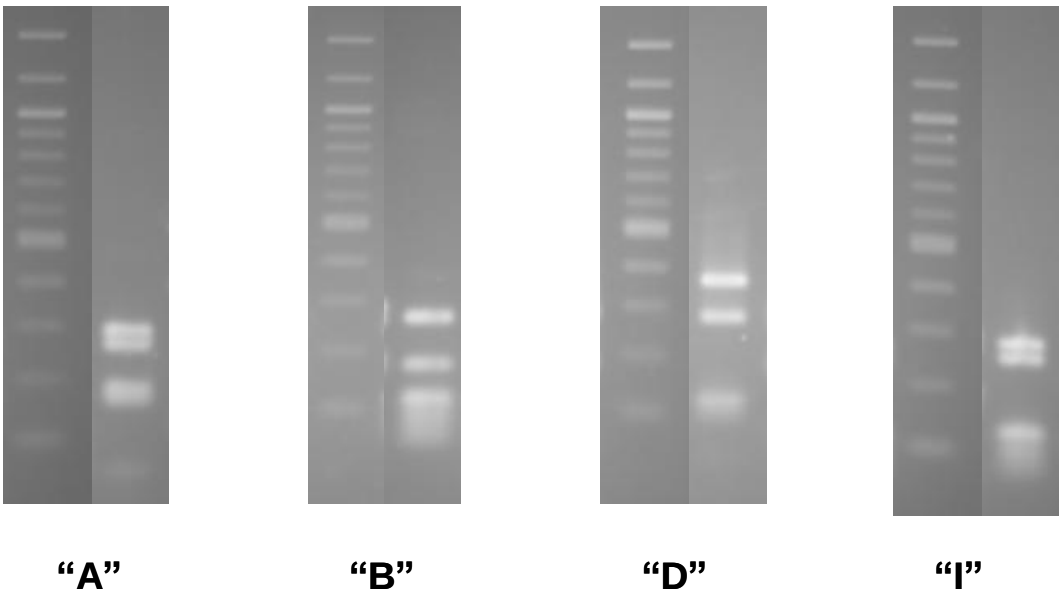


Figure A-5. RFLP digest patterns with *HaeIII* of sequenced M1 MFT Archaeal clones from Table 3.3. DNA ladders are shown on the left of each digest pattern.

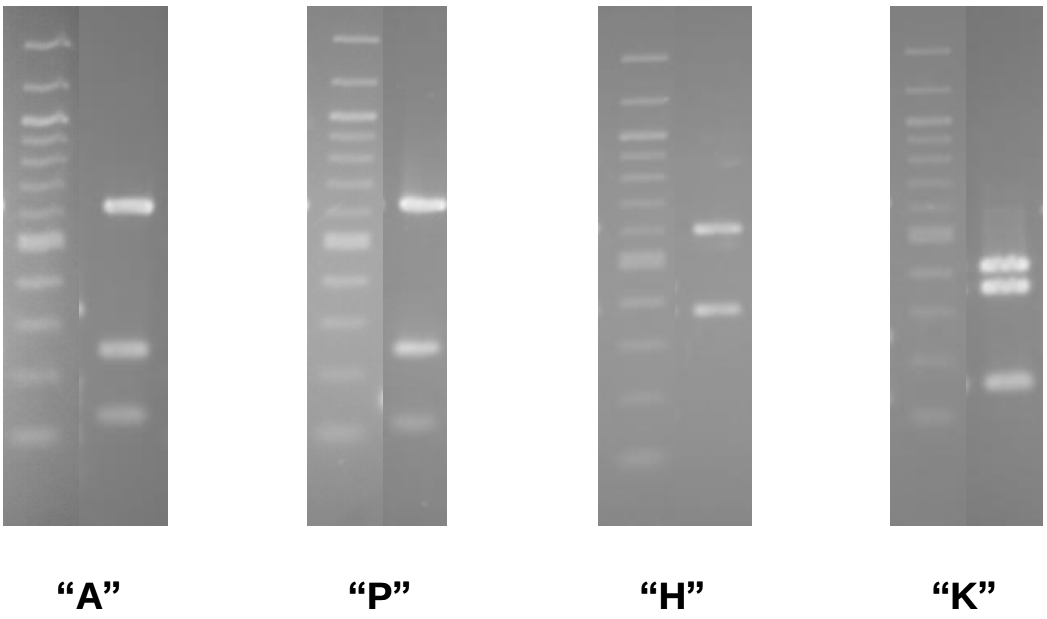
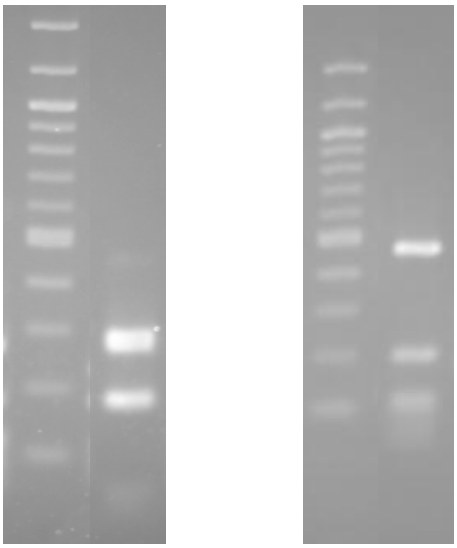
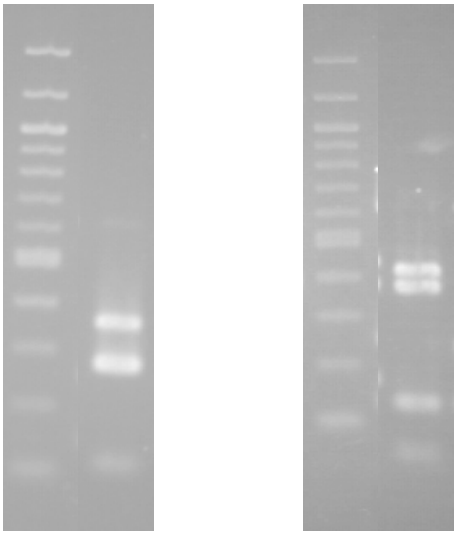


Figure A-6. RFLP digest patterns with *HhaI* of sequenced M1 MFT Archaeal clones from Table 3.3. DNA ladders are shown on the left of each digest pattern.



“C” **“E”**

Figure A-7. RFLP digest patterns with *HaeIII* of sequenced M2 MFT Archaeal clones from Table 3.3. The *HaeIII* digest patterns of M2 MFT Archaeal clones that are the same as the *HaeIII* digest patterns of sequenced M1 MFT Archaeal clones have not been included. Refer to Figure A-5 to see those. DNA ladders are shown on the left of each digest pattern.



“D” **“F”**

Figure A-8. RFLP digest patterns with *HhaI* of sequenced M2 MFT Archaeal clones from Table 3.3. The *HhaI* digest patterns of M2 MFT Archaeal clones that are the same as the *HhaI* digest patterns of sequenced M1 MFT Archaeal clones have not been included. Refer to Figure A-6 to see those. DNA ladders are shown on the left of each digest pattern.

7.2 APPENDIX B – ADDITIONAL PHOTOS OF SETTLING COLUMN TAILINGS

Figure B-1. Photos of duplicate settling column tailings amended with citrate: a) thin cracks are dispersed throughout tailings in one and b) many small spherical bubbles are seen dispersed throughout tailings in the other.

Figure B-2. Photos of duplicate settling column tailings amended with citrate plus polyacrylamide: a) small but numerous bubbles are dispersed throughout the tailings in one column and b) large gas pockets and some thin cracks are observed in the tailings of the other column.

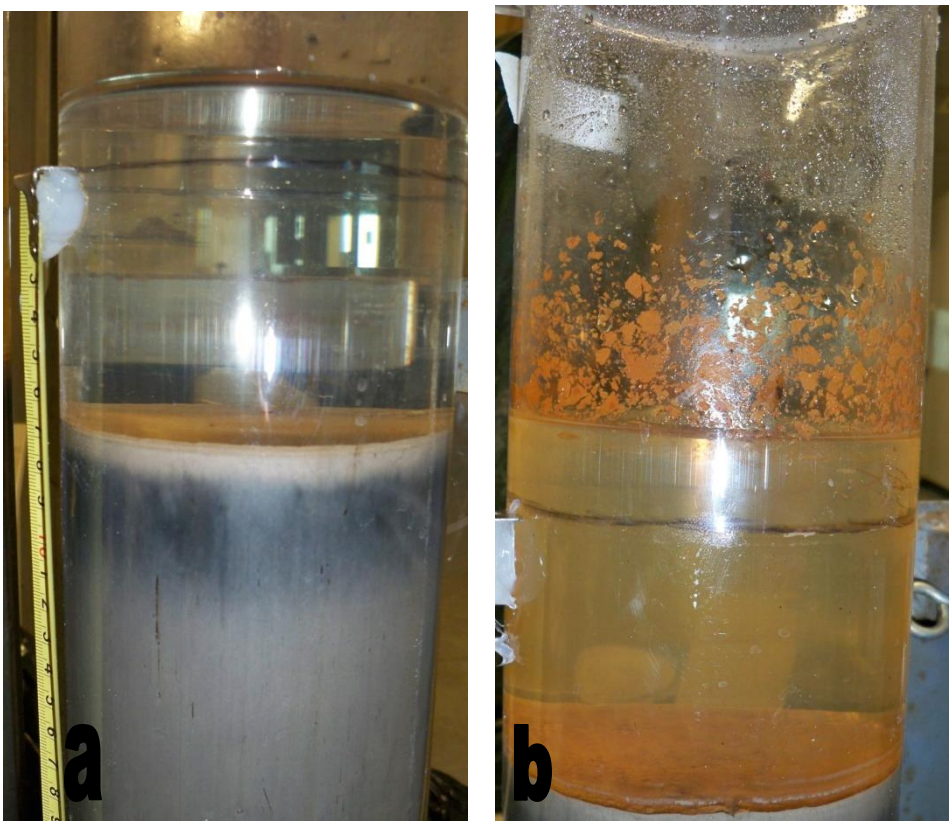


Figure B-3. Photos of water caps in duplicate settling column tailings amended with citrate plus polyacrylamide: a) a very clear water cap is observed on the tailings surface of one column and b) a very turbid water cap is observed on the tailings surface of the other column.