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Methane Production in Oil Sands Tailings under Nitrogen-Depleted Conditions

by

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Abstract

Surface mining for bitumen extraction results in production of tailings that are deposited into large ponds. Tailings in the ponds support diverse microbial communities capable of metabolizing organic compounds and producing biogenic gases (methane, CH_4 and carbon dioxide, CO_2). Because of low endogenous concentrations of bioavailable nitrogen (N), tailings ponds might be deficient in available N (NO_3^-/NH_4^+). This study examined the potential of N_2-fixation mediated methanogenic degradation of citrate and petroleum hydrocarbons in oil sands tailings under N-depleted conditions. Anaerobic primary cultures were set up with available N or N-deficient medium under N_2 headspace using mature fine tailings (MFT) and amended with citrate as a carbon source. Citrate was metabolized to CH_4 under both N available and deficient conditions suggesting N_2-fixation mediated degradation of citrate. Acetylene reduction assay and incorporation of $^{15}N_2$ stable isotope into microbial biomass supported N_2-fixation during citrate metabolism.

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1.1 Introduction to the Oil Sands

The Alberta oil sands account for 99% of Alberta's total oil reserves and currently make up about 40%-50% of Canada's total oil production which is projected to increase over the next few years (Government of Alberta, 2012; American Energy Forum, 2012; CAPP, 2012; EIS, 2012; Net Resources International, 2011). Comprising an estimated 170 billion barrels of crude bitumen, Alberta holds one of the world's largest oil reserves following Saudi Arabia with 260.1 billion barrels and Venezuela with 211.2 billion barrels (Government of Alberta, 2012).

1.1.1 Bitumen Extraction from the Oil Sands

Several techniques are employed to mine bitumen from the oil sands. These include surface mining and *in-situ* mining which can be done using several different processes (The Oil Sands Developers Group, 2009).

Approximately 60% of all the bitumen recovered in Alberta currently is accessed through surface mining. This process is known as open pit mining where trucks and shovels are used to remove the oil sands ores; the bitumen is then separated using a hot water process which is discussed later in this chapter (The Oil Sands Developers Group, 2009; ECRB, 2008). Bitumen deposits are considered to be surface minable if they lie under 100 m of overburden or less but this accounts for only 20% of the total minable bitumen formation in northern Alberta

(Grant et al., 2008). By contrast, as much as 40% of the bitumen is currently recovered using *in-situ* methods. There are five types of *in-situ* mining practices, the most commonly used being steam assisted gravity drainage (SAGD) (Oil Sands InfoMine, 2012; Oil Sands Developers Group, 2009).

The main characteristic of SAGD is how the steam is introduced and utilized using a two well system. Two horizontal wells are drilled: One stretches to the bottom of the bitumen formation and the other ends 5 m above. Steam is injected through the upper well forming a steam-saturated zone known as a steam chamber. The steam condenses and the heat from the steam is transferred by gravity to the bitumen formation below. This causes the bitumen to "melt" and as the steam chamber expands, bitumen is able to flow into the lower well for extraction. This extraction method is currently the most popular *in-situ* technique because it can recover up to 60% of the bitumen in a formation and is currently being used by Suncor, Petro-Canada and Encana (Al-Bahlani and Babadagli, 2009; Oil Sands InfoMine, 2012).

It is estimated that surface mining produced approximately 140,000 m^3 (~1.2 million barrels) and *in-situ* mining produced 89,000 m^3 (~0.75 million barrels) of crude bitumen per day in 2010 (~1.9 million barrels total) (ECRB, 2010). This accounts for 60% and 40% of Alberta's bitumen production respectively (ECRB, 2008)).

1.1.2 Environmental Impact of Bitumen Extraction

The total area of the oil sands is estimated to encompass approximately 142,200 km^2. Of this, only about 4,200 km^2 will be disturbed using open pit mining but will yield as much as 20% of the total recoverable bitumen. The reservoirs in the remaining area contain approximately 80% of the total oil sands bitumen and are only accessible using *in-situ* techniques (Government of Alberta, 2012).

Open pit and *in-situ* mining have different environmental impacts. In open pit mining, vast quantities of land are disturbed resulting in the destruction of habitat and ecosystems. Currently, over 715 km^2 of boreal forest have been lost due to this activity (Government of Alberta, 2012). Before mining can begin, 1-3 m of topsoil or muskeg is removed and up to 100 m of overburden lying above the ore deposit. The overburden is stored in overburden dumps, used to fill mined-out pits or compacted into tailings dykes (Grant et al., 2008). Open pit mining produces significant quantities of tailings which are toxic to aquatic and terrestrial life and are difficult to remediate and reclaim (van den Heuvel et al., 2000; Farrell et al., 2004; Colavecchia et al., 2003; Colavecchia et al., 2006; Rogers et al., 2002; Quagraine et al., 2005). Though *in-situ* mining appears to create less disturbance than open pit mining, this opinion does not take into account land fragmentation or infrastructure (Jordaan et al., 2009). A publication, entitled, "Death by a Thousand Cuts" by the Pembina Institute and CPAWS describes this issue in detail. Their 2006 case study examined the proposed SAGD OPTI-Nexen Long Lake project which was in the early stages of its development. The approved project called for 89 km of roads, 522 wells and 1 km^2 land cleared for a central facility on a 106 km^2 land parcel. This web of

development would place approximately 80% of the land parcel within 250 m of an industrial feature once construction is complete. If the 36,000 km^2 area that has been approved for this type of *in-situ* development is developed using the same design as the Long Lake project, it will result in over 2,960 km^2 of forest being cleared and over 30,000 km of roads being constructed. This degree of land fragmentation raises serious concerns regarding the populations of sensitive and endangered species such as caribou, which are heavily impacted by industrial development (Schneider and Dyer, 2006).

Oil sands activities also consume a significant amount of natural resources such as natural gas and water. It is estimated that 28 m^3 of natural gas (National Energy Board, 2013) and 2.5-4 barrels of fresh water are consumed for every barrel of bitumen produced by *in-situ* extraction (Government of Alberta, 2012). For open pit mining, 20 m^3 of natural gas (National Energy Board, 2013) and 3-4 barrels of fresh water are consumed per barrel of bitumen produced (Government of Alberta, 2012). Currently, the oil sands industry consumes approximately 21 million m^3 per day (water use data unavailable); by 2015, it is estimated that bitumen production in the oil sands will consume 60 million m^3 of natural gas per day (National Energy Board, 2013). In 2015, it is anticipated that bitumen production will exceed 3 million barrels per day (National Energy Board, 2013), resulting in extremely high water consumption, however, 40-70% will be recycled (Government of Alberta, 2012). Despite this, about 1.5 barrels of water per barrel of bitumen end up in mature fine tailings (MFT) in tailings ponds (Grant et al., 2008). With the current rate of bitumen production resulting from open pit mining, it is estimated that over 0.2 million m^3 MFT are already being produced each day (Pembina, 2013).

1.2 Oil Sands Tailings

Tailings are produced during bitumen extraction from surface mined ores. For every barrel of bitumen, approximately two tonnes of oil sand ores are mined and extracted using the modifications of the Clark Hot Water Extraction process (CHWE) (Figure 1-1) (Government of Alberta, 2012, Masliyah et al., 2004). Water is heated to 50 - 80 °C and combined with sodium hydroxide or sodium salts of weak acids and the crushed oil sands ores (Hepler and Smith, 1994; Scott et al., 2010). Because the sand is hydrophilic and the bitumen is hydrophobic, the bitumen is able to float to the surface where it can be collected (Takamura et al., 1982; 1983). Naphthenic acids and partially aromatic asphaltic acids containing oxygen in carboxylic, sulphonic and phenolic functional groups act as surfactants at elevated pH; this promotes the efficiency of bitumen recovery by reducing surface and interfacial tension. This process can incorporate the use of compounds such as naphtha or paraffinic diluents (C_5-C_6) which reduce the emulsion stability, allowing for greater bitumen recovery (Chalaturnyk et al., 2002; Scott et al., 2010; Masliyah et al., 2004). Shell Albian's paraffinic diluent also partially precipitates asphaltenes in the bitumen froth trapping water and solids thereby enhancing gravity separation resulting in improved bitumen quality and leaving residual asphaltenes in the slurry (Masliyah et al., 2004; Total E&P Joslyn Ltd., 2010). Sodium citrate is also utilized by Shell Albian Sands with difficult-to-process ores. The citrate molecule bonds with divalent ions such as magnesium and calcium, which inhibits their bridging ability between fine particles and bitumen (Zhang et al., 2009).

After bitumen recovery, the remaining slurry consists of about 55 wt% solids in solution. These solids are made up of 82 wt% sand and 17 wt% fine particulates (smaller than 44 μm). The majority of the solvent is removed by distillation. Consolidation agents such as gypsum (Syncrude and Suncor) and polyacrylamide (Albian) are added to thicken the tailings and the slurry is then pumped into settling basins or "tailings ponds" for containment (Masliyah et al., 2004; Total E&P Joslyn Ltd., 2010; Chalaturnyk et al., 2002). The coarse sand quickly separates from the slurry, forming beaches and dykes leaving only fine tailings of 6-10 wt% in the fresh tailings. Over the course of a few years, the fine tailings settle forming a stable slurry structure (around 30 wt%) known as mature fine tailings (MFT; Table 1-1). These tailings are very difficult to consolidate further and as such, will remain in a fluid state for decades (Chalaturnyk et al., 2002). Some of the water in the suspension collects at the surface of the pond forming a "water cap". This process-affected water is continually withdrawn and re-used in processing (Allen, 2008)

1.2.1 Tailings Water Quality

Tailings water quality (Table 1-2) is poor compared to environmental guidelines such as Canadian Environmental Quality Guidelines (CEQG) and United States Environmental Protection Agency (USEPA) guidelines (Allen, 2008; CCME, 2005; USEPA, 1999). Poor water quality is the result of the lack of water treatment before it is recycled from the tailings pond and reused in the bitumen extraction process; this has caused problems in bitumen recovery. High concentrations of soluble salts have led to increased water hardness; this neutralizes surfactants added to aid in bitumen recovery. High concentrations of carbonate, sulphate,

silica, phosphate and iron oxides also contribute to scaling on heat exchangers, and sulphate, chloride, bicarbonate, naphthenic acids, copper, and dissolved oxygen contribute to corrosion of the processing facilities. This continual recycling with lack of treatment compounds problems and increases contamination, which can hinder reclamation efforts due to high toxicity (Allen, 2008).

1.2.2 Reclamation Challenges of MFT and Tailings Water

Reclamation efforts are further hindered by additional contaminants in MFT solids and tailings water such as heavy metals, salts, chemical additives such as polyacrylamide, residual bitumen and unrecovered organic solvents such as naphtha or diluents (Cymerman et al., 1999; Quagraine et al., 2005; Grant et al., 2008; Allen, 2008; Tang et al., 2011). These compounds are often present in tailings materials at sufficient concentrations to be toxic to plants, and aquatic and terrestrial biota (van den Heuvel et al., 2000; Farrell et al., 2004; Colavecchia et al., 2003; Colavecchia et al., 2006; Rogers et al., 2002; Tang et al., 2011; Croser et al., 2001; Renault et al., 1999; Grant et al., 2008). Studies performed on vegetation grown on tailings solids found extremely poor survival rates. In one such study examining boreal trees grown in soil containing fine tailings, a mixture of only 15% fine tailings inhibited growth and caused tree death due to high salinity (Renault et al., 2000). In addition, the concentration of naphthenic acids in tailings (between 49 mg L^{-1} in Syncrude's Mildred Lake Settling Basin and 68 mg L^{-1} in Suncor tailings ponds as compared to Athabasca river water that is generally < 1 mg L^{-1}, Holowenko et al., 2002; Golder Associates Limited, 2001), is sufficiently high to be toxic to aquatic organisms and

animals (Rogers et al., 2002; van den Heuvel et al., 2000; Farrell et al., 2004; Colavecchia et al., 2003; Colavecchia et al., 2006; Grant et al., 2008). Several studies performed on fish and amphibian embryos have found high mortality rates and severe deformities when cultured in tailings water (Peters et al., 2006; Melvin and Trudeau, 2012). This is one of the many reasons that the biodegradation of hydrocarbons is of such importance.

1.2.3 Biogeochemical Processes

Tailings are composed of sand, silt, clay, unrecovered bitumen, residual solvent and trace metals. The sand fraction consists of 97.5-99% $Si0_2$, 0.5-0.9% Al_20_3, and 0.1-0.9% Fe and the clay consists of secondary minerals such as kaolinite and illite with traces of vermiculite, smectites, chlorite, and mixed-layer clays as well as oxide minerals (Yong and Sethi, 1978; Longstaffe, 1983; Roberts et al., 1980; Scott et al., 1985; Lane et al., 1984). The heavy metals Ti, Zr, Fe, V, Mg, Mn, Al, Pb, Zn, Nb, and Mo have also been detected in tailings in addition to common ions such as K^+, Ca^{2+}, Mg^{2+}, Na^+, Cl^-, SO_4^{2-} and HCO_3^- which originate from connate water (water trapped within minerals upon their formation), river water and chemical water treatment (Kramers and Brown, 1976; MacKinnon and Retallack, 1982; Trevoy et al., 1976; Kessick, 1980; Jonasson et al., 1991; Kupchanko, 1968; MacKinnon, 1989). These ions are important as the nature of the clay minerals can affect MFT densification of tailings ponds, which is described later, and bitumen extraction in oil sand ore processing. Clays such as montmorillonite have a synergistic effect with calcium ions which activate interactions between the clay and bitumen droplets. This results in the creation of a hydrophilic coating on the bitumen droplet surface which negatively impacts bitumen recovery (Liu et al., 2002; Kessick,

1980). The concentration of ions can be affected by ion exchange between free and surface adsorbed cations as well as microbial interactions which can affect solid phase mineralogy and metal distribution (MacKinnon, 1989; Burchfield and Hepler, 1979; Salloum et al., 2002). Ferric iron (Fe^{III}), for example, is utilized as an electron acceptor prior to the onset of methanogenesis by the genera *Geobacter* and *Geothrix* (Anderson et al., 1998; Coates et al., 1999). This interaction can cause the dissolution of Fe^{III} minerals (ferrihydrite, lepidocrocite, goethite and hematite), decrease clay swelling and collapse the phyllosilicate structure. This decreases the surface area of the clay particle and increases the surface charge density via cation exchange (Kostka et al., 1999). This type of microbial activity can cause mineral transformations and can affect distribution of metals by changing their solubility (Lovley, 1995; 2006). Evidence suggests that some Fe^{III}-reducing microorganisms are also capable of directly reducing and immobilizing toxic metals in contaminated aquifers (Lovley and Anderson, 2000).

Oil sand tailings harbour a diverse population of microorganisms including anaerobic heterotrophs, sulphate-reducing prokaryotes and methanogens. Methanogens are of particular interest because of their ability to produce methane (CH_4) by metabolizing simple organic compounds (Penner and Foght, 2010). After the bitumen extraction processes, residual bitumen, naphthenic acids, diluents such as naphtha and chemical additives like trisodium citrate are present in the tailings ponds; some of these compounds can be toxic to the environment (van den Heuvel et al., 2000; Farrell et al., 2004; Colavecchia et al., 2003; Colavecchia et al., 2006; Rogers et al., 2002). Anaerobic bacteria may decompose these hydrocarbons and organic compounds resulting in the formation of acetate, hydrogen and CO_2;

methanogens are then able to consume these compounds and produce CH_4 (Siddique et al., 2012; Zengler et al., 1999).

1.2.3.1 Hydrocarbon Degradation under Anaerobic Conditions

Anaerobic respiration can utilize inorganic electron acceptors in the following order of preference: NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-}, and CO_2 (Peters and Conrad, 1996; Chidthaisong and Conrad, 2000). These electron acceptors can be used for the anaerobic oxidation of organic compounds. Terminal electron acceptors such as N-oxides (NO_3^-, NO_2^-, N_2O) can be used to totally oxidize various organic compounds in catabolic CO_2-producing reactions. It has also been shown that Mn- and Fe-oxides can be used in microbial respiration in addition to metalloids, toxic metals and radionuclides, which can all be used as electron acceptors in contaminated environments. During fermentation, formate can be utilized in the production of CO_2 and H_2, and during acetogenesis, acetate and H_2 can be produced through the fermentation of hydrocarbons, alcohols and aminoacids. S and oxidized sulphur compounds such as SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$ can also be used to oxidize fermentation products resulting in the formation of CO_2, acetate and H_2S (Dassonville and Renault, 2002). Some examples of terminal electron acceptors are shown in Figure 1-2.

Tailings contain many different hydrocarbons originating primarily from residual solvent but also unrecovered bitumen. Depending on the solvent used in extraction, these can include low molecular weight *n*-alkanes and monoaromatic hydrocarbons ranging from C_3-C_{12}, BTEX (benzene, toluene, ethylbenzene, and xylenes) compounds, low molecular weight *iso*-paraffins

and naphthenes from the naphtha diluent or C_5-C_6 aliphatic hydrocarbons used in Albian diluent (Albian Sands Energy Inc.); residual bitumen contributes to small amounts of PAHs such as naphthalene, fluorenes, dibenzothiophenes, phenanthrene and pyrenes and higher molecular weight aliphatic hydrocarbons (C_{12}-C_{21}) (Siddique et al., 2006; 2007). Some hydrocarbons are degraded preferentially or are more difficult to degrade due to their structure, such as methyl branching or complex aromatic structures which can make hydrocarbons more resistant to microbial attack (Siddique et al., 2006; 2007). The majority of the residual bitumen is composed of non-biodegradable or very slowly degraded asphaltenes such as PAHs with five rings or more (U.S. Congress, 1991; National Research Council, 1985). The general degradation pathway of organic compounds is depicted in Figure 1-3.

Under anaerobic conditions, hydrocarbon degradation is considered to occur via three main activation pathways (Figure 1-4): Fumarate addition, carboxylation and hydroxylation (Foght, 2008; Wentzel et al., 2007). Fumarate addition can occur at either the terminal methyl group of hydrocarbon molecules or more commonly, the sub-terminal carbon group; for alkanes both mechanisms result in products which are then linked to SCoA and converted to acyl-CoA which can then be further metabolized by β-oxidation (Rojo, 2009). Fumarate addition is known to occur under most anaerobic conditions such as iron, sulphate and nitrate-reducing conditions (Callaghan et al., 2006; Beller and Spormann, 1997; Kane et al., 2002; Mbadinga et al., 2011). Fumarate addition has also been found for aromatic hydrocarbon degradation under methanogenic conditions but the mechanisms of *n*-alkane degradation under methanogenic conditions have yet to be resolved (Beller and Edwards, 2000; Aitken at al., 2013). In carboxylation, a CO_2 molecule is bound to the hydrocarbon; in alkanes, this occurs at the C_3,

cleaving the hydrocarbon at the C_2 position (Aeckersberg et al., 1991; 1998; Pirnik et al., 1974; Grossi et al., 2000; Mbadinga et al., 2011). Carboxylation is known to occur under sulphate-reducing conditions and is suspected under denitrifying conditions (So and Young, 1999; Callaghan et al., 2009; Mbadinga et al., 2011). Hydroxylation, which has only been demonstrated anaerobically for aromatic hydrocarbons, binds an OH- group to aromatic molecules such as ethylbenzene targeting the carbon closest to the ring. This process is known to occur under both nitrate-reducing and methanogenic conditions and is suspected for *n*-alkane degradation under nitrate-reducing conditions (Vogel and Grbić-Galić, 1986; Szaleniec at al., 2010; Zedelius et al., 2011).

Aromatic fumarate addition occurs under methanogenic conditions as well as sulphate-, iron- and nitrate-reducing conditions; examples are depicted in Figure 1-5 (Callaghan et al., 2006). In toluene degradation, fumarate is added to the sub-terminal methyl group by benzylsuccinate synthase resulting in the formation of benzylsuccinate (Leuthner et al., 1998). The benzylsuccinate molecule is then activated by a succinyl-CoA-dependent CoA-transferase resulting in the formation of benzylsuccinyl-CoA. This molecule is then degraded to benzoyl-CoA and succinyl-CoA though a β-oxidation pathway (Leuthner, and Heider, 2000). Similar pathways have been identified for the activation of the methyl groups on 2-methylnaphthalene and some xylene isomers (Heider, 2007).

Hydroxylation has been demonstrated in the degradation of ethylbenzene under methanogenic and denitrifying conditions (Vogel and Grbić-Galić, 1986; Grbić-Galić and Vogel, 1986; Szaleniec at al., 2010). Ethylbenzene is activated by hydroxylation of the side chain

carbon closest to the ring; this reaction, which uses water as the hydroxyl donor, is catalyzed by ethylbenzene dehydrogenase. It is suggested that ethylbenzene hydroxylation occurs via two single-electron transfer reactions (Szaleniec at al., 2010). This chain of reactions results in the formation of acetyl-CoA and benzoyl-CoA (Fuchs et al., 2011).

Carboxylation has been demonstrated under sulphate-reducing conditions and is suspected under denitrifying conditions (So and Young, 1999; Callaghan et al., 2009; Fuchs et al., 2011). Benzene can be activated by direct carboxylation followed by CoA ligation and cleavage resulting in the formation of acetyl-CoA and benzoyl-CoA. Benzoyl-CoA is then further degraded to acetyl-CoA and CO_2 (Fuchs et al., 2011; Edwards and Grbić-Galić, 1992).

Benzoyl-CoA degradation can occur via two different pathways, ATP-dependent or ATP-independent reduction of the aromatic ring as depicted in Figure 1-6, however both reaction pathways produce the same molecules resulting in the production of three acetyl-CoA molecules and CO_2 (Fuchs et al., 2011). Both aliphatic and aromatic hydrocarbon degradation result in the formation of acetyl-CoA which can be further degraded to the methanogenic substrates H_2 and CO_2 (Ferry, 1993).

1.2.3.2 Methanogenesis

Methanogenesis utilizes the anaerobic hydrocarbon degradation products of fermenters and syntrophs such as acetate (acetyl-CoA), H_2 and CO_2 as seen in Figure 1-3 (Siddique et al., 2012; Ferry, 1993). Using zero- and first-order kinetics based on the concentration of naphtha in MFT and the volume of MFT in Mildred Lake Settling Basin (MLSB), Siddique et al. (2008) calculated that 8.9-400 million L/day of CH_4 could be produced from Syncrude's MLSB. The

estimation was higher than the measured surface flux of 3-43 million L CH_4/day and the calculated predicted CH_4 flux of 42-84 million L/day, however this is likely due to the ideal incubation conditions including concentrations of naphtha, and other nutrients in the laboratory cultures upon which the calculation was based. Both the measured and predicted values fall within the calculated range and it can therefore be used to approximate the potential CH_4 production (Siddique et al., 2008).

Several metabolic pathways exist for the formation of CH_4: hydrogenotrophic, methylotrophic and acetoclastic methanogenesis (Figure 1-7). Hydrogenotrophic metabolism converts CO_2 or HCO_3^- to CH_4 through a series of two-electron reductions using H_2 as an electron donor. H_2 is most commonly produced by other microorganisms as a by-product of their metabolism and is an important extracellular metabolite. Most H_2-oxidizing methanogens such as *Methanococcus* and *Methanopyrus* can also utilize formate. Some methanogens can use secondary alcohols and some primary alcohols as reducing agents though it is much less common (Rother, 2010).

Methylotrophic metabolism utilizes methyl group-containing compounds such as methanol, dimethylsulphide, monomethylamine and dimethylamine, trimethylamine (Hippe et al., 1979; Mah, 1980; Van der Maarel and Hansen, 1997). Unlike the hydrogenotrophic pathway, CO_2 is produced rather than consumed. This results in one mole of methyl-CoM being oxidized to CO_2 for every three moles of methyl-CoM reduced to CH_4 (Rother, 2010).

The final form of methanogenic metabolism occurs via the acetoclastic pathway. In this pathway, acetate is phosphorylated to acetyl-CoA which is then reduced to CH_4 and CO which is

oxidized to CO_2 (Rother, 2010; Ferry, 1997). Because the primary source of CH_4 production in Syncrude tailings is naphtha, and because hydrocarbon degradation commonly produces acetyl-CoA, it is likely that the primary CH_4 producers are acetoclasts followed by hydrogenotrophs (Siddique et al., 2011; Ferry, 1993). This is supported in a study by Siddique et al. (2011) that identified the microbial composition of MFT in long chain *n*-alkane degrading MFT cultures and found the dominant Archaea to be Methanosarcinales, specifically the genus *Methanosaeta* of the family Methanosaetaceae, and Methanomicrobiales, specifically the genus *Methanoculleus* of the family Methanomicrobiaceae. Methanosaetaceae are known to be obligate acetoclasts and Methanomicrobiaceae are hydrogenotrophic while formate and secondary alcohols can be used as electron donors by some species.

1.2.3.3 Biodensification of MFT

Fedorak et al. (2003) found that tailings densification rates were faster in methanogenic MFT than in non-methanogenic MFT. These data suggest that methanogenic microbial activities may play a role in enhancing tailings densification and increasing water recovery.

Water recovery from tailings is very important in tailings management both for reuse in the bitumen extraction process (thereby minimizing the amount of water which must be drawn from the Athabasca River) and for increasing the shear strength of tailings for subsequent use in reclamation (List and Lord, 1997; ERCB, 2009). If tailings were left to naturally dewater, it could take more than one hundred years before the shear strength of the tailings solids was sufficient for reclamation (10 kPa; ERCB, 2009). This is because of the interactions between the colloidal

fines, bitumen and pore water resulting in the formation of a stable suspension (Eckert et al. 1996).

A recent study by Arkell (2012) examining densification rates of tailings with organic amendment proposed a pathway for the consolidation of tailings that involved biological, chemical, and mineralogical interactions. In the first step, organic substrates are anaerobically metabolized under methanogenic conditions resulting in the production of CH_4 and CO_2. The resulting CO_2 then dissolves in the pore water producing HCO_3^- and protons (H^+); this results in a decrease in pH and an increase in ionic strength. As the pH decreases, carbonate minerals ($CaCO_3$ and $CaMg(CO_3)_2$) are dissolved releasing Ca^{2+} and Mg^{2+} ions into the pore water. These ions can exchange Na^+ on the surface of the clay particles which, in combination with the increased ionic strength, cause a decrease in the diffuse double layer (the layer of negative charge on the clay surface and positively charged ions surrounding it) of the clay particles in suspension. This in turn leads to increased densification and water release. After adding two organic amendments, acetate and canola to MFT, Arkell (2012) observed an increased volume of pore water released from MFT compared to unamended MFT. After 82 d of incubation, 31 vol% water recovery was observed in acetate-amended MFT columns compared to unamended baseline controls with only 3 vol% water recovery. After 212 d of incubation, 35 vol% water recovery was observed in canola-amended columns versus unamended baseline controls with 25 vol% water recovery. Both experiments documented a decrease in pH and an increase in HCO_3^-, Ca^{2+} and Mg^{2+} as well as a decrease in exchangeable Na^+ in the pore water. These results support the proposed pathway and indicate that the metabolism of organic compounds and resulting methanogenesis results in increased tailings densification (Arkell, 2012).

1.2.4 Microbial Community

Tailing ponds are known to support a diverse population of largely anaerobic microorganisms capable of hydrocarbon degradation and methanogenesis (Penner and Foght, 2010; Siddique et al., 2006; 2007; 2011). These processes are valuable for biodensification (Arkell, 2012; Fedorak et al., 2003) and bioremediation of oil sand tailings, providing environmental benefits such as water recycling to decrease the demand for fresh water withdrawal from the Athabasca River and reducing the toxicity of tailings for reclamation (Quagraine et al., 2005; Whitby, 2010; Leahy and Colwell, 1990; Chikere et al., 2011).

The microbial communities involved in these processes tend to vary between the tailings ponds of different operators and sampling sites. This variance is attributed to the different composition of the tailings at the sampling site. Factors such as total petroleum hydrocarbons, total straight chain hydrocarbons, total aromatic hydrocarbons, polycyclic aromatic hydrocarbons, naphthenic acids (NA) and clay content can vary between sampling sites with clay and NA having the most significant influence on community structure (Yergeau, et al., 2012). A recent study by Yergeau et al. (2012) examined the community composition of MFT from both Suncor and two sites in Syncrude's tailings pond, MLSB. They found that Suncor's MFT had a more diverse bacterial composition whereas Syncrude had a wider range of archaeal genera. They also found significant differences in samples taken from the two sites in MLSB highlighting the impact of tailings composition on microbial communities and the metabolic activity that can occur at those sites. Because both Syncrude and Suncor use naphtha as a diluent and gypsum as a consolidation agent, their community composition is more likely to resemble one another whereas Albian, which uses paraffinic solvents (C_5-C_6) as a diluent,

polyacrylamide as a flocculating agent and trisodium citrate as a dispersant, is likely to have more variance in community composition and their resulting metabolic processes (Masliyah et al., 2004; Zhang et al., 2009).

1.2.4.1 Overcoming Nutrient Deficiencies

For microbial metabolism to occur, sufficient macro-nutrients must be present. The nutrient requirement for anaerobic metabolism for C:N is considered to range between 50:1 and 100:1 in wastewater treatment however, ratios as high as 180:1 have been reported (Droste, 1997; USEPA, 1995; Ammary, 2004). The viability of these ratios however, is dependent on biomass yield and C removal efficiency because low yield or low efficiency cultures require a lower nutrient ratio (Ammary, 2004). Interestingly, metabolic processes continue to occur in tailings ponds under nutrient-limited conditions; nitrogen (N) is one such limiting nutrient. Whereas ammonium is a known contaminant in MLSB (14 mg L^{-1}), bioavailable N (NH_3^+, NH_4, NO^{2-}, NO^{3-}) is deficient in other tailings ponds such as those belonging to Albian and Suncor (Allen, 2008; Widdison and Burt, 2008; Anderson et al., 2010; Siddique, 2008). Two hypotheses have been developed to explain how this deficiency has been overcome: The use of alternative N containing molecules such as polyacrylamide (PAM), and N_2-fixation.

PAM is a high molecular weight polymer with amide and carboxylic functional groups and is used as an anti-erosion agent in irrigation water, a flocculent in drinking water and to remove particulates and consolidate tailings in tailings ponds (Cymerman et al., 1999; Wheale, 2002). Several studies examining agricultural use and wastewater treatment have indicated

that microorganisms are able to survive in N-depleted aerobic and anaerobic, methanogenic conditions with PAM added as the sole N source. These recent studies were conducted by Kay-Shoemake et al. (1998a) and Haveroen et al. (2005) respectively. Kay-Shoemake et al. (1998b) also found that amidase enzyme concentrations were elevated in samples containing PAM indicating that the amide group was likely being removed from the PAM backbone and utilized by the microbial population (Figure 1-8). Another study, conducted by Bao et al. (2010) conclusively demonstrated the ability of aerobic microorganisms in oil field water to utilize the amide groups on partially hydrolyzed polyacrylamide (HPAM) by converting it to a carboxyl group on the HPAM backbone. This was demonstrated using size exclusion chromatography and HPLC analysis.

The low solubility of N_2 in water (~1.8 g Kg^{-1}) means it is unlikely that N_2 will diffuse into wet, anaerobic environments however, the N_2 needed for N_2-fixation may be present in MFT as a result of denitrification reactions, which will be discussed later (Engineering Toolbox, 2012; Swerts et al., 1996). Nitrogen fixation is an oxygen-sensitive process in which N_2 is taken up from the environment by diazotrophs and converted via the nitrogenase enzyme into "fixed" N which is bioavailable (Cabello et al., 2009). This process is known to occur in many bacteria and archaea including methanogens (Belay et al., 1984; Leigh, 2000). Multiple *nif* genes (e.g., 17 genes in *Klebsiella pneumoniae*) are required for this process. Transcription is negatively regulated in the presence of molecular oxygen or available N such as ammonium or utilizable amino acids like glutamine (Ausubel, 1984; Kranz and Haselkorn, 1986; Kranz and Cullen, 2004; Fischer, 1994). Although N_2-fixation can occur at very low concentrations of bioavailable N (0.4 μM NO_3^- or 2 μM NH_4^+), 10 μM NO_3^- or 250 μM NH_4^+ can significantly reduce or completely

inhibit N_2-fixation (LaRoche and Breitbarth, 2005; Vintila and El-Shehawy, 2007). Earth's largest N reservoir is in the form of N_2, thus N_2-fixation is an essential part of the N cycle (Francis et al., 2007).

1.2.4.1.1 Nitrogen Cycle

The complete N cycle (Figure 1-9) couples aerobic and anaerobic processes. Aerobic processes include nitrification (NH_4^+ -> NH_2OH -> NO_2^- and NO_2^- -> NO_3^-) and nitrate (NO_3^-) assimilation (NO_3^- -> NO_2^- -> NH_4^+). The first step in this pathway involves ammonium (NH_4^+) assimilation from the degradation of organic matter and oxidation of (NH_4^+) to hydroxylamine (NH_2OH) by ammonia monooxygenase (Amo) by ammonia-oxidizing bacteria by coupling NH_4^+ and O_2 with H_2. NH_2OH and H_2O are then converted to nitrite (NO_2^-) and $5H^+$ via a hydroxylamine oxidoreductase (Hao) in the periplasm. Finally, NO_2^- and H_2O are converted to NO_3^- and $2H^+$ in nitrite-oxidizing bacteria via Nxr (nitrite oxidoreductase), a membrane-bound enzyme (Cabello et al., 2009).

Nitrate (NO_3^-) assimilation begins with the uptake of NO_3^- through ATP-dependent transporters. Nitrate is then reduced via Nas (assimilatory nitrate reductase) that catalyzes the reduction of NO_3^- to NO_2^- through the transfer of two electrons. Nitrite is further reduced to ammonium via ferredoxin-dependent Nir (as found in eukaryotes, prokaryotes and some haloarchaea) or NADH-dependent Nir (found in fungi, heterotrophic bacteria and some phototrophs) (Cabello et al., 2009).

The oxygen-sensitive reactions in the N cycle include N_2-fixation ($N_2 + 8e^- + 10H^+ + 16ATP \rightarrow 2NH_4^+ + H_2 + 16ADP + 16P_i$) as discussed above, denitrification ($NO_3^- \rightarrow NO_2^-$ and $NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) and anammox ($NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$). Denitrification is sequentially catalyzed by a series of enzymes beginning with the nitrate reductases, Nar or Nap. Nar is a membrane-bound enzyme reductase that reduces $NO_3^- + 2H^+$ to $NO_2^- + H_2O$. Nap enzymes are found among Gram-negative bacteria and serve the same function as Nar, however some denitrifiers are able to couple Nap enzyme with NO_3^- and nitric oxide (NO) to perform aerobic denitrification (Cabello et al., 2009). NO_2^- is further reduced with $2H^+$ to NO and H_2O via the nitrite reductase, Nir. Nitric oxide reductase (Nor) and nitrous oxide reductase (Nos) then reduce NO to N_2O and N_2O to N_2 (Cabello et al., 2009).

Like denitrification, the anammox reaction results in the formation of N_2. This process includes aspects of nitrification and denitrification and includes many of the same pathways as denitrification: NO_2^- is reduced to NO by Nir; NO is then combined with NH_4^+ via a Hao-like protein, hydrazine hydrolase (Hzh) to N_2H_4 where it is oxidized by Hzo (hydrazine-oxidizing enzyme) to N_2 (Cabello et al., 2009).

The occurrence of N cycling in tailings ponds would indicate the presence of rudimentary nutrient cycling, which is important to support multicellular life and complex microbial communities. Nutrient cycling will be essential once the tailings are reclaimed in order to establish a functional ecosystem (Harrison, 2003; Bradshaw and Chadwick, 1980).

1.3 Summary

The oil sands are a valuable resource producing a significant quantity of bitumen for upgrading and export however, they are also costly to the environment. Massive expanses of forests are being disturbed and considerable volumes of fresh water are being taken from the Athabasca River every year, resulting in large quantities of tailings being produced. These tailings contain many toxic contaminants, which make them difficult to reclaim, and contribute to global warming through the release of greenhouse gasses. However, microbial activity has proven capable of reducing the environmental impact through biodensification and natural attenuation resulting in less fresh water being drawn from the Athabasca River and the reduction of toxic hydrocarbons in tailings for use in reclamation.

1.4 Hypothesis

Nutrients are essential for microbial metabolism: Without the correct combination of micro and macronutrients, microbial activity is severely hindered. Although most micronutrients are available in trace amounts in tailings ponds, there is no readily available source of available N, but is N the limiting factor in MFT methanogenesis? This study examined the possibility of anaerobic N_2-fixation as the primary N source in mature fine tailings in conjunction with the degradation of hydrocarbons using citrate as an easily degradable hydrocarbon substitute. The potential of PAM serving as an alternative N source was also investigated.

The results of this study serve to evaluate the bioremediation potential of oil sands tailings ponds, increase our understanding of *in-situ* processes in oil sands tailings ponds and give us greater insight into the nutrient cycling occurring in Alberta's tailing ponds and reclaimed wetland sites.

Table 1-1: General characteristics of mature fine tailings (MFT) from Mildred Lake Settling Basin (MLSB). Table adapted from Siddique et al., 2006.

General Characteristics of Mature Fine Tailings	**Values**
Solids (% by weight)	39.5
Bitumen (% by weight)	4.4
Naphtha (% by weight)	0.4
pH	7.8
Conductivity ($\mu S\ cm^{-1}$)	4200
Alkalinity (as ppm of $CaCO_3$)	1570

Table 1-2: Environmental Pollutants Present in Tailings Ponds. Table adapted from Allen, 2008.

Pollutant (mg L^{-1})	Oil sands tailing pond water	Water Quality Guidelines		
Inorganic		**CEQG**	**USEPA**	**Other**
Bicarbonate	775-950	[a]	[a]	500[b]
Chloride	80-540	[a]	[a]	150[c]
Sulphate	218-290	[a]	[a]	50 [c]
Total dissolved solids	1900-2221	[a]	[a]	1340[d]
Organic				
Benzene	<0.01-6.3	0.37	[a]	[a]
Cyanide	0.01-0.5	0.005	0.005	[a]
Oil and grease	9-92	No odour or visible sheen	[a]	5-10[e]
Phenols	0.02-1.5	0.004	0.001	[a]
Toluene	<0.01-3	0.002	1.3	[a]
Naphthenic Acids	80-110[f]	[a]	[a]	[a]
PAHs	0.01	0.00001-0.00006	[a]	[a]
Trace Metals				
Aluminum	0.07-0.5	0.1	0.75	[a]
Arsenic	0.006-0.015	0.005	0.15	[a]
Chromium	0.003-2	[a]	0.074	[a]
Copper	0.002-0.9	0.002	1	[a]
Iron	0.8-3	0.3	1	[a]
Lead	0.04-0.19	[a]	0.0025	[a]
Nickel	0.006-2.8	[a]	0.052	[a]
Zinc	0.01-3.2	[a]	0.12	[a]

a: No regulation under this agency

b: $NaHCO_3$ toxicity (zooplankton) of 10 milliequivalents HCO_3/L (Mount et al. 1997)

c: Ambient water quality guidelines from the Government of British Columbia (2000)

d: General guideline cited by SETAC (2004)

e: EPEA; Environmental Protection and Enhancement Act; example maximum discharge limits for various Alberta industries (Alberta Environment 1999)

f: Data from FTFC, 2005

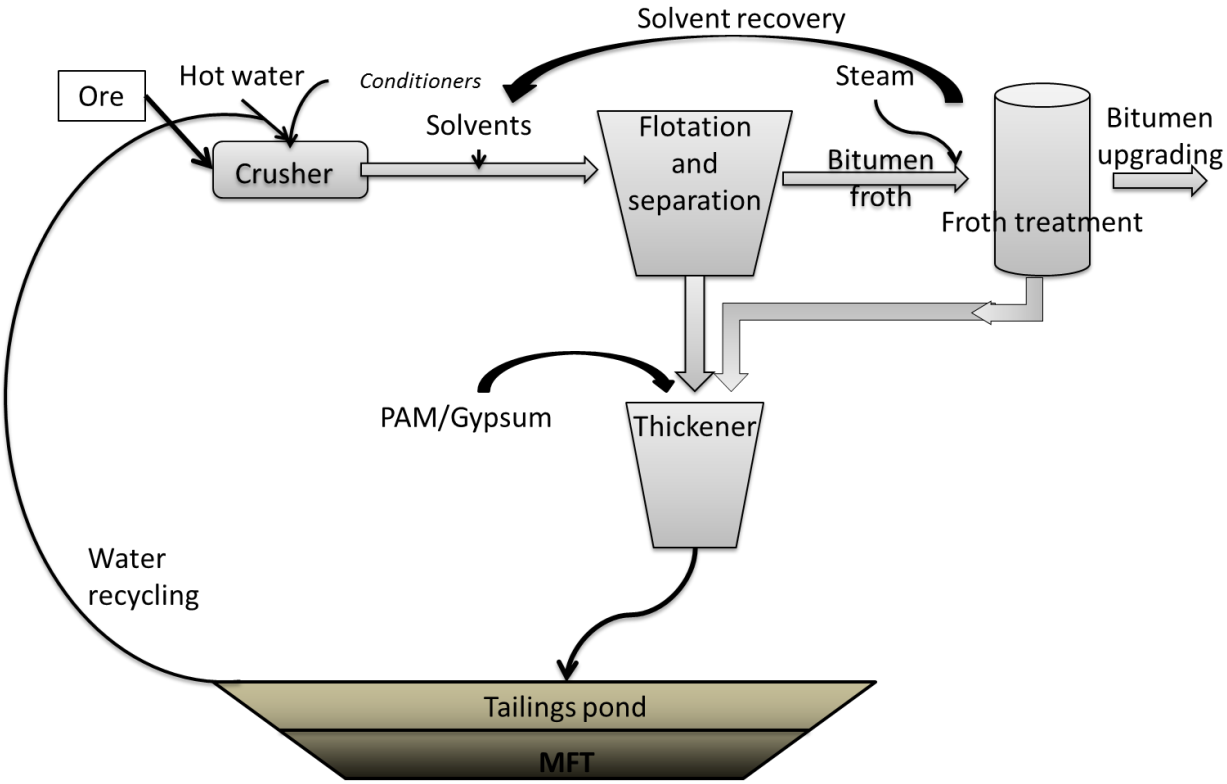

Figure 1-1: Simplified example of modified Clark hot water extraction process (Masliyah et al., 2004).

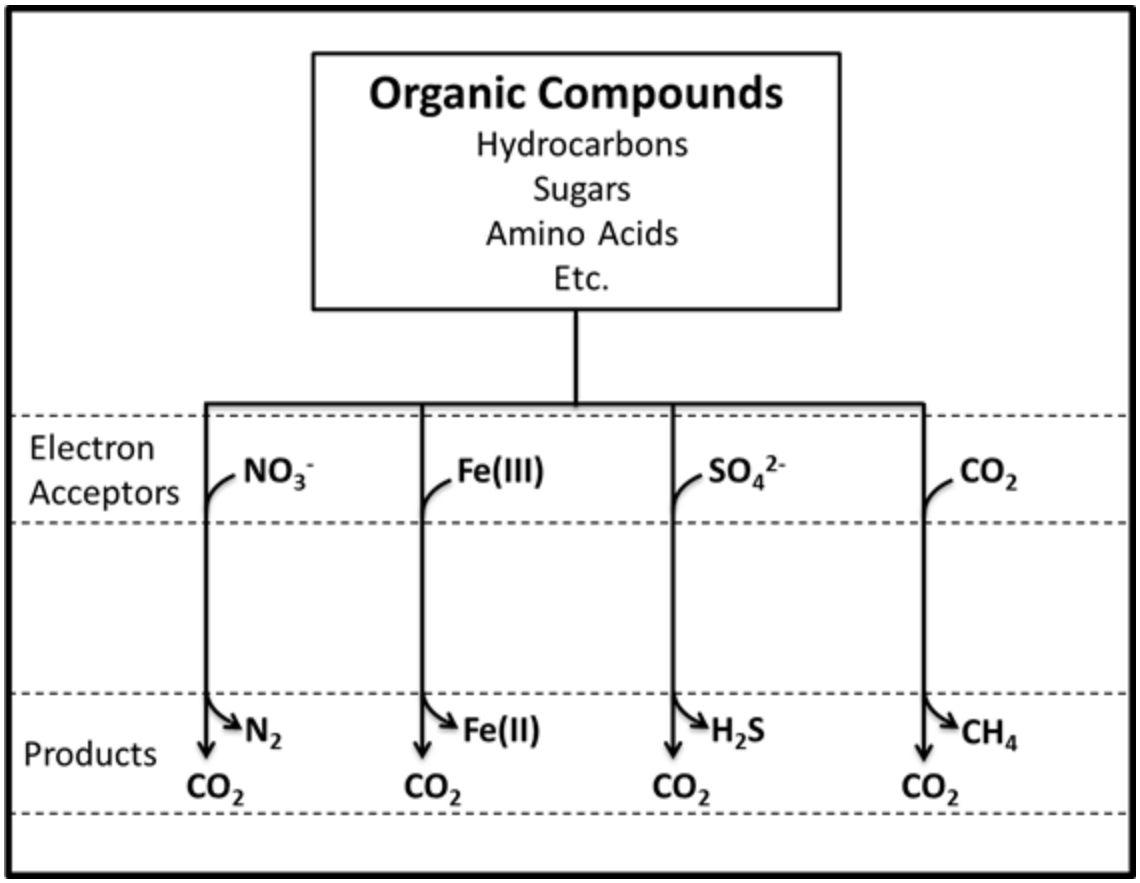

Figure 1-2: Example of anaerobic terminal electron acceptor reactions involved in the degradation of organic compounds (Widdel and Rabus, 2001).

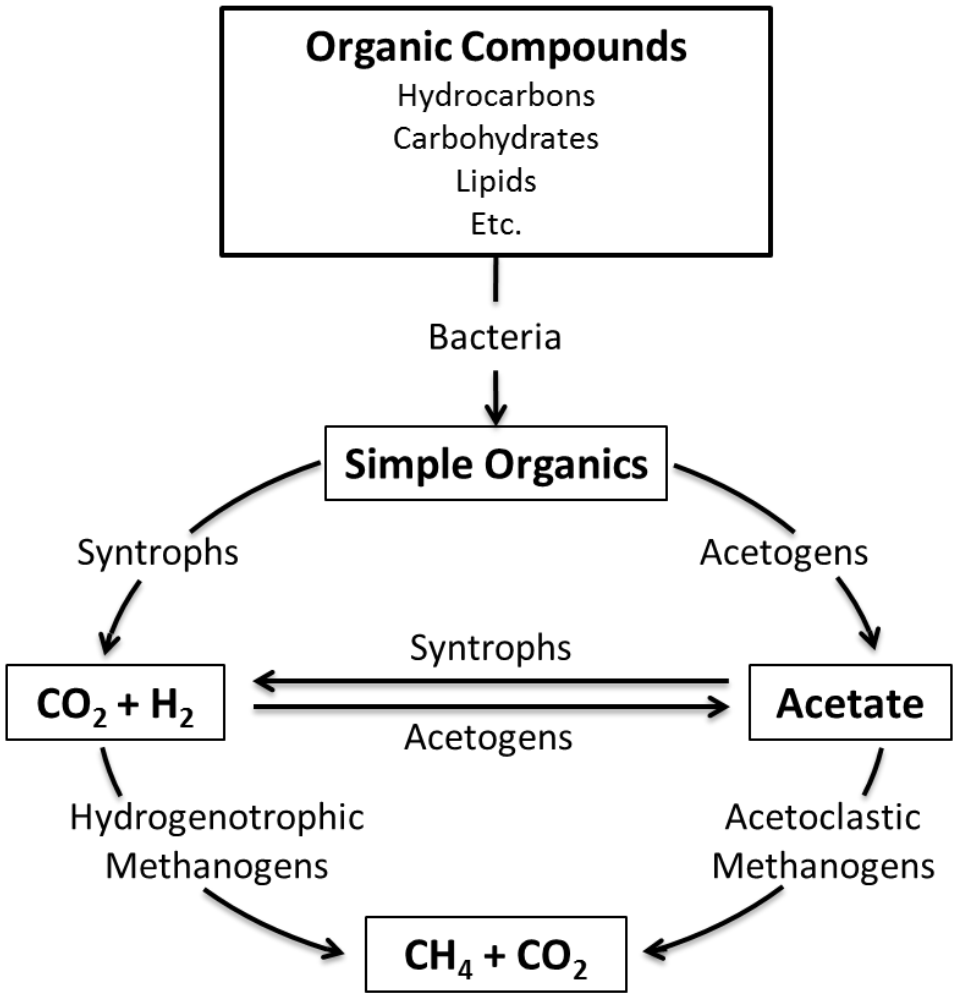

Figure 1-3: General degradation pathway of organic compounds (Siddique et al., 2012; Dassonville and Renault, 2002).

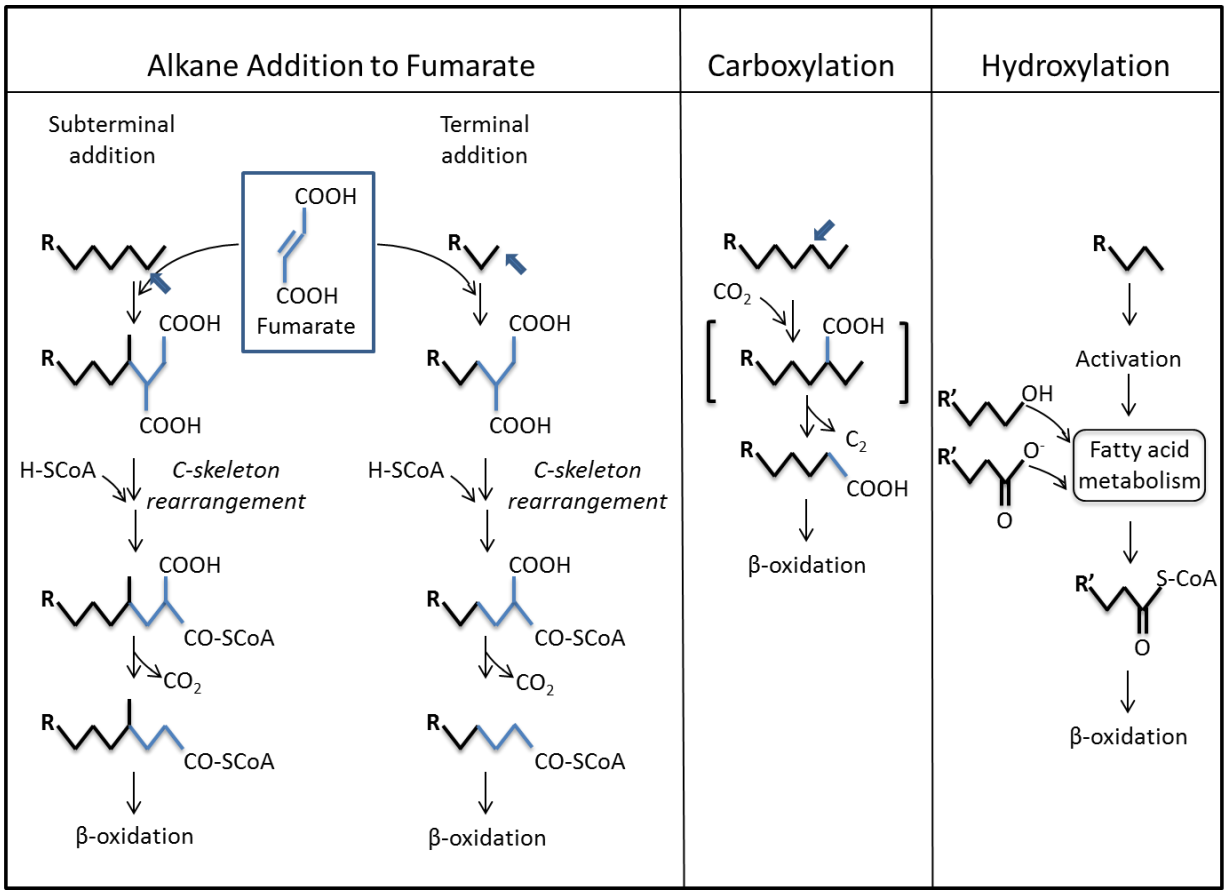

Figure 1-4: Examples of alkane addition to fumarate under iron-, sulphate-reducing and denitrifying conditions, carboxylation under sulphate-reducing and possibly denitrifying conditions and possible mechanism for hydroxylation under nitrate-reducing conditions. Figure adapted from Rojo (2009) and Zedelius et al. (2011) (Kniemeyer et al., 2007).

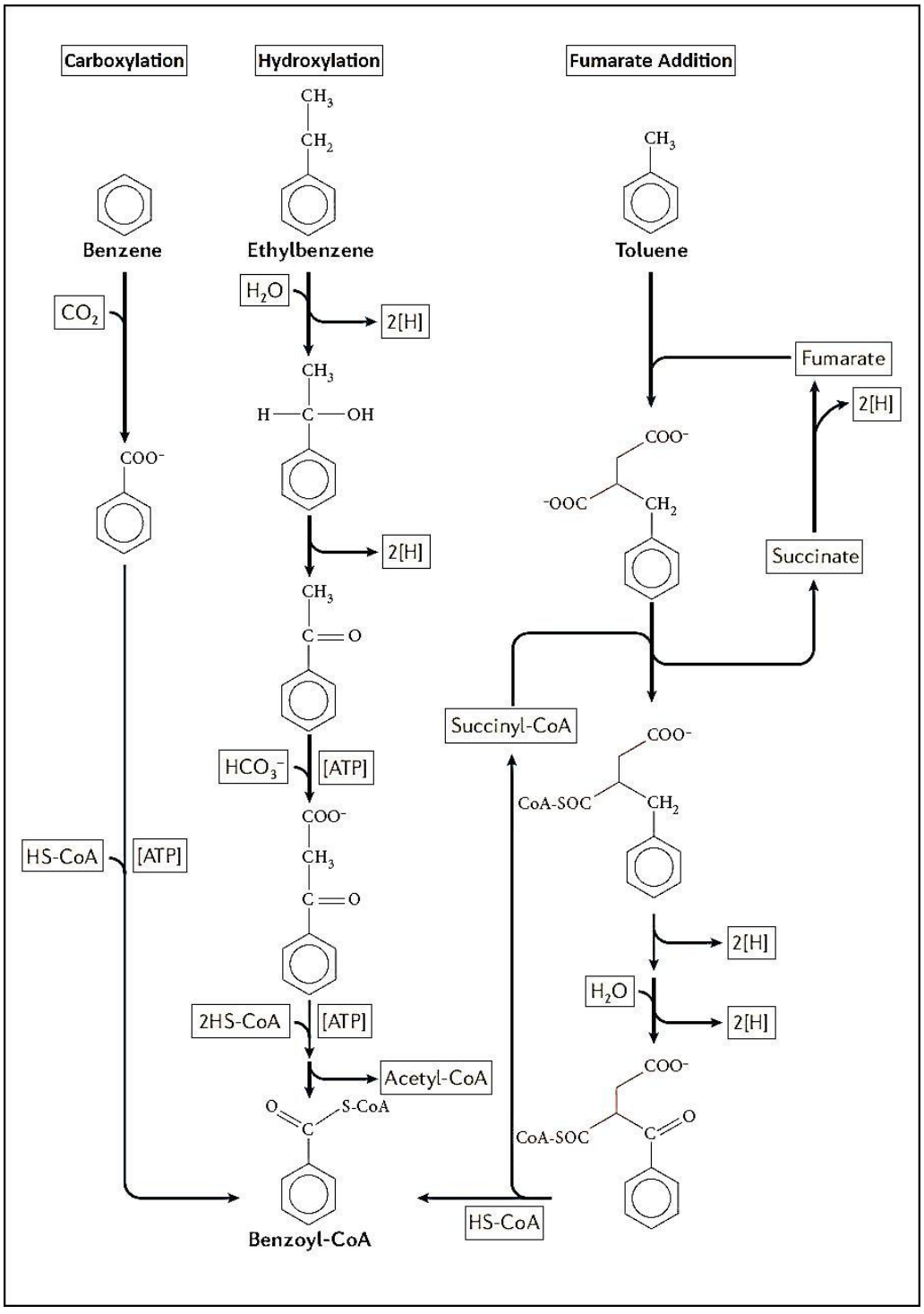

Figure 1-5: Examples of aromatic hydrocarbon degradation under anaerobic conditions. Hydroxylation occurs under methanogenic and denitrifying conditions, carboxylation occurs under sulphate-reducing conditions and is suspected under denitrifying conditions, and fumarate addition occurs under methanogenic, sulphate-, iron- and nitrate-reducing conditions. Figure adapted from Fuchs et al. (2011).

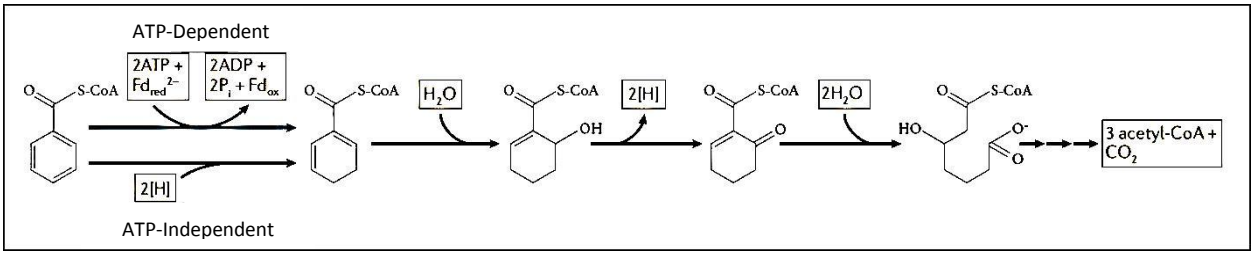

Figure 1-6: Benzoyl-CoA degradation pathway indicating ATP-dependent and ATP-independent pathways. Figure adapted from Fuchs et al. (2011).

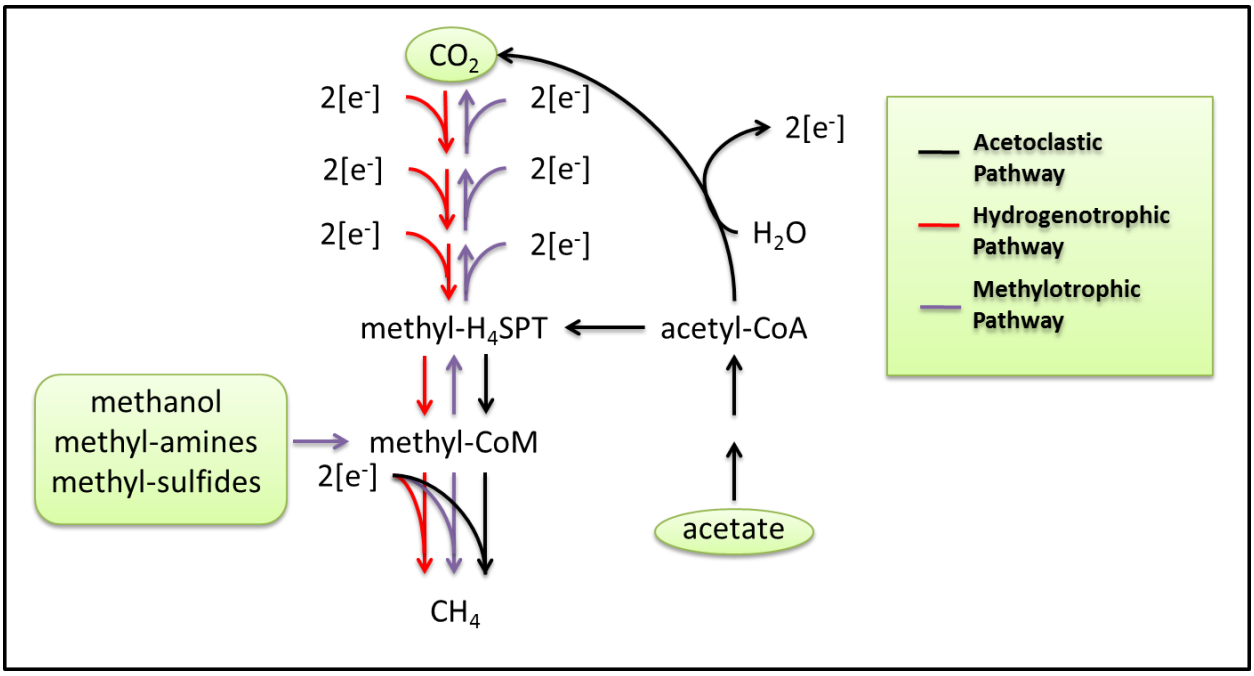

Figure 1-7: Degradation of methanogenic substrates. Hydrogenotrophic pathway shown in red, acetoclastic pathway in black and methylotrophic pathway in purple with intermediates omitted. Electrons provided by H_2. Figure adapted from Rother (2011).

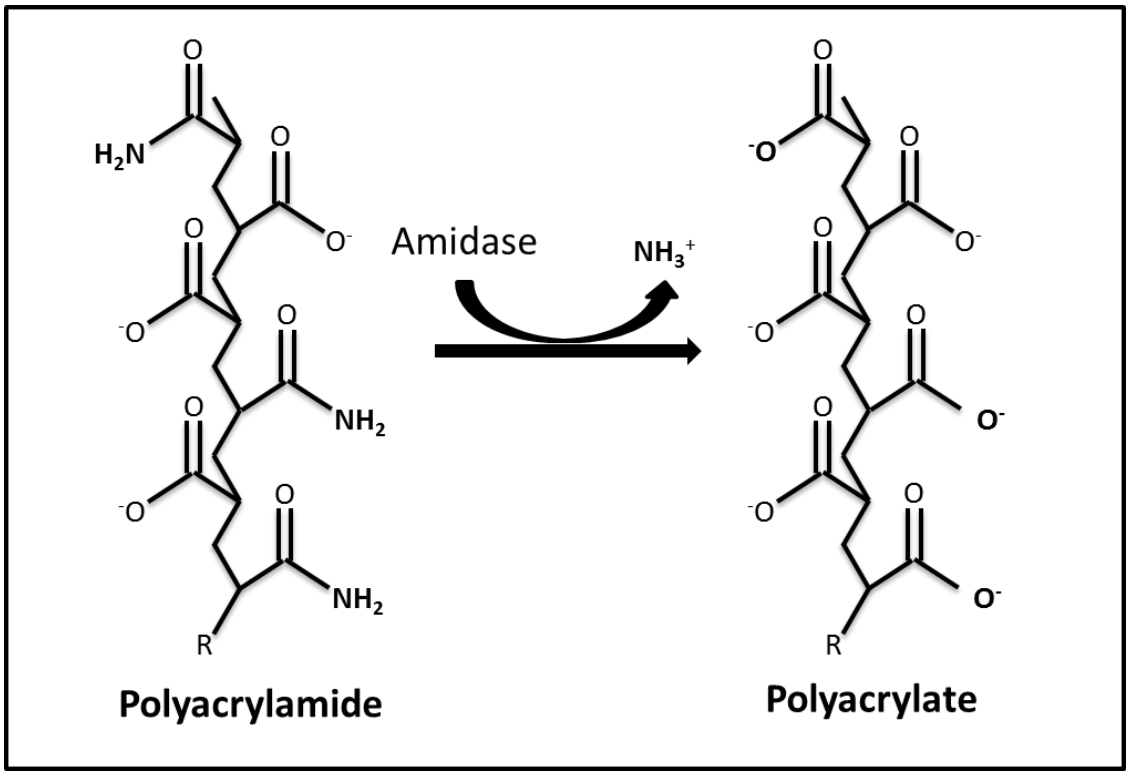

Figure 1-8: Proposed mechanism of PAM hydrolysis. Amine group position in bold. Figure modified from Kay-Shoemake et al. (1998b).

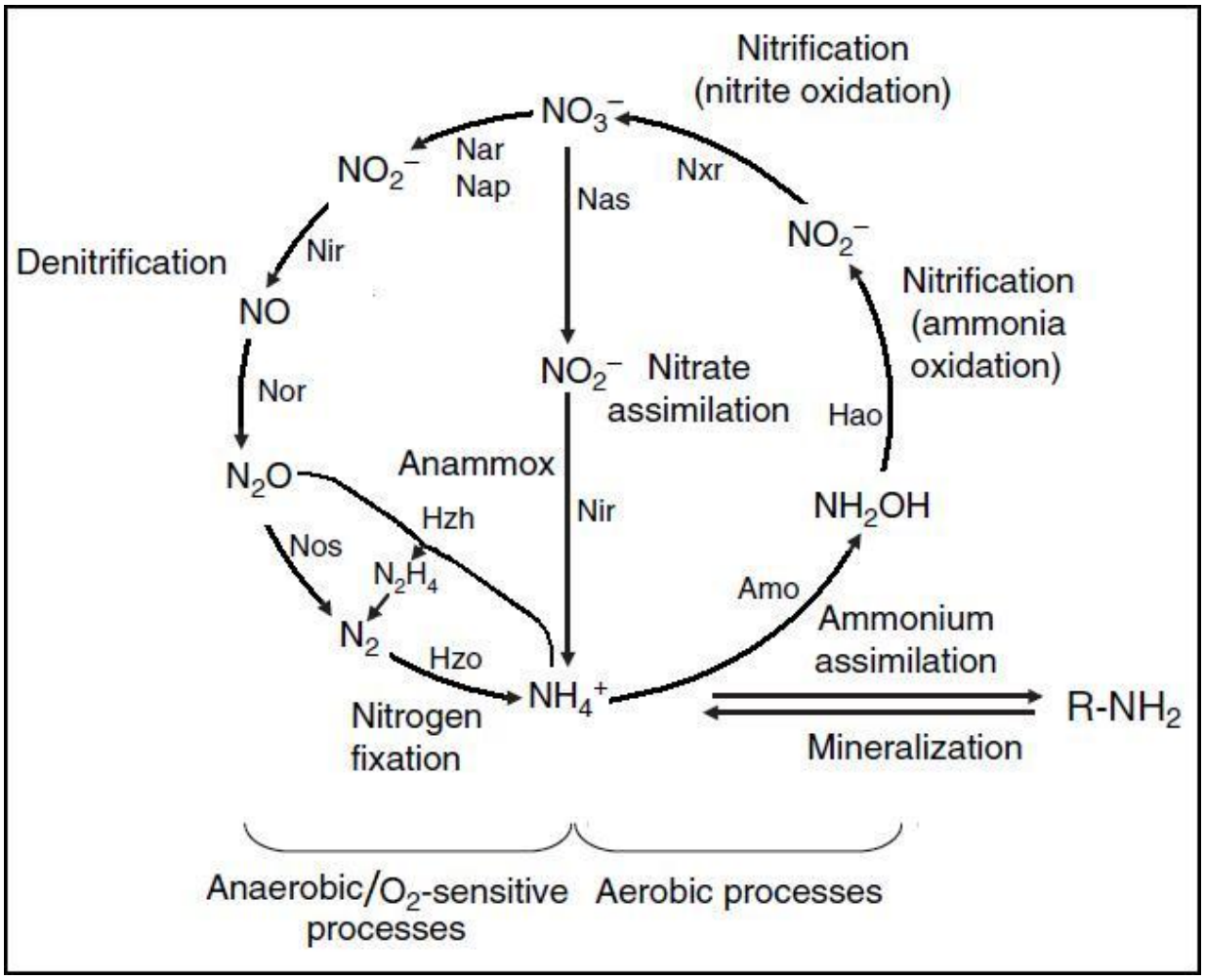

Figure 1-9: Overview of the N cycle, anaerobic and aerobic processes. Amo, ammonia monooxygenase; Hao, hydroxylamine oxidoreductase; Nxr, nitrite oxidoreductase; Nas, assimilatory nitrate reductase; Nir, nitrite reductase; Nar and Nap, nitrate reductase; Nor, nitric oxide reductase; Nos, nitric oxide synthase; Hzh, hydrazine hydrolase; Hzo, hydrazine-oxidizing enzyme. Modified from Cabello et al. (2009).

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Chapter 2. Are Methanogenic Consortia Fixing N_2 During Organic Carbon Metabolism in Alberta's Oil Sands' Tailings Ponds?

2.1 Introduction

The Alberta oil sands in northern Alberta are one of the largest oil reserves in the world containing an estimated 170 billion barrels of crude bitumen. While only 20% of oil sands formations are accessible through open pit mining, surface mining is still the dominant method of bitumen extraction in the region (Government of Alberta, 2012a). Bitumen is recovered from the ores using modified variants of the Clark hot water extraction process which uses heated water, diluents and chemicals such as surfactants to separate the bitumen from the sands (Masliyah et al., 2004). The remaining slurry is piped into large tailings ponds; as of 2010, these ponds have accumulated a total 830 million m^3 and covered an estimated 176 km^2 area (Government of Alberta, 2013). The larger particles of the slurry materials quickly settle out on dykes and in the ponds leaving a colloidal suspension composed of sand, clay, residual bitumen and unrecovered diluents (6-10 wt% solids) (Allen, 2008; Chalaturnyk et al., 2002). These fine tailings are left to settle, consolidate and release water which can be recycled; this process eventually results in the formation of a stable slurry (~30 wt% solids) known as mature fine tailings (MFT) (Chalaturnyk et al., 2002). It is estimated that further densification of MFT to a mechanically stable soil-like material for reclamation could take up to 150 years (Eckert et al., 1996; Voordouw, 2012), however efforts have been made to shorten this time. The addition of thickening agents such as gypsum, used by Syncrude and Suncor, results in the formation of composite or consolidated tailings (CT), which greatly reduces the time required for dewatering and consolidation (List and Lord, 1997; Mamer, 2010). Shell Albian Sands uses the flocculant

polyacrylamide (PAM) (as reviewed by Li, 2010), which binds with particles forming heavy aggregates that quickly settle out resulting in water release and formation of thickened tailings (Kindzierski, 2001). Both of these methods involve the addition of thickening agents, however microbial processes have also been found to play a similar role by changing pore water chemistry and clay surface properties (Siddique et al., In review).

Since the early 1990's, methane (CH_4) bubbles have been visible on the surface of tailings ponds beginning with Syncrude's Mildred Lake Settling Basin (MLSB). Since then, MLSB has been releasing millions of litres of CH_4 per day (Holowenko et al., 2000; Siddique et al., 2008). This CH_4 is the product of the degradation of residual hydrocarbons present in the MLSB (Siddique et al., 2006; 2007). Albian's paraffinic solvent (C_5-C_6) and organic compounds such as citrate, used as a dispersant during bitumen recovery, can both be utilized in the formation of CH_4 (Li, 2010; Masliyah et al., 2004; Zhang et al., 2009). In 2003, Fedorak et al. found that methanogenesis in MFT and CT cultures increased the rate of tailings' densification. Evidence suggests that the formation of de-watering channels created by escaping CH_4 bubbles plays a significant role in water release from MFT (Guo, 2009; Brown et al., 2012); this water can then be recycled in the extraction process decreasing the draw of fresh water from the river (Government of Alberta, 2012b). This process presumably is mediated by syntrophs and fermenters which degrade the hydrocarbons and other organic compounds into acetate, H_2 and CO_2; these products can then be utilized by methanogens (Ferry, 1993; Penner and Foght, 2010; Siddique et al., 2012). However, for microbial metabolism to occur, sufficient nutrients must be present. The C:N ratio for anaerobic metabolism is considered to range between 50:1 and 100:1 under anaerobic conditions but it has been reported as high as 180:1 (Droste, 1997; USEPA,

1995; Ammary, 2004). Although the concentrations of NH_4^+ are high in Syncrude's MLSB and Suncor's pond 2/3 (14 mg L^{-1} and 19.6 mg L^{-1} respectively, Allen, 2008; Mah et al., 2011), Albian tailings ponds were deficient in available N (Supplementary Materials Figure A-1). Despite this deficiency, the Albian Muskeg River Mine tailings pond is actively producing methane (Li, 2010). This nutrient deficiency is therefore being met by means other than available N. PAM deamination or N_2-fixation might be the possible mechanisms for meeting N requirements.

Haveroen at al. (2005) examined the possibility of PAM as a N source under anaerobic conditions. They established a series of transfer cultures using domestic sludge as well as consolidated tailings and MFT from MLSB. They found that PAM stimulated methanogenesis under N-depleted conditions indicating that PAM was likely serving as a N source. These results did not support N_2-fixation as a N source because the N deficiency in cultures without PAM could not be overcome by the microbial community resulting in much lower CH_4 generation. Nitrogen fixation, however, has been well documented in methanogens and as such, N-fixing methanogens may play a role in Albian's microbial community (Bomar et al., 1985; Belay et al., 1984; Lobo and Zinder, 1990; Buckley et al., 2008). As of yet, there have been no published studies examining N_2-fixation in conjunction with methanogenesis in oil sands tailings ponds, and as such, this possibility was investigated in this research study.

2.2 Materials and Methods

2.2.1 Chemicals and Reagents

Polyacrylamide (Magnafloc LT27AG) was purchased from Ciba Specialty Chemicals Ltd, Mississauga, Ontario, Canada and Tri-sodium citrate dihydrate (purity >99.8%) was purchased from BDH, Halifax, Nova Scotia, Canada. All other chemicals (analytical reagent grade) were purchased from Fisher Scientific, Ontario, Canada. For a full list of chemicals and reagents, their manufacturers and grade, refer to Supplementary Materials C-1.

2.2.2 Gases

$^{15}N_2$ (98% purity) was purchased from Sigma-Aldrich, and acetylene (scientific grade), 30% CO_2/70% N_2 and 30% CO_2/70% Ar (scientific grade) and other gasses (certified standard) were purchased from Praxair, Edmonton, Alberta, Canada.

2.2.3 Sample Collection

Mature fine tailings (MFT) were collected in bulk in June, 2008 from the Muskeg River Mine Tailings Pond operated by Shell Albian Sands Inc. at a depth of 7 m below the surface (UTM coordinates 0465371E 6342304N) and stored in a sealed plastic pail in the dark at room temperature (Li, 2010). Dry weight was determined to be 25% by drying 1 mL in an oven using two replicates. These tailings had been used in the preliminary experiment by Siddique in 2008 (Supplementary Figure A-2). In 2011, tailings in the pail were thoroughly homogenized by hand mixing with an autoclave sterilized metal rod (under open air) and transferred using an autoclave-sterilized 500 mL beaker to 3-L wide mouth plastic jugs which had been rinsed with

70% ethanol and UV-irradiated for 24 h. These samples were then used immediately in the culture setup in 158-mL serum bottles.

2.2.4 Preparation of Media

The nutrient media were prepared using a modified version of the methanogenic medium described by Fedorak and Hrudey (1984). In the medium containing N, a 100-fold stock of Mineral Solution I was prepared in ddH_2O that contained the following (per litre): NaCl (50 g), $CaCl_2.2H_2O$ (10 g), NH_4Cl (50 g), and $MgCl_2.6H_2O$ (10 g). The 1000-fold stock of Mineral Solution II contained: $(NH_4)_6Mo_7O_{24}.2H_2O$ (10 g), $ZnSO_4.7H_2O$ (0.1 g), H_3BO_3 (0.3 g), $FeCl_2.4H_2O$ (1.5 g), $CoCl_2.6H_2O$ (10 g), $MnCl_2.4H_2O$ (0.03 g), $NiCl_2.6H_2O$ (0.03 g), and $AlK(SO_4)_2.12H_2O$ (0.1 g). In the N-deficient medium, the 100-fold stock of Mineral Solution I contained the following salts: NaCl (50 g), $CaCl_2.2H_2O$ (10 g), and $MgCl_2.6H_2O$ (10 g) whereas the following salts were added to prepare the 1000-fold stock of N-free Mineral Solution II: $Na_2Mo_7O_{24}.2H_2O$ (13.7 g) ($Na_2Mo_7O_{24}$ was substituted in place of $(NH_4)_6Mo_7O_{24}$ and the quantity was adjusted to ensure that the concentration of molybdenum remained consistent between the two types of medium), $ZnSO_4.7H_2O$ (0.1 g), H_3BO_3 (0.3 g), $FeCl_2.4H_2O$ (1.5 g), $CoCl_2.6H_2O$ (10 g), $MnCl_2.4H_2O$ (0.03 g), $NiCl_2.6H_2O$ (0.03 g), and $AlK(SO_4)_2.12H_2O$ (0.1 g) (Fedorak and Hrudley 1984). Vitamin B solution was omitted from all media due to its N content (Haveroen et al., 2005) and 2-methyl-*n*-butyric acid was also omitted as it can be utilized in carboxylation reactions by certain microorganisms (Robinson and Allison, 1969), or possibly serve as a carbon source. The solutions were stored at room temperature for several weeks as we waited for the serum bottles to arrive prior to combining the solutions to make media.

Solutions were combined to make media as follows: boiled ddH_2O 1 L; Mineral Solution I 14 mL L^{-1}; Mineral Solution II 1.4 mL L^{-1}; resazurin 14 mL L^{-1} (0.1% resazurin sodium salt); phosphate solution 14 mL L^{-1} (50 g L^{-1} KH_2PO_4); $NaHCO_3$ 5.7 g L^{-1}. Because the N-free Mineral Solution II would not fully dissolve even after vigorous shaking and use of low heat and magnetic stir-bar apparatus for 48 h, it had to be vigorously shaken immediately before it was used in the medium to ensure that all minerals were re-suspended.

Media were prepared using a magnetic stir-bar apparatus (no heat) and sparged with 30% CO_2 balance N_2 gas until pH of ~7.2-7.4 was achieved (about 1.5 hours). The medium (100 mL) was transferred to 158 mL clear glass serum bottles (158-mL actual volume, Wheaton 125-mL glass bottles, #223748) using serological pipettes while continuously sparging the flasks containing the media with CO_2/N_2 until all the media had been transferred from the flasks to the serum bottles. The bottles were sealed with autoclave-sterilized blue butyl stoppers (Bellco Glass Inc. #2048-11800) and crimped with aluminum caps (Wheaton unlined aluminum tear-off seals, #22419306). The headspace in the bottles was flushed with CO_2/N_2 gas for 5 min using Becton Dickinson 18G 1 ½ needles (ref: 305196) followed by autoclaving for 30 min using the liquid cycle. After the medium had cooled for 24 h, 1 mL filter-sterilized (Millex-GS 0.22 µm sterile filter ref:SLGS03355) anaerobic 2.5% sodium sulphide solution was added to each bottle using a sterile BD 1 mL syringe with luer-lok tip (ref: 309628) connected to BD 25G 5/8" needle (ref: 305122), which had been flushed with CO_2/N_2, to remove O_2.

2.2.5 Culture Setup for Experiment

Using an anaerobic chamber, 50 mL MFT was transferred to autoclaved 158-mL serum bottles using 60 mL sterile syringe (BD luer-lok tip syringe ref:309653). Because the MFT coated the inside of the syringes after the first use, a weigh scale was used to ensure all bottles were consistent by weight (± 0.1 g). Bottles were sealed with autoclaved blue butyl stoppers cut to a depth of 1/3" and crimped using aluminum caps.

Fifty milliliters of prepared methanogenic medium was added to each sealed anaerobic bottle containing MFT using a 60-mL sterile syringe with 18G 1 ½ needles (BD ref: 305196). Medium injection was paused twice during the addition to allow the pressure to equilibrate between the syringe and bottle and avoid over pressurizing the serum bottle. The bottles were prepared according to the experimental design given in Table 2-1. Cultures were shaken by hand and then flushed with CO_2/N_2 or CO_2/Ar for 5 min, alternating flushing and removing headspace at 30-sec intervals using a vacuum pump. Flushing was done using 18G needle tips and 0.22 µm sterile filters on the incoming gas tube. All cultures were incubated in the dark for 2 weeks at room temperature (22°C) preceding the experiment to acclimatize the microbial communities to the new media and deplete residual background concentrations of any available N in the N-deficient cultures (Supplementary Figures A-1, A-3). At the start of the experiment (Day 0), the presence of CH_4 was verified in the headspaces of bottles to ensure the viability of the cultures and the headspaces were flushed again. For the preparation of amendments, both trisodium citrate and PAM were dissolved in sterile milliQ water in autoclaved glass bottles (0.933 g in 15 mL and 0.05 g in 20 mL, respectively) and the headspaces

were flushed with CO_2/N_2 or CO_2/Ar to ensue anaerobic conditions. The citrate was then filter-sterilized, however the PAM could not be filter sterilized nor was it autoclaved, to avoid possible thermal degradation (Haveroen et al., 2005). The citrate was added to the bottles at a final concentration of 200 mg L^{-1} (0.5 mL per culture) while 1.25 mg PAM (0.5 mL, 100 mg L^{-1} dry weight) was also added according to the experimental design to mimic *in-situ* conditions (Shell Albian Sands, personal communication, March 7, 2008). Cultures were incubated in the dark at room temperature. Citrate and PAM concentrations were chosen to mimic *in-situ* concentrations. Anaerobic nanopure sterile water was used as an amendment control in cultures receiving no citrate or PAM. All cultures were shaken thoroughly by hand to ensure homogeneity and the exposed surfaces of stoppers were sterilized with ethanol wipes prior to syringes being inserted. Sterilized control cultures were autoclaved twice per day for 99 min on each of four successive days prior to citrate addition.

2.2.6 Culture Sampling

Seven-milliliter MFT samples were taken from each serum bottle using needle and syringe and placed in 8-mL sample vials (National Scientific, ref: 03393A) at Day 0, Week 10 and Week 16 of incubation and stored at -20°C for chemical and molecular analyses.

2.2.7 Methane (CH_4) Analysis

CH_4 analysis was performed by injecting 0.1 mL of headspace gas into a gas chromatograph equipped with flame ionization detector (GC-FID Hewlett Packard 5890; column: Poropak Type R; oven temperature: 30°C and flow rate of He: 12.5 mL min^{-1}) using 28G ½" 1/2cc insulin syringes (BD ref:329461). Readings were made on triplicate headspace samples

for each culture. Standards (0.16%, 4%, 8%, 15% and 25% of CH_4 in 158-mL serum bottles) were prepared and used to construct a standard curve to quantify CH_4 production. Cultures were shaken by hand before each reading to ensure that all trapped CH_4 bubbles were released from the MFT into the headspace. Readings were taken weekly for the duration of the experiment. Gas pressure inside the bottles was also measured at the time of CH_4 analysis to calculate µmoles of CH_4. This was done by measuring the concentration of CH_4 (%) in the headspace and converting it to total µmoles using the Ideal Gas Law (Supplementary Materials Calculation A-1).

The theoretical maximum of CH_4 production was calculated using the following equation derived from the Symons and Buswell equation (Roberts, 2002):

Citrate: $C_6H_5O_7 + 1.25\ H_2O \rightarrow 4.125\ CO_2 + 1.875\ CH_4$

2.2.8 Citrate Analysis

Citrate analysis was done by diluting 0.5 mL of the culture liquid phase in 0.5 mL monobasic ammonium phosphate eluent solution in 1.5 mL clear glass HPLC vials (National Scientific, ref:C4000-1W) (Supplementary Methods B-1). Eluent was prepared by dissolving 10 g monobasic ammonium phosphate in 900 mL milliQ water and adjusting the pH to 2.8 with 1 M phosphoric acid. The solution was then diluted with milliQ water to the 1 L mark on the flask and vacuum filtered using a 0.45 µm HA filter (hydrophilic, Millex). All samples were filtered with 0.22 µm Millex filters to remove particulates and analyzed on an automated Agilent 1100 series HPLC system (Agilent 1100 series HPLC system equipped with degasser, pump,

autosampler, thermostatted column compartment, guard frits and UV detector) using blanks (0 mg L^{-1}) and prepared citrate standards from 2 to 200 mg L^{-1}.

2.2.9 Acetylene Reduction Assay

One experimental culture bottle from each citrate-amended culture was sacrificed and divided into three 60-mL serum bottles (60-mL actual volume, Wheaton 50-mL glass serum bottle, #223745). For a positive control, the N_2-fixing bacterium *Pseudomonas sp.* 5.1b (Eckford et al, 2002), was cultured in N-deficient medium containing sucrose, mannitol, and sodium lactate as carbon sources (Supplementary Methods B-2). Negative controls were prepared by adding NH_4Cl in place of NaCl to *Pseudomonas sp.* 5.1b cultures (Rennie, 1981). Sterile negative controls were prepared by sacrificing a sterile MFT culture replicate described in section 2.2.5. All bottles were sealed using butyl stoppers and tear away caps. After flushing the headspace with CO_2/N_2, acetylene was injected into the headspace of all bottles to a final concentration of 3 vol% (74.4 µmoles) acetylene in the headspace, and citrate was added to the tailings culture to a final concentration of 200 mg L^{-1}. Sterile nanopure water was used as an amendment in controls compensating for the volume of citrate solution added to other bottles. The production of ethylene during acetylene reduction was measured daily for 4 d using GC-FID. Standard curves for acetylene and ethylene were constructed using acetylene and ethylene standards prepared at 0.017%, 0.1%, 0.8%, 1.6% and 3.3% concentrations in empty 60 mL serum bottles. Gas pressure inside the cultures was also measured at the time of analysis to calculate µmoles of ethylene (Supplementary Materials Calculation A-1).

2.2.10 $^{15}N_2$ Incorporation Analysis

After the completion of the acetylene reduction assay, only the cultures with CO_2/N_2 headspaces were selected and flushed with CO_2/N_2. The cultures were incubated in the dark at ~22°C for two weeks to deplete any remaining citrate and restore CH_4 production. CH_4 was measured to ensure the presence of methanogenic activity, and then 1000 mg L^{-1}citrate was added to the cultures. The headspace in the bottles was evacuated using 60 mL syringes and 20G 1" needle tips (BD ref:305175) until such a strong vacuum existed that the syringe plunger could not be further drawn. Then, 60 mL $^{15}N_2$ gas was injected into the headspace. The cultures were incubated for 3 weeks in the dark at room temperature, after which time 0.5 mL of the tailings were removed, dried in an oven and 5 mg were ground and combusted using a Costech ECS 4010 Elemental Analyzer System connected to a Conflo III & continuous Flow Delta Plus Advantage IRMS. The combusted samples were separated by gas chromatography and detected by a thermal conductivity detector (TCD), which was calibrated against the International Reference Scale for air, to determine the percentage of ^{15}N. Analysis was conducted by the Natural Resources Analytical Laboratory in the Department of Renewable Resources (AOAC, 2001; Sparks, 1996; Costech, 2001; de Groot, 2004). CH_4 production was confirmed by GC-FID at the end of the experiment but due to over pressurization, CH_4 concentrations could not be accurately determined.

2.2.11 Anions, Cations, Metals, pH and Nitrogen (N) concentrations

Nitrate (NO_3^-), nitrite (NO_2^-) and ammonium (NH_4^+) concentrations in each liquid phase sample were determined for all the treatments through colorimetric analysis using a

SmartChem Discrete Wet Chemistry analyzer (Maynard and Kalra, 1993); the analysis was performed by the Natural Resources Analytical Laboratory. Total N was determined in oven dried MFT from cultures by Dumas Combustion using a Costech Elemental Analyzer (AOAC International, 2000), as performed by the Natural Resources Analytical Laboratory. Anion, cation and trace metals samples were prepared for analysis by digesting 0.3 g of oven dried MFT slurry from cultures in HNO_3 and 30% H_2O_2. Anion concentrations were determined using a Dionex DX600 Ion chromatograph (Pfaff, 1998), performed by the Biogeochemical Analytical Service Laboratory at the University of Alberta. Cations and trace metals were determined by inductively coupled plasma – optical emission spectrometer (Creed et al., 1994), performed by the Biogeochemical Analytical Service Laboratory. The pH was estimated for the tailings suspension by adding a drop of slurry to pH measuring paper strips (Sigma Chemical Company pH test strips, ref: P-4536, P-3536) from samples taken at Day 0, week 10 and Week 16. The pH of the tailings liquid phase was measured using a pH meter (Fisher Scientific Accumet Research AR 20 pH/conductivity meter) in samples of the liquid phase separated from the slurry which were also used in the citrate analysis and available N analyses.

2.2.12 Microbial Community Analysis

For the characterization of microbial communities, DNA was extracted from MFT samples collected at Day 0 and Week 10 from cultures of all the treatments (MFT mixed with or without N media and amended with citrate and/or PAM under CO_2/N_2 headspaces) using the bead beating extraction protocol (Li, 2010). For DNA extraction, two 300-µL MFT samples were withdrawn from each culture and placed in premeasured, autoclaved 2-mL screw cap tubes

(Sarstedt ref:72.692.007) containing 0.5 g of 0.1 mm and 0.5 g of 2.3 mm diameter zirconium-silica beads (Biospec Products Inc. cat:11079101z/11079125z). After this, 300 µL of 0.1 M phosphate buffer (prepared by adding monobasic solution [1.3799 g $NaH_2PO_4.H_2O$ + 100 mL milliQ H_2O] to dibasic solution [1.4196 g Na_2HPO_4 + 100 mL milliQH_2O] until the solution reaches pH 8 and then filter sterilized) and 300 µL of 10% sodium dodecyl sulphate (SDS) solution (48 mL milliQ H_2O, 2 mL 5M NaCl, 50 mL 1M Tris-HCl and 10 g SDS) were added to each tube followed by 300 µL 24:1 chloroform:isoamyl alcohol solution. Tubes were shaken (beadbeaten) in a Mo-Bio homogenizer at 600 reciprocations per min (rpm) and centrifuged for 5 min at max speed (15,000 rpm) in an Eppendorf microfuge. After centrifugation, the supernatant was transferred into a sterile 2-mL Eppendorf tube. These steps were repeated with fresh reagents, beadbeating at 2000 rpm and again at 6000 rpm for each sample to ensure complete DNA extraction. Three different speeds were used to ensure that all cells had been lysed; this was done in stages to remove DNA extracted at lower speeds and avoid shearing the DNA. Ammonium acetate was added to the pooled supernatants to obtain a final concentration of 2.5 M. The tubes were mixed by inverting by hand and were then centrifuged for 7 min at max speed. The supernatants were transferred to new tubes and 0.54 volume of isopropanol was added to each supernatant and incubated at -20°C for ~24 h. DNA was recovered through precipitation by centrifuging the tubes for 30 min at maximum speed and draining the isopropanol. After air drying the DNA pellets for 2 h, DNA pellets were dissolved in 10 µL autoclaved nanopure water and all replicates were combined to form composite samples. A region of the 16S rRNA gene was amplified by PCR using the Pyrotag Sequencing Protocol (Supplementary Methods B-3) (Li, 2010). Forward primer 5'-CTATGCGCCTTGCCAGCCCGCTCAG

aaa ctY aaa Kga att gRc gg, reverse primer acg ggc ggt gtg tRc for the 16S rRNA gene and linker sequence 5'-CCATCTCATCCCTGCGTGTCTCCGAC were used in combination with sample-specific barcodes. The PCR products were sent to Genome Quebec for 454-pyrosequencing and resulting files were processed using mothur (Supplementary Methods B-4) (Schloss et al., 2009) and the Phoenix Visual Genomics pipeline for sequence quality control (Visual Genomics, 2012). The SILVA database was used for identification of the microbial community.

2.3 Results

2.3.1 Methanogenesis

CH_4 production from microbial metabolism was measured in different MFT cultures described in Table 2-1 and the data are presented in Figure 2-1. Cultures with N-deficient methanogenic medium contained effectively no available N with the exception of low background concentrations of NO_3^- (below 0.35 mg L^{-1}) (Supplementary Figures A-3).

In the cultures with a CO_2/N_2 headspace, the presence or absence of N did not have an effect on CH_4 production in the unamended treatments (Figure 2-1a). Methane production remained low in baseline controls with and without N over the course of the experiment reaching only 15.2 (±1.5) and 26.1 (±3.7) µmoles of CH_4, respectively by Week 6 and 25.9 (±4.8) and 38.4 (±2.8) µmoles of CH_4 by Week 16. Because these treatments were not amended with a labile carbon source, this CH_4 likely represented the degradation of endogenous hydrocarbons, metabolites or biomass in the MFT.

Methane concentrations in citrate-amended treatments quickly surpassed those of the unamended baseline controls. This was due to the availability of a labile carbon source which was added to cultures at Day 0, Week 6, Week 10 and Week 13. Initially, the rate of CH_4 production in amended cultures with N surpassed the amended cultures without N due to the availability of a fixed source of N in the medium (Supplementary Figure A-4). However, after the citrate was depleted, amended treatments with and without N had produced similar amounts of CH_4 at 204.9 (±3.2) and 216.0 (±25.0) µmoles of CH_4, respectively. Following the citrate amendment at Week 6, the rate of CH_4 production was the same in all amended treatments regardless of whether or not available N was present in the media indicating that the N-deficient cultures were overcoming N deficiency presumably by fixing N_2 in the headspace. By the end of the experiment, amended treatments had produced 692.3 (±5.6) and 736.4 (±29.0) µmoles of CH_4, respectively.

Cultures with PAM exhibited the same trends as the amended and unamended treatments. The unamended treatment with PAM produced CH_4 at a low rate similar to the other baseline controls producing 22.6 (±4.8) µmoles of CH_4 by Week 6 and a total of 33.1 (±0.6) µmoles of CH_4 by the end of the experiment. Amended cultures with PAM exhibited the same trend in CH_4 production as the amended treatment without N. After an initial delay in CH_4 production, CH_4 concentrations were similar to those in the other amended treatments reaching 204.4 (±4.4) µmoles of CH_4 at Week 6. After citrate addition, amended cultures with PAM produced CH_4 at the same rate as the other amended treatments and reached 736.3 (±3.1) µmoles of CH_4 at Week 16.

Interestingly, cultures with a CO_2/Ar headspace followed the same trend in CH_4 production as those with CO_2/N_2 (Figure 2-1b). Unamended baseline controls maintained low rates of CH_4 production over the course of the experiment at similar concentrations to treatments with CO_2/N_2 headspaces. A similar delay in CH_4 production was also seen in the first few weeks between amended treatments with and without N (Supplementary Figure A-4). This indicated the possibility that residual N_2 was present in the Ar headspace treatments either due to N_2 contamination in the Ar gas or incomplete purging of the headspace with CO_2/Ar. In all citrate-amended cultures, citrate depletion was concomitant with CH_4 production resulting in the complete degradation of amended citrate in all living cultures (Figure 2-2). Neither CH_4 production nor citrate depletion was observed in sterile control cultures; citrate accumulated in the sterile controls after subsequent citrate additions. Because only the liquid phase could be analyzed, the small decrease where citrate appears to represent less than 200 mg L^{-1} is likely due to either adsorption to MFT solids or the spiking method for citrate addition to the sealed bottles.

2.3.2 Acetylene Reduction

Only citrate-amended cultures were used in the acetylene reduction experiment due to their greater metabolic activity, apparent from their higher rates of methanogenesis compared to the baseline controls as seen in Figure 2-1. Citrate (200 mg L^{-1} final concentration) was added to each culture at Day 0 and monitored for ethylene production from acetylene.

Figure 2-3 shows ethylene production by acetylene reduction in N-deficient citrate-amended treatments. No ethylene was observed in the sterile negative controls or the cultures

containing N. Ethylene was detected in the positive control reaching 3.3 µmoles (± 0.64) after 4 d, and the ethylene in N-deficient cultures reached 0.8 µmoles (± 0.12) under N_2 headspace and 0.7 µmoles (± 0.03) in the culture with Ar in the headspace. Ethylene in the PAM-amended cultures reached 1.4 µmoles (± 0.02) with N_2 headspace and 0.6 µmoles (± 0.04) under Ar. Those containing PAM reached 1.4 µmoles (± 0.02) in the culture treatment with N_2 headspace and 0.6 µmoles (± 0.04) in the Ar treatment. Replicates containing PAM exhibited higher enzyme activity in the cultures with CO_2/N_2 headspaces likely because PAM is a flocculant and encourages aggregate stability which provides a better matrix for microbial growth (Casear-TonThat et al., 2008), however this trend was not observed in cultures under Ar headspace.

During the study, methanogenesis was significantly reduced in the presence of acetylene (which made up 3% of the total headspace; Supplementary Figure A-7) as previously observed by Belay et al. (1988). After the acetylene reduction experiment, the headspaces were flushed with CO_2/N_2 or CO_2/Ar and the cultures were incubated for 2 weeks. After the 2 weeks, the CH_4 concentrations had spiked indicating recovery from methanogenesis inhibition (data not shown). Because the results of the CH_4 analysis were so similar between the N_2 and Ar headspace treatments, and the acetylene reduction assay suggested the presence of N_2-fixation in the Ar headspace, analysis of the Ar headspace treatments were discontinued. It was theorized that residual N_2 was present in the Ar headspace providing sufficient N_2 for N_2-fixation however, due to the short retention time between N_2 and Ar on the gas chromatography thermal conductivity detector (GC-TCD), this could not be confirmed.

2.3.3 $^{15}N_2$ Incorporation

After the 2 weeks of incubation following the acetylene reduction assay, the same cultures were amended again with citrate (1000 mg L^{-1}) for microbial metabolism and the headspaces were removed and completely replaced with $^{15}N_2$ gas. The sterile control used in this study also contained N-deficient medium.

Figure 2-4 shows the $^{15}N_2$ incorporation into the microbial biomass in MFT solids after 3 weeks of incubation. Live citrate-amended treatments, N-deficient medium and N-deficient plus PAM, incorporated the same significant ($p<0.01$) amount of ^{15}N into the MFT solids at 2.7% (± 0.13) ^{15}N whereas the cultures containing ^{14}N in their medium only exhibited background ^{15}N (0.37 ± 1.6x10^{-3} %) at similar concentrations to the sterile control (0.37± 2.4x10^{-5} %) (natural abundance ≈0.366% ^{15}N, De Bievre and Taylor, 1993). This evidence strongly suggests the presence of N_2-fixation in the N-deficient culture treatments including those containing PAM. These results indicate that it is unlikely that the PAM-supplemented cultures were using PAM as a N source.

2.3.4 Characterization of Microbial Communities by 16S rRNA Gene Analysis

Microbial community composition in MFT cultures was determined by 454 pyrosequencing analysis of 16S rRNA genes. Mothur software and Phoenix Visual Genomics pipeline (hereafter referred to as Phoenix) were used to process (removal of sequence noise, homopolymers longer than 8 repeats, short reads and chimeras) the same set of data obtained from Genome Quebec to confirm the microbial diversity and assess the variation in the data processed using two different bioinformatics software platforms. Genomic DNA was extracted

from samples at Day 0 and Week 10 which was the time point where the depletion of the second dose of added citrate occurred (Figure 2-1a).

Figures 2-5 and 2-6 show the community compositions of Bacteria and Archaea, respectively at Day 0 and Week 10. At Day 0, all samples from all treatments were similar in diversity and abundance. In data derived from mothur software, Proteobacteria had the highest abundance in all treatments followed by Chloroflexi, Acintobacteria, Firmicutes and Spirochaetes. Of the Proteobacteria, Deltaproteobacteria were dominant in unamended cultures with N and in citrate-amended cultures without N. All other treatments were dominated by Alphaproteobacteria.

At Week 10, unamended baseline treatments were similar. In both treatments, Anaerolineales was the dominant order with 26% in unamended cultures with N and 27% in unamended cultures without N, followed by Rhizobiales with 14% and 13% in unamended cultures with and without N, respectively. In with N treatments, the largest increase was in the Alphaproteobacteria, particularly the Rhizobiales (increased 8%), and the largest decrease was in the Deltaproteobacteria, particularly the Desulfuromondales (decreased 7%). In the treatment without N, the largest increase was in the Betaproteobacteria, (Burkholderiales increased 3%) and the largest decrease was in Alphaproteobacteria (Rhizobiales decreased 2%) compared to Day 0. Bacillales decreased in both treatments and there was little overall change in the percent composition of Proteobacteria, however, Alphaproteobacteria had become the dominant order in both baseline treatments. Due to the lack of replicate data, the significance is unknown.

The bacterial composition of the citrate-amended treatment without N was similar in composition to the baseline control but had a higher percent composition of Clostridiales (8% as compared to 2% in the unamended without N treatment). The abundance of Clostridiales remained relatively unchanged in the unamended treatments and increased in all amended treatments. The amended treatment with N increased 5% and the amended treatment without N increased 6%. The largest increase in Clostridiales was in the amended treatment without N plus PAM which increased from 3% to 35%. Despite this increase in all amended cultures, Clostridiales were dominated by different genera. In the amended treatment with N, there was an increase in the genus *Acidaminobacter*. At least one species (*Acidaminobacter hydrogenoformans*) within this genus is capable of fermenting citrate and other organic compounds to acetate, formate, CO_2 and H_2 in pure culture and in mixed cultures with sulphate-reducers and methanogens (Stams and Hansen, 1984). *Clostridium* sensu stricto increased in all treatments but most predominantly in the citrate-amended treatment without N plus PAM where it became the dominant genus. *Clostridium* sensu stricto is known to contain several species of N_2-fixers as well as mixed alcohol and acid fermenters capable of converting sugars to pyruvate, an intermediate in the citrate fermentation pathway (Chen, 2005; Wiegel et al., 2006; Li, 2010).

In the citrate-amended treatment with N, the abundance of Rhizobiales dropped by 7% in contrast to the unamended treatment with N which increased 8%. In the citrate-amended treatment without N, the proportion of Rhizobiales almost tripled (from 7% at Day 0 to 17% at Week 10). This increase was also in contrast to unamended cultures without N where the abundance of Rhizobiales decreased by 2%; the Rhizobiales population in the amended

treatment without N containing PAM also decreased in abundance by 5%. These changes occurred within an unclassified genus in the family Xanthobacteraceae which are known to contain anaerobic hydrocarbon-degraders and N_2-fixers which can either be free-living or nodule-forming (Chaphalkar and Salunkhe, 2005). All citrate-amended treatments showed a decrease in Bacillales.

The citrate-amended treatment with N had the largest community shift with an increase in Lactobacillales. At Day 0, Lactobacillales represented fewer than 10 reads in the baseline treatments however, by Week 10, Lactobacillales in the citrate-amended treatment with N was at 62%. The majority (99.8%) of the Lactobacillales were of the genus *Trichococcus,* best known for causing the coagulation of materials in activated sludge plants. *Trichococcus* is also known to contain several citrate-fermenting species capable of fermenting citrate to acetate and formate (Stams et al., 2009). As these microorganisms have also been found in soils as well as denitrifying and methanogenic batch cultures (Viviantira et al., 2012), it is likely that Lactobacillales are present in low numbers in Albian tailings and are outcompeted by microorganisms better adapted to N-deficient conditions.

All cultures, as analyzed by both mothur and Phoenix, data comprised sequences related to Proteobacteria, including alpha, beta, delta and gamma, Chloroflexi, Firmicutes, Acinobacteria and “other”, which comprised sequences that made up less than 2% of the total sequence reads in any one treatment. Spirochaetes were also identified by both mothur and Phoenix analyses, however they represented less than 2% of the community in all treatments in the mothur analysis (highest abundance 2%) and so were combined with “other” in Figure 2-5a

but appear in the Phoenix data (highest abundance 2%) (Figure 2-5b). Unlike mothur which produced unclassified sequences, Phoenix categorized all sequences. This resulted in an increase in phyla above 2% in Proteobacteria, Actinobacteria and Spirochaetes and a large increase in the orders Coriobacteriales and Anaerolineales in Phoenix data compared to the mothur data. The data analyzed by Phoenix generally followed the same trends as the mothur data with a few exceptions likely caused by assigning many of the unclassified sequences into the existing orders found by mothur. Phoenix found that Deltaproteobacteria were dominant in treatments with N, citrate-amended with N, and citrate-amended without N, whereas mothur did not find Deltaproteobacteria to be dominant in amended cultures with N. All other treatments were dominated by Alphaproteobacteria. Mothur also found little overall change in the percent composition of Proteobacteria whereas Pheonix data suggested a decrease of 10% in Proteobacteria in unamended cultures with N and an increase of 4% in the baseline treatment without N.

In the mothur data, the archaeal community was composed of Methanomicrobiales, Methanosarcinales and Methanobacteriales (Figure 2-6a). Methanobacteriales were dominant in most treatments, however the citrate-amended treatment with N had a much larger population of Methanosarcinales. Because these samples were taken at Day 0 immediately after citrate and PAM were added, all treatments without N medium were expected to be similar, as such, this difference can be explained by sample variance.

The archaeal population in unamended baseline treatments showed an increase of 22% in Methanobacteriales in the treatment with N and a decrease of 24% in the treatment without

N. Methanomicrobiales also experienced a decrease of 13% in the treatment with N and an increase of 16% in the treatment without N, however the archaeal community remained similar between the two baseline treatments (Figures 2-6).

All amended treatments had experienced a change in abundance by Week 10. The citrate amendments resulted in very similar archaeal community compositions between amended treatments. The number of unclassified reads dropped and Methanosarcinales became highly dominant amongst amended treatments. In amended cultures with N, there was a population shift in Methanosarcinales increasing by 53%. Increases of 28% and 30% were also seen in N-deficient citrate-amended cultures and N-deficient citrate-amended cultures with PAM respectively. The largest increase in Methanosarcinales was in the genus *Methanosaeta* in all treatments, however, the citrate-amended cultures without N also saw a large increase in the genus *Methanosarcina*. Both these genera are composed of acetoclastic methanogens but employ different enzymes to catalyze acetoclatic methanogenesis (Smith and Ingram-Smith, 2007). The shift clearly indicates a preference for acetoclastic methanogenesis in the citrate-amended cultures. Amended treatments with and without N were more similar in abundance than the amended treatment with PAM suggesting that PAM may be affecting archaeal distribution, however the similarity likely indicates that the methanogenic population does not distinguish between the available N present in the medium or N_2-fixation under N-depleted conditions.

Phoenix data were very similar to mothur data and followed all the same trends. Phoenix categorized some of the unclassified Archaea as Methylococcales, increasing the read

number above 2%. At Week 10, the numbers of Methylococcales dropped below 0.6% in all treatments.

2.3.5 Anions, Cations, Metals, pH and N concentrations

Anions, cations, metals and pH concentrations in the cultures remained relatively unchanged over the course of the study (Supplementary Figures A-5 and A-6, and Supplementary Table A-1). Chloride (Cl^-) concentrations were higher in the cultures containing N in the medium, as they contained NH_4Cl which was omitted from N-deficient cultures. Sulphate (SO_4^{2-}) concentrations were below the detection limit (0.04 mg L^{-1}). Ammonium (NH_4^+) concentrations decreased over time in live N-containing cultures; from 4.13 (± 0.93) mg L^{-1} to 1.64 (± 0.08) mg L^{-1} between Day 0 and Week 16, and 2.4 (± 0.36) mg L^{-1} to 1.35 (± 0.21) mg L^{-1} in N-containing cultures amended with PAM (Supplementary Figure A-3c). Little to no detectable NH_4^+ was found in all N-deficient cultures, and NO_3^- was detectable in low concentrations (between 0.04 and 0.22 mg L^{-1}) in the cultures. Data for treatments with CO_2/Ar headspace were excluded from figures due to the similarity with CO_2/N_2 treatments, however, data have been included to illustrate this in Supplementary Figures A-3a and A-5a; only the sterile control with N was included in most figures due to the similarity with the sterile control without N except in Supplementary Figures A-3 and A-5a, and Supplementary Figure A-5k where the chlorine concentrations differ between the sterile control with N and the sterile control without N medium.

Trace metals and other cations data were unreliable due to variations in the second and third replicates between the sterile controls at Day 0 and the samples from the treatment with

N plus citrate at Week 10. Concentrations then dropped closer to the expected range for the remainder of the samples from Week 10 and were consistent through Week 16. Because of this discrepancy, significance could not be reliably determined, however, concentrations appear unchanged over the course of this study.

2.4 Discussion

This study examined the possibility of nitrogen (N_2)-fixation and polyacrylamide (PAM) as N sources for the methanogenesis occurring in the Shell Albian Sands Muskeg River Mine tailings pond, which is severely limited in available N (~ 1 mg L^{-1}, Supplementary Figure A-1). Nitrogen (N_2)-fixation in oil sand tailings has not been previously examined and while many studies have examined N_2-fixation in association with methanogenesis in pure culture (Belay et al., 1984;1988 Lobo and Zinder, 1990; Blank et al., 1995; Leigh, 2000; Kessler et al., 2000), it is has not been well documented in environmental samples. A preliminary study by Siddique (2008) indicated that citrate-stimulated methanogenesis was possible under N-deficient conditions in Albian tailings culture (Supplementary Figure A-2). The study included treatments with PAM (1000 mg L^{-1} dry weight), with and without citrate (1000 mg L^{-1}) and with and without N methanogenic media. Like the study by Haveroen et al. (2005), vitamin B solution and 2-methyl-*n*-butyric acid were also omitted from the methanogenic medium due to the N content of the amino acids in the vitamin B solution and the potential use of the short-branched fatty acid methyl-*n*-butyric acid, by the microbial population (Robinson and Allison, 1969). Though 2-methyl-*n*-butyric acid is a growth factor for some specific species of methanogens (*Methanobacterium ruminantium*) and the vitamin B solution contains essential

growth factors such as biotin and para-aminobenzoic acid, the parameters of the study required their exclusion. This current study was conducted to further examine the findings of Siddique (2008) using PAM and citrate concentrations closer to those found *in-situ* and incorporated a second anaerobic headspace containing CO_2/Ar in addition to CO_2/N_2. This current study measured CH_4 production with citrate degradation, acetylene reduction and $^{15}N_2$ incorporation to determine N_2-fixation was occurring and if PAM was being utilized as an N source.

Methane production and citrate degradation were measured to determine if methanogenesis was occurring and if citrate was being utilized as a methanogenic substrate. In unamended baseline controls, both with and without N, CH_4 concentrations remained low for the duration of the experiment suggesting that the microbial communities were degrading endogenous hydrocarbons in the MFT. In the citrate-amended treatments, the results of the CH_4 and citrate analysis (Figures 2-1 and 2-2) clearly indicated the occurrence of concurrent citrate depletion with CH_4 production in all living treatments including N-deficient cultures, both with and without PAM. The depletion of citrate was also observed after CH_4 had reached a plateau at Week 10. This stop in CH_4 production coincided with the complete depletion of citrate in all amended cultures and supported the hypothesis that citrate was being degraded in conjunction with methanogenesis.

Amended cultures with N initially began producing CH_4 at a faster rate than N-deficient amended treatments both with and without PAM. The lack of available N resulted in a delay in CH_4 production in N-deficient cultures (Supplementary Figure A-4). This delay served as an

acclimatization period in the N-deficient cultures after which both N-deficient cultures with and without PAM achieved the same rate of methanogenesis as the N-containing cultures. The delay in CH_4 production in N-deficient cultures with and without PAM was also observed by Siddique (2008) however, his PAM-containing cultures suffered a longer delay in CH_4 production likely because of the higher PAM concentration which might have limited nutrient availability by reducing mobility within the MFT slurry or had an inhibitory effect on microbial activities. Li (2010) also observed a delay in CH_4 production in cultures with PAM during her experiment to examine the contribution of citrate, diluent and PAM on methanogenesis in Albian MFT; it was suggested that the delay in CH_4 production in PAM-containing cultures was due to reduced accessibility to citrate molecules.

The presence of methanogenesis in the N-deficient treatments suggested the presence of N_2-fixation. To test this hypothesis, an acetylene reduction assay was conducted. Acetylene reduction assays have been used to determine if nitrogenase activity, the enzyme responsible for N_2-fixation, is present in a culture (Belay et al., 1984;1988). Only amended cultures were used due to their higher rate of methanogenesis stimulated by the presence of a labile carbon course. In the assay, amended cultures with N produced no ethylene, this was likely due to the inhibition of the nitrogenase enzyme in the presence of available N (NH_4^+) (Kessler et al., 2000; Ruppel and Merbach, 1995). In contrast, both citrate-amended, N-deficient cultures reduced acetylene to ethylene indicating the possibility of N_2-fixation. Interestingly, the concentrations of ethylene produced were higher in treatments with PAM than those without, possibly because the flocculation of the slurry provided a better growth matrix for N_2-fixing microbes.

During the acetylene reduction assay (Figure 2-3), severe inhibition of methanogenesis was observed (Supplementary Figure A-7). This is because acetylene can inhibit the ability of methanogens to maintain a transmembrane pH gradient by interfering with H^+-flux (Sprott et al., 1982). Despite this, nitrogenase activity was observed in N-deficient treatments. In their examination of pure cultures of N_2-fixing methanogens, Belay et al. (1984; 1988) observed that, at low acetylene concentrations (0.1-0.5%), N_2-fixing methanogens could concurrently reduce acetylene and produce CH_4, however, both functions were inhibited at higher concentrations (1-10%). This was not the case in the current experiment as nitrogenase activity was occurring independently of methanogenesis. This might indicate that nitrogenase activity and methanogenesis were likely not occurring in the same microorganism.

The cultures with Ar headspaces performed very similarly to those with N_2 headspaces across all treatments in both the CH_4 production and acetylene reduction assay (Figures 2-1, Supplementary Figure A-4 and Figure 2-3). The concentrations of N in the N-deficient cultures for the treatments with CO_2/Ar headspaces (Supplementary Figures A-3) had insufficient residual N to inhibit nitrogenase activity as determined by the acetylene reduction assay, therefore it is unlikely that there would have been sufficient N present to support the sustained methanogenesis observed in Figure 2-1b without the presence of N_2 in the culture headspaces. Because of this, it was decided not to continue the Ar treatments in further experiments as they likely contained N_2 contamination and would not contribute valuable information to this study.

Incorporation of the stable isotope molecule $^{15}N_2$ was used to conclusively determine if N_2-fixation was occurring in the MFT cultures. No $^{15}N_2$ incorporation above background ^{15}N

concentrations (natural abundance ≈0.366% ^{15}N, De Bievre and Taylor, 1993) was observed in the amended cultures with N. This likely was due to the inhibition of the nitrogenase enzyme in the presence of available N (NH_4^+) (Kessler et al., 2000; Ruppel and Merbach, 1995). However, both amended N-deficient cultures with or without PAM incorporated high concentrations of ^{15}N compared to the background levels in the sterile control and treatments with N where N_2-fixation was inhibited. Cultures with PAM incorporated the same concentrations of ^{15}N as those without PAM in the $^{15}N_2$ assay (Figure 2-4), indicating that both N-deficient treatments with and without PAM were fixing N_2. This is interesting because earlier work by Haveroen et al. (2005) found evidence that PAM could be used as a N source in N-deficient MFT cultures. In her thesis, Haveroen (2005) had attempted to rule out N_2-fixation, however, the positive controls for her acetylene reduction would not perform as expected so the data could not be used. She also attempted to determine if the amino-groups had been removed from PAM molecules but was unsuccessful. Despite this, the data do indicate that after three serial dilutions, methanogenic activity was greater in N-deficient cultures with PAM compared to N-deficient cultures without PAM, supporting the use of PAM as an alternate N source. This study did not share these findings. Instead, after the second citrate addition at Week 6, all citrate-amended cultures produced CH_4 at the same rate. Further analysis with acetylene and $^{15}N_2$ clearly indicated that nitrogen fixation was taking place in N-deficient cultures with PAM. The difference between these findings and those of Haveroen at al. (2005) likely resulted from the combination of several factors. First, while the media she used was very similar to that used in this study, she omitted $(NH_4)_6Mo_7O_{24}.2H_2O$ entirely as opposed to substituting with $Na_2Mo_7O_{24}.2H_2O$. Because molybdenum is the most predominant nitrogenase cofactor (Leigh,

2000), it may have been required for the microbes present in the cultures to allow them to fix N_2 as opposed to utilizing PAM as in the Haveroen study. Haveroen also performed several serial dilutions resulting in a final MFT to media ratio of 1:614 whereas this study maintained a constant ratio of 1:1. It is possible that any existing N_2-fixing consortium in her cultures were lost over the course of the serial dilutions or that other micronutrients were limited thereby inhibiting the growth of N_2-fixers. Different labile carbon sources were also used between the two studies, but more importantly Haveroen was using MFT from Syncrude whereas this study used MFT from the Shell Albian Sands Muskeg River Mine tailings pond. The microbial community in tailings ponds also varies widely between operators (Yergeau et al., 2012); because of the difference in ore processing, the use of naphtha as opposed to paraffinic solvents, the absence of citrate in Syncrude tailings ponds and the comparatively high concentrations of NH_4^+ in Syncrude, the microbial communities in these two studies were likely very different which could also account for the use N_2-fixation as opposed to PAM in this study.

The microbial communities were characterized to determine how the communities differed between treatments, which microorganisms were present and which members might be responsible for the observed methanogenesis and N_2-fixation in the different treatments. In the unamended baseline controls with and without N, very little change occurred with time. The community compositions in the unamended treatments were also very similar indicating that the presence of a labile carbon source has a more profound effect on the distribution of the microbial community than the presence or absence of N. This is likely because organic compounds that are easily degraded such as low molecular weight hydrocarbons are consumed comparatively quickly (Siddique et al., 2006) and are no longer present in MFT at high

concentrations; only recalcitrant compounds or those that are degraded slowly likely remain. Without the citrate amendment, microbial metabolism remained low due to the lack of easily degradable carbon even though stimulating nutrients such as available N were present. In citrate-amended cultures, there was a change in abundance in all treatments. In the amended treatment with N, the proportions of all orders dropped in comparison to the baseline controls, especially Anaerolineales which initially was the dominant order; only the Firmicutes, Lactobacillales and Clostridiales increased and became dominant. The percentage of Clostridiales also increased in the amended treatment without N along with Rhizobiales, in contrast to the baseline controls which remained relatively unchanged. In amended cultures with PAM, the percent composition of Clostridiales increased over 21-fold compared to the Clostridiales represented in the unamended baseline control without N; the abundance of Anaerolineales was also lower than the baseline controls. Methanosarcinales also became dominant in all amended treatments and the unamended treatment without N. In the unamended baseline control with N, Methanobacteriales was dominant, however, because the archaeal community in the unamended treatments are so similar, the difference is likely not significant.

The genus *Trichococcus* of the order Lactobacillales was dominant in the amended treatment with N. *Trichococcus* contains many species of soil bacteria and has been found in denitrifying and methanogenic batch cultures (Viviantira et al., 2012; Park et al., 2010); it also contains several species capable of fermenting citrate to acetate and formate (Stams et al., 2009). Lactobacillales is dominant in the citrate-amended treatment with N but is present as less than 10 reads in other treatments. This suggests that both an available N and labile carbon

source are required for *Trichococcus* to outcompete other microorganisms in the community such as Anaerolineales (Chloroflexi). The decrease of Anaerolineales in the citrate-amended treatment with N is likely due to increased competition with *Trichococcus*.

Anaerolineales contains several fermentative species capable of producing acetate, pyruvate, H_2 and formate from sugars and have been shown to grow syntrophically with methanogens (Yergeau, 2012). Many community analyses have reported the presence of Chloroflexi in tailings and MFT. However, sequence abundance varies widely between operators, sampling sites and sampling depths (Yergeau, 2012; Ramos-Padrón, 2011; Siddique, 2011); as such, trends in abundance are difficult to analyse.

In the order Clostridiales, *Clostridium* sensu stricto increased in all citrate-amended treatments however, the increase was most pronounced in the N-deficient treatment with PAM. The high percentage of *Clostridium* in amended cultures with PAM potentially occurred because some species of *Clostridium* are fermenting, N_2-fixing aggregators and PAM has been shown to promote growth of aggregating microorganisms (Zoutberg et al., 1989; Caesar-TonThat et al., 2008; Kuhner et al., 2000). In the citrate-amended cultures with N, the number of *Clostridium* sensu stricto was evenly matched with *Acidaminobacter* which has been shown to ferment citrate syntrophically with methanogens (Stams and Hansen, 1984). Firmicutes have been found in both Syncrude and Suncore MFT and generally comprise 5-25% of the uncultured MFT microbial community (Yergeau, 2012; Ramos-Padrón, 2011). Siddique et al. (2012) also observed an increase in Clostridial populations but they were composed primarily of sulphur-reducing bacteria.

While amended cultures without N only showed a small increase in the percent composition of *Clostridium*, it was the only treatment to exhibit a large increase in the population of Rhizobiales. This increase occurred within the family Xanthobacteraceae which are known to contain hydrocarbon degraders and N_2-fixing species (Chaphalkar and Salunkhe, 2005).

Methanosarcinales was the dominant Archaeon in all amended treatments and was also found to be a dominant methanogen after incubation in long-chain *n*-alkane degradation studies by Siddique et al. (2011) in MFT from Syncrude's MLSB. Yergeau et al. (2012) also found Methanosarcinales in both Syncrude and Suncor MFT but found Methanomicrobiales, a hydrogenotrophic methanogen, to be dominant. A study by Siddique et al. (2012) demonstrated that the carbon amendment plays a large role in the dominance of methanogens within the same MFT. They found that Methanosarcinales were dominant in *n*-alkane-amended cultures and Methanomicrobiales were dominant in BTEX (benzene, toluene, ethylbenzene, and xylenes)-amended cultures. As such, it is possible that the use of a different organic amendment such as BTEX in the Albian MFT cultures from this study would have resulted in the dominance of Methanomicrobiales over Methanosarcinales. Because the archaeal populations were similar between amended treatments with or without N in the media, it is unlikely that any of the abundant methanogens were fixing N_2 in the MFT cultures.

The increase in Rhizobiales in the family Xanthobacteraceae which are known to contain hydrocarbon-degraders and N_2-fixing species (Chaphalkar and Salunkhe, 2005) suggests that Rhizobiales are performing the majority of the N_2-fixing activity in amended cultures without N.

This increase was not seen in the amended treatment with N as N_2-fixing activity was not necessarily due to the presence of available N, allowing *Trichococcus* to outcompete many of the other microbial populations as well as the less efficient fermenters. The number of Rhizobiales also decreased in the amended cultures with PAM as PAM has been shown to promote the growth of aggregate-forming *Clostridium* (Zoutberg et al., 1989; Caesar-TonThat et al., 2008; Kuhner et al., 2000) which may be capable of fixing N_2 thereby fulfilling the N requirement and overcoming the N deficiency. Anaerolineales were also present in all cultures. Anaerolineales contains several fermentative species capable of producing acetate, pyruvate, H_2 and formate from sugars and growing syntrophically with methanogens (Yamada et al., 2006). Once the N deficiency was overcome in N-deficient cultures, fermenters such as Anaerolineales were able to utilize the labile carbon source and the acetoclastic methanogen Methanosarcinales was able to fully establish and produce CH_4 at the same rate as cultures with N present in the medium.

Metal and ion concentrations appeared to remain relatively constant over the course of the study however, results were highly inconsistent between replicates for some samples making it difficult to analyse the results reliably. Despite this, even the highest concentrations of heavy metals were below the soil remediation guidelines for all land uses (Government of Alberta, 2010).

Due to the low solubility of N_2 in water (~1.8 g Kg^{-1}, Engineering Toolbox, 2012), the N_2 needed for N_2-fixation more likely originates from low levels of denitrification activity as denitrifying bacteria are present in MFT even under low concentrations of NO_3^- (Penner and

Foght, 2010; Sobolewski, 1992). Unfortunately, NO_3^- data were also highly variable. Denitrification reactions would have led to a decrease in NO_3^- and due to the variability in results, no conclusions can be drawn from this data.

Total N also remained relatively unchanged over the course of the study (Supplementary Figure A-3a) even though N_2-fixation was occurring, which should have caused an increase in total N in the slurry. This is possibly because anaerobic metabolism can require very little N; C:N ratios have been reported as high as 180:1 in wastewater (Ammary, 2004). Given low N requirements for anaerobic metabolism and lack of representation of the amino groups from PAM in the PAM treatments (Supplementary Figure A-3a), the increase in total N from N_2-fixation may have been too small to be sufficiently represented and was likely overcome by sample variability as represented by the error bars.

2.5 Conclusions

In this study, methanogenesis was observed under N-limiting conditions. The N limitations were overcome after a short delay in CH_4 production where the microbial communities adapted to fix N_2. Once N_2-fixation was established, the rate of methanogenesis was comparable to cultures containing available N. The presence of nitrogenase activity required for N_2-fixation was confirmed through acetylene reduction under N limiting conditions with and without PAM. Finally, N_2-fixation itself was observed through the incorporation of $^{15}N_2$ into the microbial biomass of the N-deficient cultures, including those with PAM. These data indicate that N_2-fixation is occurring in conjunction with methanogenesis in the MFT cultures and their fast adaptation to N_2-fixation upon exposure to a readily degradable carbon source

suggests a predisposition to fixing N_2. As such, it can be suggested that N_2-fixation may be occurring in N-deficient oil sands tailings ponds when sufficient labile carbon is present. If paraffinic solvent had been used in this study as opposed to citrate, it is possible that N_2-fixation-mediated methanogenesis would still occur, as many groups of fermentative bacteria are present in Albian MFT such as Xanthobacteraceae which are also known to contain hydrocarbon degraders and N_2-fixing species (Chaphalkar and Salunkhe, 2005).

This study suggests that CH_4 production in N-deficient tailings ponds is supported by N_2-fixation. Additionally, these data also suggest that, over time, the microbial communities in tailings ponds may be capable of establishing N and C cycling. This is significant with respect to current reclamation efforts that focus on using oil sand tailings to establish functional ecosystems, and contributes to our knowledge of the development of nutrient cycling in these reclaimed sites.

Table 2-1. Experimental design for culture bottles containing Albian MFT mixed 1:1 with either N or N-deficient methanogenic nutrient medium and amended, with or without citrate or polyacrylamide (PAM). Treatments without citrate were baseline controls; sterile treatments were negative controls.

Treatment	Medium Without N	Medium With N	Citrate 200 mg L^{-1}	Sterilized MFT	PAM 100 mg L^{-1} (1.25 mg d.wt)	N_2 Headspace	Ar Headspace
With N		4				2	2
With N + Citrate		6	6			3	3
Sterile With N + Citrate		2	2	2		2	
Without N	4					2	2
Without N + Citrate	6		6			3	3
Sterile Without N + Citrate	2		2	2		2	
Without N + PAM	4				4	2	2
Without N + Citrate + PAM	6		6		6	3	3

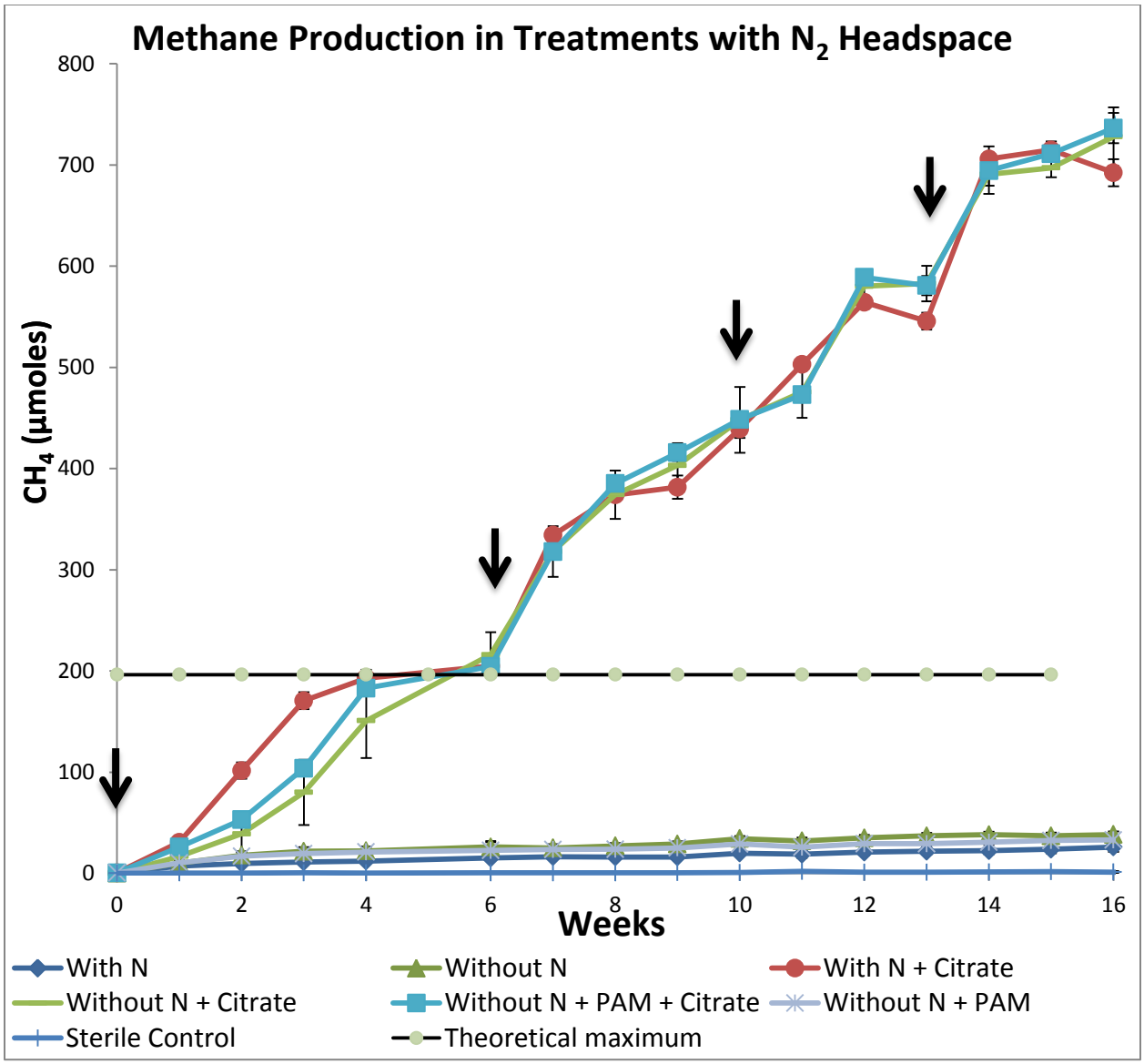

Figure 2-1a: Cumulative methane (CH_4) production in treatments with 30% CO_2/70% N_2 headspace coupled with citrate metabolism by indigenous microorganisms in MFT, incubated at ~22°C for 16 weeks (see Supplementary Figure A-4 for close-up on the delay in CH_4 production). Arrows indicate citrate (200 mg L^{-1}) addition at Day 0, Week 6, Week 10 and Week 13 (see Table 2-1 for treatments). The horizontal line represents the theoretical maximum CH_4 concentrations produced by the initial 200 mg L^{-1} of citrate. Error bars represent standard deviation for three replicates in citrate-amended treatments and two replicates in unamended and heat-killed sterile cultures. Only one sterile control is shown because none of the sterile controls produced CH_4. With or without N indicates the presence or absence of available N in the medium.

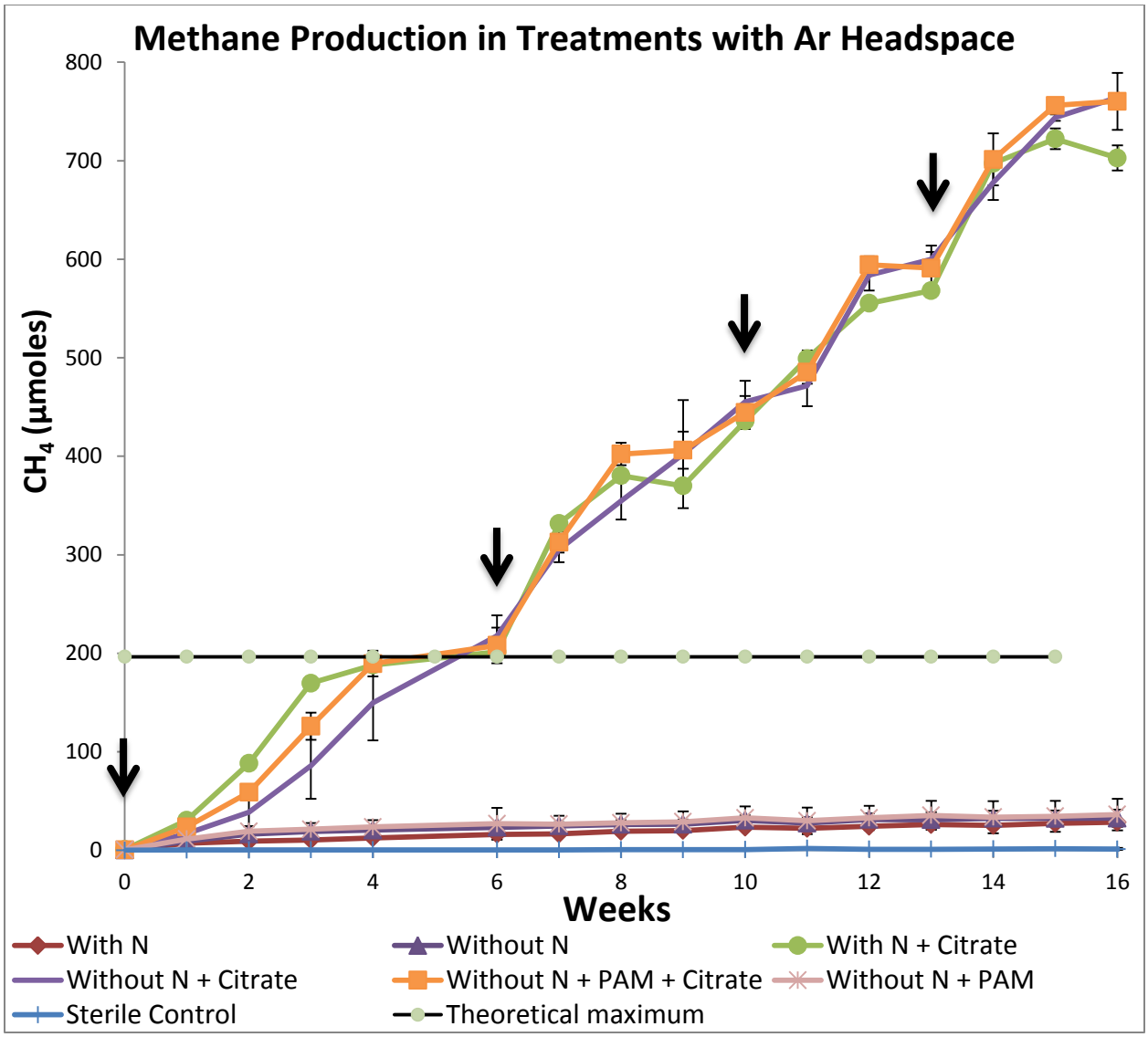

Figure 2-1b: Cumulative methane (CH_4) production in treatments with 30% CO_2/70% Ar headspace coupled with citrate metabolism by indigenous microorganisms in MFT, incubated at ~22°C for 16 weeks (see Supplementary Figure A-4 for close-up on the delay in CH_4 production). Arrows indicate citrate (200 mg L^{-1}) addition at Day 0, Week 6, Week 10 and Week 13 (see Table 2-1 for treatments). The horizontal line represents the theoretical maximum CH_4 concentrations produced by the initial 200 mg L^{-1} of citrate. Error bars represent standard deviation for three replicates in citrate-amended treatments and two replicates in unamended and heat-killed sterile cultures. Only one sterile control is shown because none of the sterile controls produced CH_4. With or without N indicates the presence or absence of available N in the medium.

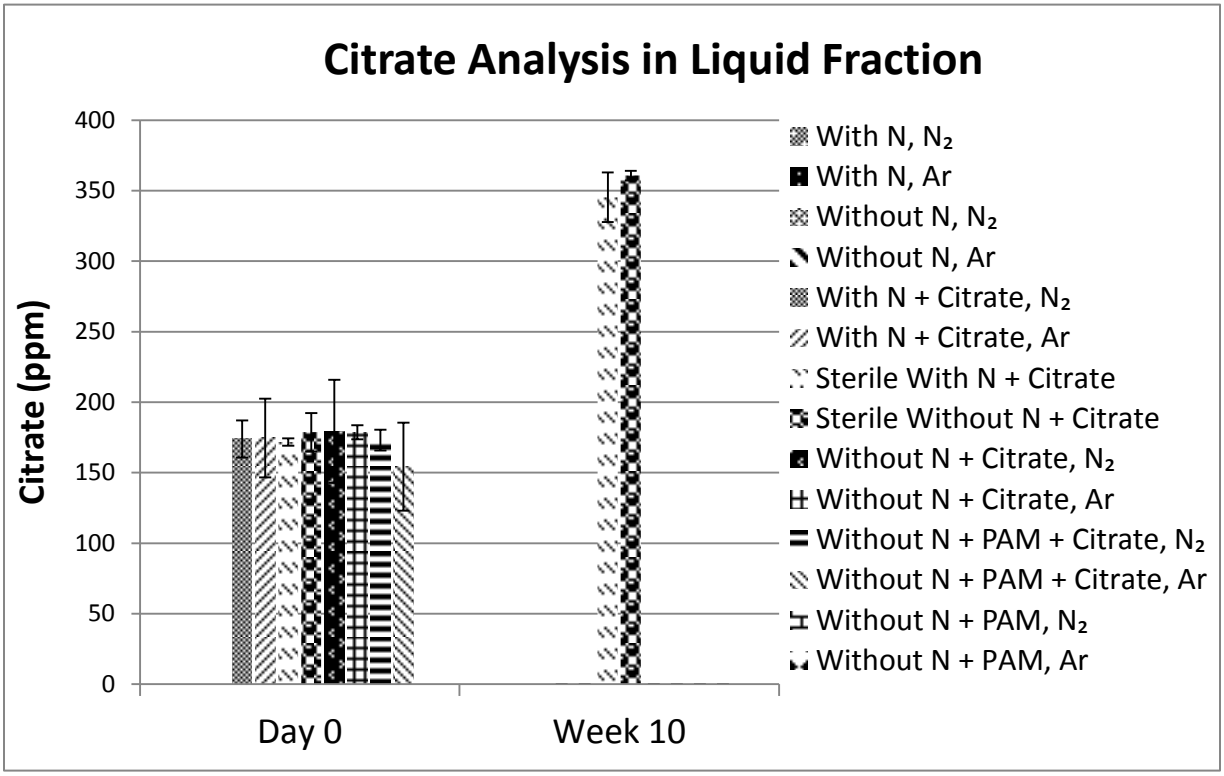

Figure 2-2: Citrate analysis conducted using HPLC. Data represents Day 0 concentrations following the first addition of citrate addition while Week 10 concentrations, before the addition of third dose of citrate, reveal depletion of citrate due to microbial metabolism in live cultures. A second dose of citrate was added at Week 6. Only the liquid fraction was analyzed. With or without N indicates the presence or absence of available N in the medium. N_2 represents 30% CO_2/70% N_2 and Ar represents 30% CO_2/70% Ar gas in the headspace. See Table 2-1 for treatment description. Error bars represent standard deviation

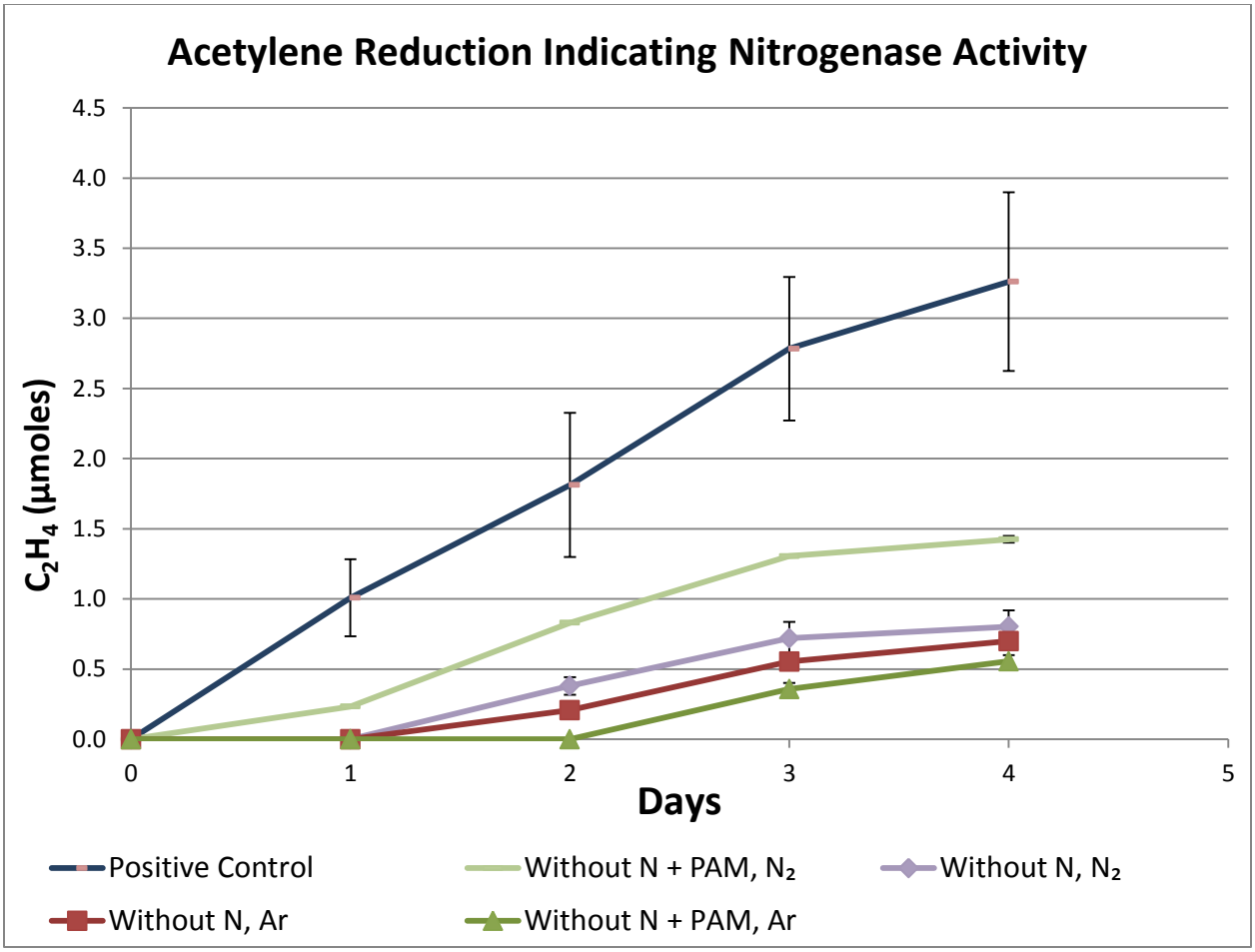

Figure 2-3: Ethylene produced from acetylene (C_2H_2) reduction by MFT cultures with 3% (74.4 µmol) C_2H_2 headspace. Cultures were incubated at ~22°C for 4 d in 60 mL serum bottles. All cultures were amended with 200 mg L^{-1} citrate at day 0. N_2 and Ar represent 30% CO_2/70% N_2 and 30% CO_2/70% Ar headspaces, respectively in the bottles. Both positive and negative controls contained known N_2-fixing *Pseudomons* sp.; negative control also contained NH_4Cl. Sterile negative control was a heat-killed MFT culture. No ethylene was produced in heat killed cultures or culture containing N medium or NH_4Cl (not shown). Error bars represent standard deviation for three replicates. With or without N indicates the presence or absence of available N in the medium.

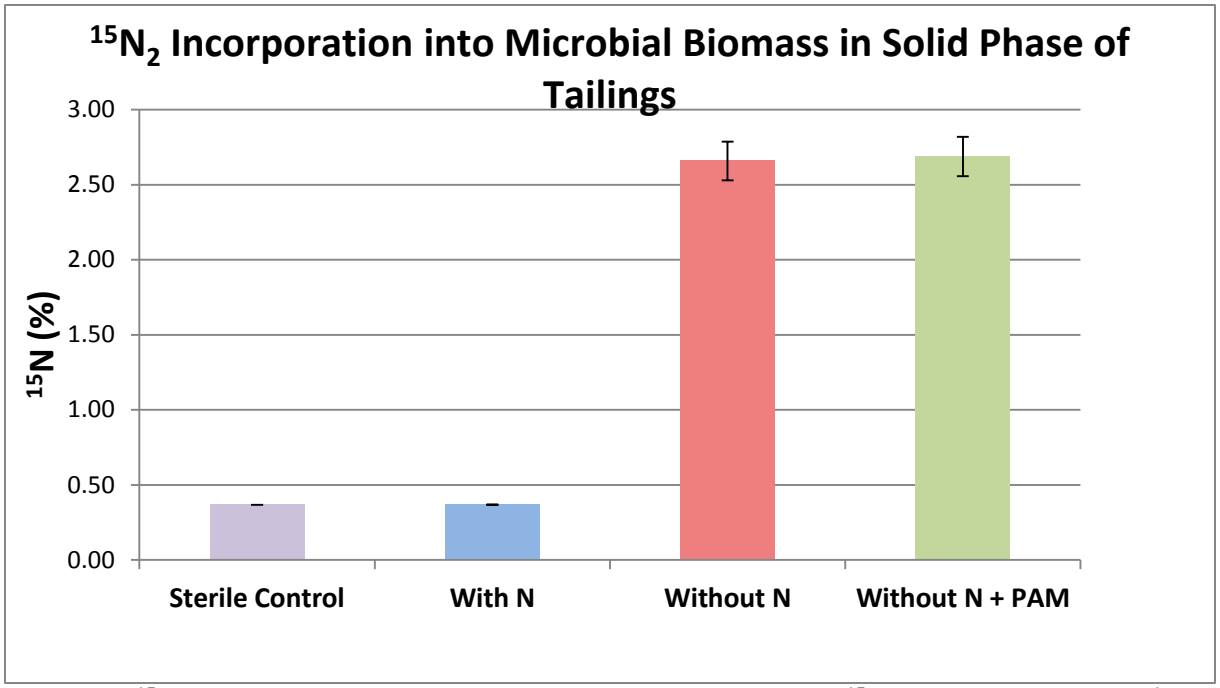

Figure 2-4: ^{15}N concentrations in MFT solids after incubation with $^{15}N_2$ gas and 1000 mg L^{-1} citrate at ~22°C, in the dark for 3 weeks. Sterile control represents background concentrations of ^{15}N in MFT solids. Error bars represent standard deviation for three replicates. $p<0.01$ for both culture treatments without N as compared to both N medium containing cultures and sterile controls. With or without N indicates the presence or absence of available N in the medium.

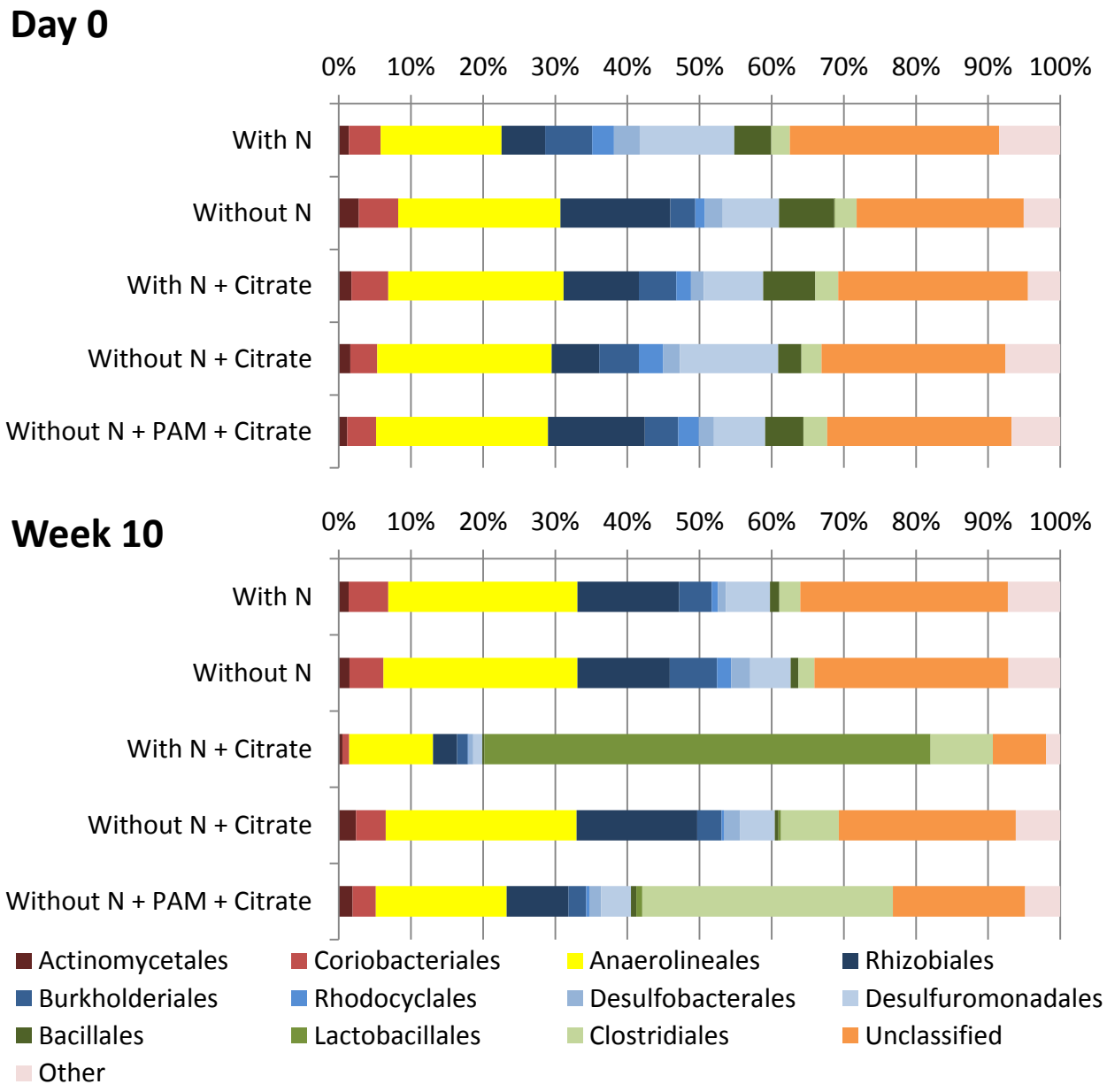

Figure 2-5a: Microbial community structure of bacterial DNA extracted from MFT cultures analyzed using 454 pyrosequencing of 16S rRNA genes and processed with mothur software. With or without N indicates the presence or absence of available N in the medium. Orders are classified by colour according to Phyla, reds: Actinobacteria; yellow: Chloroflexi; blues: Proteobacteria; greens: Firmicutes.

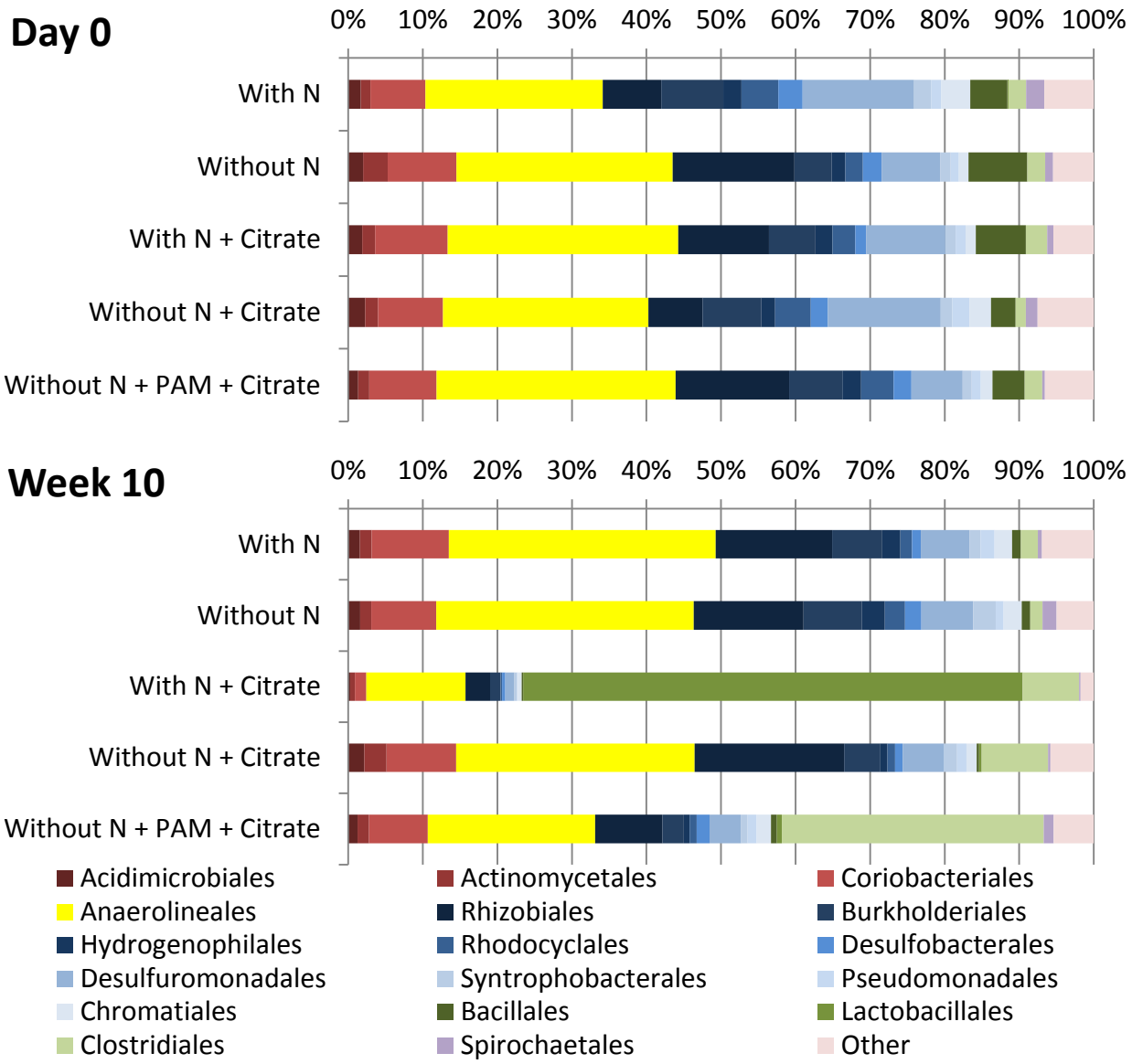

Figure 2-5b: Microbial community structure of Bacteria from MFT cultures analyzed using 454 pyrosequencing of 16S rRNA genes and processed with Phoenix software. With or without N indicates the presence or absence of available N in the medium. Orders are classified by colour according to Phyla, reds; Actinobacteria, yellow; Chloroflexi, blues; Proteobacteria, greens; Firmicutes.

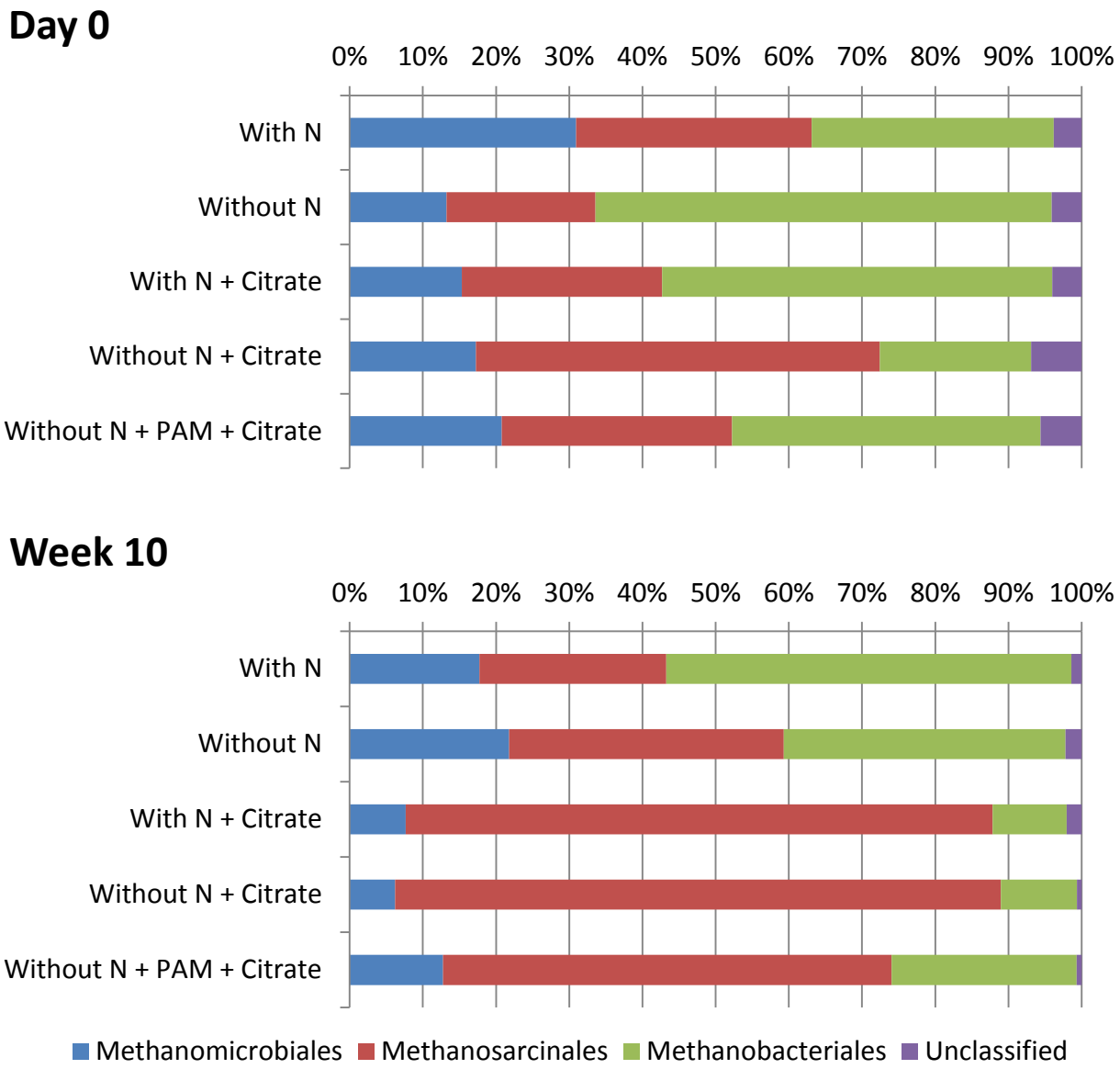

Figure 2-6a: Microbial community structure of Archaea from MFT cultures analyzed using 454 pyrosequencing of 16S rRNA genes and processed with mothur software. With or without N indicates the presence or absence of available N in the medium. All orders from the phylum Euryarchaeota.

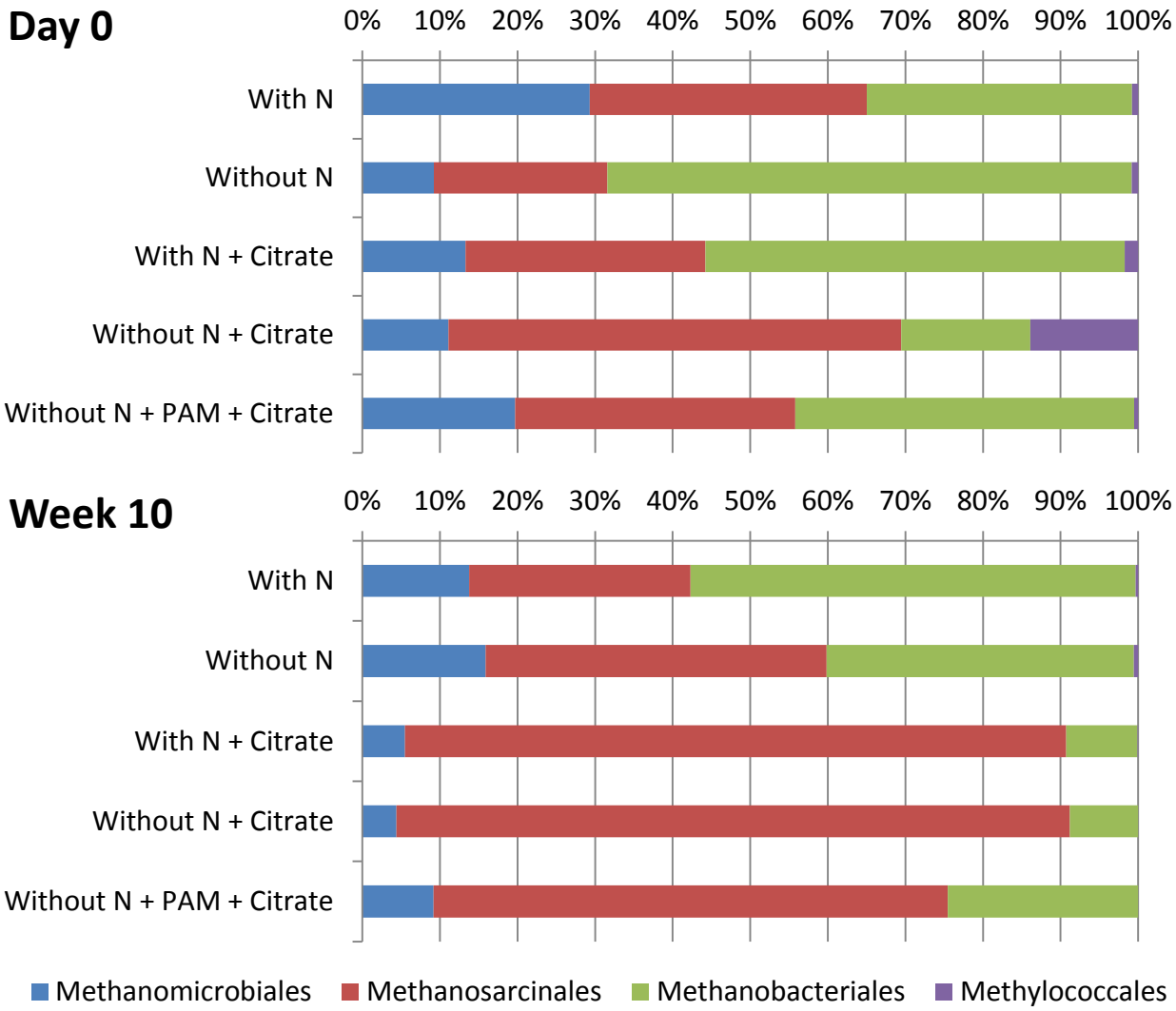

Figure 2-6b: Microbial community structure of Bacteria from MFT cultures analyzed using 454 pyrosequencing of 16S rRNA genes and processed with Phoenix software. With or without N indicates the presence or absence of available N in the medium. All orders from the phylum Euryarchaeota.

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Chapter 3. General Conclusions

3.1 Research Summary

The goal of this research was to investigate how methanogenesis was sustained in tailings ponds under N-limiting conditions. Our hypothesis was that N_2 and/or PAM were being used as a N source to overcome the deficiency in available N in Shell Albian (Albian) tailings. The research objectives were to: (1) study N_2 fixation as a possible source of N, (2) examine PAM as a possible source of N; and (3) characterize the indigenous microbial community to infer the microbial potential of microorganisms in sustaining methanogensis under N-limiting conditions by fixing N or utilizing PAM as N source. The conclusions drawn from the results were as follows: (1) the concurrent degradation of citrate with methanogenesis, production of ethylene from acetylene and incorporation of ^{15}N in cultures incubated in N-deficient medium in the presence of a labile carbon source (citrate) supported the hypothesis that N_2-fixation can support methanogenesis in N-deficient mature fine tailings (MFT). (2) The experiments examining PAM using Albian MFT generated results that were different from the previous literature that PAM was being used as an N source (Haveroen, 2005). Instead we found that N_2-fixation was more favourable when PAM was present in the MFT. (3) The analysis of the microbial communities suggested that different microbial consortia were responsible for N_2-fixation in citrate-amended N-deficient cultures with and without PAM and that the bacterial population, and not the archaeal population, were responsible for N_2-fixation.

To date, no studies have been published examining N_2-fixation in oil sand tailings or N dynamics in MFT with respect to the methanogenic consortium or microbial community. The

presence of N_2-fixation in N-deficient oil sands tailings ponds explains how methane production is possible and suggests the possibility of N-cycling in tailings ponds. This may be of particular importance for future reclamation and remediation efforts which seek to utilize tailings materials in the establishment of reclaimed wetlands (Harrison, 2013).

(1) The ability of the amended N-deficient cultures with or without PAM to achieve the same rate of methanogenesis as the amended cultures with available N indicated that N requirements were being met through other means. Results from the acetylene reduction assay supported this hypothesis by indicating the presence of nitrogenase activity, the enzyme responsible for N_2-fixation (Belay et al., 1984;1988), in both N-deficient treatments with or without PAM. Because treatments intended to have no N_2 in the headspace (CO_2/Ar) were also producing methane at the same rate and producing ethylene in the acetylene reduction assay, it was theorized that residual N_2 was present in the headspace. To confirm that N_2-fixing activity was present, $^{15}N_2$ was added to the headspace of amended treatments, incubated, and the MFT was analyzed for ^{15}N incorporation into the microbial biomass. Concentrations of ^{15}N were found to be more than 7-fold higher in treatments with N-deficient medium, both with and without PAM, whereas the biomass in the sterile control and the amended treatment with N contained background concentrations of ^{15}N.

(2) These results clearly indicated that N_2-fixation was taking place not only in amended, N-deficient cultures but also in amended, N-deficient cultures with PAM. This contrasts with the results of Haveroen et al. (2005) which suggested that PAM

could be used as a N source under N-depleted conditions. However, although Haveroen (2005) was unable to rule out N_2-fixation and was unable to determine if the amino-groups had been removed from the PAM molecules, her data indicated increased methanogenesis in N-deficient cultures containing PAM compared to those without PAM. The results of Haveroen (2005) were different from the current study which found the rate of methanogenesis to be the same in all amended treatments after acclimatization to N-deficient conditions and supported N_2-fixation. The results of this study however, are not in conflict with Haveroen (2005) as the culture setup differed and the MFT and carbon amendment in the cultures were from another operator; as such, they very likely supported a different microbial community with a preference for PAM degradation over N_2-fixation.

(3) There was a community shift in the bacterial population affected by the presence or absence of N in the amended treatments. In the amended treatment with N, there was a large increase in the citrate-fermenting genus *Trichococcus* (Stams et al., 2009), which became the dominant bacterial population due to the availability of a labile carbon source and available N. In the amended treatments without N, there was an increase in family and genera capable of N_2-fixation that differed based on the presence of absence of PAM such as the family Xanthobacteraceae in N-deficient cultures and the genus *Clostridium* sensu stricto in N-deficient cultures with PAM. These results strongly support the hypothesis that a different microbial community was present under conditions with sufficient available N versus N-deficient conditions. However, the similarity in abundance of Archaea by Week 10 between

unamended treatments with and without N, and the similarity between amended treatments with N, without N and without N and PAM, indicated that the presence or absence of N did not affect the archaeal mode of metabolism in methanogenesis. These data strongly suggest that the N_2-fixation occurred in the bacterial population and not in the archaeal population.

The dominance of Methanosarcinales in all amended cultures likely resulted from the choice of labile carbon source used in this study. Methanosarcinales were also found to be the dominant class of Archaea when long-chain *n*-alkanes were used as culture amendments in MFT from another operator (Siddique et al., 2011). When BTEX was used instead, the hydrogenotrophic methanogens in Methanomicrobiales were dominant (Siddique et al., 2012) indicating the ability of the microbial community to shift in order to best utilize the available carbon sources and their metabolites. As such, it is possible that amendment with the paraffinic solvents used by Albian, which contains mostly linear and branched alkanes (C_5-C_6) (as reviewed by Li, 2010), would result in the dominance of a different archaeal population and possibly a different bacterial population with a higher percentages of Xanthobacteraceae of the order Rhizobiales (Proteobacteria), as Xanthobacteraceae contains N_2-fixers as well as hydrocarbon degraders (Chaphalkar and Salunkhe, 2005).

The analysis of ions and metals proved inconclusive due to the lack of phase separation and determination of metals in each phase (liquid and solid). A relatively larger sample size was

needed to separate the phases. However even the highest concentrations of heavy metals was below Alberta's reclamation guidelines for soil. We are unable to comment on the metal concentrations in pore water in MFT and discuss water quality guidelines because we could not analyze the metals in the pore water. This could be cause for concern as tailings materials are being used as reclamation materials in the construction of wetlands (Harrison, 2013). Before any conclusions can be drawn regarding what is naturally occurring in the area, however, metal analysis must first be conducted on undisturbed wetlands in the area for comparison.

In conclusion, N_2-fixation in MFT under N-deficient conditions serves as a N source even in the presence of PAM in Albian MFT cultures and may also serve as an N source *in-situ*. This indicates that PAM is not being used as a N source in the N-deficient Albian MFT cultures used in this study. It is also likely that a different microbial community is present depending on the treatment and suggest that there would be a change in the microbial community if hydrocarbons such as the C_5-C_6 paraffinic solvents used by Albian were the primary source of available N in MFT cultures. While it is possible that Methanosarcinales would not be the dominant methanogens, it is also possible that the family Xanthobacteraceae would be present in greater numbers under N-deficient conditions as Xanthobacteraceae is known to contain N_2-fixers as well as hydrocarbon degraders (Chaphalkar and Salunkhe, 2005).

3.2 Future Research

Future research on N_2-fixation in MFT should determine if the microbial community is capable of fixing N_2 while utilising hydrocarbons as a carbon source: this will advance our understanding of anaerobic biodegradaton processes. Because the microbial community may

vary when different carbon amendments are used, it is possible that N_2-fixation may not be supported by a different microbial population or that different microorganisms will be responsible for N_2-fixing activity. Nitrogen cycling should also be examined in MFT. This is of particular importance because MFT is being used in wetland reclamation (Harrison, 2013). The success or failure of a newly reclaimed ecosystem is dependent on the establishment of nutrient cycling, if N-cycling is found in MFT, then it may also occur in reclaimed wetlands that have incorporated MFT from the same operator. To examine this possibility, MFT cultures must first be established, monitored for N-cycling intermediates and probed for N-cycling genes followed by a community analysis. If N-cycling genes are present, wetlands that incorporated MFT, such as Syncrude's new end-pit lake, should also be examined for N-cycling intermediates and genes. A community analysis should also be conducted to determine if similar microbial populations are present. These data can then also be compared to community analyses on undisturbed wetlands in the area. The results can be used to determine how much of the microbial population is maintained from the MFT in the end-pit lake, if the microorganisms responsible for N-cycling are present and if they are the same as those found in MFT. These data would also give insight into the health of the new ecosystem by comparing the microbial population and N-dynamics to undisturbed wetlands. Given enough time, this study could also be extended to monitor the health of the end-pit lake over time as compared to natural lakes in the area. If the end-pit lake project is successful, then the results could provide a monitoring scheme to determine where new end-pit lakes are in their progress towards sustainable ecological health.

Hydrocarbon degradation needs to be examined in end-pit lakes. End-pit lakes were proposed as a method of detoxifying tailings materials, however, this relies heavily on the composition of the microbial community. While a community analysis and comparison would help to determine if the same hydrocarbon degraders from the MFT are present in the new end-pit lakes, hydrocarbon degradation analyses must also be performed to determine if the population is active and serving the intended purpose. This could be analysed by taking core samples from the end-pit lake for large scale studies or for smaller scale studies, creating cultures in bottles containing some of the MFT material from the lake basin and water from closer to the surface. An analysis of hydrocarbons should be performed and cultures, or columns, monitored for degradation. Additionally, some columns, or cultures, could be amended with labile or recalcitrant hydrocarbons and monitored for degradation in order to gain more easily quantifiable data.

It would be very interesting to study the functionality and progress of end-pit lakes as a reclamation option as it may become the principal reclamation technique for decommissioning tailings ponds in the near future. As such, it will also be important to determine what the reclamation process is and if there is anything that can be done to make remediation efforts more successful. It would also be interesting to consider the possible role that aerobic microorganisms may play in N-dynamics when MFT is used in landscape developments.

3.3 References

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Supplementary Materials: Appendix A

Table A-1: pH of mature fine tailings suspension estimated using pH paper. With or without N indicates the presence or absence of available N in the medium.

MFT Culture	Day 0	Week 10	Week 16
With N	7.7-8	7.7-8	8
Without N	8	8	8
With N + Citrate	7.7-8	7.7-8	7.7-8
Sterile	7.7	7.7-8	8
Without N + Citrate	8	8	8
Without N + PAM + Citrate	8	7.7-8	7.7-8
Without N + PAM	7.7-8	8	7.7-8

Calculation A-1: Calculation to determine µmoles of CH_4 and C_2H_4. Formula (1) was used to determine the percent of CH_4 and ethylene in the headspace (*hdsp*); $\bar{X}$ indicates the mean average of three injections from a single culture replicate and *RF* indicates the response factor which is the ratio of the calibration curve between the known concentrations of the standards and the measured value. Formula (2) is a manipulation of the Ideal Gas Law where *n* is the number of moles, *P* is the pressure (kPa), *R* is the gas constant ($8.3144\ L \cdot kPa \cdot mol^{-1}K^{-1}$) and *T* is the temperature in Kelvin. In the working formula (3), *14.696 psi* represents atmospheric pressure, P_{psi} represents the measured pressure in the culture headspaces, $V_{hdsp\ (L)}$represents the volume of headspace in litres in the culture headspaces, $T_{°C}$ represents the temperature in Celsius and *273 K* is the conversion factor from Celsius to Kelvin; $8.3144\ L \cdot kPa \cdot mol^{-1}K^{-1}$is the gas constant and *1,000,000 µmol ·mol*$^{-1}$ is the conversion factor from moles to µmoles. Formula (4) was used to calculate the total number of moles in the headspace using the results of formulae (1) and (3).

(1) $$\%CH_4\ or\ \%C_2H_{4_{hdsp}} = \bar{X}_{\ injections} * RF$$

(2) $$\mathrm{n} = \frac{PV}{RT}$$

(3)
$$\text{Total } \mu\text{mol}_{hdsp} = \left(\frac{\left((14.696\ psi + P_{psi}) * 6.89\ kPa \cdot psi^{-1} \right) * V_{hdsp\ (L)}}{(273\ K + T_{°C}) * 8.3144\ L \cdot kPa \cdot mol^{-1}K^{-1}} \right) * 1{,}000{,}000\ \mu mol \cdot mol^{-1}$$

(4) $$CH_4\ or\ C_2H_4 \mu\text{mol}_{hdsp} = \ \%CH_4\ or\ \%C_2H_{4_{hdsp}} * Total\ \mu\text{mol}_{hdsp}$$

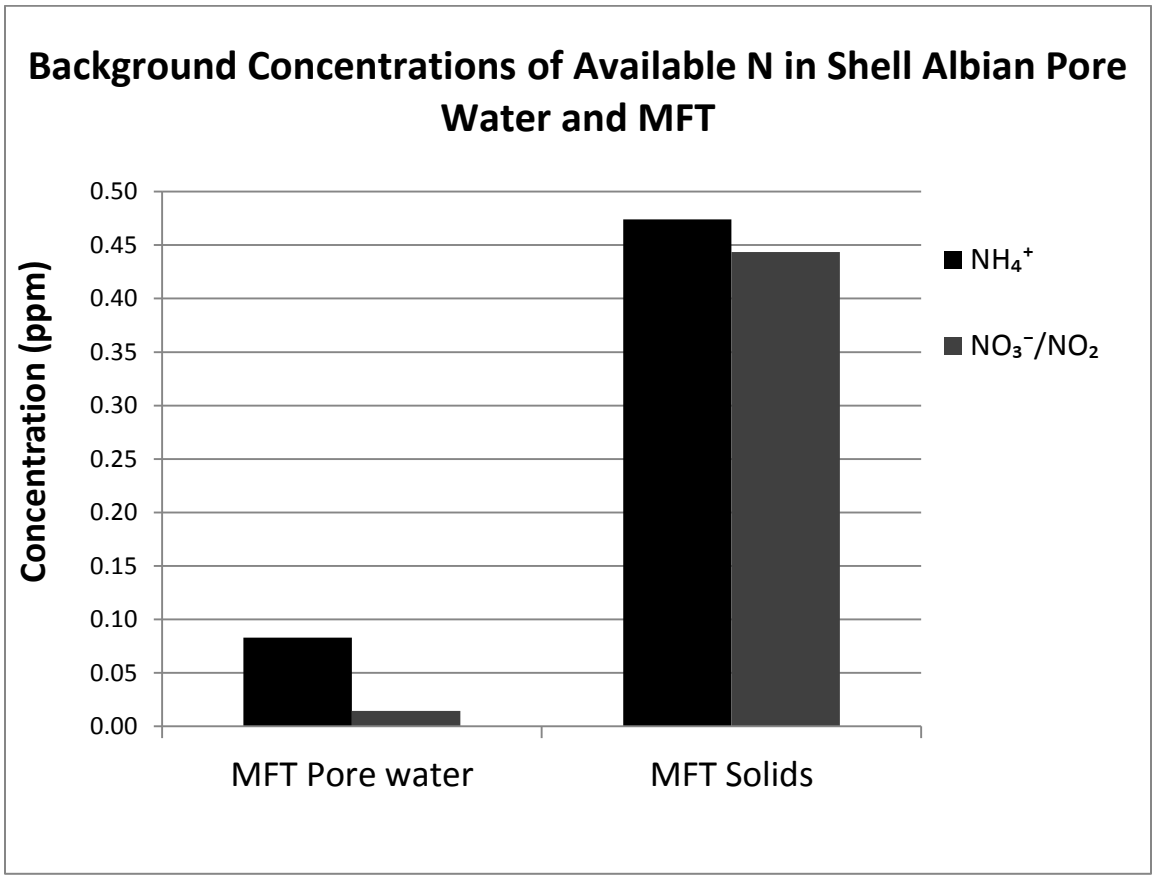

Figure A-1: Total available N in Shell Albian MFT before the addition of media. Both solid and pore water phases are represented; concentrations were determined using colorimetric analysis.

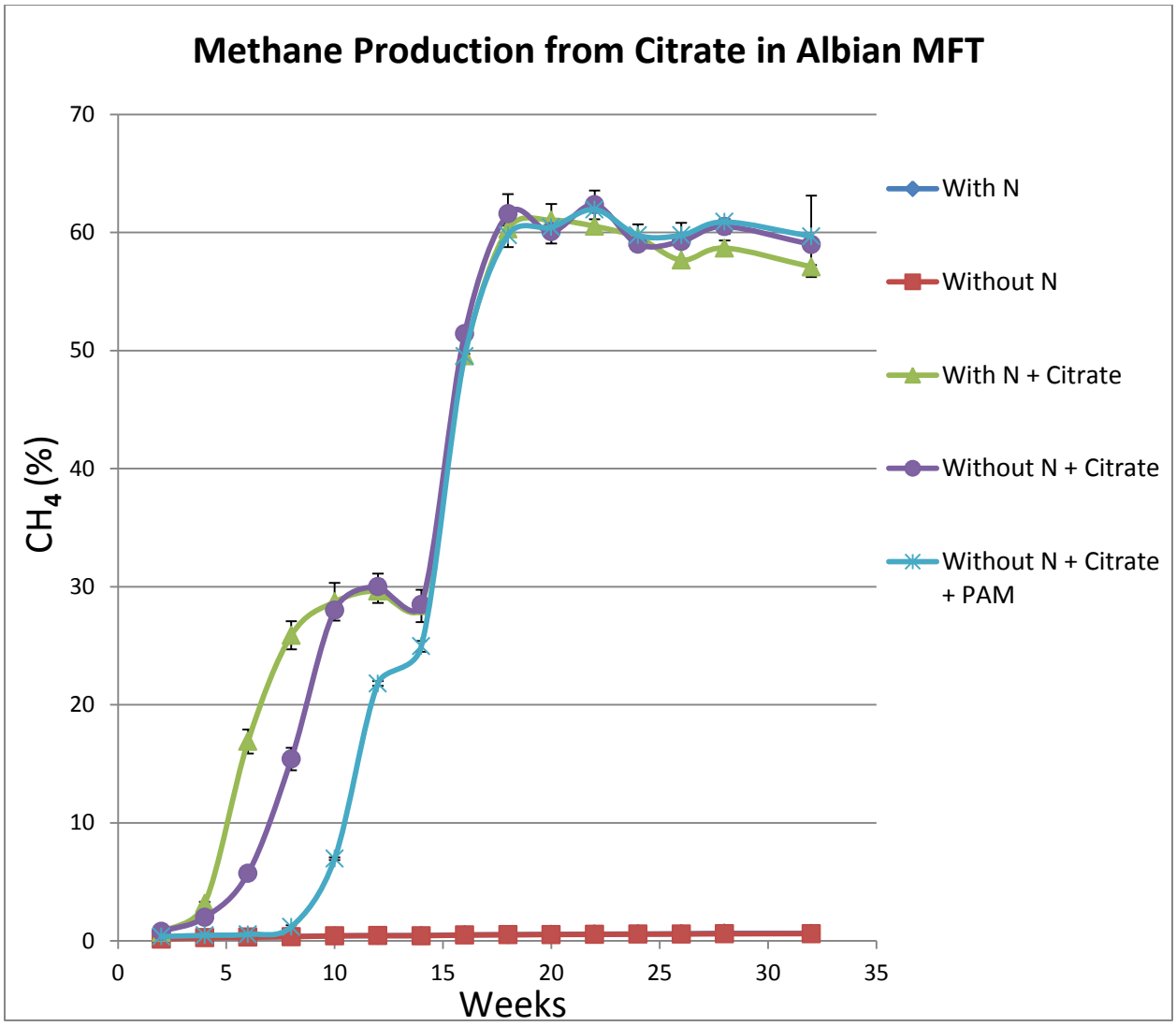

Figure A-2: Unpublished data from Siddique (2008) showing methane production in citrate-amended cultures under N-deficient conditions (without N + citrate) at volumes comparable to N-containing cultures (with N + citrate). The cultures were composed of 50 mL medium and 50 mL MFT under a 70% N_2/30% CO_2 headspace. With or without N indicates the presence or absence of available N in the medium. Plus citrate and/or plus PAM indicates that one or both chemicals were added to the cultures. Citrate-amended cultures contained 1000 mg L^{-1} citrate and PAM-containing cultures had 1000 mg L^{-1} PAM. Error bars represent standard deviation.

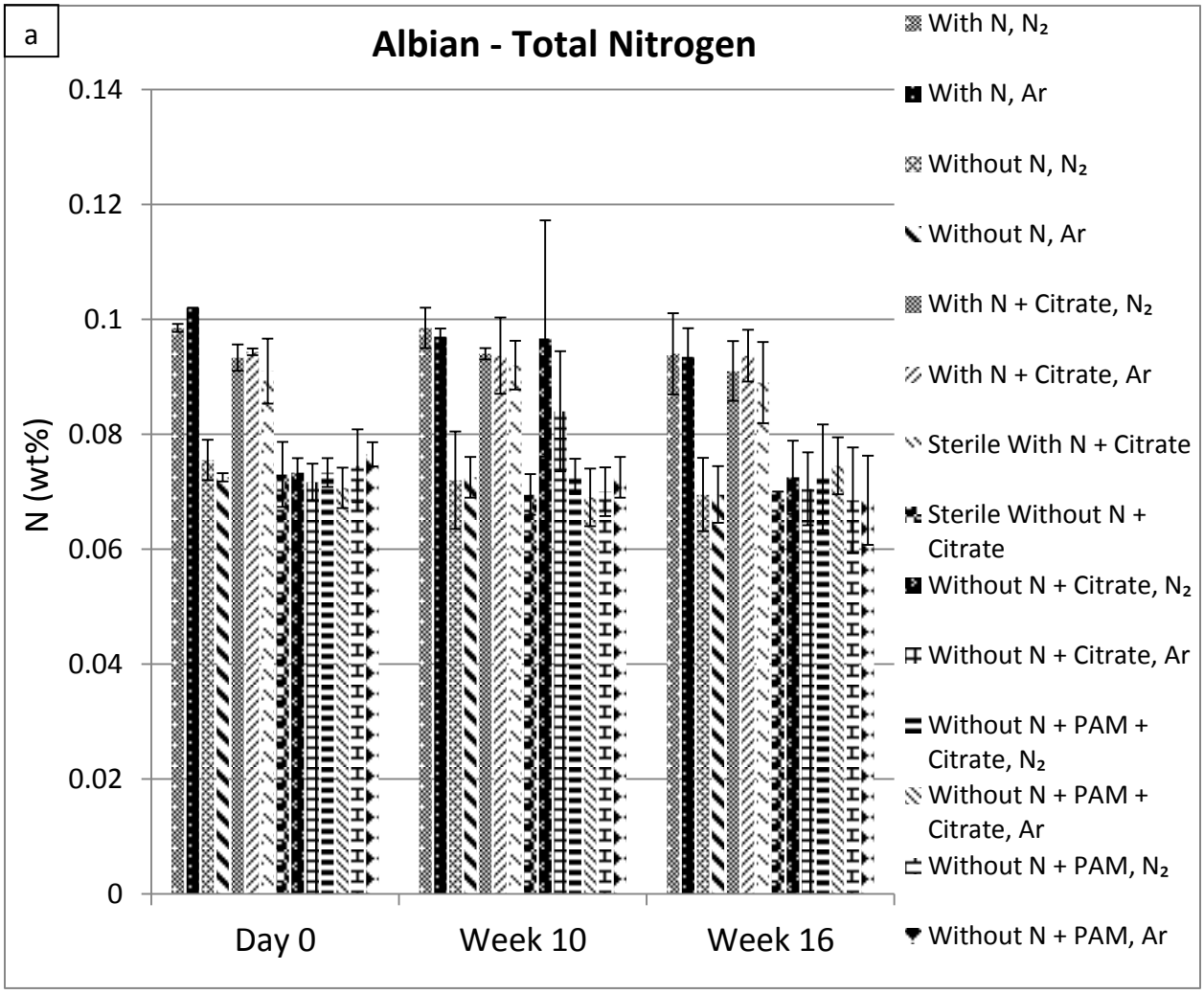

Figure A-3a: N concentrations in cultures at Day 0 (following two weeks of pre-incubation), Week 10 and Week 16. **Figure a**. analysis was performed using Dumas Combustion analysis of oven dried MFT and represents all N sources including organic and microbial biomass. With or without N indicates the presence or absence of available N in the medium. N_2 represents 30% CO_2/70% N_2 and Ar represents 30% CO_2/70% Ar gas in the headspace. See Table 2-1 for treatments. Error bars represent standard deviation.

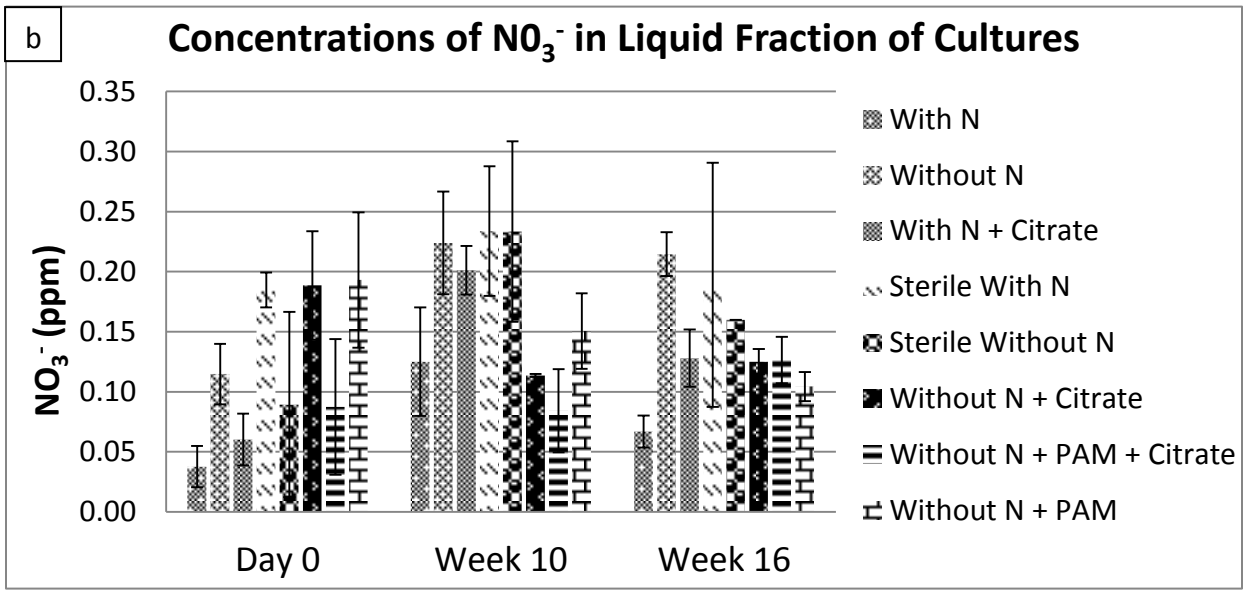

Figure A-3b: N concentrations in culture bottles at Day 0 (following two weeks of pre-incubation), Week 10 and Week 16. **Figure b.** represents all NO_3^- in the cultures, determined using colorimetric analysis. Only the liquid fraction was analyzed due to the small sample volume. With or without N indicates the presence or absence of available N in the medium. See Table 2-1 for treatments. Error bars represent standard deviation.

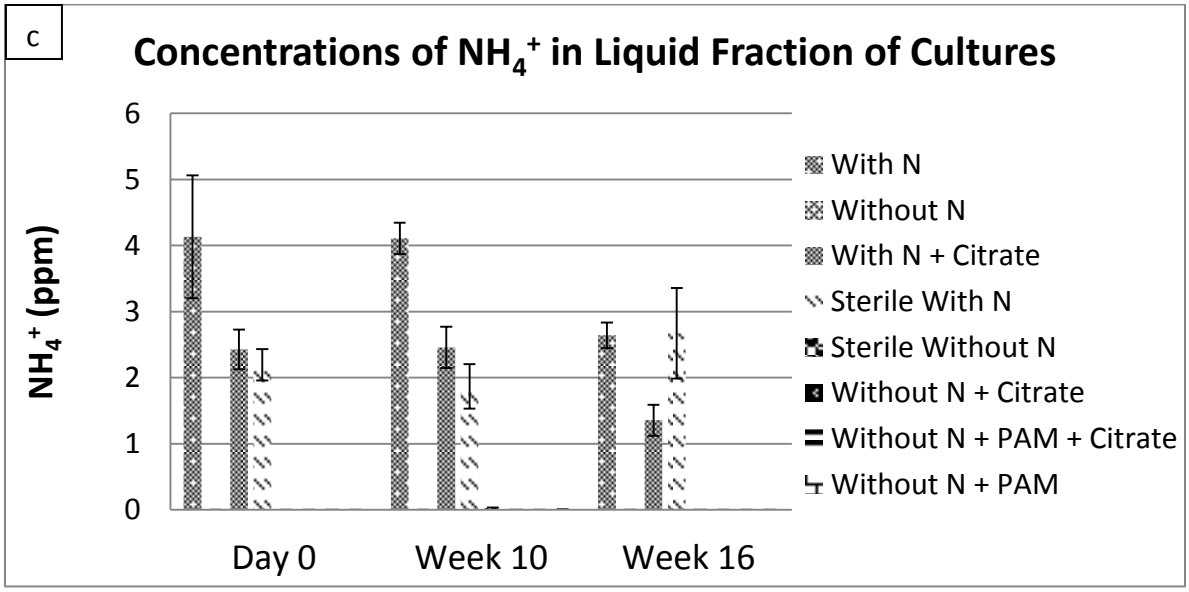

Figure A-3c: N concentrations in culture bottles at Day 0 (following two weeks of incubation), Week 10 and Week 16. **Figure c.** represents all NH_4^+ in cultures, determined using colorimetric analysis. Only the liquid fraction was analyzed due to the small sample volume. With or without N indicates the presence or absence of available N in the medium. See Table 2-1 for treatments. Error bars represent standard deviation.

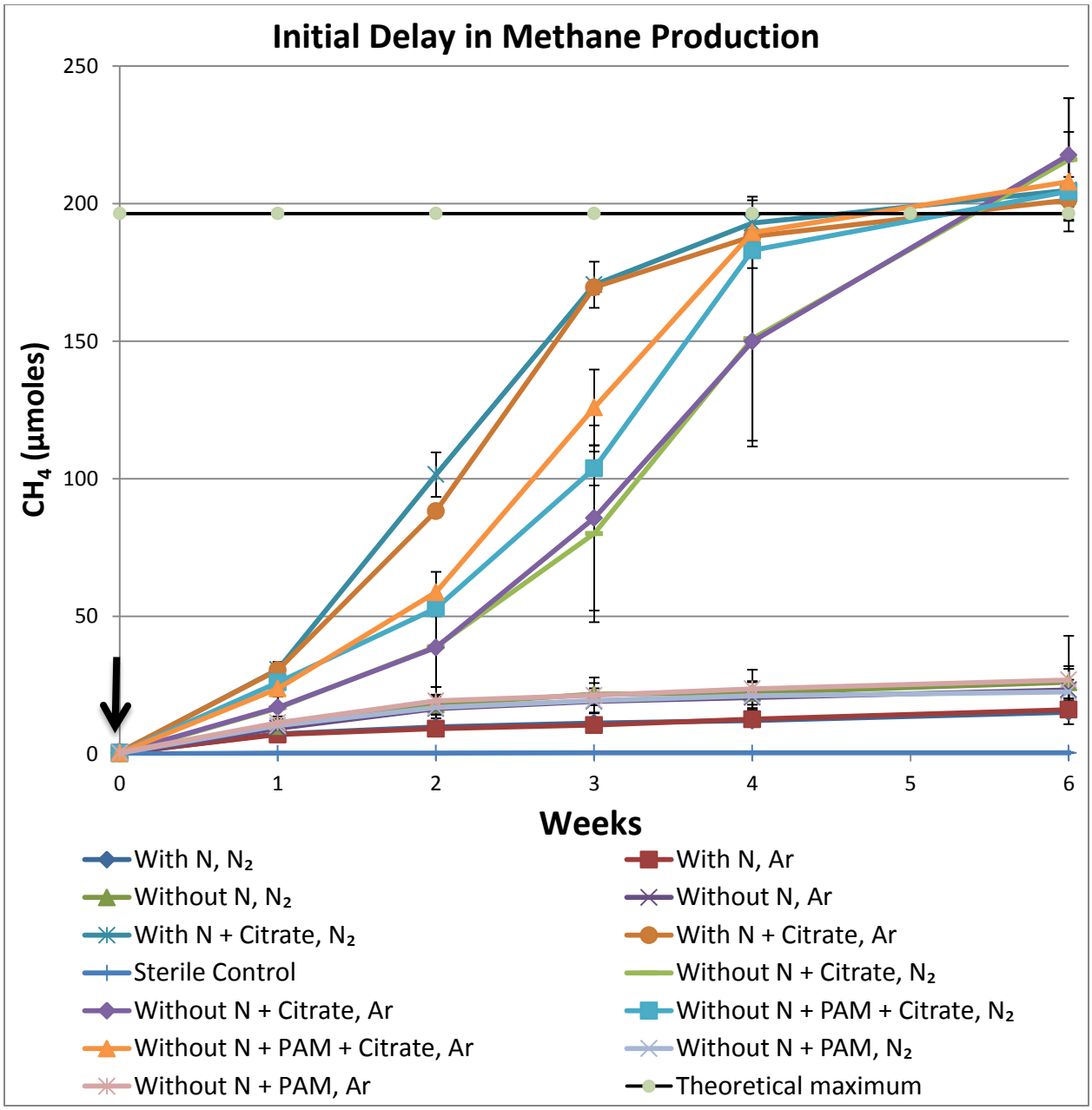

Figure A-4: Initial delay in cumulative methane (CH_4) production coupled with citrate degradation by MFT, incubated at ~22°C with citrate added to a concentration of 200 mg L^{-1} at Day 0. Arrow indicates citrate addition. Error bars represent standard deviation for three replicates in citrate-amended treatments and two replicates in unamended and heat-killed sterile cultures. Only one sterile control is shown as none of the sterile controls produced CH_4. With or without N indicates the presence or absence of available N in the medium. N_2 represents 30% CO_2/70% N_2 and Ar represents 30% CO_2/70% Ar gas in the headspace. The horizontal line represents the theoretical maximum CH_4 concentrations produced from the initial 200 mg L^{-1} of citrate. See Table 2-1 for treatments. Error bars represent standard deviation.

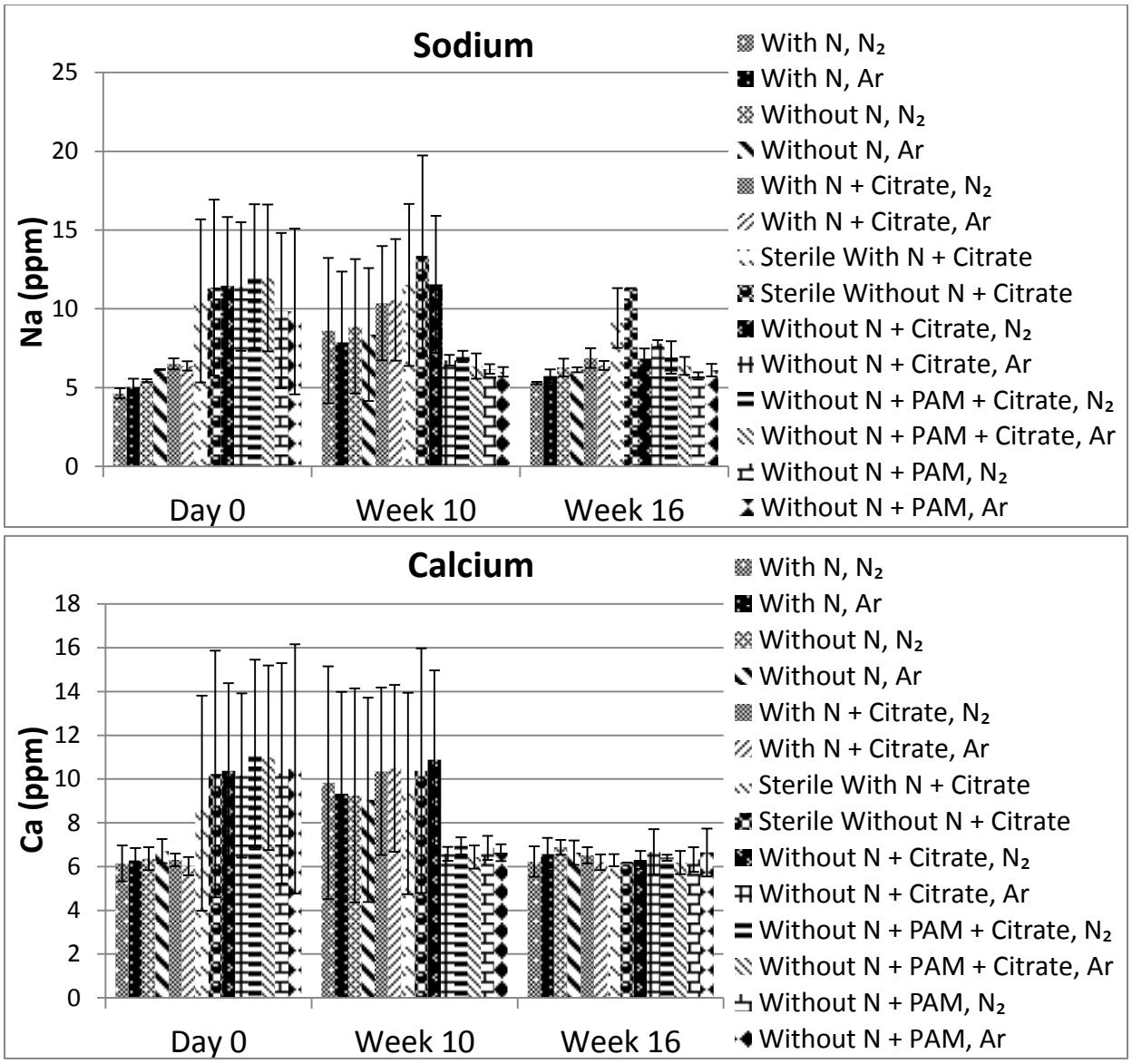

Figure A-5a: Concentrations of calcium and sodium in culture MFT. With or without N indicates the presence or absence of available N in the medium. N_2 represents 30% CO_2/70% N_2 and Ar represents 30% CO_2/70% Ar gas in the headspace. See Table 2-1 for treatments. Error bars represent standard deviation.

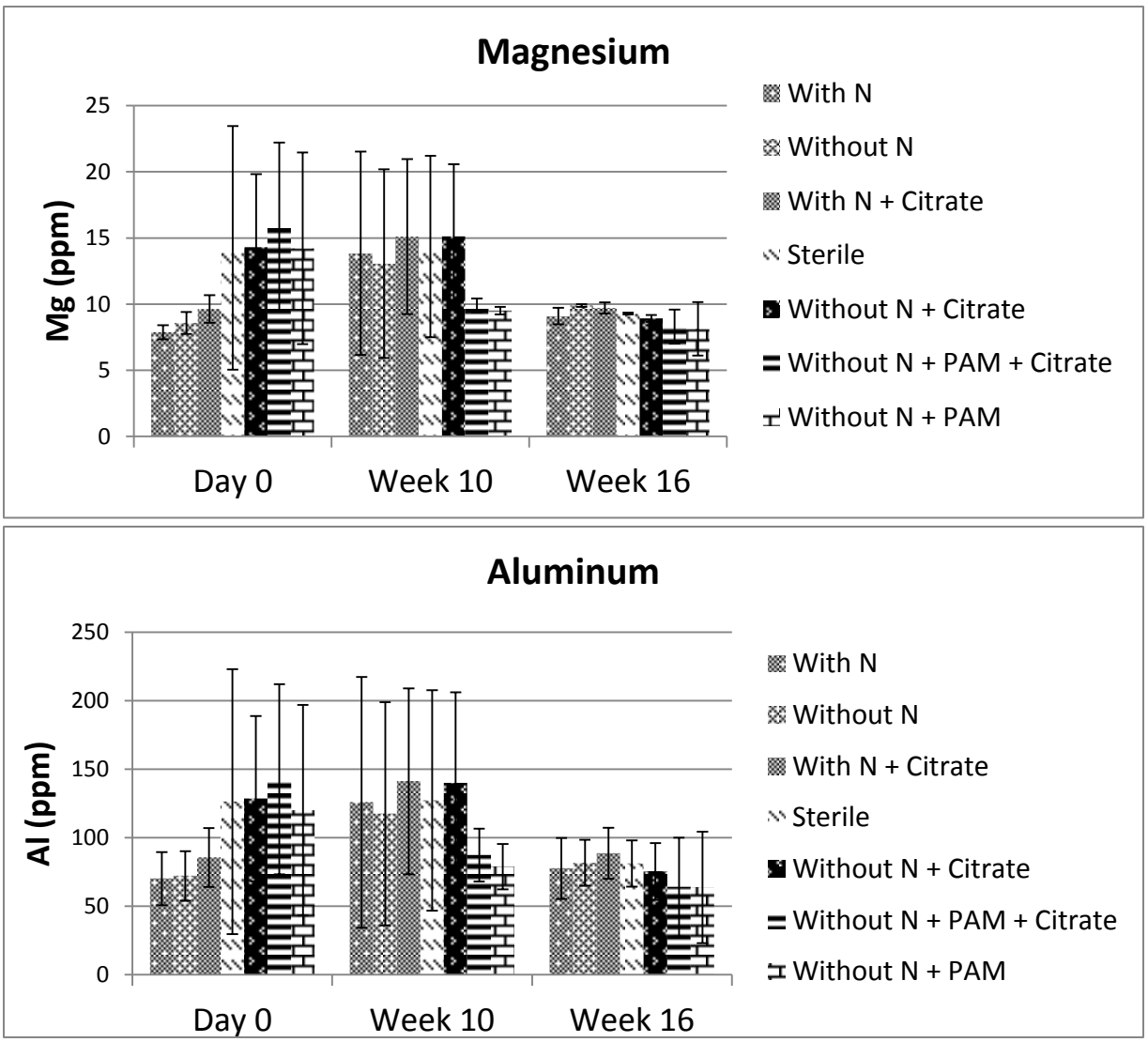

Figure A-5b: Concentrations of magnesium and aluminum in culture MFT. With or without N indicates the presence or absence of available N in the medium. See Table 2-1 for treatments. Error bars represent standard deviation.

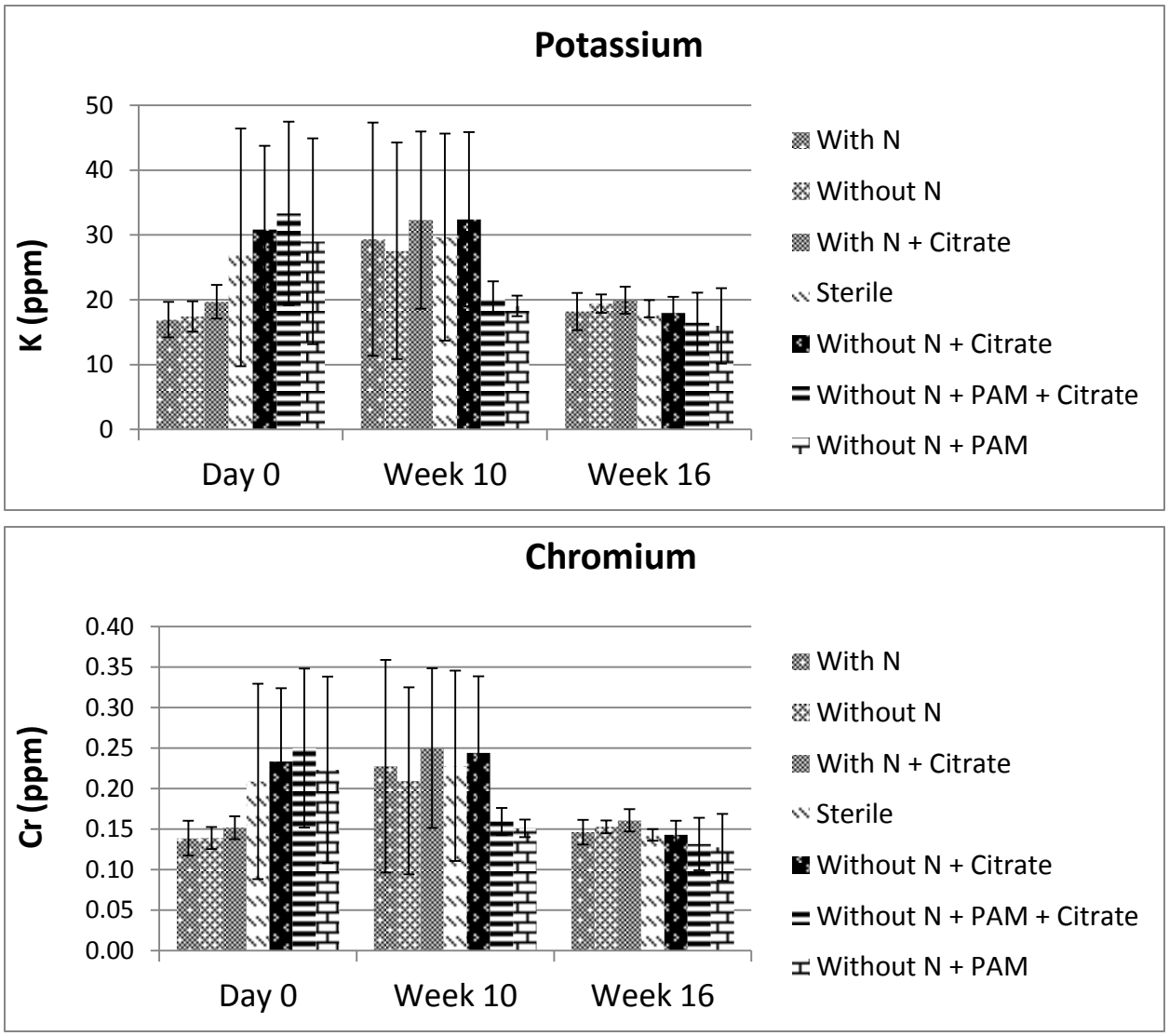

Figure A-5c: Concentrations of potassium and chromium in culture MFT. With or without N indicates the presence or absence of available N in the medium. See Table 2-1 for treatments. Error bars represent standard deviation.

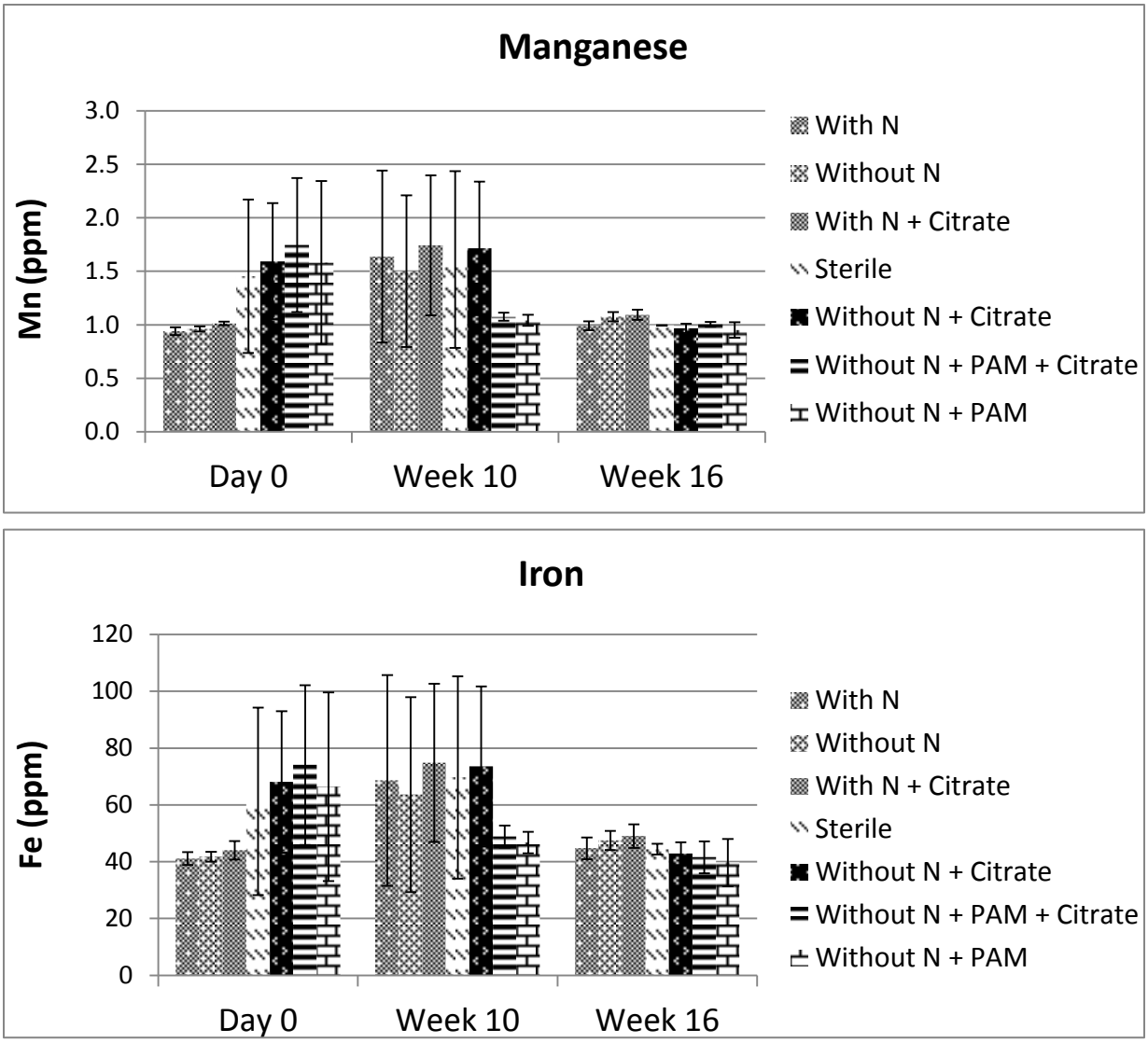

Figure A-5d: Concentrations of manganese and iron in culture MFT. With or without N indicates the presence or absence of available N in the medium. See Table 2-1 for treatments. Error bars represent standard deviation.

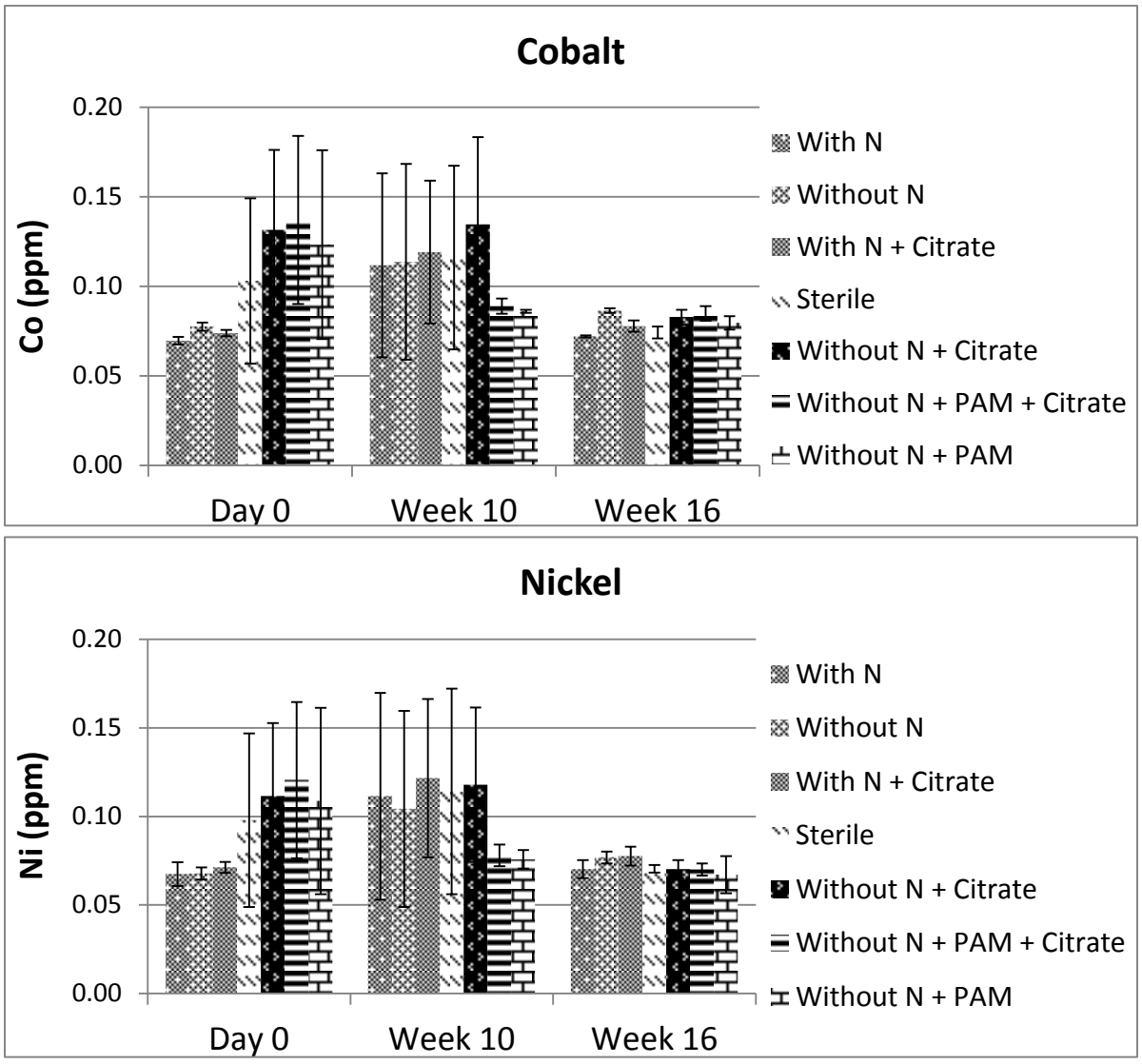

Figure A-5e: Concentrations of cobalt and nickel in culture MFT. With or without N indicates the presence or absence of available N in the medium. See Table 2-1 for treatments. Error bars represent standard deviation.

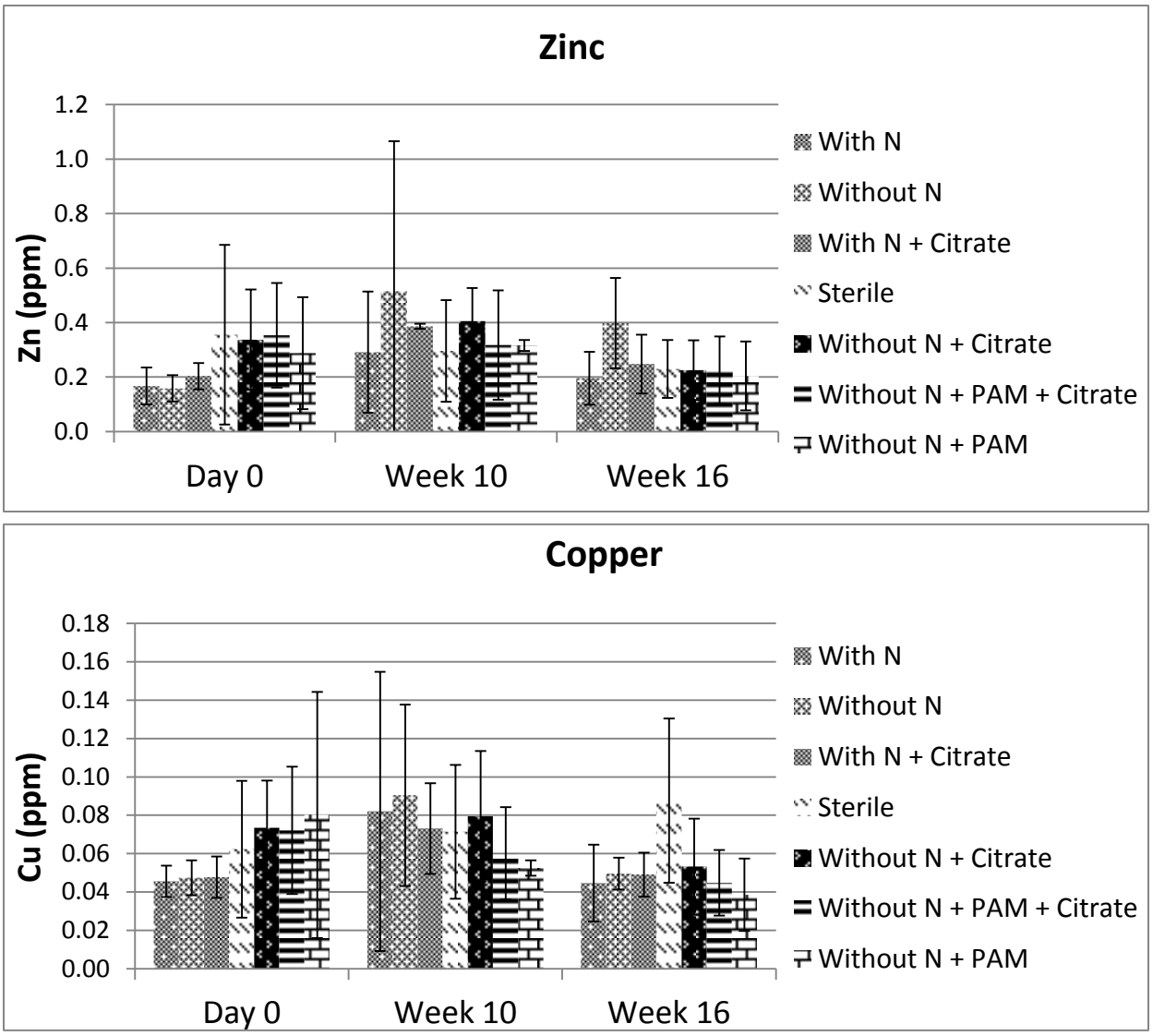

Figure A-5f: Concentrations of zinc and copper in culture MFT. With or without N indicates the presence or absence of available N in the medium. See Table 2-1 for treatments. Error bars represent standard deviation.

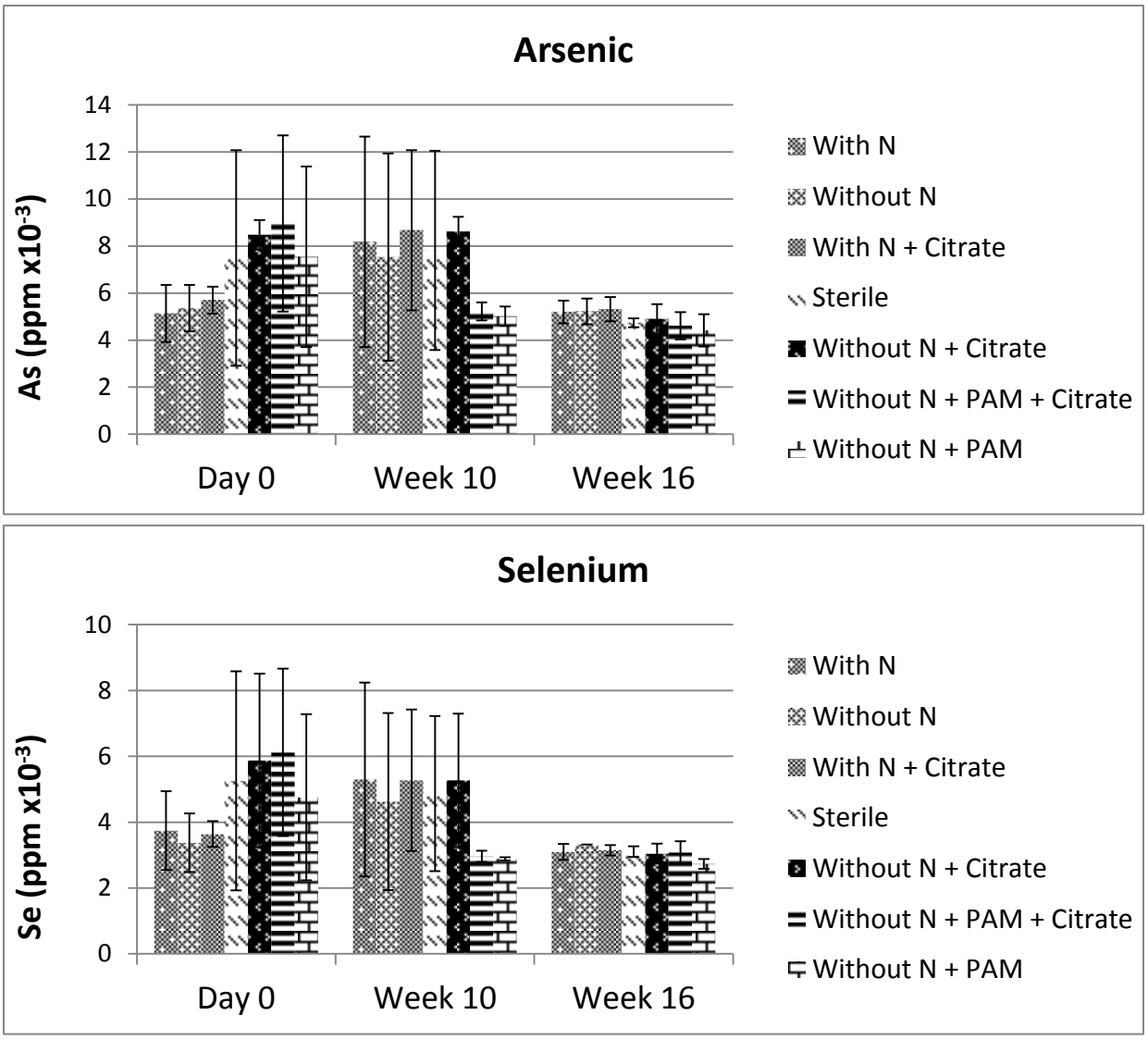

Figure A-5g: Concentrations of arsenic and selenium in culture MFT. With or without N indicates the presence or absence of available N in the medium. See Table 2-1 for treatments. Error bars represent standard deviation.

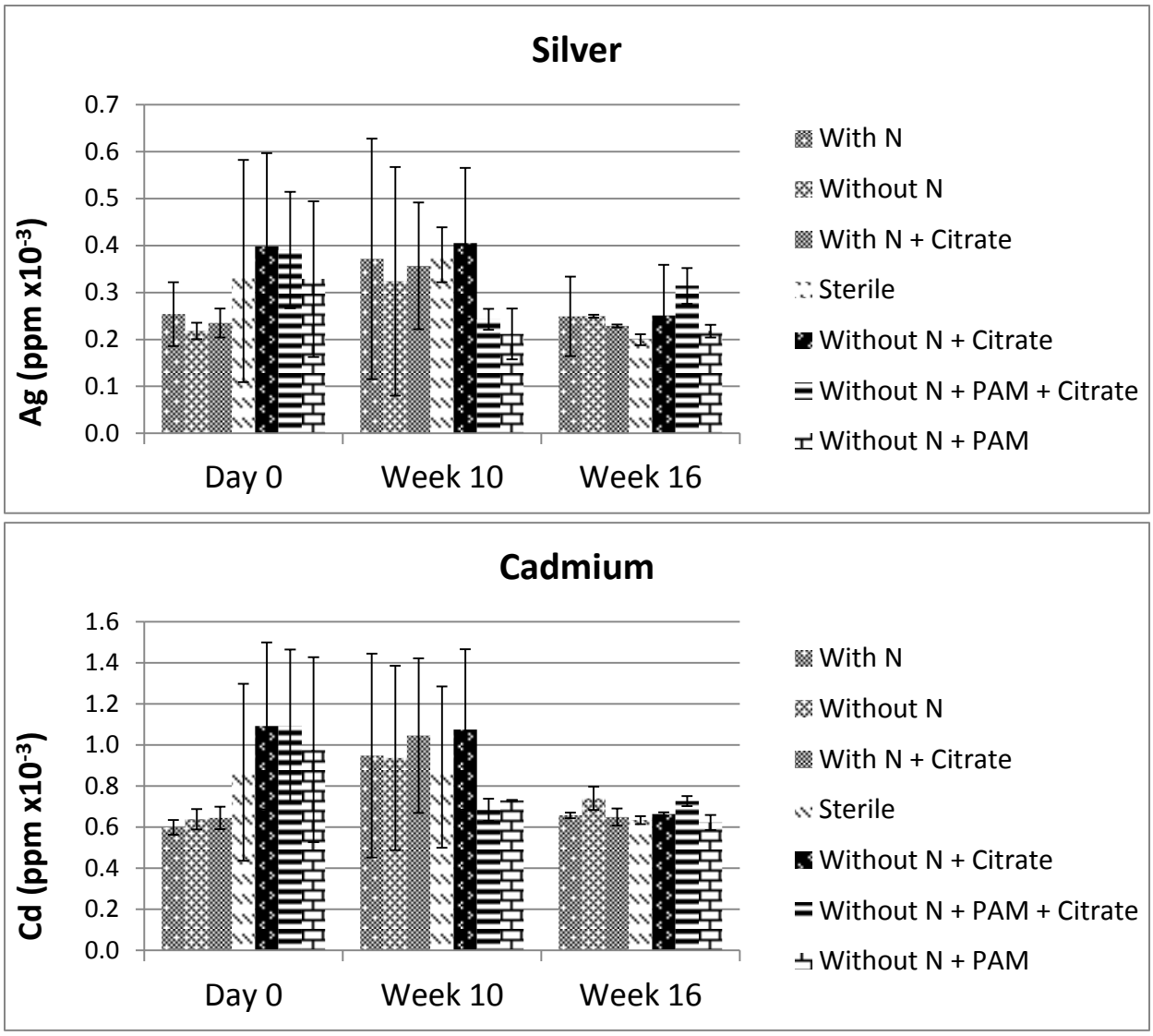

Figure A-5h: Concentrations of silver and cadmium in culture MFT. With or without N indicates the presence or absence of available N in the medium. See Table 2-1 for treatments. Error bars represent standard deviation.

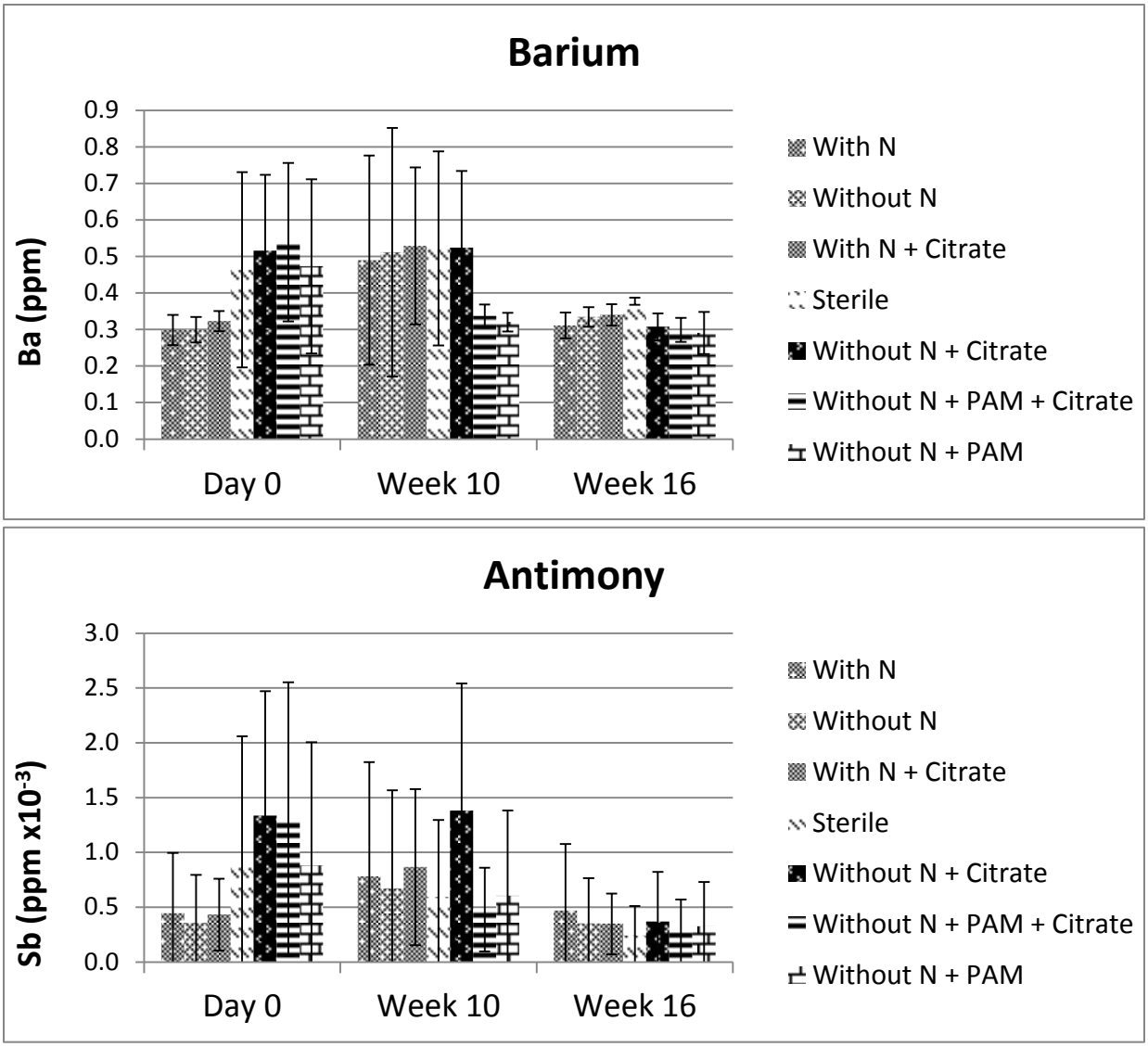

Figure A-5i: Concentrations of barium and antimony in culture MFT. With or without N indicates the presence or absence of available N in the medium. See Table 2-1 for treatments. Error bars represent standard deviation.

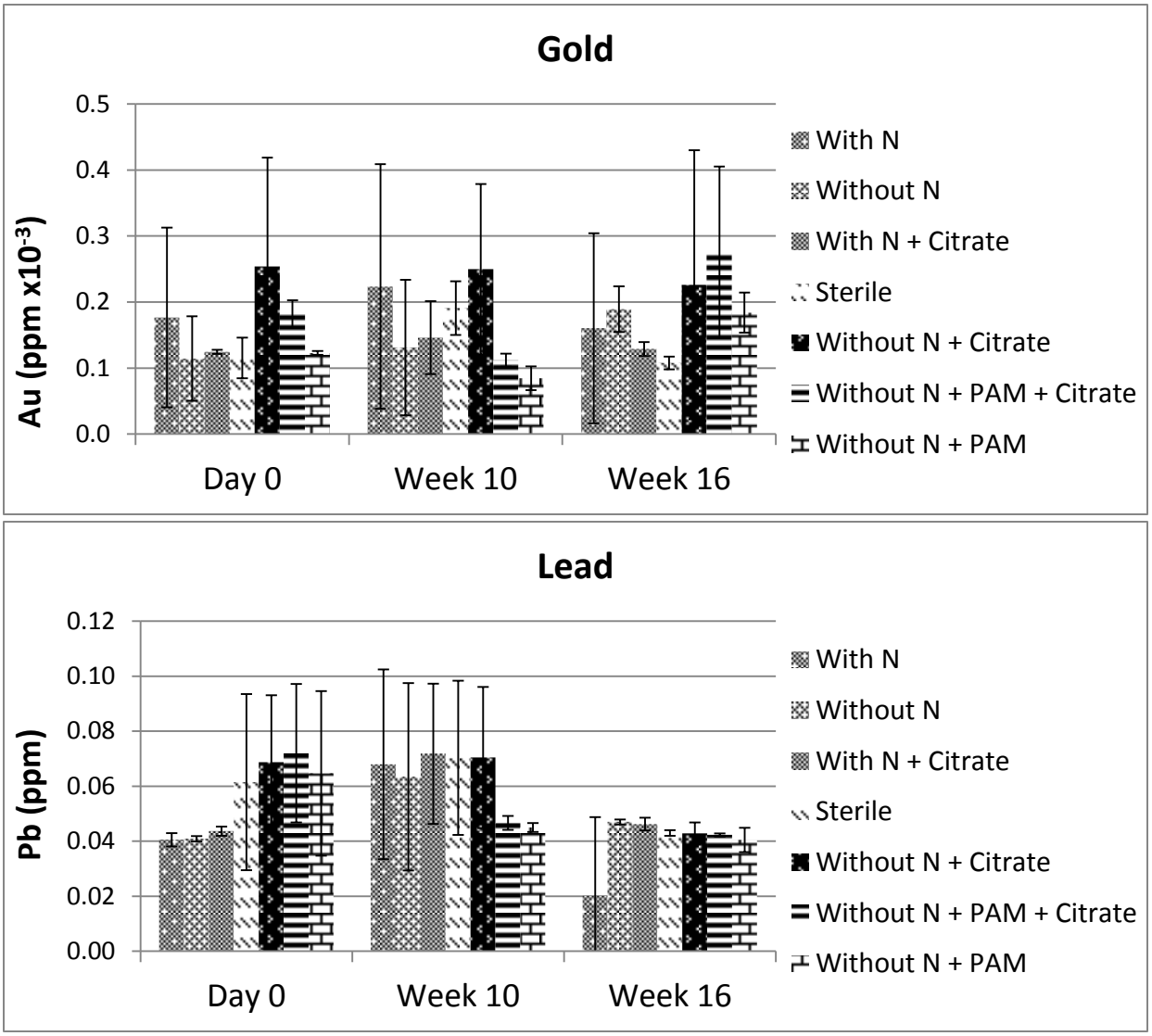

Figure A-5j: Concentrations of gold and lead in culture MFT. With or without N indicates the presence or absence of available N in the medium. See Table 2-1 for treatments. Error bars represent standard deviation.

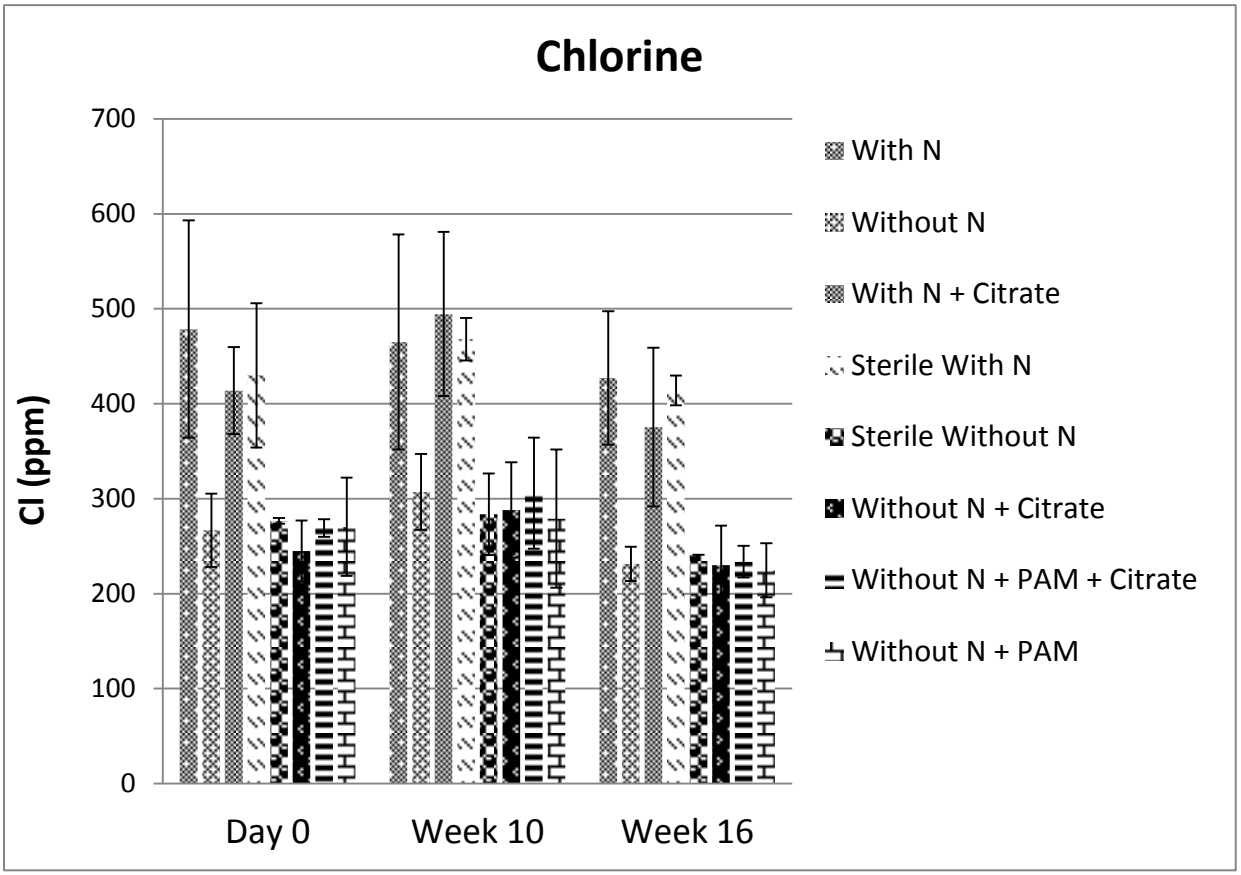

Figure A-5k: Concentration of chlorine determined in culture MFT. With or without N indicates the presence or absence of available N in the medium. See Table 2-1 for treatments. Error bars represent standard deviation.

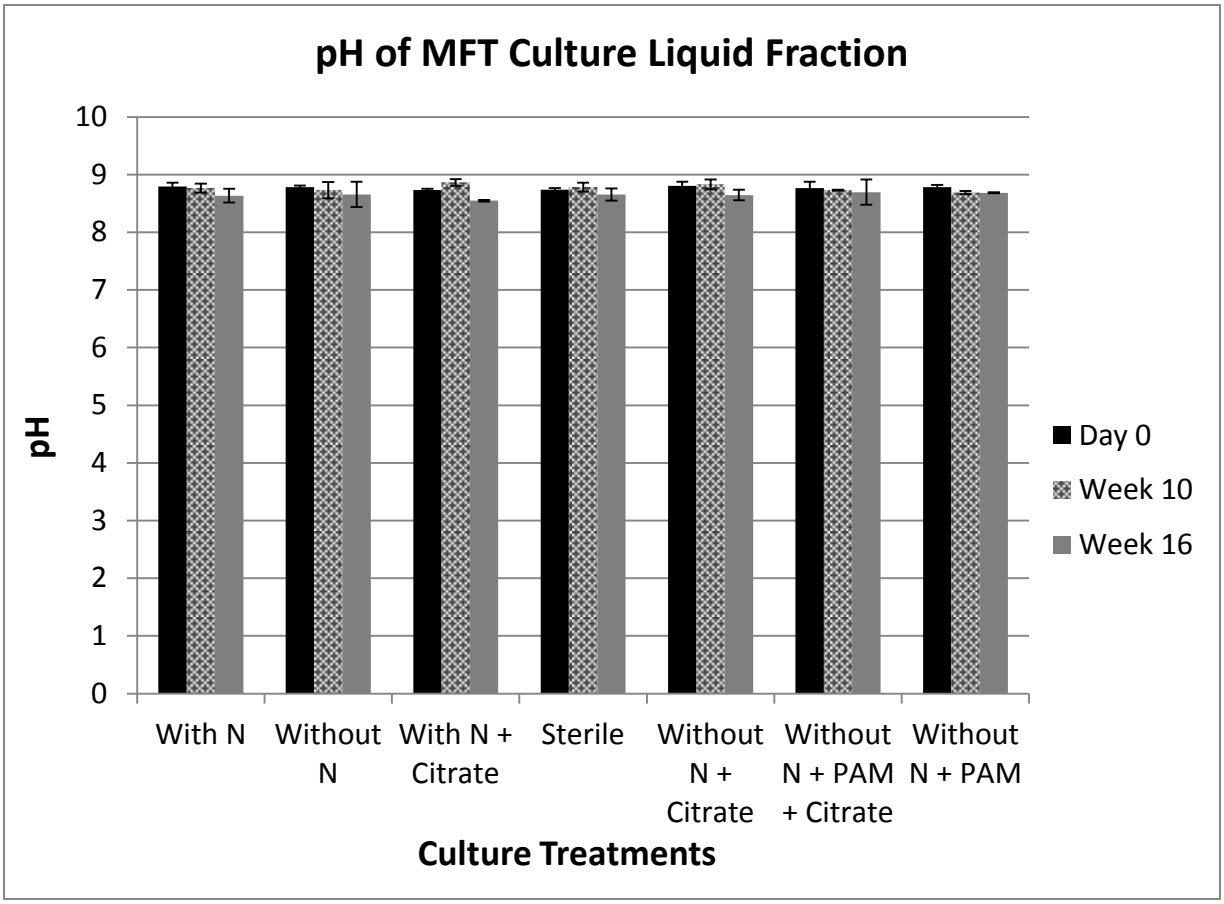

Figure A-6: pH of liquid fraction in culture bottles during incubation, determined by pH meter. With or without N indicates the presence or absence of available N in the medium. See Table 2-1 for treatments. Error bars represent standard deviation.

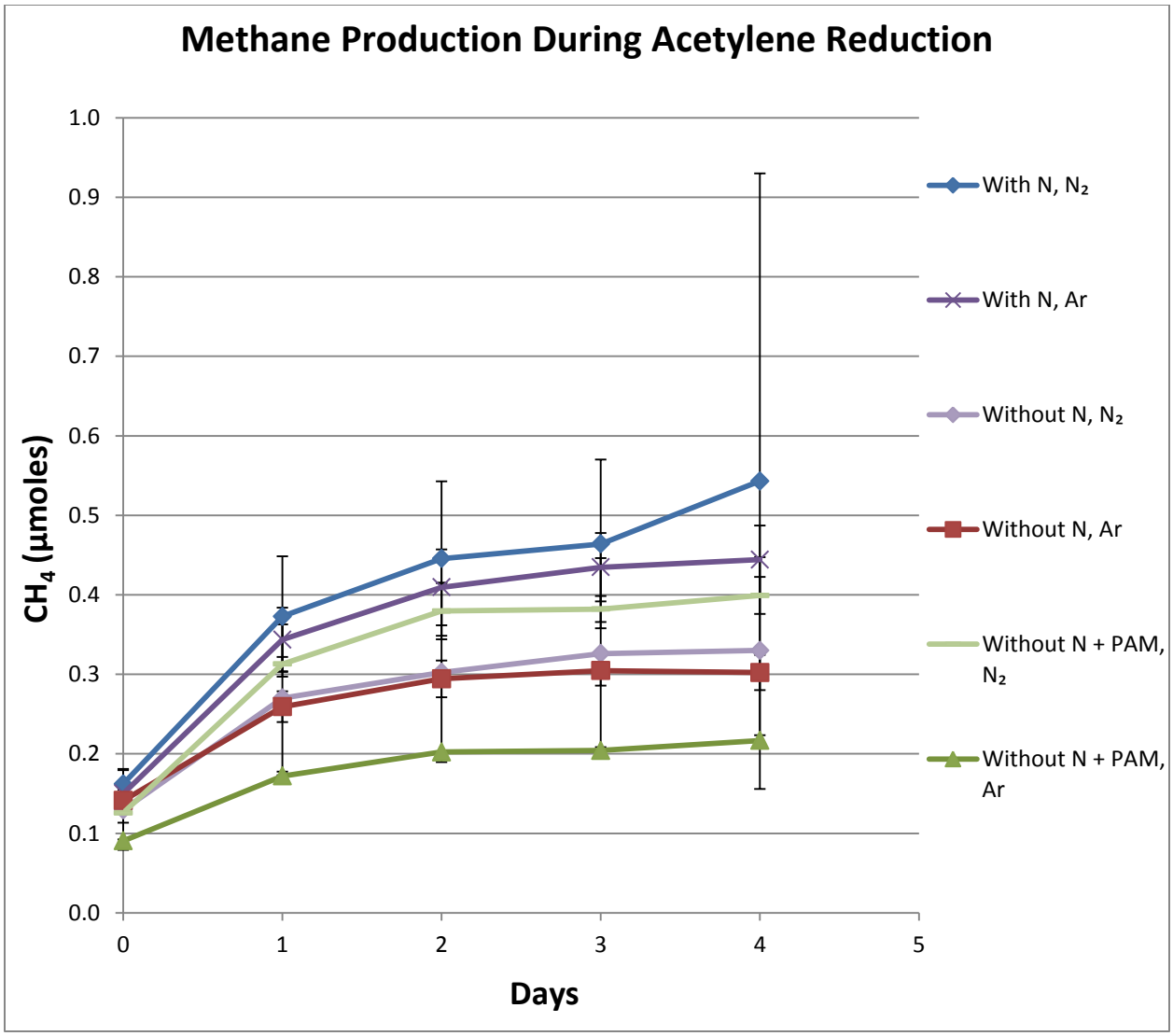

Figure A-7: Methane production in acetylene reduction cultures. With or without N indicates the presence or absence of available N in the medium; N_2 and Ar indicate the presence of CO_2/N_2 or CO_2/Ar in the headspace. Error bars represent standard deviation; positive controls and negative controls produced no CH_4.

Supplementary Materials: Appendix B

Methods B-1: Determination of Citrate by Reverse-Phase HPLC

The eluent was prepared by accurately weighing 10.0 g monobasic ammonium phosphate and transferring it to a 1 L volumetric flask where it was dissolved in 900 mL deionized water. The pH was adjusted to 2.80 with 1 M phosphoric acid and diluted to the mark with water. The eluent was then vacuum-filtered through a 0.45 µm Millex HA filter (Millipore Carrigtwohill Co.) and transferred to a HPLC eluent (carrier liquid) bottle.

To prepare 1000 mg L^{-1} stock solution for citrate, 0.3888 g trisodium citrate dihydrate was added to a 250.0-mL volumetric flask and diluted to the mark with eluent. Using this solution, working standards of 2, 10, 50, 100 and 200 mg citrate L^{-1} were prepared.

Samples were prepared by filtration through a 0.22 µm Millex filter (Millipore Carrigtwohill Co.) and 0.5 mL eluent was mixed with 0.5 mL sample: this brought the sample pH to ~2.8. The samples were then transferred to 2-mL HPLC vials for analysis.

The HPLC parameters were:

Flow rate:	1.0 mL min^{-1}
Injection volume:	50 µL
Column temperature:	24.0°C
Sample run time:	5.0 min
Detection wavelength 1:	214 nm

Column: Lichrospher 100, RP-18, 5 µm, part: 799250D-5643

Hulecki, J. 2009. Unpublished method: Determination of citrate by reverse-phase HPLC. University of Alberta, Edmonton, Alberta, Canada.

Methods B-2: Nitrogen-Deficient Combined Carbon Medium

The medium was prepared in two solutions.

Solution I:
K_2HP0_4, 0.8 g
KH_2P0_4, 0.2 g
NaCl, 0.1 g
NaFeEDTA, 28.0 mg
$Na_2MoO_4.2H_20$, 25.0 mg
Sodium vanadate, 25.0 mg
Yeast extract, 100.0 mg
Mannitol, 5.0 g
Sucrose, 5.0 g
Sodium lactate, 0.5 mL (60%, v/v)
Purified agar, 7 g
Distilled H_20, 900 mL

Solution II:
$MgS0_4.7H_20$, 0.2 g
$CaCl_2$, 0.06 g
Distilled H_20, 100 mL

The solutions were autoclaved and combined aseptically after they were cool. The following compounds were then added (final concentrations):
Filter-sterilized biotin (5 µg/L)
Para-aminobenzoic acid (PABA) (10 µg/L)

The pH was then aseptically adjusted to 7.0.

Rennie, R. 1981. A single medium for the isolation of acetylene-reducing (dinitrogen-fixing) bacteria from soils. Canadian Journal of Microbiology, 27:8-14.

Methods B-3: 16S rRNA Gene Pyrotag Sequencing Protocol

The following components were combined in a 0.2-mL PCR tube for each barcode (per reaction) to form a master mix: 2.5 µM Forward primer (1.25 µl), 2.5 µM Reverse primer (1.25 µl), 10X reaction buffer (2.5 µl), DMSO (1.25 µl), Taq polymerase (0.625 µl) and PCR-grade water (17.625 µl).

Each mastermix was vortexed and dispensed into three replicate PCR tubes; the remainder was kept in the original tube. This became the no-template negative control. One-half microliter of the same template DNA was then added to each of the three PCR tubes.

All tubes were placed in the thermocycler (Eppendorf Mastercycler Pro S) and the following program was run:

1 cycle of 5 min denaturation at 95°C
10 cycles of 30 s at 95°C, 30 s at 60°C, decreasing 0.5°C/cycle, and 30 s at 72°C
30 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C.
1 cycle of 10 min at 72°C

The pure products in the replicate template-containing PCR tubes were pooled and 4 µl of each template-containing and no-template control reaction were run on an agarose gel to confirm specific amplification.

The template-containing pooled PCR products were purified using the Qiagen QIAquick spin kit, then quantified using Nanodrop, performing 2 readings for each sample. Samples were mixed well before measuring.

The purified PCR products were diluted with PCR-grade water (sterile, nuclease free; Promega) to the concentration of 30 ng/µl in a 96-well plate. Two microliters of each diluted PCR product was run on an agarose gel to confirm equal concentrations and purity, and suitably diluted PCR products were sent for pyrotag sequencing.

This method was designed by Jonathan Klassen, 2010, University of Alberta.

Methods B-4: Mothur Commands

Note, all samples were in separate *.sff files and were processed accordingly. Example file names are given here.

merge.files(input=silva.archaea.fasta-silva.bacteria.fasta, output=silva.fasta)

sffinfo(sff=fileA.sff-fileB.sff-fileC.sff)

summary.seqs(fasta=fileA.fasta)

trim.flows(flow=fileA.flow, oligos=oligosname1.txt, pdiffs=2, bdiffs=1, processors=2)

shhh.flows(file=fileA.flow.files, processors=2)

trim.seqs(fasta=fileA.shhh.fasta, name=fileA.shhh.names, oligos=oligosfileA.txt, pdiffs=2, bdiffs=1, maxhomop=8, minlength=200, flip=T, processors=2)

merge.files(input=fileA-fileB-fileC, output=fileABC)

unique.seqs(fasta=fileABC.shhh.trim.fasta, name=fileABC.shhh.trim.names)

summary.seqs(fasta=fileABC.shhh.trim.unique.fasta, name=fileABC.shhh.trim.unique.names)

align.seqs(fasta=fileABC.shhh.trim.unique.fasta, reference=silva.fasta, flip=T)

summary.seqs(fasta=fileABC.shhh.trim.unique.align, name=fileABC.shhh.trim.unique.names)

screen.seqs(fasta=fileABC.shhh.trim.unique.align, name=fileABC.shhh.trim.unique.names, group=fileABC.shhh.groups, end=41790, optimize=start, criteria=95)

summary.seqs(fasta=fileABC.shhh.trim.unique.good.align, name=fileABC.shhh.trim.unique.good.names)

filter.seqs(fasta=fileABC.shhh.trim.unique.good.align, vertical=T, trump=., processors=2)

unique.seqs(fasta=fileABC.shhh.trim.unique.good.filter.fasta, name=fileABC.shhh.trim.unique.good.names)

pre.cluster(fasta=fileABC.shhh.trim.unique.good.filter.unique.fasta,
name=fileABC.shhh.trim.unique.good.filter.names, group=fileABC.shhh.good.groups, diffs=2)

summary.seqs(fasta=fileABC.shhh.trim.unique.good.filter.unique.precluster.fasta,
name=fileABC.shhh.trim.unique.good.filter.unique.precluster.names)

chimera.uchime(fasta=fileABC.shhh.trim.unique.good.filter.unique.precluster.fasta,
name=fileABC.shhh.trim.unique.good.filter.unique.precluster.names,
group=fileABC.shhh.good.groups, processors=2)

remove.seqs(accnos=fileABC.shhh.trim.unique.good.filter.unique.precluster.uchime.accnos,
fasta=fileABC.shhh.trim.unique.good.filter.unique.precluster.fasta,
name=fileABC.shhh.trim.unique.good.filter.unique.precluster.names,
group=fileABC.shhh.good.groups)

summary.seqs(name=current)

classify.seqs(fasta=fileABC.shhh.trim.unique.good.filter.unique.precluster.pick.fasta,
template=trainset7_112011.pds.fasta, taxonomy=trainset7_112011.pds.tax, cutoff=80,
processors=2)

remove.lineage(fasta=fileABC.shhh.trim.unique.good.filter.unique.precluster.pick.fasta,
name=fileABC.shhh.trim.unique.good.filter.unique.precluster.pick.names,
group=fileABC.shhh.good.pick.groups,
taxonomy=fileABC.shhh.trim.unique.good.filter.unique.precluster.pick.pds.taxonomy,
taxon=Mitochondria-Cyanobacteria_Chloroplast-unknown)

summary.seqs(fasta=fileABC.shhh.trim.unique.good.filter.unique.precluster.pick.pick.fasta,
name=fileABC.shhh.trim.unique.good.filter.unique.precluster.pick.pick.names)

system(copy fileABC.shhh.trim.unique.good.filter.unique.precluster.pick.pds.pick.taxonomy
final.taxonomy)

system(copy fileABC.shhh.trim.unique.good.filter.unique.precluster.pick.pick.names

final.names)

system(copy fileABC.shhh.trim.unique.good.filter.unique.precluster.pick.pick.fasta final.fasta)

system(copy fileABC.shhh.good.pick.pick.groups final.groups)

dist.seqs(fasta=final.fasta, cutoff=0.15, processors=2)

cluster(column=final.dist, name=final.names)

make.shared(list=final.an.list, group=final.groups, label=0.03)

count.groups(shared=final.an.shared)

sub.sample(shared=final.an.shared, size=1489)

classify.otu(list=final.an.list, name=final.names, taxonomy=final.taxonomy, label=0.03)

phylotype(taxonomy=final.taxonomy, name=final.names, label=1)

make.shared(list=final.tx.list, group=final.groups, label=1)

sub.sample(shared=final.tx.shared, size=1489**)**

classify.otu(list=final.tx.list, name=final.names, taxonomy=final.taxonomy, label=1)

Schloss, P.D., et al., Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology, 2009. 75(23):7537-41

Supplementary Materials: Appendix C

Materials C-1: Chemical Reagents and Gasses

Chemicals/Reagents

Methanogenic media and culture amendments: Polyacrylamide (Magnafloc LT27AG, Ciba Specialty Chemicals Ltd), tri-sodium citrate dihydrate (analytical grade, BDH), sodium chloride (GR ACS, BDH), calcium chloride dehydrate (GR ACS, Fisher Scientific), ammonium chloride (GR ACS, EMD), magnesium chloride hexahydrate (GR ACS, Fisher Scientific), ammonium molybdate (reagent grade, Fisher Scientific), sodium molybdate (GR ACS, EM Science), zinc sulphate (GR ACS, Merck), boric acid (GR ACS, Fisher Scientific), ferrous chloride (analytical grade, Chemical Works), cobalt (II) chloride hexahydrate (reagent grade, Sigma Aldrich), magnesium chloride (reagent grade, Fisher Scientific), nickel chloride (reagent grade, Fisher Scientific), aluminum potassium disulphate (reagent grade, BDH), sodium bicarbonate (reagent grade, Fisher Scientific), sodium sulphate (reagent grade, EMD), resazurine sodium salt (GR ACS, MPBiomedicals), potassium phosphate, monobasic (GR ACS, Anachemia), double distilled water (ddH_2O).

Nitrogen-deficient medium and carbon sources: Di-potassium hydrogen orthophosphate (analytical grade, BDH), potassium phosphate monobasic anhydrous (reagent grade, Sigma Aldrich), sodium chloride (GR ACS, BDH), disodium ferrous ethylenediaminetetraacetate (reagent grade, Sigma Aldrich), sodium molybdate (GR ACS, EM Science), yeast extract (Difco), mannitol (analytical grade, SchwarzMann Biotech), sucrose (GR ACS, BDH), sodium lactate (GR ACS, Fisher Scientific), magnesium sulphate (GR ACS, EMD), calcium chloride (anhydrous)

(reagent grade, Fisher Scientific), Biotin (D) (reagent grade, Sigma Aldrich), para-aminobenzoic acid (PABA) (reagent grade, Sigma Aldrich), ammonium chloride (GR ACS, EMD).

DNA Extraction and PCR: Tris-HCl (reagent grade, Baker), sodium chloride (biology grade, Fisher), monobasic phosphate (GR ACS, Fisher), dibasic phosphate (GR ACS, Fisher Scientific), sodium dodecyl sulphate (SDS) (electrophoresis purity, Biorad), ammonium acetate (GR ACS, EMD), chloroform (GR ACS, Fisher Scientific), isoamyl alcohol (GR ACS, MP), isopropanol (GR ACS, Fisher Scientific), sterile milliQ water (MQH_2O), magnesium chloride (biology grade, BioSciences), ß-mercaptoethanol (biology grade, Calbiochem), ammonium sulphate (biology grade, Fisher Scientific), dNTPs (Invitrogen), Bovine Serum Albumen (BSA) (Roche), DMSO (biology grade, Alfa aesar), Taq polymerase (DNA-free, Invitrogen, ref:10342-053).

Gases

Acetylene (scientific grade, Praxair), $^{15}N_2$ (98% purity, Sigma-Aldrich), 30% CO_2/70% N_2 and 30% CO_2/70% Ar (certified standard, Praxair). GC-FID: Helium (certified standard, Praxair), hydrogen (certified standard, Praxair), compressed air (certified standard, Praxair).