Methane Production and Emission Mitigation in Oil Sands Tailings Concurrent with Hydrocarbon Degradation under Nitrogen Limited Conditions

by

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Abstract

Alberta's oil sands generate large volumes of tailings from bitumen ore processing. These tailings ponds produce biogenic methane, which can be measured across 60-80% of the tailings surface. Based on current surface area data and emissions studies, tailings ponds could account for 8% of Canada's methane emissions. With a government mandate to reduce methane emissions by 45% of 2012 levels, understanding the dynamics of methanogenesis in tailings is highly important. Methane production from oil sands tailings is driven by hydrocarbon metabolism by the microbial community. For community growth and metabolism to occur, sufficient nutrients such as nitrogen (N) must be available in the environment. Several tailings ponds as well as reclaimed wet landscapes that incorporate mature fine tailings (MFT) are deficient in bioavailable N (NH_4^+, NO_2^-/NO_3^-) and yet continue to produce methane. Understanding this process with hydrocarbon amendments is important as it is generally assumed methane production will halt in the absence of nutrients. Additionally, literature suggests that methanogenesis is inhibited in the presence of amorphous Fe(III). This implies amendments such as amorphous Fe(III) oxides (rust) may be effective at reducing methane emissions in tailings ponds and potentially in reclaimed wet landscapes.

In Chapter 2, we investigate methane production resulting from the degradation of *n*-alkanes and toluene in the presence and absence of fixed N in microcosms under an N_2/CO_2 headspace. Acetylene reduction assays indicated that N_2-fixation was present in the absence of fixed N concurrent with *n*-alkane and toluene degradation, and methane production. Community gene analysis using 16S rRNA indicated that the bacterial community was dominated by fermentative bacteria and the archaeal community consisted primarily of *Methanoseata* in amended cultures. Functional gene analysis (DNA and mRNA) for the N_2 fixing enzyme, nitrogenase (*nifH*) indicated genes can be expressed disproportionately to the coding DNA. In *n*-alkane cultures, *nifH* mRNA was predominantly expressed by *Methanosaeta* and in sequenced

toluene cultures, *nifH* expression was detected in both *Desulfovibrionales* and *Methanosaetaceae*. These results suggested Archaea were responsible for most of the nitrogenase expression, and therefore N_2-fixation in *n*-alkane amended N depleted treatments and in nearly equal proportions with *Desulfovibrionales* in toluene amended N depleted CNRL cultures. The presence of these genes in MFT suggest that anaerobic N transformations such as N_2-fixation are possible *in-situ*. The results of this study support our hypothesis that N_2-fixing microorganisms within the microbial communitycan support hydrocarbon degradation and methanogenesis in oil sands tailings communities.

In Chapter 3, we investigated the use of amorphous Fe(III) as a methane inhibitor in conjunction with hydrocarbon degradation under N depleted conditions. As in chapter 2, we established cultures using tailings from three operators active at the time of this work, Albian, CNRL, and Syncrude. Cultures were established with and without fixed N and amended with toluene or *n*-alkanes in the presence of $Fe(OH)_3$. 16S rRNA and functional genes were sequenced to define the microbial community. While no *n*-alkane degradation was observed, toluene degradation was recorded. In all cases, methanogenesis was inhibited. As observed in Chapter 2, N_2-fixation as determined using an acetylene reduction assay was present in N deficient cultures alongside *nifH* expression. These data support our hypothesis that amorphous Fe(III) can be effectively used as a treatment to mitigate methane emissions by oil sands tailings communities while maintaining detectable rates of hydrocarbon degrading activity under N deficient conditions.

In Chapter 4, we sought to determine if $Fe(OH)_3$ could be used to inhibit methane production from the metabolism of citrate, a dispersant and labile methanogenic substrate found in some tailings ponds. Acetylene reduction assay revealed that N_2-fixation was occurring in N deficient cultures, both with and without Fe(III). Methanogenesis was observed in the absence of Fe(III) as expected, however, methane was not inhibited in cultures treated with amorphous $Fe(OH)_3$. We theorized this was due to abiotic reactions between citrate and $Fe(OH)_3$ resulting in the formation of ferric citrate complexes. Unlike amorphous $Fe(OH)_3$ ferric citrate complexes are not known to inhibit methanogenesis. These data suggest

that $Fe(OH)_3$ may not effectively treat methane emissions in ponds that contain a continuous source of citrate.

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Abbreviations

CNRL	Canada Natural Resources Limited
DNRA	Dissimilatory nitrate reduction to ammonium
GC-FID	Gas chromatography flame ionization detector
GC-MS	Gas chromatography mass spectrometer
MFT	Mature fine tailings
MLSB	Mildred Lake Settling Basin
N	Nitrogen
nifH	Encodes for nitrogenase enzyme
nirS	Encodes for nitrite reductase
nosZ	Encodes for nitrous oxide reductase
nrfA	Encodes for nitrite reductase
PCR	Polymerase chain reaction
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid

1. Introduction

1.1 Oil Sands Tailings

1.1.1 Industry and tailings production

The Oil Sands occupy an area of 142,000 km^2 in the Athabasca and Cold Lake regions of northern Alberta (Canadian Association of Petroleum Producers, 2015). These formations are composed primarily of sand and clay and contain deposits of organic matter known as kerogen. Kerogen is derived from the ancient deposition of bacteria, lignin from terrestrial plants (Meslé et al., 2013), and potentially lignin precursors found in unicellular and multicellular algae (Labeeuw et al., 2015). In addition to recalcitrant nitrogen and organics, kerogen contains solvent soluble bitumen, which is extracted for upgrading (Meslé et al., 2013).

Bitumen is refined into crude oil that makes up 60% of all oil production in Canada (Canada's Oil Sands, 2016). The oil sands region contains an estimated 166 billion barrels of extractable oil, which is recovered at 2.8 million barrels per day (Alberta Energy, 2018). Canada holds the third largest oil reservoir in the world because of the oil sands, however not all bitumen deposits are considered accessible to mining.

Bitumen is extracted through two primary methods, *in-situ* extraction, and open pit mining. In 2017, 55% of bitumen was mined using *in-situ* methods and 45% was accessed through open pit mining (Alberta Energy, 2018). The extraction method employed depends on the depth of the deposit. *In-situ* methods must be used for deposits located more than 70 m below the surface, whereas shallower deposits can be accessed through open pit mining (Canada's Oil Sands, 2016). The most common *in-situ* methods are SAGD (steam assisted gravity drainage) and CSS (cyclic steam stimulation). These extraction methods operate by pumping steam into the deposit to melt the bitumen. Once the viscosity of the bitumen has been reduced, it can be pumped out for upgrading and refining. The water or solvents used in this processed are recovered and reused thereby producing little waste materials. In open pit mining, the surface materials are stripped off and the deposit materials are excavated by truck and shovel. The bitumen materials are then crushed and processed using a modified Clark Hot Water extraction process.

The Clark Hot Water Process is the method by which bitumen is separated from sand and clay particles using a combination of mechanical processing, heat, and surfactants (Mikula et al., 1996). In some cases, caustic soda (NaOH) is added to increase the release of surfactants. However, this practice has been largely discontinued by most operators due to resulting problems with alkalinity in wastewater and downstream processes (Allen, 2008). The slurry is piped to a separation vessel where the bitumen floats

to the surface forming a froth that is skimmed. Some operators (former Shell Albian, hereafter referred to as Albian), added dispersants such as trisodium citrate to further increase the release of bitumen from the solid particles (Devenny, 2009). Solvents such as naphtha used by Syncrude, CNRL, and Suncor, or paraffinic diluent (C_5-C_6) used in former Albian processes to reduce emulsion stability and precipitate asphaltenes. The froth is then pumped off for further upgrading and eventual processing to crude oil. Solvents are recovered and the remaining slurry is combined with consolidating agents before it's pumped into setting basins known as tailings ponds (Masliyah et al., 2004).

Fresh tailings are composed of sand, silt, clay, unrecovered solvents, residual bitumen, and water (Allen, 2008). Once released into tailings ponds, the heavier particulates settle out forming sand dykes and beaches. The tailings suspension settles to 20 wt% solids in weeks but can take years to reach 30-35 wt% solids. This sludge material is known as mature fine tailings (MFT). Further consolidation to 44 wt% solids will take an additional 10 years (Allen, 2008 and references therein). As of 2015, there were ~1.075 billion m^3 of oil sands tailings stored for future reclamation (http://osip.alberta.ca/map/).

There are two proposed methods for tailings reclamation, terrestrial reclamation, and aquatic consisting of wetlands and end-pit lakes. It is estimated that 50-70% of tailings will be reclaimed to terrestrial land (Sobkowicz, 2012). For terrestrial reclamation, tailings that have been consolidated either through settling or with the addition of flocculating agents are added to mined out pits. Processed oil sands is then added followed by overburden (BGC Engineering Inc., 2010). The tailings are then capped with an additional 50 to 120 cm materials such as glacial till followed by peat mineral mix (Sobkowicz, 2012). The site is then suitable for plants such as barley, which die after a single season and contribute to both soil structure and carbon reserves. Trees and shrubs can be planted on the surface the following year.

In aquatic reclamation, between 20 and 40% of disturbed land will be reclaimed as wetlands (Sobkowicz, 2012). Wetlands may be one of several types including bogs, fens, marshes, open water, and littoral zones around end-pit lakes. These biomes will primarily be built on beds of tailings in areas of topographic gradients less than 0.3%. The design of these wetlands will vary to accommodate hydrology and topography but the base of the wetland will generally contain about 30 cm of peat. The other form of aquatic reclamation is water capping in the form of end-pit lakes. In this model, tailings are added to a mine-out pit and capped with at least 5 meters of water (BGC Engineering Inc., 2010). Riparian and littoral zones are then prepared to surround the lake. Approximately 30 end pit lakes are planned for the oil sands region and will account for 5-10% of the reclaimed landscape (Sobkowicz, 2012).

Due to the importance of water quality in the surface waters, water treatment will likely be necessary for 20-50 years to remove contaminants as they are released from the tailings in the basin and other tailings material in landforms within the lake's watershed. Both wetland and end-pit lake tailings reclamation landforms are expected to naturally attenuate naphthenic acids and eventually support an ecosystem (BGC Engineering Inc., 2010). Currently, there are concerns that methane production from the underlying tailings combined with continued microbial metabolism may result in additional contaminants being released and transported to the cap water where plants and animals may be affected.

1.1.2 Methanogenesis from tailings

After 15 years in operation in the early 1990s, a tailings pond owned by Syncrude known as Mildred lake settling basin (MLSB) began producing methane with emission production estimated at 12 g CH_4/m^2 or 40 million L/day (Holowenko, 2000). Since then other tailings ponds such as those belonging to Suncor, CNRL and Albian have also become methanogenic. Based on recent pond surface area data (103 km^2; osip.alberta.ca) and previous estimates (Kong et al., 2019), the combined tailings ponds could be producing 956 tCH_4/day or more making up close to 8% of Canada's methane emissions (4,300 kt CH_4; ec.gc.ca). Studies have suggested methane production resulted from the degradation of solvents. These include *n*-alkane and some BTEX compounds found in naphtha as well as *n*-alkanes and some iso-alkanes found in the paraffinic solvent (Mohamad Shahimin and Siddique, 2017a, 2017b, Siddique et al., 2015, 2007, 2006).

Studies have suggested methane production from MLSB could be far more prolific but is inhibited due to the addition of gypsum, a sulfur containing mineral used in the treatment of tailings (Salloum et al., 2002). Gypsum is used by both Syncrude and Suncor to produce consolidated tailings (CT), these tailings are approximately 60% solids and can be used more readily in dry landscape reclamation instead of waiting for tailings to consolidate to MFT (BGC Engineering Inc., 2010). Depending on sample depth, SO_4^{2-} concentrations have been reported between 0.1 – 36 mg/L (Fedorak et al., 2002; Penner and Foght, 2010), whereas methanogenesis is inhibited until SO_4^{2-} concentrations are reduced below 20 mg/L (Salloum et al., 2002). Other factors known to inhibit methanogenesis includes the presence of poorly crystalline Fe(III) in clay minerals (Lovley and Phillips, 1987).

1.1.3 Microbiology of tailings ponds

Below the oxic capwater, tailings ponds are highly stratified and dominated by both facultative and obligate anaerobes (Holowenko et al., 2000; Penner and Foght, 2010). Tailings ponds contain diverse

anaerobic microbial communities including methanogens, sulfate reducing bacteria, denitrifiers, and iron reducing bacteria (Penner and Foght, 2010; Salloum et al., 2002). In the deeper layers of MFT (5-15 m below the surface) where most terminal electron acceptors have been depleted, there is a greater abundance of sulfate-reducing bacteria, fermenters, syntrophs and methanogens (Ramos-Padrón et al., 2011). Among archaea, methanogens dominate. In a study by Penner and Foght (2010), the microbial community in MLSB was examined at various depths. The archaeal population was primarily composed of *Methanosarcinales*, and *Methanomicrobiales* where *Methanosaeta* was dominant at all depths. The bacterial community varied with depth and was more diverse.

In MFT taken from MLSB and Suncor's tailings pond 6 (TP6), Gamma- and Betaproteobacteria, were abundant (An et al., 2013; Penner and Foght, 2010). This was also true in laboratory studies using unamended MFT control cultures from MLSB (Siddique et al., 2012). Betaproteobacteria consist of aerobic and facultative groups with highly versatile degradative capabilities (https://en.wikipedia.org/wiki/Betaproteobacteria) such as the bacteria *Thauera* and *Azoarcus* found in MLSB (Penner and Foght, 2010), which are capable of coupling hydrocarbon degradation with denitrification (Foght, 2008). The reduced presence of this taxon in laboratory studies (Siddique et al., 2012) using *n*-alkane, BTEX and naphtha amended cultures suggests that this taxon is outcompeted by enriched taxon when labile hydrocarbons are present in the absence of nitrate. The observation of a higher proportion of denitrifiers *in-situ* is interesting as MFT typically has very low nitrate concentrations (Fru et al., 2013). In contrast, the percentage of Firmicutes increased in hydrocarbon amended cultures suggesting Firmicutes, most notably the order Clostridia (Siddique et al., 2012), may play an important role in hydrocarbon degradation *in-situ* when sufficiently labile carbon is present.

1.2 Microbial Metabolism

1.2.1 Anaerobic hydrocarbon degradation

A wide range of hydrocarbons including aliphatic and aromatic molecules can be biodegraded under aerobic conditions (Leahy and Colwell, 1990). This process is executed by monoxogynase enzymes that utilize oxygen to activate otherwise stable carbon-carbon bonds by generating reactive oxygen species (Rojo, 2009). Generally, the oxygen is incorporated at the terminal methyl group to form an alcohol, which is subsequently converted to an aldehyde. The molecule is then converted to carboxylic acid and metabolized via β-oxidation to acetyl-CoA. Aerobic hydrocarbon degradation is well documented and can be further studied through available literature.

Tailings ponds are primarily anaerobic therefore, microbial activity is primarily carried out by anaerobic Bacteria and Archaea. Under anaerobic conditions, molecules such as NO_3^-, Fe^{3+}, SO_4^{2-}, and CO_2 are used as terminal electron acceptors (Chidthaisong and Conrad, 2000; Peters and Conrad, 1996). Reduction of these molecules provides energy for the oxidation of organic molecules such as hydrocarbons and organic acids like citrate. Electron acceptors with the highest reduction potential are used preferentially as they yield more energy. Therefore, NO_3^- is used preferentially before Fe^{3+} followed by SO_4^{2-}, and CO_2.

Studies have found alkane and BTEX biodegradation under methanogenic conditions (Abu Laban et al., 2015a, 2015b, Mohamad Shahimin and Siddique, 2017a, 2017b, Siddique et al., 2015, 2012, 2011, 2007, 2006), and under NO_3^-, SO_4^{2-} reducing conditions (Abu Laban et al., 2009; Mbadinga et al., 2011 and references therein; as reviewed by Weelink et al., 2010). BTEX degradation has also been reported under Fe^{3+} reducing conditions however, alkane degradation has never been observed under this reducing condition (Mbadinga et al., 2011 and references therein). This thesis will focus primarily on iron reducing and CO_2 reducing (methanogenic) conditions.

Hydrocarbon degradation occurs via three major pathways under anaerobic conditions, fumarate addition, carboxylation, and hydroxylation (Foght, 2008; Wentzel et al., 2007). In fumarate addition, fumarate is added to the methyl group of an alkylbenzene like toluene to form benzylsuccinate (Fuchs et al., 2011). This metabolite is further processed to benzoyl-CoA and, through a series of steps, yields acetyl-CoA. Fumarate addition in aromatics has been found under methanogenic, NO_3^-, Fe^{3+}, and SO_4^{2-} reducing conditions. In *n*-alkanes, fumarate addition occurs at the terminal or sub-terminal carbon forming alkylsuccinates (Rojo, 2009). These compounds are then added to SCoA to form acyl-CoA, which can then be degraded via β-oxidation. This process is known to occur under NO_3^-, SO_4^{2-} reducing, and methanogenic conditions (Mbadinga et al., 2011), and has been demonstrated with *n*-alkanes ranging from C_3 to C_{26} by the presence of alkylsuccinates in culture (Agrawal and Gieg, 2013; Toth and Gieg, 2018).

An alternative method to fumarate addition is carboxylation. In aromatic carboxylation, CO_2 is bound directly to the benzene ring resulting in the formation of benzoyl-CoA (Fuchs et al., 2011). In *n*-alkanes, CO_2 is ligated to the C_3 of *n*-alkanes cleaving the molecule at C_2 resulting in the formation of alkylsuccinates, which are further degraded through β-oxidation (Callaghan et al., 2009, 2006). Carboxylation has been demonstrated under SO_4^{2-} reducing conditions and is suspected under NO_3^- reducing conditions. While the degradation of *n*-alkanes are well documented under methanogenic conditions (Abu Laban et al., 2015a; Aitken et al., 2013; Gieg et al., 2008; Holowenko et al., 2000; Li, 2010; Siddique et al., 2012, 2007, 2006; Yagi et al., 2010; Zengler et al., 1999; Zhou et al., 2012), alkylsuccinates

have only been detected in methanogenic cultures relatively recently (Berdugo-clavijo and Gieg, 2014; Toth and Gieg, 2018).

The final major pathway is hydroxylation. Hydroxylation is mediated by a molybdenum dependent enzyme that attacks the C_2 position of ethylbenzene (Johnson et al., 2001), and has been documented in methanogenic and NO_3^- reducing conditions (Fuchs et al., 2011). This pathway has not been demonstrated in *n*-alkane degradation, however this pathway is suspected in the presence of NO_3^- (Zedelius et al., 2011).

1.2.2 Methanogenesis and methanogens

It is generally accepted that biogeneic methane production occurs via three major pathways, methylotrophic, hydrogenotrophic, and acetotrophic methanogenesis. Methylotrophic methanogens produce methane by reducing single carbon compounds such as methanol, and methylated carbon compounds that contain no carbon-carbon bonds such as dimethylamine and dimethyl ether (Chistoserdova et al., 2009). In hydrogenotrophic methanogenesis, hydrogen is used as an electron donor to reduce CO_2, whereas acetate is reduced in acetoclastic methanogenesis (Hedderich and Whitman, 2013; Schink, 1997). Because methanogens cannot degrade organic polymers, such as lipids, proteins, and polysaccharides, methanogens depend on fermenting bacteria to metabolize these compounds. Primary fermenters break these molecules into monomers and oligomers, which are further metabolized to hydrogen, CO_2, fatty acids, alcohols, and organic acids such as acetate, succinate, and lactate (Meslé et al., 2013). Remaining molecules longer than two carbons are further degraded by secondary fermenters, also known as syntrophs, yielding acetate, hydrogen, and CO_2, which can then be utilized by methanogens.

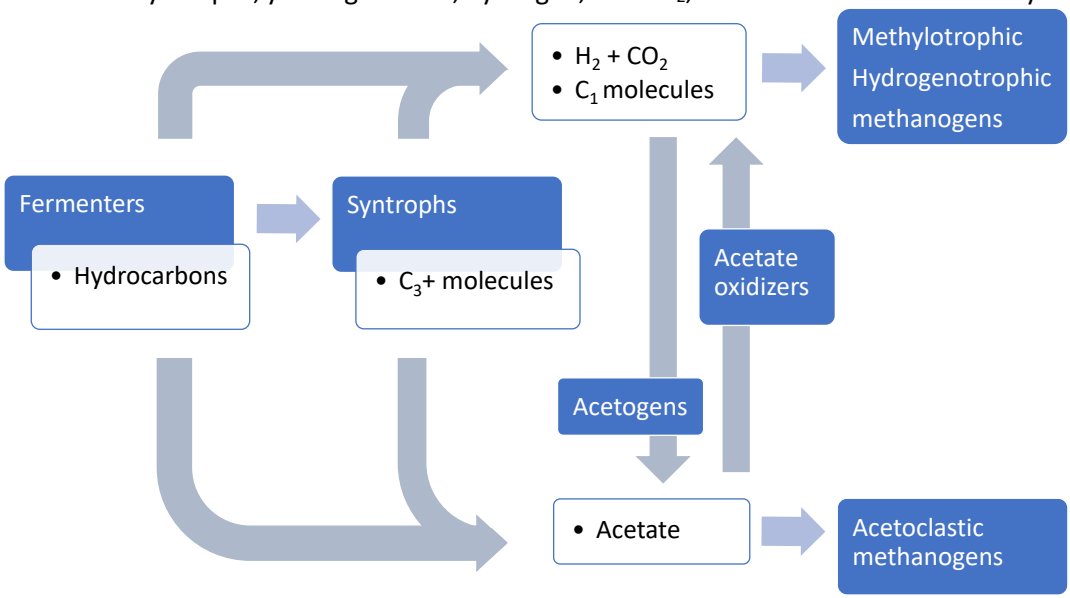

Figure 1-1: Degradation of organic polymers under methanogenic conditions (Meslé et al., 2013).

Interestingly, fermentation metabolism, and subsequently acetoclastic methanogens, are dependent on the presence of hydrogenotrophs. During fermentation, hydrogen is produced and will accumulate in the absence of hydrogenotrophic activity, thereby inhibiting hydrogenase activity in fermenters (Garcia et al., 2000). Fermentation metabolism becomes endergonic under these conditions, and becomes favourable when hydrogen concentrations are low (Schink, 1997; Schink and Stams, 2006). The co-culture, "*Methanobacillus omelianskii*" is an example of this syntrophy (Barker, 1940; Bryant et al., 1967). In this culture (as reviewed by Schink and Stams, 2006), Strain S (syntroph) fermented ethanol to acetate and H_2, and Strain M.o.H (*Methanobacillus omelianskii* hydrogenotroph, now *Methanobacterium bryantii*), metabolized H_2 and CO_2 to methane. Because the fermentation reaction is endergonic under standard conditions, it can only proceed if the partial pressure of hydrogen is kept below 10^{-3} bar by the hydrogenotrophic methanogen. However, neither strain could survive on ethanol independently.

1.2.3 Iron reducing conditions

Iron reduction typically occurs in conjunction with the anaerobic oxidation of organic compounds including both naturally occurring organic matter in sediment and hydrocarbon contamination. These degradation processes have been observed in the natural attenuation of oil spills. A study was conducted on one such spill that occurred in Minnesota, USA in 1979 (as reviewed by Vodyanitskii, 2011), where 400,000 L of oil was released into the environment. Researchers modelled the aerobic and anaerobic hydrocarbon biodegradation using the kinetics of organic matter decomposition and the influence of aerobes, Mn and Fe reducers, and methanogens. Over the course of the study, 60% of the aromatic hydrocarbons were degraded anaerobically. Of this, 19% was degraded in conjunction with iron hydroxide reduction. These values were determined by examining the accumulation of light weight CO_2 isotopes, depletion of Fe(III) in soil, and accumulation of Fe(II).

Biodegradation of toluene, phenol, and benzene via Fe(III) reduction in the environment have also been reported, this activity has been attributed to members of the *Geobacteraceae* family including *Geobacter metallireducens* (Vodyanitskii, 2011, and references therein). While the degradation of alkanes has not been observed under iron reducing conditions (Mbadinga et al., 2011; Zwolinski et al., 2001), toluene, phenol, cresol, and naphthalene degradation have been documented (H R Beller et al., 1992; Coates et al., 2001; Kleemann and Meckenstock, 2011; Kunapuli et al., 2007, 2010; Lovley et al., 1993; Lovley and Lonergan, 1990).

The majority of iron reducing activity in sediment occurs during the oxidation of fermentation products (Lovley, 1991). *Geobacter metallireducens* is one species capable of oxidizing acetate with Fe(III) reduction, as well as various alcohols and fatty acids. *Desulfuromonas acetoxidan* is a closely related marine relative, and is capable of oxidizing acetate in conjunction with the reduction of both Fe(III) and S^0 (Roden and Lovley, 1993). In addition to organic ligand metabolism, several organisms are also known to grow while respiring Fe(III) and oxidizing H_2. These include a *Pseudomonas* species, *Shewanella putrefaciens,* and BrY (Lovley, 1993 and references therein). Interestingly, *Desulfovibrio* can reduce Fe(III) at equivalent rates while oxidizing H_2 but does not accumulate biomass.

Examples of reactions catalyzed by Fe(III) reducers (Lovley, 1993).

Organic ligand oxidation

$lactate^- + 4\ Fe(III) + 2\ H_20 \rightarrow acetate^- + HCO_3^- + 4\ Fe(II) + 5\ H^+$

$pyruvate^- + 2\ Fe(III) + 2\ H_20 \rightarrow acetate^- + HCO_3^- + 2\ Fe(II) + 3\ H^+$

$formate^- + 2\ Fe(III) + H_20 \rightarrow HCO_3^- + 2\ Fe(II) + 2\ H^+$

$acetate^- + 8\ Fe(III) + 4\ H_2O \rightarrow 2\ HCO_3^- + 8\ Fe(II) + 9\ H^+$

Inorganic ligand oxidation

$H_2 + 2\ Fe(III) \rightarrow 2\ H^+ + 2\ Fe(II)$

$S^0 + 6\ Fe(III)\ 4\ H_2O \rightarrow HSO_4^- + 6\ Fe(II) + 7\ H^+$

The potential to oxidize H_2 during Fe(III) reduction is interesting. Just as hydrogenotrophic metabolism is needed to support fermentation, and therefore acetoclastic methanogenesis, it is possible that H_2 oxidation under Fe(III) reducing conditions may also facilitate fermentation metabolism by reducing the partial pressure of hydrogen. Fe(III) reducers capable of oxidizing both fermentation products and H_2 are especially well suited to this energetic system.

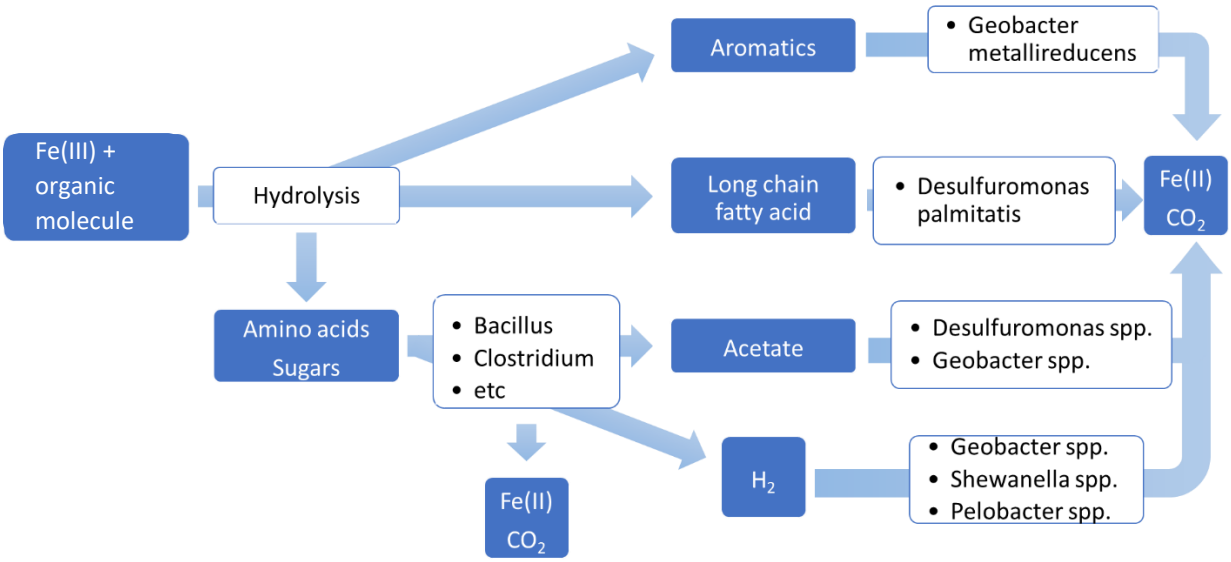

Figure 1-2: Simplified model for the oxidation of organic matter coupled with dissimilatory Fe(III) reduction in sediment. Modified from Lovley, 1997.

In soils and sediment where oxygen has been depleted, Fe(III) is one of the most commonly available electron acceptors (Lovley, 2011). Fe(III) is generally insoluble between pH 6.5-7.5 and can be found in the form of Fe(III) oxides and clay minerals. For a long time, it was thought Fe(III) reducing bacteria required direct contact with iron minerals for use in microbial metabolism. This hypothesis was supported by several studies that found Fe(III) was not reduced by Fe(III) reducers when separated by a permeable membrane in solution (Lovley, 1997 and references therein). However, these studies neglected to include positive controls. It was found that *Shewanella putrefaciens* was capable of producing soluble Fe(III) chelating agents to support metabolism (Dobbin et al., 1995).

Numerous studies have found Fe(III) reducing bacteria can reduce chelated Fe(III) faster than insoluble Fe(III) oxides and minerals (Lovley, 1991). This increase in respiration is also observed when Fe(III) is chelated with ethanoldiglycine (EDG), ethylenediaminetetraacetic acid (EDTA), and *N*-methylliminodiacetic acid (MIDA) (D. Lovley et al., 1996). Addition of these compounds also increased the rate for aromatic hydrocarbon degradation. Other mechanisms of Fe(III) reduction include electron shuttling compounds, humic substances (Nevin and Lovley, 2002 and references therein), and direct interspecies electron transfer (DIET) (Lovley, 2011). Electron shuttles are molecules that can accept an electron from a Fe(III) reducing bacteria and transfer it to the surface of a Fe(III) oxide (Nevin and Lovley, 2002). These molecules can be oxidized and reduced multiple times. Electron shuttles could be particularly useful when Fe(III) oxides are otherwise inaccessible to Fe(III) reducing microorganisms.

Humic acids work similarly to microbial produced electron shuttles (D. Lovley et al., 1996). Quinone moieties within the humic substances accept electrons from the Fe(III) reducing bacteria and abiotically transfer electrons to Fe(III) oxides thereby returning the molecules to an oxidized state (Nevin and Lovley, 2002). Other extracellular quinones, such as anthraquinone-2,6-disulfonate (AQDS) are similarly capable of electron shuttling (Lovley et al., 1996; Lovley et al., 1998). These processes rely on molecule to molecule interactions, however some microorganisms, such as *Geobacter* species, are capable of DIET. *Geobacter* utilizes a network of conductive filaments capable of transferring electrons with metal-like conductivity (Lovley, 2011). This process allows the direct transfer of electrons from the Fe(III) reducing bacteria to insoluble Fe(III) oxides and minerals.

1.2.4 Anaerobic nitrogen transformations

Labile carbon sources are limiting in MFT and essential nutrients such as nitrogen (N) may be deficient (Collins et al., 2016; Penner and Foght, 2010). For anaerobic metabolism, sufficient N must be present. This C:N ratio is generally considered to range between 50:1 and 100:1, however, it has been reported as high as 180:1 (Ammary, 2004; Droste, 1997; USEPA, 1994). These ratios are largely dependent on biomass and will vary based on the efficiency of carbon metabolism (Ammary, 2004). Despite these nutrient requirements, the methanogenic degradation of carbon compounds can occur under N limiting conditions suggesting the presence of anaerobic N transformations (Collins et al., 2016).

Nitrogen is brought into a biological system through the fixation of N_2 to NH_4^+ and lost again from the system through denitrification. Nitrogen-fixation is a process by which N_2 gas from the environment is "fixed" into a bioavailable form by N_2-fixing prokaryotes known as diazotrophs (Cabello et al., 2004). The nitrogenase enzyme which mediates this oxygen sensitive process is regulated by multiple *nif* genes which are negatively regulated in the presence of O_2 or bioavailable N sources such as amino acids, ammonium nitrate (Ausubel, 1984; Fischer, 1994; Kranz and Cullen, 1995; Kranz and Haselkorn, 1986). While concentrations of 10 µM NO_3^- or 250 µM NH_4^+ can inhibit N_2-fixation, this process has been observed at very low N concentrations (0.4 µM NO_3^- or 2 µM NH_4^+) (LaRoche and Breitbarth, 2005; Vintila and El-Shehawy, 2007).

There exist many examples of N_2-fixation in anaerobes in both bacteria and archaea. Some exist symbiotically with multicellular organisms or in a microbial community, and others fix N independently to support their metabolic activity. In the realm of symbiosis, N_2-fixers can be found in the guts of termites. This N_2-fixing community supplements the otherwise N-deficient termite diet in a symbiotic relationship whereby the termite supplies an anaerobic environment and a steady supply of organic compounds to

support microbial metabolic activity. Despite this beneficial relationship, the termites also pay a hefty cost. As they often live in N-deficient habitats, they serve as an excellent N-source to other predators (Ohkuma et al., 1999).

Sharing N obtained through N_2-fixation is also common in the microbial world where it is often exchanged for structural proteins or energy rich metabolites within a microbial community (Udvardi and Day, 1997). An example of this type of relationship can be observed between anaerobic N_2-fixing, methane oxidizing archaea, and sulfate-reducing bacteria. In the study by Dekas et al. (2009), aggregates composed of methane oxidizing archaea and sulfate-reducing bacteria were visualized with fluorescent *in-situ* hybridization (FISH) and nanoscale secondary ion mass spectrometry (nanoSIMS). The images clearly depicted the incorporation of $^{15}N_2$ in the N_2-fixing methane oxidizing archaea within the aggregates, and the presence of the fixed N, $^{15}NH_4^+$, in the outer shell of sulfate-reducing bacteria. The researchers concluded that the symbiotic relationship likely provides fixed N to the sulfate-reducers, which in turn provide energy for methane oxidation. Despite this, the authors were unable to rule out the possibility of N_2-fixing activity in the sulfate-reducing bacteria of which several species are capable (Bertics et al., 2010; Zehr et al., 1995).

To support their own metabolism, many microbes are also able to fix N independently under N-deficient conditions. Examples of this can be found in the hyperthermophilic hydrogenotrophic methanogens, *Methanocaldococcus* and *Methanothermococcus*. These strains, which were isolated from hydrothermal vents, have been found to metabolize N_2 and NH_4^+. In a study by Nishizawa et al. (2014), one of these strains was found to suffer growth inhibition in the absence of molybdenum. Molybdenum is the most common cofactor necessary for the function of nitrogenase, the N_2-fixing enzyme. More interestingly, both strains were found to produce ^{15}N depleted biomass utilizing environmental N_2. ^{15}N-depleted biomass is characteristic in geological samples of ancient organic material found in hydrothermal environments indicating the likely ancient origin of this process. These strains are thought to utilize dissolved N_2 for microbial growth as dissolved N_2 is more abundant than other N sources such as NO_2^- and NH_4^+ in hydrothermal environments. Other studies of *nifH* genes in hydrothermal fluids have indicated the presence of methanogenic archaea, all of which are known to use NH_4^+ as a N source, and anaerobic bacteria such as sulfate-reducers and clostridia as potential N_2-fixers (Mehta et al., 2003).

The denitrification process operates through several subsequent intermediates including NO (nitric oxide) and the greenhouse gas N_2O (nitrous oxide) and requires low oxygen tension and the presence of an N-oxide. In this anaerobic process, NO_3^- (nitrate), NO_2^- (nitrite), NO, and N_2O are utilized in place of O_2 as

terminal electron acceptors in the electron transport chain. This can occur in chemolithotrophs via autotrophic denitrification where an inorganic reductant is oxidized, or heterotrophic denitrification using organic reductants. Dissimilatory denitrification resulting in the transformation of NO_3^- to a gas is an example of N autotrophy, this contrasts with dissimilatory nitrate reduction to ammonia (DNRA, ammonification) where the same products are reduced to NH_4^+ and excreted. Both processes utilize respiratory nitrate reduction and conserves energy, however there are no known bacteria capable of both functions. Nitrate can also be reduced through assimilatory nitrate reduction. This pathway utilizes the steps as DNRA but NH_4^+ is retained by the microbe for the biosynthesis of N-containing compounds (Zumft, 1997).

1.2.5 Methanogenesis and Fe(III)

It is well known that amorphous Fe(III) can inhibit methanogenesis (Lovley and Phillips, 1986; Roden and Wetzel, 1996; Van Bodegom et al., 2004). This inhibition is generally attributed to competition between the Fe(III) reducers and methanogens for acetate and H_2 (Lovley and Phillips, 1986; Roden and Wetzel, 1996). However, competition does not sufficiently explain the total inhibition of methanogenesis in the presence of amorphous Fe(III).

In a study by Van Bodegom et al. (2004), three methanogens were investigated in pure culture, the obligate acetoclastic methanogen, *Methanosaeta concilii*, *Methanospirillum hungatei* a H_2/CO_2 and formate using methanogen, and *Methanosarcina barkeri*, a methanogen that can utilize H_2/CO_2, acetate, and methanol. The study clearly demonstrated methanogenic inhibition in the presence of Fe(III) when Fe(III) reducing microorganisms were absent thereby eliminating the possibility of competition. *Methanospirillum hungatei* suffered almost total inhibition whereas *Methanosarcina barkeri* experienced partial inhibition, with greater inhibition experienced when growing on H_2/CO_2 than on acetate. The obligate acetoclastic methanogen, *Methanosaeta concilii*, was also grown on acetate and was not inhibited by Fe(III). The authors suggest this difference can be explained by the activation pathway for acetyl-CoA, which differs between the two organisms. Their study also demonstrated Fe(III) reduction by *Methanosarcina barkeri*. The authors suggest this activity as an explanation for the reduced inhibition of methanogenesis in the presence of Fe(III).

Direct reduction of Fe(III) by methanogens has since been demonstrated in numerous studies (Bond and Lovley, 2002; Liu et al., 2011; Zhang et al., 2013, 2012). The methanogens studied were *Methanothermobacter thermautotrophicus* (Zhang et al., 2013), *Methanosarcina voltaei* (Bond and Lovley, 2002), *Methanosarcina mazei* (Zhang et al., 2012), and *Methanosarcina barkeri* (Bond and Lovley,

2002; Liu et al., 2011; Van Bodegom et al., 2004). These methanogens reduced the iron oxides in the iron poor smectite Wyoming montmorillonite (Zhang et al., 2013), ferric iron in the iron rich smectite nontronite (Liu et al., 2011; Zhang et al., 2013), illite-smectite (Zhang et al., 2012), and amorphous iron (Bond and Lovley, 2002; Van Bodegom et al., 2004).

In methanogens capable of hydrogenotrophic metabolism in the presence of H_2, the hydrogenase enzyme can oxidize H_2 to $2H^+$ and $2e^-$ (Hedderich and Whitman, 2013). When no other electron acceptor is available, these electrons are transferred to a reductase enzyme in the methanogenic pathway. However, when Fe(III) is bioavailable, these electrons could instead be diverted to Fe(III) (Bond and Lovley, 2002). When studying *Methanosarcina barkeri* under a H_2/CO_2 headspace, Liu et al. (2011) found that the Fe(II) produced was stoichiometrically equivalent to the amount of H_2 consumed, and methanogenesis was inhibited. Yet ferric iron-oxide minerals have also been found to stimulate methane production (Jiang et al., 2013; Kato et al., 2019, 2012; Kato and Igarashi, 2019; Pan et al., 2017).

In a study using poorly crystalline akageneite (β-FeOOH) and acetate, Jiang et al. (2013) observed a significant increase in methanogenesis as compared to cultures without akageneite. The researchers found the microbial community was primarily comprised of the methanogen *Methanosarcina barkeri* and an acetate oxidizing *Clostridium*. They observed a significant increase in H_2 production as acetate concentrations decreased in cultures containing the poorly crystalline iron due to acetate oxidation and Fe(III) reduction by the *Clostridium*. This was followed by a decrease in H_2 and increase in methane indicating that *Methanosarcina barkeri* was utilizing hydrogenotrophic metabolism for methane production rather than Fe(III) reduction. When the known methanogen inhibitor 2-bromoethanosulfonate (BES) was added, both Fe(III) reduction and H_2 production slowed indicating the metabolic activity of *Methanosarcina barkeri* was necessary to facilitate acetate fermentation and Fe(III) reduction. This observation reinforces the energetic model requiring low H_2 partial pressure for fermentation metabolism (Schink and Stams, 2006). While *Methanosarcina barkeri* has been previously found capable of acetoclastic methanogenesis (Van Bodegom et al., 2004), these methanogens were not able to produce methane using acetate in the absence of akageneite under these experimental conditions (Jiang et al., 2013). Enhanced methanogenesis in the presence of akageneite has also since been demonstrated by Pan et al. (2017).

In a separate study by Kato et al. (2012), hematite and magnetite were found to facilitate methanogenesis whereas ferrihydrite (amorphous iron oxyhydroxide) did not. In this study, *Methanosarcina* spp. were also dominant but in contrast to Jiang et al. (2013), they were found to initially metabolize acetate to methane

(Kato et al., 2012). *Methanosarcina* spp. were thought to syntrophically partner with *Geobacter*. In this arrangement, both *Methanosarcina* and *Geobacter* initially utilized acetate before *Methanosarcina* switched to hydrogenotrophic metabolism. The authors suggested that *Geobacter* was transferring electrons to *Methanosarcina* via the conductive iron oxides and using methanogenesis as an electron sink. Kato and Igarashi (2019), have recently demonstrated this phenomenon now known as electric syntrophy, where electrons are flowed between microorganisms through a conductive mineral, such as iron sulfide. Where sulfate and ferrihydrite independently were found to inhibit methanogenesis, the two in combination formed poorly crystalline iron sulfide capable of facilitating electric syntrophy between chemoheterotrophic bacteria (*Geobacter*, and *Desulfotomaculum*) and methanogenic archaea (*Methanosarcina*) thereby promoting methanogenesis (Kato and Igarashi, 2019, and references therein). Methanogenesis via electric syntrophy has also since been demonstrated in the presence of magnetite (Kato et al., 2019).

Regardless of the method of Fe(III) mediated inhibition of methanogenesis, it seems capacity for methanogenesis in the presence of Fe(III) is dictated by the conductivity of the Fe(III) oxides.

1.3 Thesis Overview

1.3.1 Research Scope

Hydrocarbon degradation under methanogenic conditions in oil sands tailings has been demonstrated and discussed in numerous studies (Abu Laban et al., 2015b, 2015a; Fedorak et al., 2002; Foght et al., 2017; Holowenko et al., 2000; Laban et al., 2015; Li, 2010, 2009; Mohamad Shahimin, 2016; Mohamad Shahimin and Siddique, 2017a, 2017b; Penner and Foght, 2010; Siddique et al., 2015, 2012, 2011, 2008, 2007, 2006), however, the method by which methanogenesis persists in tailings depleted in available N (NH_4^+, NO_2^- /NO_3^-) has not been well examined.

Our previous study (Collins et al., 2016), suggested that N_2-fixation may be responsible for meeting the N demand necessary for methanogenic metabolism of citrate in oil sands tailings. Nitrogen fixation has also been examined in conjunction with methanogenesis (Bae et al., 2018; Dekas et al., 2009; Harada et al., 2001; Harper et al., 2004; Kessler et al., 2001; Leigh, 2005, 2000; Lilburn et al., 2001; Murray and Zinder, 1984; Nishizawa et al., 2014; Swerts et al., 1996) but literature relating to nitrogen fixation supporting methanogenesis and hydrocarbon degradation is scarce. This begs the question, can methanogenesis and hydrocarbon degradation be sustained by N_2-fixation in oil sands tailings? Additionally, Fe(III) (amorphous

and certain clay minerals) is known to inhibit methanogenesis (Reiche et al., 2008; Roden and Wetzel, 1996; Sivan et al., 2016a; Van Bodegom et al., 2004; Zhang et al., 2012).

The rapid transition of methanogenesis to iron reduction (Sivan et al., 2016b) suggests Fe(III) could potentially serve as a tailings amendment to reduce fugitive methane emissions. It is therefore beneficial to determine if methanogenesis is inhibited through the addition of Fe(III) in the microbial community present in oil sands tailings, and if N_2-fixation microorganisms would continue to support hydrocarbon degradation. There are however, certain organic compounds such as cysteine, glutamate, EDTA, and citrate, which can chelate metals such as iron thereby reducing the effect of biological interactions (as reviewed by Gadd and Griffiths, 1978). Citrate is used as a dispersant in some bitumen ore extraction processes. Citrate is a known siderophore for ferric iron and can solubilize $Fe(OH)_3$ at neutral pH by forming ferric citrate complexes (Silva et al., 2009). In rumen studies, ferric citrate was not found to impact methanogenesis (Hales et al., 2018; Wu et al., 2016). It is therefore also valuable to determine if methanogenesis in oil sands tailings can be inhibited by inducing iron reducing conditions in the presence of the dispersant, citrate.

In this research, we evaluated N_2-fixation as a N source to support *n*-alkane and aromatic hydrocarbon degradation under methanogenic conditions in mature fine tailings (MFT) from three different oil sands operators as this has not been examined previously and may explain how methanogenesis continues in-situ when bioavailable N is deficient to support continued hydrocarbon metabolism. We were also interested in determining the pathways by which N_2 may be replenished *in-situ* and determining if these processes continue when iron reducing conditions are induced and citrate is present. This research was designed to explore methanogenesis and hydrocarbon degradation in a microbial community enriched from MFT under N depleted conditions in tailings materials obtained from operators CNRL, Syncrude, and a tailings pond formerly operated by Shell Albian. Three operators were selected to allow for a broader investigation of microbial processes as the microbial population is known to vary between operators (Yergeau et al., 2012).

1.3.2 Research Objectives

Objective 1: Investigate N_2-fixation mediated methanogenesis from the metabolism of *n*-alkane and aromatic hydrocarbons by microorganism endogenous to oil sands tailings and identify the active microbial community capable of N_2-fixing activity (Chapter 2).

Objective 2: Determine if methanogenesis under the conditions studied in Chapter 2 can be inhibited by amending the microbial community with amorphous Fe(III), and examine the changes both in the microbial community and the active N_2-fixing community (Chapter 3).

Objective 3: Evaluate the potential of Fe(III) to inhibit methanogenesis under N-deficient conditions in the presence of the iron chelating molecule citrate, which may be found in some tailings ponds, and determine how the presence of this compound affects the microbial community and active N_2-fixing microorganisms (Chapter 4).

1.3.3 Thesis Outline

Chapter 1: Introduction

Chapter 2: Nitrogen Fixation Mediated Hydrocarbon Degradation by The Methanogenic Community in Oil Sands Tailings

This Chapter describes the degradation of *n*-alkanes and the model aromatic hydrocarbon toluene, under methanogenic conditions and in the absence of bioavailable inorganic N (NH_4^+, NO_2^-/NO_3^-). Cultures were established using inoculum enriched from MFT sourced from three different oil sands operators, CNRL, Syncrude, and former oil sands operator, Albian. These cultures were evaluated for hydrocarbon degradation, methanogenesis, N_2-fixation, and the community composition was examined including functional gene presence and expression for anaerobic N-cycling processes. Both groups of hydrocarbons underwent biodegradation with resulting methanogenesis concurrent with N_2-fixation in cultures containing tailings from at least one of the three culture sets (Albian, Syncrude, CNRL) suggesting the observed metabolism was supported by N_2-fixing activity. The community composition was also determined using 16S rRNA and the presence and expression of functional genes for N_2-fixation were examined to identify the key members of the active community.

Chapter 3: Reducing Methane Emissions in Oil Sands Tailings Using Amorphous Iron Concurrent with Hydrocarbon Degradation Under Nitrogen Limited Conditions

Due to the heightened political focus on reducing GHG emissions, this Chapter explores the potential of modifying the reducing condition in MFT from methanogenic to iron reducing using an amorphous Fe(III) amendment. Cultures were established using MFT inoculum from three different tailings stock (Albian, Syncrude, and CNRL) as outlined in Chapter 2, both in the presence and absence of bioavailable fixed N (NH_4^+, NO_2^-/NO_3^-) to allow for direct comparison. While toluene degradation occurred in at least one culture, *n*-alkanes persisted thereby concurring with the body of scientific literature which has yet to

report *n*-alkane degradation under iron reducing conditions. Here, the inhibition of methanogenesis and the reduction of Fe(III) to Fe(II) was evaluated and the microbial community was examined to determine how the change in reducing conditions affected both the total community (16S rRNA), and the expression of N_2-fixing genes using the Illumina sequencing platform.

Chapter 4: Citrate Promotes Methane Emissions in Oil Sands Tailings Under Methanogenic and Iron Reducing Conditions

This Chapter focuses on the feasibility of using amorphous Fe(III) as an amendment to inhibit methanogenesis when citrate is present in tailings ponds. Only inoculum from Albian cultures were employed in culture assembly due to Albian's previous use of trisodium citrate as a dispersing agent during bitumen ore processing. Cultures were established in the presence and absence of fixed N to allow for direct comparison of data between other chapters in this document, and evaluated for methanogenesis, N_2-fixation, and reduction of Fe(III) to Fe(II). The microbial community was evaluated using MiSeq Illumina sequencing of the 16S rRNA gene and both the presence and expression of N_2-fixing functional genes were examined to determine how the addition of citrate may affect the community.

Chapter 5: General Conclusions

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2. Nitrogen Fixation Mediated Hydrocarbon Degradation by the Methanogenic Community in Oil Sands Tailings

2.1 Introduction

Bitumen extraction from Alberta's oil sands makes up 60% of oil production in Canada (Canada's Oil Sands, 2016). The oil sands region contains an estimated 166 billion barrels of extractable oil and is being recovered at 2.4 million barrels per day (Canadian Association of Petroleum Producers, 2016). In 2014, 45% of bitumen recovery was accessed through open pit mining (Alberta Energy, 2015). The process for extracting bitumen from oil sands ores is known as the Clarks hot water extraction (Mikula et al., 1996). This process uses heat and surfactants to separate bitumen from sand and clay. The slurry is then processed in a separation vessel where bitumen is skimmed off for upgrading and eventual processing to crude oil. Solvents such as naphtha used by CNRL and Syncrude, and the paraffinic diluent (C_5-C_6) employed by Albian are added to reduce emulsion stability and precipitate asphaltenes (Devenny, 2009). Solvents are recovered, and the remaining slurry is pumped out to tailings ponds to settle. These tailings are composed of sand, silt, clay, residual bitumen, unrecovered solvent, and water (Allen, 2008). The tailings suspension settles to 20 wt% in a few weeks but can take a few years to reach 30 wt% forming a material known as mature fine tailings (MFT). Further consolidation to 44 wt% will take an additional 10 years (MacKinnon et al., 2001).

As of 2015, the total surface area of oil sands tailings ponds was 100 km^2 and occupied a volume of over 1 billion Mm^3 (http://osip.alberta.ca/map/). In the early 1990s after 15 years of operation, a tailings pond owned by Syncrude known as Mildred Lake Settling Basin (MLSB) became methanogenic. This pond was estimated to produce 40 million L/day in 2000 (Holowenko, 2000). Currently, all tailings ponds produce biogenic methane. The rate of methanogenesis differs in each pond and is dependent on the availability of readily degradable carbon. For example, froth tailings produce the most GHG due to the high concentration of anaerobically degradable hydrocarbons (Foght et al., 2017). Methane production is primarily derived from the microbial metabolism of unrecovered solvents. Hydrocarbon biodegradation results in the formation of the methanogenic substrates, acetate, and CO_2 and H_2, which are further metabolized by acetoclastic and hydrogenotrophic methanogenesis, respectively (Foght, 2008).

Several studies have examined the degradation of hydrocarbons in oil sands tailings cultures under methanogenic conditions. These include *n*-alkanes ranging from C_5-C_{18} (Mohamad Shahimin, 2016; Siddique et al., 2011, 2006), toluene and xylene isomers (Siddique et al., 2007), branched and cyclic

alkanes (Abu Laban et al., 2015b; Siddique et al., 2015; Tan et al., 2015), naphtha (Mohamad Shahimin and Siddique, 2017c), and paraffinic solvents (Mohamad Shahimin and Siddique, 2017a). However, microbial metabolism of hydrocarbons requires the presence of essential nutrients such as N. It is generally considered that the C:N ratio needed for anaerobic growth and metabolism ranges between 50:1 and 100:1 however, some studies have found anaerobic communities supported on ratios as high as 180:1 (Ammary, 2004; Droste, 1997; USEPA, 1994). N is depleted in some tailings such as those found in Shell Albian pond 6 (hereafter referred to as Albian; Collins et al., 2016) and yet these tailing continue to produce methane.

In our previous study (Collins et al., 2016), we examined this phenomenon by establishing a series of MFT cultures that were either deficient in bioavailable inorganic N or contained N (NH_4^+) in the media. Cultures were composed of 50:50 Albian MFT and culture media with citrate amendment and incubated under an N_2/CO_2 headspace for 16 weeks. The results indicated N_2-fixation was occurring in citrate amended, N depleted Albian MFT cultures concurrent with methanogenesis suggesting N_2-fixation was supporting the methanogenic microbial community.

The objective of the current study was to determine if N_2-fixation could support hydrocarbon degradation in tailings communities. This research is relevant to reclaimed wet landscapes that incorporate nutrient deficient tailings materials or tailings that become N deficient through microbial N cycling and release in the form of N_2 as it is generally expected that microbial metabolism will slow or stop in the absence of bioavailable nutrients. Once fixed N (NO_2^-/NO_3^-, NH_4^+) is depleted, the microbial community may turn to N_2-fixation to support methanogenic activity. Continued microbial metabolism can threaten the long-term sustainability of end pit lakes by creating channels for bitumen release and increasing turbidity. As such, understanding methanogenesis under nutrient limited conditions is important for emissions modeling and informing policy on reclamation practices and appropriate end land use.

2.2 Methods and Materials

2.2.1 Chemicals, gasses, and materials

Hydrocarbons *n*-pentane (>99% purity), *n*-hexane (>96% purity), and *n*-octane (>99% purity), *n*-heptane, toluene (>99% purity) were purchased from Fisher Scientific, (Ontario, Canada), and *n*-decane (>99% purity) was purchased from Sigma-Aldrich (Ontario, Canada). Chemicals (analytical reagent grade or higher) were purchased from Fisher Scientific. Acetylene and 70% N_2/30% CO_2 (scientific grade) and all other gasses (certified standard) were purchased from Praxair (Alberta, Canada). MFTs used for Syncrude

and CNRL primary enrichment cultures were collected using a wireline fluid sampler from MLSB at a depth of 20.75 M and ETF from a depth of 16 M respectively, in July 2013. Fresh 2013 Albian tailings (hereafter known as A2013) were collected from below the mudline at an unknown depth by site operators using a wireline fluid sampler however, cultures made using this MFT were discarded during the preliminary phases of work in favour of using inoculum from existing Albian cultures previously described (Collins et al., 2016; Mohamad Shahimin, 2016). All tailings were stored in sealed pails without headspace at 4˚C for four months before use.

2.2.2 Primary enrichment cultures from Syncrude and CNRL tailings

Enrichment cultures used as inoculum were established using 50 ml methanogenic media with or without inorganic N (hereafter, referred to as "with N" or "without N"), and 50 ml MFT in 158 ml serum bottles with butyl stoppers. This proportion of MFT to culture media was used to ensure sufficient nutrients were present to promote microbial activity and reduce the viscosity of the tailings material such that culture samples can be taken via syringe. Cultures were established in an anerobic chamber and incubated under an 70% N_2/30% CO_2 headspace as described previously (Collins et al., 2016) by alternating flushing the cultures with gas and then removing the headspace with vacuum at 30 second intervals for 2 minutes followed by flushing with gas for 5 minutes prior to incubation or amendments with hydrocarbons. Methanogenic media with N (0.18 mg/L inorganic N) contained NaCl, $CaCl_2$, NH_4Cl, $MgCl_2$, $(NH_4)_6Mo_7O_{24}$, $ZnSO_4$, H_3BO_3, $FeCl_2$, $CoCl_2$, $MnCl_2$, $NiCl_2$, $AlK(SO_4)_2$, $NaHCO_3$, KH_2PO_4, Resazurin (anaerobic indicator), and Na_2S (reducing agent; Collins et al., 2016). The N containing salt NH_4Cl was omitted from the N deficient methanogenic media. In place of $(NH_4)_6Mo_7O_{24}$, Na_2MoO_4 was used to ensure that the concentration of molybdenum, a key element required for N_2-fixing activity, was consistent between the two types of media (7.26 mg/L). Molybdenum is present in oil sands tailings at concentrations of 0.006 to 0.073 mg/L in the liquid phase and 0.74 to 1.54 mg/L in the solid phase (T. Siddique, personal communication, May 4, 2020).

Cultures were pre-incubated for two weeks in the dark to allow the microbial community to acclimate to the media before amendment with hydrocarbons at day 0. Enrichment cultures containing Syncrude and CNRL MFT were amended with 200 mg L^{-1} toluene or octane and were incubated in the dark for 140 days for monitoring methane (**Figure S 1**, **Figure S 2**). Albian tailings collected in 2013 were used to prepare "A2013" cultures. A2013 cultures were amended with 200 mg L^{-1} citrate or hexane and were incubated in the dark for 99 days with methane monitoring (**Figure S 3**). All culture sets (Syncrude, CNRL, A2013) and subsequent culture sets contained the following treatments; hydrocarbon amended with N and

hydrocarbon amended without N, unamended with N and unamended without N, and heat-killed sterile amended cultures with and without N. Unamended cultures accounted for methanogenesis from endogenous carbon sources. Unamended and sterile cultures were established in duplicate based on previous studies where little to no methane production was observed in these treatments (Collins et al., 2016; Siddique et al., 2006), whereas live amended cultures were established in triplicate. A2013 cultures were not used in the rest of this study.

2.2.3 Transfer cultures from Albian enrichments

Transfer cultures were established in 60 ml serum bottles using 25 ml methanogenic media and 5 ml inoculum from previously described Albian cultures (Collins et al., 2016). These volumes were selected to continue to propagate the microbial community from the primary cultures without introducing confounding factors by adding more tailings from another source. These cultures were amended with 200 mg L^{-1} hexane and incubated in the dark. Due to lack of methane production after 55 days (data not shown), cultures were inoculated with 3 ml of an *n*-hexane/*n*-pentane degrading Albian culture enriched from the same Albian tailings sample retrieved in 2008 (Mohamad Shahimin, 2016). This inoculum was sampled in an anaerobic hood, then centrifuged to remove the liquid phase and soluble N. The inoculum was then anaerobically resuspended in methanogenic media and added to cultures via syringe. Cultures were stored in the dark for 4 months then analyzed for methane production before use as inoculum in *n*-alkane cultures.

2.2.4 n-Alkane cultures

n-Alkane culture sets were established in 158 ml serum bottles with 90 ml media and 5 ml inoculum from Syncrude or CNRL primary enrichment cultures, or 5 ml inoculum from 30 ml Albian transfer cultures. Inoculum was first centrifuged to remove the liquid phase and soluble N (**Table S 1**), then resuspended as described above. Volumes were selected to further dilute residual N that may have remained in the inoculum. Cultures were stored for two weeks in the dark before day 0 amendments and were setup both with or without N as described for primary enrichment cultures. At day 0, Syncrude and CNRL cultures were amended with a 1:1:1 by volume mixture of *n*-heptane, *n*-octane, and *n*-decane (final concentrations 114, 117, and 122 mg L^{-1} respectively) to represent a more complex mixture of hydrocarbons such as those found in naphtha. Albian cultures were amended with a 1:1 by volume solution of *n*-pentane and *n*-hexane (final concentrations 157 and 165 mg L^{-1} respectively) as model compounds for paraffinic solvent. Samples (5 ml) were taken at day 0 and day 499 for DNA and mRNA extraction and analysis, and stored at -20˚C. Additional liquid samples were taken at day 518 for hydrocarbon analysis.

2.2.5 Toluene cultures

Toluene culture sets were created by transferring 20 ml from each *n*-alkane culture at day 316 to a sealed, anaerobic 60 ml serum bottle via syringe. Cultures were flushed with N_2/CO_2 for 10 minutes to volatilize any remaining *n*-alkanes and were subsequently amended with 200 mg L^{-1} toluene, then placed in the dark at room temperature (25˚C). Cultures were moved to 30˚C between days 80 and 140 to promote microbial metabolism. Day 0 samples were also taken within 48 hours of culture amendment and stored for hydrocarbon analysis. To preserve volatile hydrocarbons in solution, 0.5 ml culture was added to 1 ml methanol in a 2 ml autosampler vial (Agilent Technologies, #51855820), and topped up with methanol such that no headspace was present once the vial was sealed. Vial threading was previously wrapped 3x with Teflon tape to create a gas-tight seal. Samples were stored at 4˚C until analysis with hydrocarbon samples taken at day 189. Sterile standards were setup alongside cultures using water instead of culture media to achieve the same MFT to liquid ratio as cultures. Samples from these standards were also preserved for later hydrocarbon analysis with day 0 samples.

2.2.6 Acetylene reduction cultures

Acetylene reduction cultures were established by transferring 20 ml culture volume from all amended cultures and sterile cultures without N, to anaerobic 60 ml serum vials under a N_2/CO_2 headspace. Cultures were flushed with N_2/CO_2 for 5 minutes and re-amended with 200 mg L^{-1} hydrocarbons. Sub-culturing of *n*-alkane cultures occurred at day 316 to preserve the cultures for continued analyses as acetylene can inhibit methanogenic metabolism (Sprott et al., 1982). Acetylene was added directly to toluene cultures at day 190 due to low culture volume. Positive controls run alongside *n*-alkane cultures were setup as previously described (Collins et al., 2016). These aerobic N_2-fixing bacterium *Pseudomonas sp*. 5.1b (Eckford et al., 2002), were cultured in N-free "combined carbon media" containing 5 g L^{-1} each of sucrose and mannitol, and 0.5 ml L^{-1} sodium lactate (Rennie, 1981). In all cases, 99.2 µmol acetylene was injected into the headspace for a final concentration of 3% vol. Due to low culture activity, *n*-alkane cultures were incubated for 27 days and toluene cultures were incubated for 48 days.

2.2.7 Chemical analysis

Periodic methane measurements were taken as previously described (Collins et al., 2016). Bottle pressure was determined using a digital pressure gauge (DPG1000B ± 15.00PSIG-5, MOD-TRONIC Instruments Limited, Brampton, ON) with a luer-lok fitting and needle. Cultures were shaken by hand prior to headspace gas direct injection (0.1 ml) into a gas chromatographer equipped with a flame ionization detector (GC-FID). Primary enrichment cultures and transfer cultures were monitored for methane using

a GC-FID Hewlett Packard 5890 (column: Poropak Type R; oven temperature: 30°C and helium flow rate: 12.5 mL min^{-1}) and quantified using external standards (0.16%, 1%, 4%, 8%, and 15% methane). Hydrocarbon concentrations (*n*-alkane and toluene) were analyzed with a Trace 1300 GC-FID (ThermoFisher, column: TG-Bond 30mx0.32mm, oven temperature 40°C, and helium flow 3 ml min^{-1}) Theoretical maximum methane production was calculated using the Symons and Buswell equation (Roberts, 2002). Ethylene produced from the reduction of acetylene was measured using GC-FID with external standards (0.00316%, 0.0158%, 0.0379%, 0.0569%, and 0.0758% ethylene).

Hydrocarbon concentrations were determined in both the bottle headspace and liquid cultures. Direct injection of 0.1 ml culture headspace into a GC-MS (TRACE 1300 gas column: TraceGold TG-5MS, 30 m by 0.25 mm internal diameter, helium flow 1.2 ml min^{-1}; Thermo Scientific) was used to determine the hydrocarbon concentration in the headspace of Syncrude and CNRL *n*-alkane cultures due to previously observed decane loss in autosampler vials potentially resulting from sorption on recycled autosampler vial septa. Albian *n*-alkane and all toluene cultures were analyzed through injecting 0.5 ml culture headspace into a 20 ml headspace autosampler vial. External standards alone were used for quantification except for Albian *n*-alkane cultures, which also contained an internal standard (1,1,3-trimethylcyclohexane) to verify equal sample representation across all cultures from autosampler vial injections. Internal standards were not used in toluene cultures. External standards for hydrocarbons were prepared as matrix standards containing 92 ml water and 3 ml tailings. Five external standards were prepared ranging from 15 mg L^{-1} to 350 mg L^{-1} for hexane and pentane, 17 mg L^{-1} to 400 mg L^{-1} for heptane, octane, and decane, and 17 mg L^{-1} to 215 mg L^{-1} for toluene.

Hydrocarbons from the liquid phase were extracted for analysis by shaking cultures to ensure homogeneity and removing 0.5 ml liquid culture volume. Culture aliquots were added to 1 ml methanol in a 2 ml tube and vortexed for 10 seconds. The tubes were then centrifuged at 21,130 RCF for 3 min to remove suspended solids and 1 ml of the solution was transferred to a 44 mL EPA glass vial containing MilliQ H_2O. Vials were filled with MilliQ H_2O and caped without headspace. The same external standards prepared for headspace hydrocarbons were used for quantification of the liquid phase. Liquid phase hydrocarbons were analyzed on a GC-FID (Hewlett-Packard 6890) equipped with a purge and trap system.

Total N analyses (inorganic, and total N; TN) were performed by the Natural Resources Analytical Laboratory (NRAL) at the University of Alberta. Samples were separated into liquid and solid fractions by centrifuge. Liquid fraction TN was analyzed using a Shimadzu TOC-V/TN instrument (Shimadzu Corporation, 2001; Williams, 2000). Liquid phase inorganic N (NO_3^-, NH_4^+) were analyzed using a

SmartChem Discrete Wet Chemistry Analyzer (Maynard and Kalra, 1993), and solid phase TN was determined using a Costech Elemental Analyzer (Sparks, 1996).

Unless otherwise noted, statistical significance was determined in Excel using a two tailed, paired T-Test.

2.2.8 Hydrocarbon normalization

To correct for varying culture and headspace volume between *n*-alkane treatments at the time of analysis, an equation was formulated to normalize the hydrocarbon data. Using the following formula (Logan, 1999), where T_n is total moles, n_w is moles of hydrocarbon in the culture volume, and n_a is moles in headspace

(2-1)

$$T_n = n_w + n_a$$

and substituting in the following equations where H (atm m^3 mol^{-1}) is Henry's Law constant, C_{nw} (mol L^{-1}) is the concentration of hydrocarbon in the liquid phase, C_a is the concentration of air or headspace (0.0415 mol L^{-1}), V_a (L) is the volume of headspace, P (atm) is pressure, and V_w (L) is the volume of the liquid phase

(2-2)

$$n_a = \frac{HC_{nw}C_aV_a \times 10^3 L/m^3}{P}$$

(2-3)

$$n_w = C_{nw}V_w$$

the following equation was obtained through substitution and factoring.

(2-4)

$$C_{nw} = \frac{T_n}{\left(\frac{HC_aV_a \times 10^3 L/m^3}{P}\right) + V_w}$$

Equation 2-3 was used to determine n_w, which could then be used to determine n_a using formula 2-1. By making T_n 1 or 100%, the percent of hydrocarbons remaining in the liquid phase, or diffused into the headspace can be determined. This value can then be used to estimate the total amount of hydrocarbons in the total culture (liquid and headspace) using formula 2-5 below where A_a is the peak area of the measured hydrocarbon in the headspace, A_w is the peak area of the measured hydrocarbon in the liquid phase, and T_a is the total corrected area reflecting the total concentration in the culture bottle.

(2-5)

$$(1 + n_w)A_a = T_a$$

$$(1 + n_a)A_w = T_a$$

2.2.9 DNA/RNA sequencing and analysis

In *n*-alkane cultures, DNA and RNA analyses were performed on samples taken at day 334 in CNRL and Albian cultures, and day 0 in CNRL cultures. In toluene cultures, DNA and RNA extractions were performed on CNRL samples taken at day 189 just prior to the addition of acetylene. Two replicate DNA and RNA coextractions for each culture sample were performed using the following procedure developed and optimized for this thesis. A TE buffer was prepared containing 100 mM Tris-HCl, 100 mM EDTA, both adjusted to pH 7.0, 1.5M NaCl, and 1% CTAB in MilliQ H_2O. TE buffer (450 µl), culture sample (400 µl), 10% SDS solution (150 µl), and fresh 24:1 chloroform:isoamyl alcohol solution (300 µl) were combined in 2 ml screw cap tubes containing 0.1 mm and 2.5 mm diameter zirconium-silica beads (0.5 g each). Tubes were shaken at 5700 RPM in a Powerlyzer 24 homogenizer (MoBio) and centrifuged for 7 minutes at max speed (21,130 RCF). The supernatant was transferred to a new tube and placed on ice. Ammonium acetate (0.56 vol) and 10% SDS (50 µl) were added to the supernatant, then tubes were inverted 10 times and centrifuged on maximum for 5 min. Supernatant was transferred to a new tube and isopropanol (1 vol) was added. Glycogen (0.05 µg/µl; Thermo Scientific, #R0551) was added and tubes were inverted 40 times before incubation at -20˚C for 2-8 hours.

Following incubation, tubes were centrifuged for 30 min at maximum speed, the supernatant was removed, and pellets resuspended in nuclease free water (100 µl; Ambion, AM9930). Ethanol (2.5 vol) and glycogen (0.05 µg/µl) were added and tubes were inverted 40 times before incubation at room temperature for 2-4 hours. Tubes were centrifuged at max speed, supernatant discarded, and pellets resuspended in 30 µl nuclease free water. The presence of DNA and RNA was verified using QuBit fluorometric quantitation, replicate extractions were pooled, and half of the pooled volume was stored at 4˚C for downstream DNA analyses for up to one week. Samples for downstream RNA analyses were treated repeatedly with RNase free DNase I (Epicenter, D9905K), according to product kit (Epicenter, Masterpure MC85200) instructions including removal of DNase enzymes with 2XT and C Lysis Solution from kit and MPC Protein Precipitation Reagent (Epicenter, MMP095H) with optional RiboGuard RNase inhibitor (Epicenter, RG90925), until a 45 cycle PCR 16S rRNA gene amplification with RNA template showed no amplification.

RNA samples were treated with SuperScript IV reverse transcriptase (Invitrogen, 18090010), with gene specific primers for *nifH, nosZ, nirS*, and *nrfA* functional genes as outlined by product protocol. RNA and DNA were then used for library preparations using the primers and optimized PCR conditions outlined in

Table 2-1 with Illumina forward or reverse overhangs (Illumina, 2013). PCR reactions were prepared in duplicate and pooled following amplification. Each reaction contained the following: 15 µl GoTaq Green Master Mix (Promega, M7122), 1.25 µl DMSO, and 1.25 µl each of forward and reverse primers. BSA concentrations were optimized for each set of primers. 16S rRNA, *nifH*, and *nirS* reactions contained 0.2 µl BSA, and *nrfA* and *nosZ* reactions contained 0.75 µl BSA. Nuclease free water was added for a final reaction volume of 25 µl. Amplicons (5 µl reaction volume) were visualized on a 1% agarose and sent to a commercial lab for MiSeq Illumina sequencing (TAGC, University of Alberta). Libraries were prepared for each gene and pooled by sample for sequencing. The concentration for 16S rRNA gene amplicons were normalized across all samples.

Table 2-1: Primers and PCR conditions

Gene	Primer pairs	PCR conditions	Primer source
16S rRNA (DNA only)	926fw: AAACTYAAAKGAATTGRCGG 1392r: ACGGGCGGTGTGTRC Approximate size without primers: 450 bp	95˚C 5min, 35x 95˚C 30s, 54˚C 45s, 72˚C 30s. 72˚C 5min	(Engelbrektson et al., 2010)
NifH	NifH-F: AAAGGYGGWATCGGYAARTCCACCAC NifH-R: TTGTTSGCSGCRTACATSGCCATCAT Approximate size without primers: 458 bp	95˚C 5min, 35x 95˚C 30s, 57˚C 30s, 72˚C 30s. 72˚C 5min	(Rösch et al., 2002)
NosZ	*NosZ*-F: CGYTGTTCMTCGACAGCCAG *NosZ*1622R: CGSACCTTSTTGCCSTYGCG Approximate size without primers: 434 bp	95˚C 5min, 35x 95˚C 30s, 58˚C 1min, 72˚C 1min. 72˚C 5min	(Kloos et al., 2001) (Throbäck et al., 2004)
NirS	Cd3aF: GTSAACGTSAAGGARACSGG R3cd: GASTTCGGRTGSGTCTTGA Approximate size without primers: 425 bp	95˚C 5min, 35x 95˚C 30s, 59˚C 45s, 72˚C 30s. 72˚C 5min	(Michotey et al., 2000) (Throbäck et al., 2004)
NrfA	NrfAF2aw: CARTGYCAYGTBGARTA NrfAR1: TWNGGCATRTGRCARTC Approximate size without primers: 269 bp	95˚C 5min, 35x 95˚C 30s, 49˚C 1min, 72˚C 1min. 72˚C 5min	(Welsh et al., 2014) (Mohan et al., 2004)

Illumina data was processed using MetaAmp 2.0 (Dong et al., 2017). Using default MetaAmp workflow, paired end analyses, a similarity cutoff of 0.97, and a maximum expected error of 1, the following specifications were applied. *NirS*, *nosZ*, and *nifH* all used a minimum overlap of 50 bp with 10 bp maximum differences in this region, and were trimmed to 350 bp, 275 bp, and 385 bp, respectively. *NrfA* was analyzed with a minimum overlap of 200 bp, 10 bp maximum overlap mismatches, no primer mismatch, and a trim length of 235 bp. 16S rRNA was analyzed with a minimum overlap of 50 bp, maximum mismatch of 10 bp, 1 bp primer mismatch, and trimmed to 425 bp. 16S rRNA community analysis was processed through MetaAmp using the SILVA 132 database and workflow as outlined in Dong et al., 2017. Functional gene sequences were processed using BLASTx and MegaBLAST (blast.ncbi.nlm.nih.gov/), excluding

uncultured/environmental sample sequences. Sequences were manually curated to remove non-target sequences, and all treatments that returned fewer than 50 target reads were discarded (**Table S 2**).

2.3 Results and Discussion

2.3.1 Methanogenesis

2.3.1.1 n-alkane experimental cultures

To determine if biogenic methane was produced from the degradation of *n*-alkanes with and without N in Albian, Syncrude, and CNRL cultures, culture headspace was monitored for 499 days (**Figure 2-1**). Theoretical maximum methane values were determined based on the Symons and Buswell (1933) stoichiometric equation. Based on complete degradation of hydrocarbons added to initial culture volumes, theoretical maximums methane production we calculated were as follows: pentane, 824.5 µmol; hexane, 862.7 µmol; heptane, 594.4 µmol; octane, 609.0 µmol; and decane, 629.5 µmol. The final readings at day 499 were taken following substantial reduction in culture volume due to sub-culturing and was analyzed to compare with hydrocarbon data.

Very little methane was produced in baseline cultures regardless of N content (<0.8 µmol) and there was no significant difference in methane production observed between baseline and sterile cultures, whereas all *n*-alkane amended cultures produced significantly more methane than sterile and unamended cultures due to the presence of hydrocarbons. Amended Syncrude cultures were the most active overall with methane production beginning at day 62. Interestingly, amended cultures without N consistently produced more methane than amended cultures with N. These differences were significant ($p < 0.05$) in Albian cultures at days 287 and 499, in Syncrude at days 63-316, and in CNRL at days 244-316. The quantity of methane produced was not consistent with theoretical methane production from the observed hydrocarbon degradation due to incomplete conversion of hydrocarbons to methane, changes in culture volume, and potential loss of pressurized headspace gas due to septa punctures. The observed increase in methanogenesis in cultures with N will be discussed later in this chapter.

2.3.1.2 Toluene experimental cultures

Methane resulting from toluene metabolism was monitored for 185 days in Albian, Syncrude, and CNRL cultures with and without inorganic N in the media (**Figure 2-2**). Theoretical maximum methane production calculated for toluene amended cultures was 176 µmol upon complete biodegradation of toluene (Symons and Buswell, 1933). Across all datasets, sterile controls produced no methane and baseline cultures produced very little methane (<2.3 µmol) regardless of N content. Syncrude cultures were the most active with an increased rate of methanogenesis beginning at day ~70. As observed in *n*-

alkane cultures, a higher rate of methanogenic activity occurred in amended cultures without N. This difference was significant ($p < 0.05$) in Albian from day 89 through day 185, and in CNRL at day 26 and 141. As seen in Albian and Syncrude *n*-alkane amended cultures, greater methanogenesis in cultures without N correlated with higher toluene degradation (discussed later in this chapter).

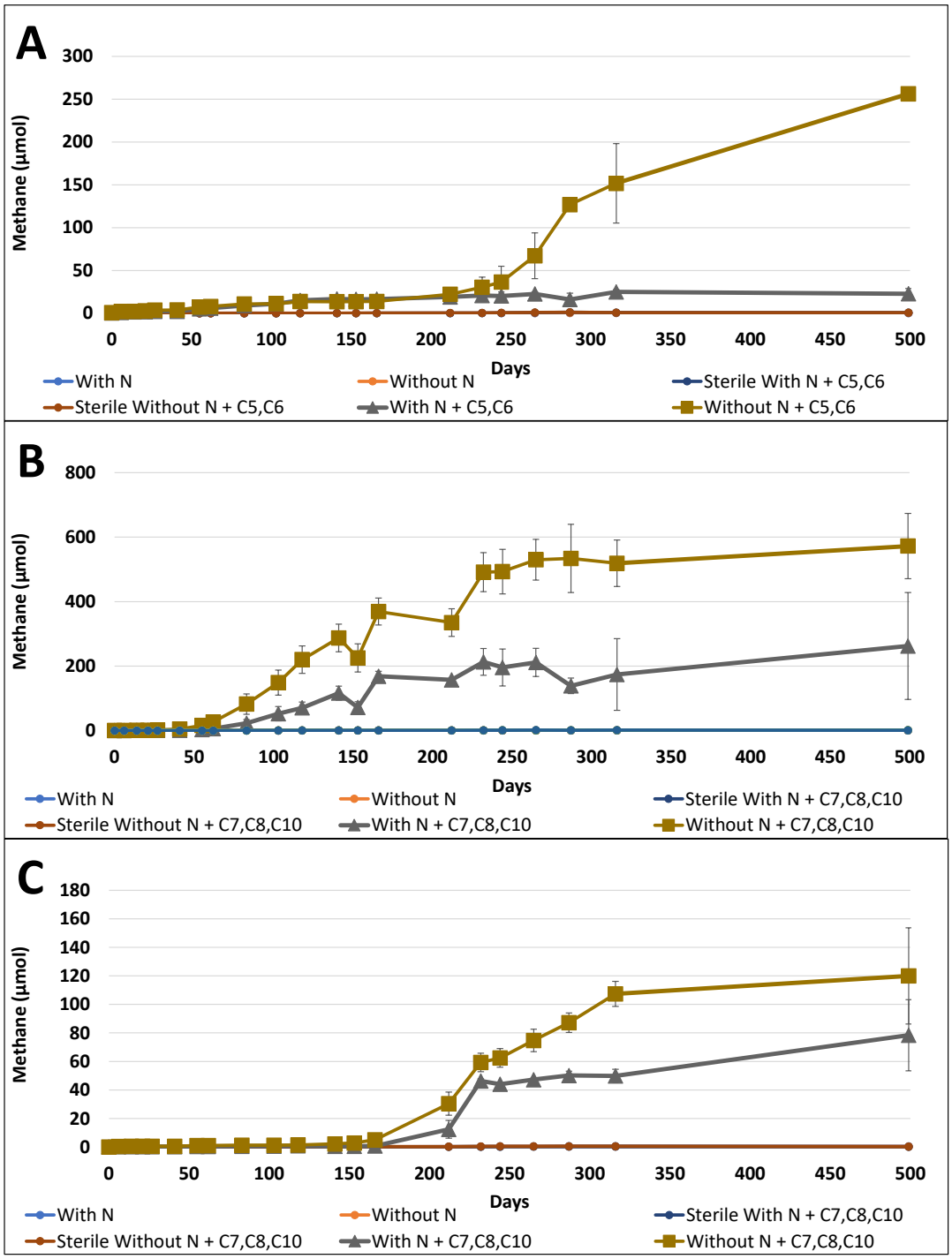

Figure 2-1: Methane production from *n*-alkane amended and baseline cultures (**A**) Albian, (**B**) Syncrude, and (**C**) CNRL. Values represent the mean µmol for triplicate amended cultures and duplicate sterile and baseline cultures. Error bars indicate ± standard deviation.

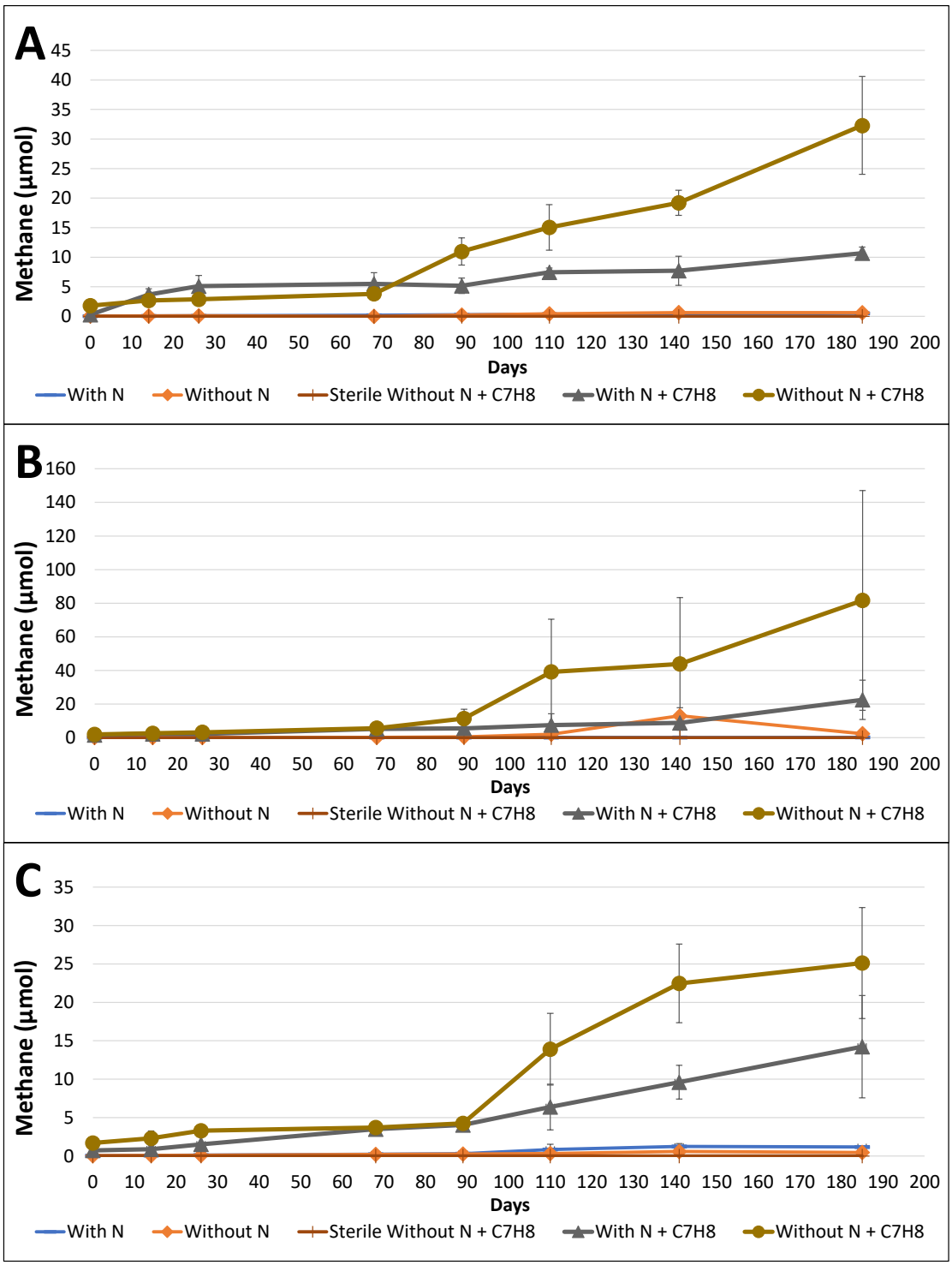

Figure 2-2: Methane production from toluene amended and baseline cultures (**A**) Albian, (**B**) Syncrude, and (**C**) CNRL. Values represent the mean µmol for triplicate amended cultures and duplicate sterile and baseline cultures. Error bars indicate ± standard deviation.

2.3.2 Hydrocarbon degradation

2.3.2.1 n-alkane experimental cultures

Hydrocarbon depletion was measured at three time points in *n*-alkane cultures however, only end-point hydrocarbon analyses results from day 518 (**Table 2-2**) are available due to failed runs. This was due to instrument failure followed by lack of instrument availability, either due to maintenance or equipment scheduling.

Table 2-2: Hydrocarbon concentration in *n*-alkane amended cultures. Cultures were prepared in triplicate amended cultures and duplicate sterile cultures.

Treatment	***n*-Pentane**		***n*-Hexane**	
Albian Liquid	mg L^{-1}	StDev	mg L^{-1}	StDev
With N + C_5,C_6	143.60	7.99	68.41	3.01
Without N + C_5,C_6	73.49	38.06	**22.14**	10.36
Sterile With N + C_5,C_6	139.95	8.94	64.73	0.01
Sterile Without N + C_5,C_6	123.64	11.25	63.54	0.001
Albian Headspace	mg L^{-1}	StDev	mg L^{-1}	StDev
With N + C_5,C_6	125.48	68.83	122.05	32.17
Without N + C_5,C_6	77.86	31.39	**60.69**	6.39
Sterile With N + C_5,C_6	144.45	13.44	100.95	2.22
Sterile Without N + C_5,C_6	114.41	27.08	89.08	8.63

Treatment	***n*-Heptane**		***n*-Octane**		***n*-Decane**	
Syncrude	mg L^{-1}	StDev	mg L^{-1}	StDev	mg L^{-1}	StDev
With N + C_7,C_8,C_{10}	88.60	62.65	55.89	34.27	48.54	13.39
Without N + C_7,C_8,C_{10}	**36.20**	1.25	**35.04**	3.06	53.78	9.99
Sterile With N + C_7,C_8,C_{10}	120.94	23.24	75.05	15.11	83.21	26.09
Sterile Without N + C_7,C_8,C_{10}	118.90	28.58	75.82	10.38	48.64	14.24
CNRL	mg L^{-1}	StDev	mg L^{-1}	StDev	mg L^{-1}	StDev
With N + C_7,C_8,C_{10}	119.48	21.75	69.36	8.44	44.87	8.05
Without N + C_7,C_8,C_{10}	94.82	19.19	58.81	8.70	56.63	4.90
Sterile With N + C_7,C_8,C_{10}	98.12	0.05	54.98	11.76	34.92	21.92
Sterile Without N + C_7,C_8,C_{10}	99.54	20.26	54.48	10.44	40.86	11.45

Hydrocarbon results indicate substantially lower hexane, octane and decane as compared to the expected values based on the initial amended concentrations, this was apparent in sterile cultures where the lighter hydrocarbons were present at disproportionally higher concentrations than the heavier hydrocarbons. This variation may have been due to the standards reading high for the heavier hydrocarbons. Because the standards contained water rather than media, it is possible the salt content in the media influenced

the extraction efficiency or hydrocarbon partitioning causing hydrocarbons to become more hydrophobic and should be considered in future studies. This may cause the heavier hydrocarbons to bind clay molecules more tightly and stay in solution or become less readily extractable in methanol. The heavier hydrocarbons may have also generally had a higher adsorption efficiency for clays and residual organics than the lighter hydrocarbons. Differences between adsorption efficiency for clays and residual organics in Syncrude and CNRL cultures may also explain the variance in hydrocarbon concentrations seen in sterile cultures. However, due to the consistency of the effect on both live and sterile cultures, changes over time can still be determined.

In *n*-alkane Albian treatments, without N cultures have significantly ($p<0.05$) lower hexane concentration in both liquid and headspace results as compared to sterile cultures. A significant difference was also observed in hexane concentrations between amended cultures with N and amended cultures without N. The average pentane concentrations were also lower in amended cultures without N, however this difference was not significant.

There were no significant differences in the concentration of *n*-alkanes in amended Syncrude cultures with N as compared to sterile cultures despite the observed methane production in those cultures. The concentration of heptane and octane were significantly lower in Syncrude cultures without N as compared to sterile cultures, however, there was no measurable difference in the concentration of decane.

Hydrocarbon degradation under anaerobic conditions has been reported to occur in order of decreasing molecular weight when studying F1 fraction of hydrocarbons (Siddique et al., 2006). Siddique et al. (2006) theorized this degradation pattern resulted from either selective membrane uptake as proposed by Kim et al. (2002), or the octanol/water partition coefficient, which increases with *n*-alkane length. In this study, our observed results varied. In Albian cultures, the longer chain, hexane was degraded before the shorter chain, pentane, however this was not observed in Syncrude cultures. Heptane and octane were degraded preferentially over decane. While interesting, this observation can be easily explained.

The primary enrichment cultures used to inoculate these *n*-alkane cultures had been previously enriched on *n*-hexane (Albian) and *n*-octane (Syncrude and CNRL). This likely would have resulted in preferential degradation of these hydrocarbons and those of similar length such as heptane, which is only one carbon shorter than octane, whereas decane is two carbons longer. More importantly, both heptane and octane were only partially degraded by day 518 while methane production slowed beginning at day 265. This is interesting as we would expect methane production to be continuous until the actively metabolized hydrocarbons were depleted. This was potentially due to hydrocarbons becoming less accessible to the

microbial community with decreasing concentration, or a build-up of inhibitory intermediates. A study by Stasik et al. (2015) found BTEX degradation and methane production could be inhibited by a buildup of acetate as a metabolic intermediate in cultures amended with a labile carbon source. Sulfate reducing bacteria were needed to mitigate this inhibition. While it is unlikely that acetate would inhibit acetoclastic methanogenesis in these cultures, it is interesting to consider a rate limiting step such as reduced growth of a bacteria required for co-metabolism of intermediates, or depletion of trace nutrients, as an explanation. Without additional data however, the persistence of heptane and octane with reduced methanogenesis in amended Syncrude cultures without N cannot be explained.

Unlike cultures from other treatment sets, there was no significant difference in the hydrocarbon concentrations across all CNRL treatments despite the observed methanogenesis. This suggests degradation of residual metabolites or carbon sources, or a slow rate of hydrocarbon degradation in these cultures that was not observed to standard deviation between cultures.

2.3.2.2 Toluene experimental cultures

Toluene concentration was determined in both the headspace and aqueous phase as outlined in the methods. Headspace analyses were conducted at day 0, day 26, day 96, and day 144 (**Figure S 4**), however the run at day 144 failed and accurate results could not be compiled. Liquid phase was analyzed at day 0 and day 189 (**Table 2-3**). Day 0 samples for liquid phase were taken between 24 and 48 hours after culture establishment and stored in methanol in tandem with matrix standards established alongside the cultures.

Table 2-3: Toluene concentration in toluene amended cultures. Values represent the mg L^{-1} toluene for triplicate amended cultures and duplicate sterile and baseline cultures.

Treatment	With N + C_7H_8	StDev	Without N + C_7H_8	StDev	Sterile Without N + C_7H_8	StDev
Day 0						
Albian	154.99	13.74	161.33	10.50	158.16	9.88
Syncrude	176.15	1.47	190.53	11.09	183.34	12.95
CNRL	175.92	6.12	164.72	8.69	170.32	22.59
Day 189						
Albian	178.40	33.10	**78.14**	18.60	158.89	16.28
Syncrude	**133.29**	5.56	**104.85**	27.30	205.84	14.18
CNRL	131.92	18.26	**80.97**	5.90	179.55	28.88

In Albian cultures at day 189, toluene amended cultures without N contained significantly lower concentrations of toluene as compared to sterile controls or toluene amended cultures with N in liquid analyses implying biodegradation of toluene. Syncrude cultures also showed a significant decrease in the concentration of toluene in both amended cultures with and without N as compared to day 0 values and sterile cultures at day 189.

Like Albian, CNRL cultures with toluene showed a significant decrease in toluene concentrations between cultures without N and sterile cultures by day 189. CNRL cultures without N also had significantly less toluene remaining than cultures with N indicating biodegradation of toluene in CNRL toluene amended cultures.

2.3.3 Acetylene reduction in culture

2.3.3.1 n-alkane experimental cultures

Acetylene reduction assay to assess N_2-fixing activity was conducted by subsampling *n*-alkane cultures at day 316 and analyzing the headspace of the subcultures periodically until ethylene production was observed after 27 days (**Figure 2-3**). Only *n*-alkane amended cultures were selected for acetylene reduction due to increased metabolic activity, as indicated by higher rates of methanogenesis. No ethylene production from acetylene reduction was observed in sterile cultures, or cultures with N in the media indicating inhibition of nitrogenase activity by inorganic N (Kessler et al., 2001). Ethylene was also absent in all Syncrude culture treatments. Interestingly, acetylene was also below detection in Syncrude cultures by day 27 (data not shown). The absence of acetylene was observed in both amended Syncrude cultures with and without N, which suggested degradation of acetylene by the microbial communities rather than reduction to ethylene followed by degradation as has been observed after several weeks of incubation following previous acetylene reduction assays (unpublished data, Collins 2013), and in other studies (Koene-Cottaar and Schraa, 1998). Acetylene metabolism has been described in several *Pelobacter* sp. (Akob et al., 2017; Schink, 1985a), a genus of the order *Desulfuromonadales*, however is not well documented in other organisms.

No Ethylene was present in tailings cultures at day 0 or at weekly intervals until day 27, at which time ethylene was recorded in the positive controls (10.6 ±10.1 µmol) and detected in Albian and CNRL cultures without N (0.15 ±0.02 µmol and 0.06 ±0.04 µmol, respectively). Albian cultures reduced significantly ($p<0.05$) more acetylene to ethylene than CNRL cultures due to higher metabolic activity as indicated by higher rates of methanogenesis. These values are lower than previously observed (Collins et al., 2016), however the cultures in this current study were not as metabolically active. Just prior to conducting the acetylene reduction assay, citrate amended cultures in Collins et al. (2016) were producing upwards of 60 µmol methane per week, whereas the most active *n*-alkane culture in the current study, amended Albian without N, had produced only 6.4 µmol methane during the same amount of time. This rate may have been sufficient to support the metabolic activity seen in this culture. Assuming 6.4 µmol methane produced during a week of Albian activity, we can back calculate to determine the molecules of hexane (4.75:1 methane to hexane) required and estimate that this would equate to ~31.18 µmol carbon utilized

in 27 days. Considering a minimum carbon to nitrogen ratio of 200:1 (Wang and Bartha, 1990), this activity would require ~0.16 µmol of nitrogen from amended Albian cultures without N. Because the acetylene reduction assay describes nitrogenase activity, which reduces N_2 to $2NH_4^+$, amended Albian cultures without N would have produced 0.3 ±0.04 µmol available N. This indicates that enough N would have been present in cultures without N to support the observed methanogenic metabolism from hydrocarbon degradation. Unfortunately, exact calculations are not possible as methane production could not be monitored over the course of the acetylene reduction assay.

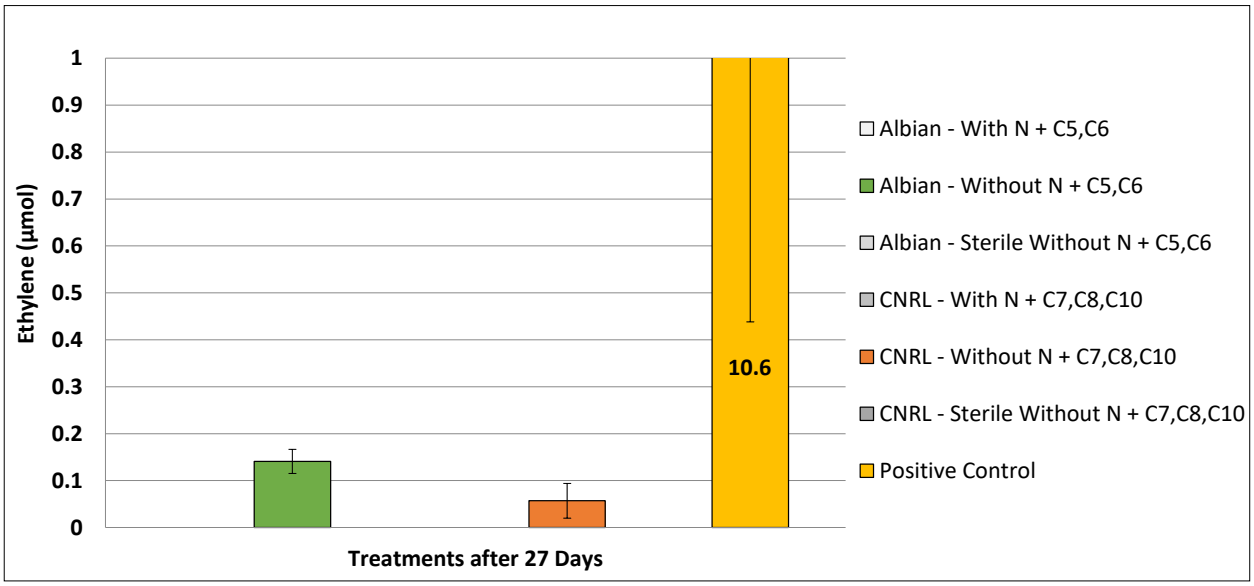

Figure 2-3: Ethylene production resulting from acetylene reduction in n-alkane amended cultures. Values represent the mean µmol in triplicate for each treatment, and error bars indicate ± standard deviation.

2.3.3.2 Toluene experimental cultures

Acetylene was injected into toluene cultures at day 190 and ethylene production was monitored periodically for 41 days(**Figure 2-4**). As with *n*-alkane cultures, only amended cultures were selected for the acetylene reduction assay. No ethylene production was observed in sterile cultures or cultures with inorganic N in the media. Unlike *n*-alkane cultures, ethylene was observed in Syncrude cultures. Because acetylene degradation was only observed in *n*-alkane degrading cultures, which were enriched for metabolizing *n*-alkanes, these data suggest acetylene is metabolized either more similarly to *n*-alkanes than toluene, by the same organisms, by members of the *n*-alkane degrading community not sufficiently present in toluene enrichments to metabolize all acetylene, or potentially via co-metabolism with *n*-alkanes.

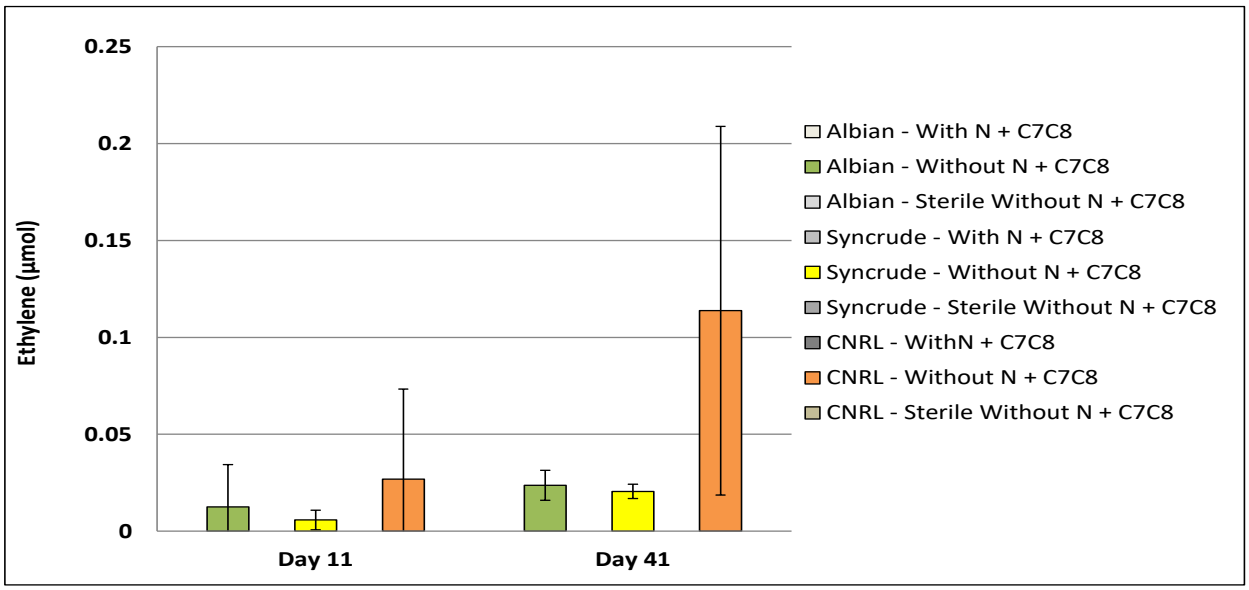

Figure 2-4: Ethylene production resulting from acetylene reduction in toluene amended cultures. Positive control was omitted due to culture death. Values represent the mean µmol in triplicate for each treatment, and error bars indicate ± standard deviation.

No ethylene was observed in cultures at day 0 or until day 11. By day 11, only a single Albian culture without N was actively producing ethylene (0.04 µmol) resulting in a low mean with high standard deviation (0.01 ±0.02 µmol). Ethylene production in Syncrude without N cultures was low (0.006 ±0.005 µmol), and CNRL cultures without N had high standard deviation (0.03 ±0.05 µmol) due to a single culture producing more acetylene than the other two replicates (0.08 µmol). By day 41, Albian cultures had produced 0.02 ±0.008 µmol ethylene, Syncrude cultures had produced 0.02 ±0.004 µmol, and CNRL cultures had produced 0.1 ±0.1 µmol. Assuming methane generation and toluene metabolism would have continued at the same rate during the acetylene reduction as in the previous 40 days, we could assume 0.3 µmol methane per day from Albian cultures. Using the methane to toluene ratio of 4.5:1, we can estimate 0.067 µmol toluene consumed per day, or 2.7 µmol over the course of the acetylene reduction in Albian cultures. Albian cultures reduced 0.02 µmol acetylene which would equate to 0.04 µmol fixed N. Assuming a minimum of 200:1 C:N, this rate of N_2-fixation is feasible.

Inhibition of methanogenesis occurred in both *n*-alkane and toluene amended cultures over the course of the acetylene reduction assay. This was likely due to inhibition of methanogenesis by acetylene and ethylene (Schink, 1985b; Sprott et al., 1982). Sprott et al. (1982) suggested this inhibition was due to disruption of the cell membrane pH gradient, which directly interfered with ATP synthesis. This is interesting as acetylene and ethylene could potentially interfere with acetylene reducing activity in N_2-fixing methanogens. N_2-fixation in Archaea has been well documented (Belay et al., 1984; Leigh, 2000). A

study by Belay et al. (1988) showed both methanogenesis and acetylene reduction was inhibited when a culture of N_2-fixing methanogens was treated with the methanogenesis inhibitor, bromoethanesulfonic acid (BES). Similarly, acetylene and ethylene could be reducing the nitrogenase activity in N_2-fixing methanogens by interfering with the proton gradient in their cell membranes. This would reduce the rates of acetylene reduction observed in this study and in our previous study thereby not reflecting the actual N being actively fixed in the cultures (Collins et al., 2016). However, these experiments still serve to confirm the presence of nitrogenase activity.

2.3.4 Microbial community and function

2.3.4.1 n-alkane experimental cultures

The observed hydrocarbon degradation in Albian cultures was supported by 16S rRNA gene data **(Figure 2-5A**) where Albian *n*-alkane amended cultures without N contained more than twice the population of the hydrocarbon degrading bacteria as compared to amended cultures with N. These were predominantly *Clostridia* and *Desulfovibrionaceae*. *Desulfovibrio* have been found to exhibit syntrophic metabolism with methanogens in sulfur limited environments (Meyer et al., 2013) and *Clostridia* spp. are known to play an active role in hydrocarbon degradation (Gieg et al., 2014). These bacterial groups were also present at much higher proportions in amended cultures than unamended cultures suggesting these bacteria were responsible for the hydrocarbon degradation in these cultures. The increased abundance of these groups in amended Albian cultures without N as compared to amended cultures with N was likely because both *Clostridia* and *Desulfovibrionaceae* contain N_2-fixing species (Cabello et al., 2009), discussed later in this chapter.

No significant hydrocarbon degradation was observed in amended CNRL treatments despite the high proportion of hydrocarbon degrading bacteria, which remained relatively unchanged from day 0 (**Figure 2-5B**). In the absence of hydrocarbon degradation, amended CNRL cultures contained a high proportion of bacteria known to play a role in hydrocarbon metabolism including *Clostridia* (Gieg et al., 2014), *Syntrophaceae* (*Smithella*; Cheng et al., 2013; Gray et al., 2011; Mohamad Shahimin and Siddique, 2017a; Paulo et al., 2018; Siddique et al., 2018, 2011), and *Spirochaetaceae*, which are known to play an indirect role in methanogenic hexadecane degradation (Cheng et al., 2013). The data suggested endogenous substrates or a very slow rate of hydrocarbon degradation was present in amended cultures to support the observed methanogenesis and the microbial community.

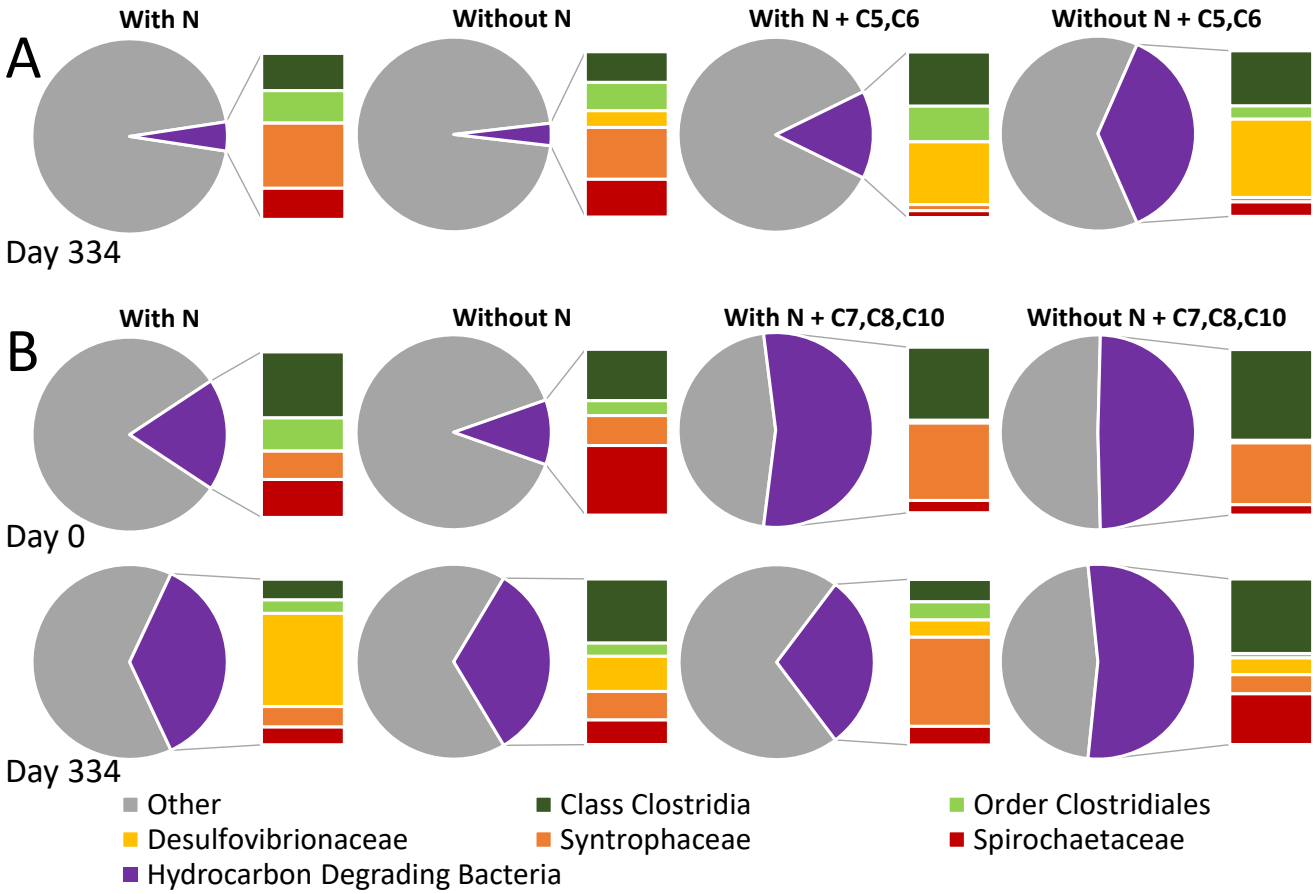

Figure 2-5: Bacterial community composition of hydrocarbon degrading bacteria in *n*-alkane amended and baseline cultures based on Illumina sequencing of the 16S rRNA gene at (**A**) from Albian cultures, and (**B**) CNRL cultures. Bacteria not known to degrade hydrocarbons and sequences with less than 2% abundance were grouped as "Other". Full Bacteria dataset in **Figure S 5**.

Amended Albian cultures produced more methane than unamended Albian cultures and contained significantly ($p < 0.05$) higher proportions of methanogens (**Figure 2-6**). Both amended cultures with and without N contained large populations of acetoclastic methanogens (*Methanosaeta*). Amended cultures without N also contained an equivalent proportion of hydrogenotrophic methanogens (predominantly *Methanolinea*; **Figure S 6**). These archaea metabolized hydrocarbon degradation products to methane in these cultures.

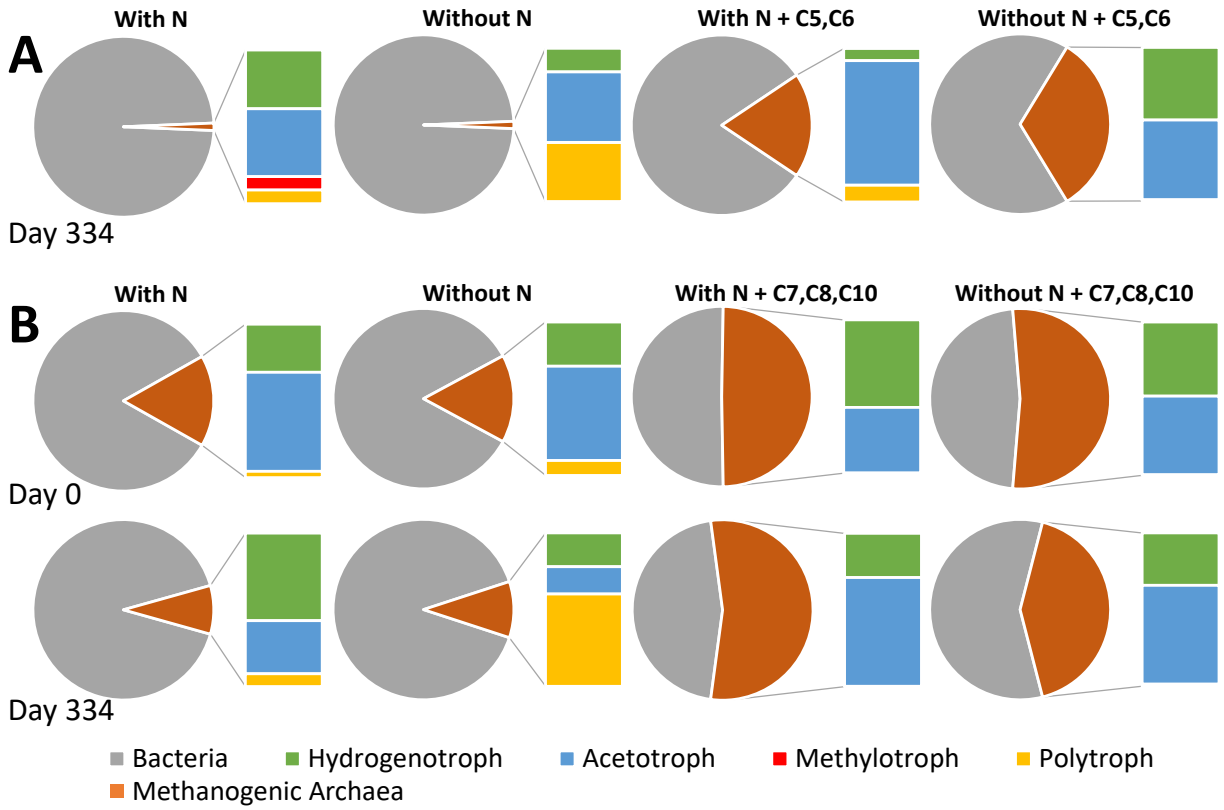

Figure 2-6: Distribution of Bacteria and methanogens from *n*-alkane amended and baseline cultures based on Illumina sequencing of the 16S rRNA gene from Albian cultures (**A**) and CNRL cultures (**B**). Polytroph indicates methanogens capable of metabolizing hydrogen, acetate, and methanol to methane. Full Archaeal dataset is presented in **Figure S 6**. The microbial community of Albian transfer culture inoculum (day 0) was previously described (Collins et al., 2016; Mohamad Shahimin et al., 2016), and contained less than 30% archaea in baseline cultures and 50% archaea in amended cultures.

The proportion of methanogens in amended CNRL cultures were higher than in amended Albian cultures and yet, the methane production between the two culture sets were comparable. Hydrocarbon degradation was also not apparent in amended CNRL cultures, however methanogenesis driven by the archaeal community continued. The proportion of methanogens did not change substantially from day 0 samples, which represented the microbial community from the primary enrichment cultures described in section **2.2.2** Primary enrichment cultures from Syncrude and CNRL tailings. However, the community shifted and the acetoclastic population (*Methanosaeta*) became dominant in both amended cultures with and without N. This indicated the presence of acetate in cultures to support acetoclastic methanogenesis and suggested the presence of residual organics or a slow rate of hydrocarbon degradation in these cultures.

The presence of N_2-fixation was examined by identifying the known N_2-fixing groups from the 16S rRNA microbial community data (**Figure 2-7**), and evaluating *nifH* genes and gene expression (**Figure 2-8**).

Nitrogenase gene expression (*nifH*) was detected in amended cultures without N, which reduced acetylene, and was absence in cultures with N, which did not reduce acetylene. This supported our hypothesis that N_2-fixation was occurring to support the N demand of the microbial community. Microbial community data also showed an increase in the abundance of N_2-fixing groups *Desulfovibrio* and *Clostridia* (Cabello et al., 2009) in amended Albian cultures without N, and an increase in *Clostridia* in amended CNRL cultures without N as compared to amended cultures with N. The abundance of the N_2-fixing methanogen *Methanosaeta* (Bae et al., 2018), remained relatively consistent regardless of N content in *n*-alkane amended Albian and CNRL cultures.

In our previous study (Collins et al., 2016), we also observed an increase in *Clostridia* in amended cultures without N and little variation in the archaeal population between cultures with and without N. Based on these observations, we suggested the majority of the N_2-fixation was performed by the bacterial population which shifted under N deficient conditions and dismissed the archaeal population, however the current results were much more interesting. In this study, we examined both the DNA and RNA (mRNA) of the *nifH* functional gene and found the DNA composition of a community was not representative of gene expression. The *nifH* genes present in the DNA from Albian cultures were predominantly Betaproteobacteriales with a dominance of *Rhodocyclaceae* (99% ident.) in amended cultures without N as compared to *Comamonadaceae* (95-98% ident.) in amended culture with N, however gene expression did not corroborate. *NifH* reads were not detected in reverse transcription samples taken from amended cultures with N (data not shown) indicating no N_2-fixation was taking place. In amended Albian cultures without N, RNA results likely indicated the majority of *nifH* gene expression was from *Methanosaetaceae* (98-99% ident., *Methanosaeta concilii* 100% ident.) with *Methanosaeta concilii* as the closest match for all sequences.

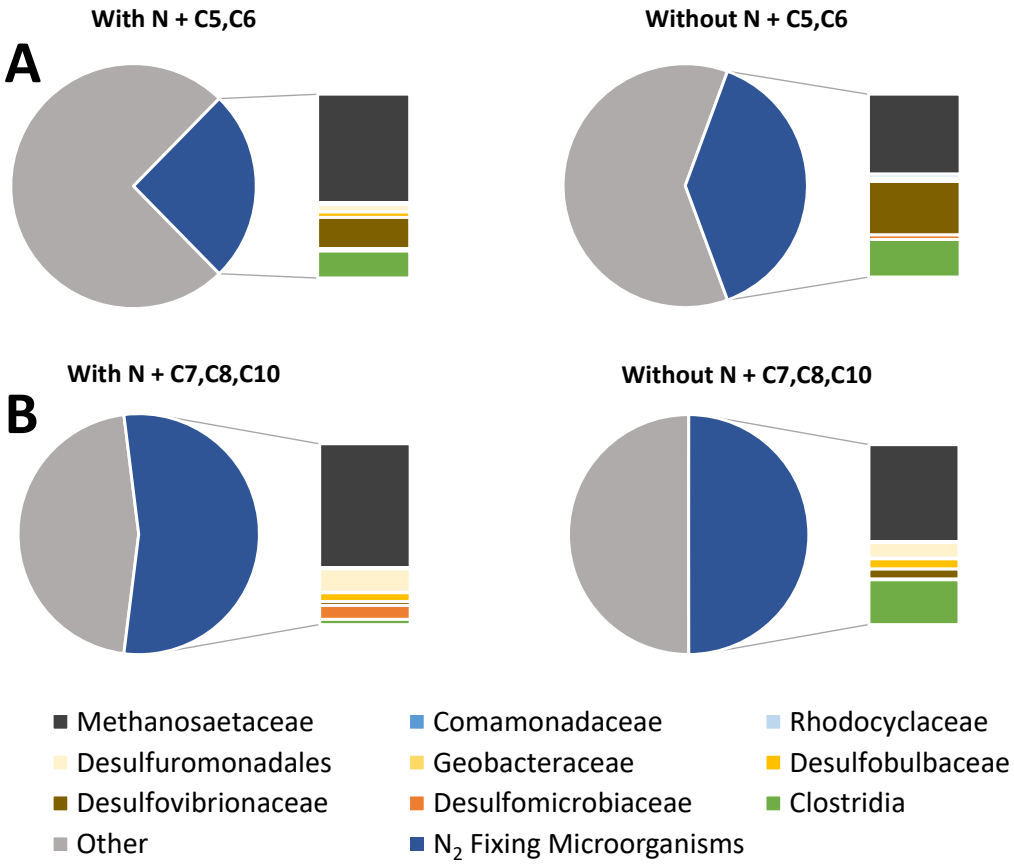

Figure 2-7: Microbial community composition of potential N_2-fixing microorganisms based on Illumina sequencing of the 16S rRNA gene *n*-alkane amended (**A**) Albian cultures, and (**B**) CNRL cultures. Prokaryotes where *nifH* genes were not detected were grouped as "Other".

CNRL results agreed with Albian data where the proportion of microorganisms possessing *nifH* genes did not correlate with gene expression. The majority of *nifH* genes present in amended cultures at day 334 originated from Deltaproteobacteria, and 37% of the *nifH* sequences in both amended cultures with and without N were *Methanosaetaceae*. *NifH* gene expression in mRNA was not detected in amended cultures with N however, cultures without N were dominated by an astonishing 89% *Methanosaetaceae*. These data likely indicate *Methanosaetaceae* is responsible for the majority of N_2-fixation in amended Albian and CNRL *n*-alkane cultures without N. The contrast between the representation of *Methanosaetaceae* in

the DNA and expression in RNA is startling, especially in Albian cultures, and begs reexamination of assumptions made regarding identification of key player for specific metabolic activity in mixed cultures.

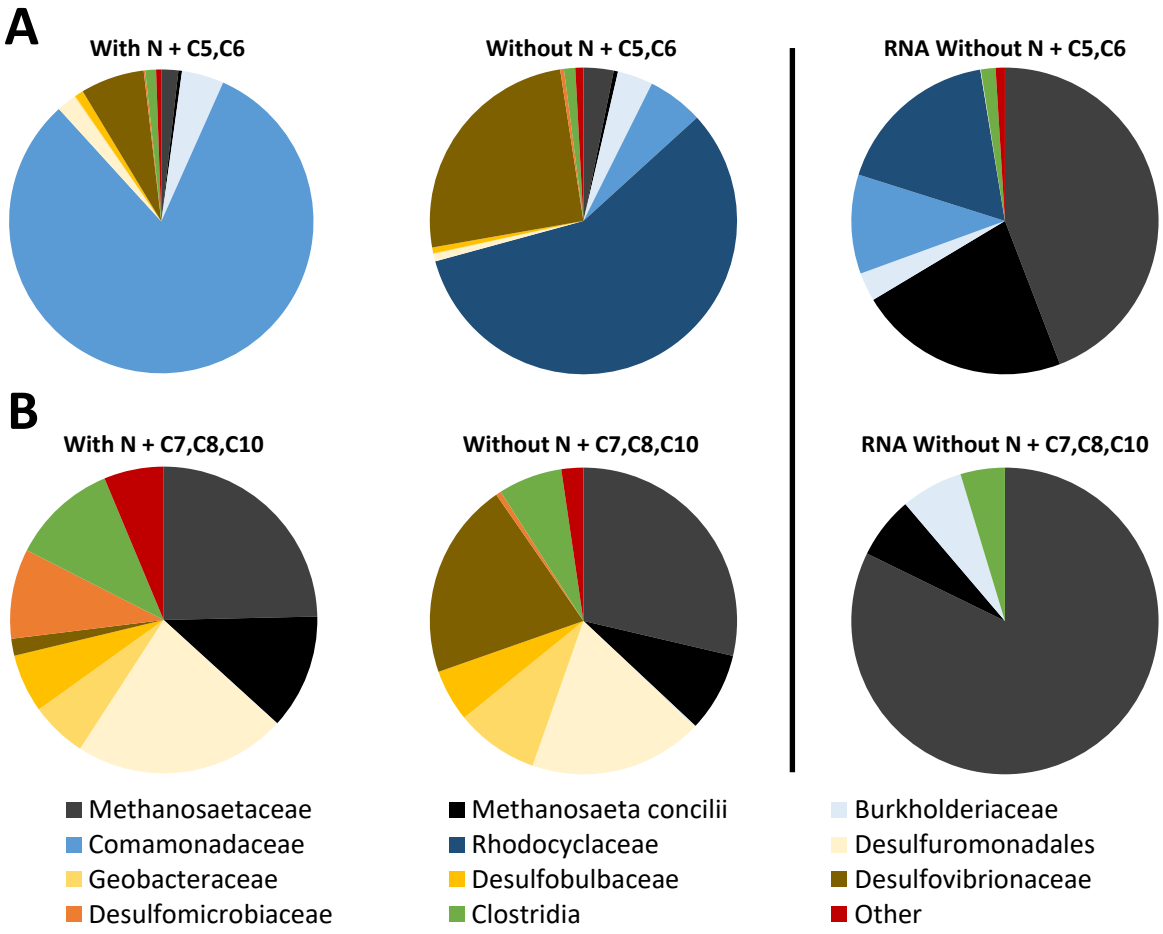

Figure 2-8: *NifH* gene abundance in *n*-alkane amended cultures based on Illumina sequencing and BLASTx match of *nifH* gene at day 334 from (**A**) Albian cultures, and (**B**) CNRL cultures. "RNA" indicates sequenced cDNA reverse transcribed from extracted RNA. Reverse transcribed RNA sequences that returned fewer than 50 reads were discarded, OTUs with no match or off-target match were discarded. The presence and expression of nifH and genes coding for other anaerobic N transformations from all live treatments are shown in **Figures S 7 – 10**.

2.3.4.2 Toluene experimental cultures

Toluene degradation was observed in amended CNRL cultures without N, these data were supported by the sequenced 16S rRNA gene community composition data (**Figure 2-9**). Unlike *n*-alkane amended CNRL cultures and toluene amended cultures with N, amended cultures without N contained a population of *Clostridiaceae* (*Youngiibacter*). These bacteria are known to play a role in the anaerobic fermentation of toluene in oil sands tailings cultures (Abu Laban et al., 2015a). While *Syntrophaceae* and *Spirochaetales* likely played a role in toluene degradation, enrichment of *Youngiibacter* in the only culture treatment to

exhibit toluene degradation suggests these *Clostridiales* were the primary toluene degraders in amended cultures without N.

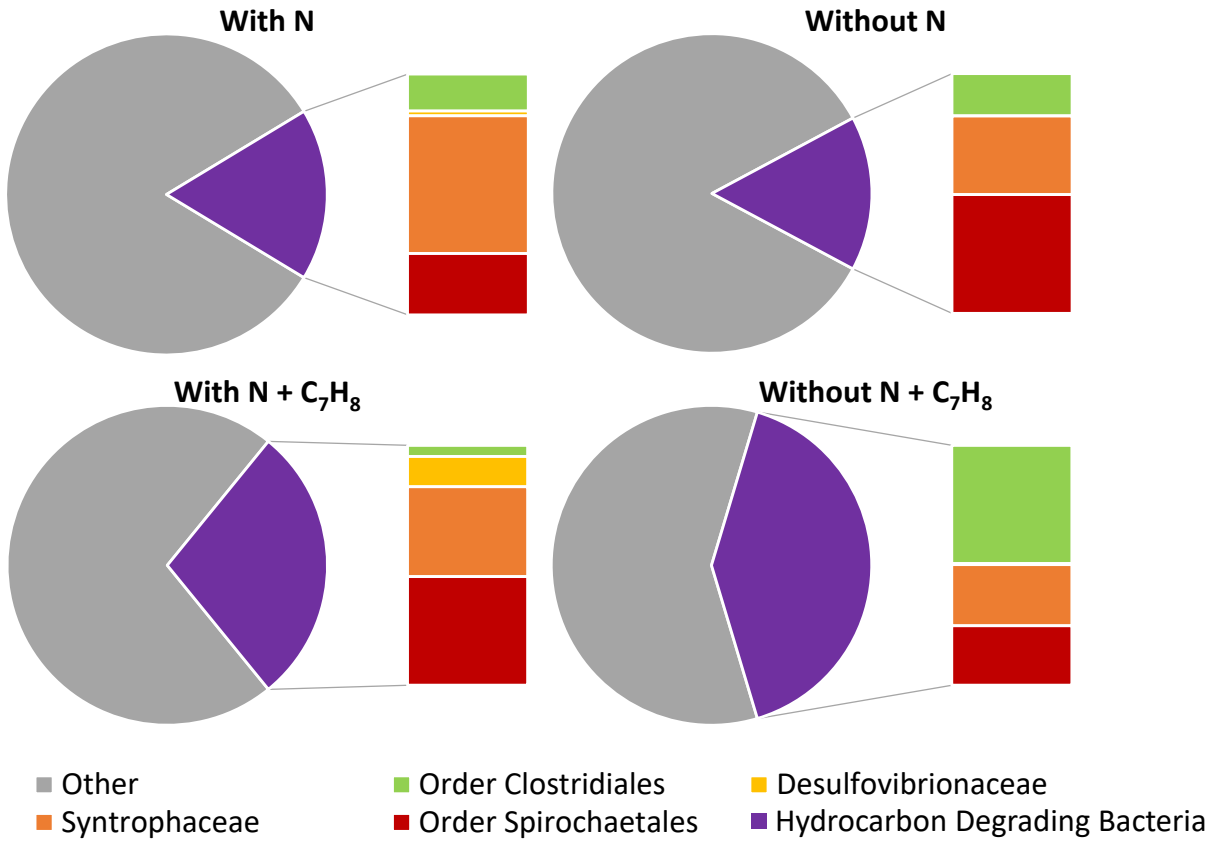

Figure 2-9: Bacterial community composition of hydrocarbon degrading bacteria in toluene amended and baseline CNRL cultures based on Illumina sequencing of the 16S rRNA gene at day 189. Bacteria not known to degrade hydrocarbons and sequences with less than 2% abundance were grouped as "Other". Full Bacteria dataset in **Figure S 11A**.

Methane production was observed in both amended CNRL cultures with and without N, this activity was due to acetoclastic methanogenesis by as supported by 16S rRNA gene data (**Figure 2-10**). Amended cultures both produced significantly more methane than unamended cultures and contained a significantly higher proportion of methanogenic archaea, however significant hydrocarbon degradation was only observed in amended cultures without N. This suggests that hydrocarbon degradation was occurring in both cultures to support acetoclastic methanogenesis and the microbial community but was below detection in amended CNRL cultures with N.

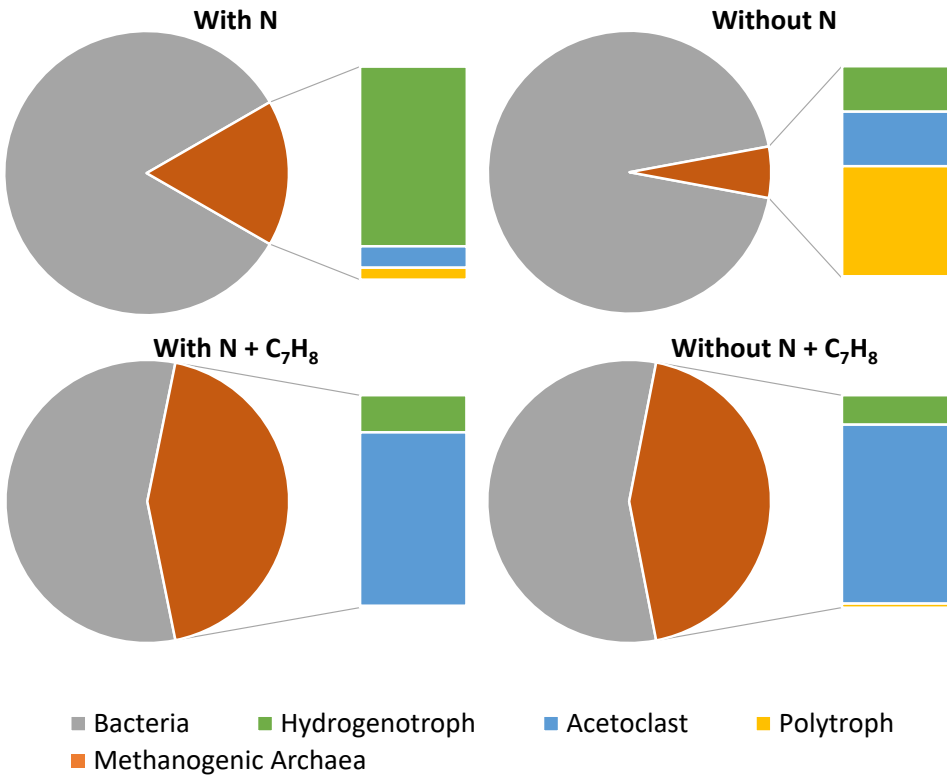

Figure 2-10: Distribution of Bacteria and methanogens in toluene amended and baseline CNRL cultures based on Illumina sequencing of the 16S rRNA gene at day 189. Polytroph indicates methanogens capable of metabolizing hydrogen, acetate, and methanol to methane. Full Archaea dataset in **Figure S 11B**.

In both *n*-alkane and toluene amended cultures, methane and hydrocarbon results indicated higher microbial metabolism in culture media without N as compared to media with inorganic N. This is unexpected as the results of our previous study using citrate (Collins et al., 2016) indicated a higher rate of methanogenesis in cultures supplemented with inorganic N as compared to cultures without N in the culture media until week 4, thereafter all treatments produced methane at the same rate. We concluded this was due to an increase in the N_2-fixing community, which subsequently provided enough available N for the citrate fermenters and methanogens. The primary difference in the culture composition in this study is lower solids content. In Collins et al., (2016), the cultures contained 50% MFT and, based on ~25% dry weight, the cultures had a final solids content of 12.5%. To reduce the concentration of bioavailable N in current cultures, only 5 ml of Albian transfer cultures, and 5 ml of Syncrude and CNRL primary enrichment cultures were used. This resulted in a final solids content of ~0.35% in Albian cultures, and ~1.3% in Syncrude and CNRL cultures. It is well known that microorganism preferentially grow on surfaces (Dunne, 2002), and aggregates offer protection against environmental factors (Gupta and Roper, 2010). Therefore, the increased microbial activity observed in hydrocarbon amended cultures without N could be due to the presence of exopolysaccharide biopolymer forming bacteria creating an artificial growth

matrix. This matrix may have helped promote the growth the microbial community including hydrocarbon degrading bacteria and methanogenic archaea. These biofilm producing prokaryotes may be capable of N_2-fixation as these effects are only seen in cultures deficient in inorganic N. Several species of N_2-fixing bacteria including *Clostridia* (Zoutberg et al., 1989), are known to create aggregates under N-depleted conditions in the presence of labile carbon regardless of whether or not they are actively fixing N_2 (Wang et al., 2017). These bacteria are then released from the biofilm in the presence of bioavailable N. Wang et al. (2017) suggested the formation of bacterial aggregates, and general excretion of exopolysaccharides may be common among N_2-fixing bacteria under N-deficient conditions. Therefore, bacteria such as *Clostridia*, *Desulfovibrionaceae* and *Syntrophaceae*, are likely also contributing to aggregate and growth matrix formation in N-deficient cultures. It is likely the formation of this growth matrix by N_2-fixing bacteria led to higher metabolic activity (methane production and hydrocarbon degradation) in amended without N cultures across all datasets, though we did not investigate this mechanism (aggregation and microbial support).

The microbial community (16s rRNA) was evaluated to assess the proportion of Bacteria and Archaea capable of N_2-fixation based on *nifH* gene detection in toluene amended CNRL cultures (**Figure 2-11**). As observed in *n*-alkane communities, *Clostridiales* increased in amended cultures without N as compared to toluene amended cultures with N whereas the proportion of *Methanosaeta* remained unchanged between the two treatments.

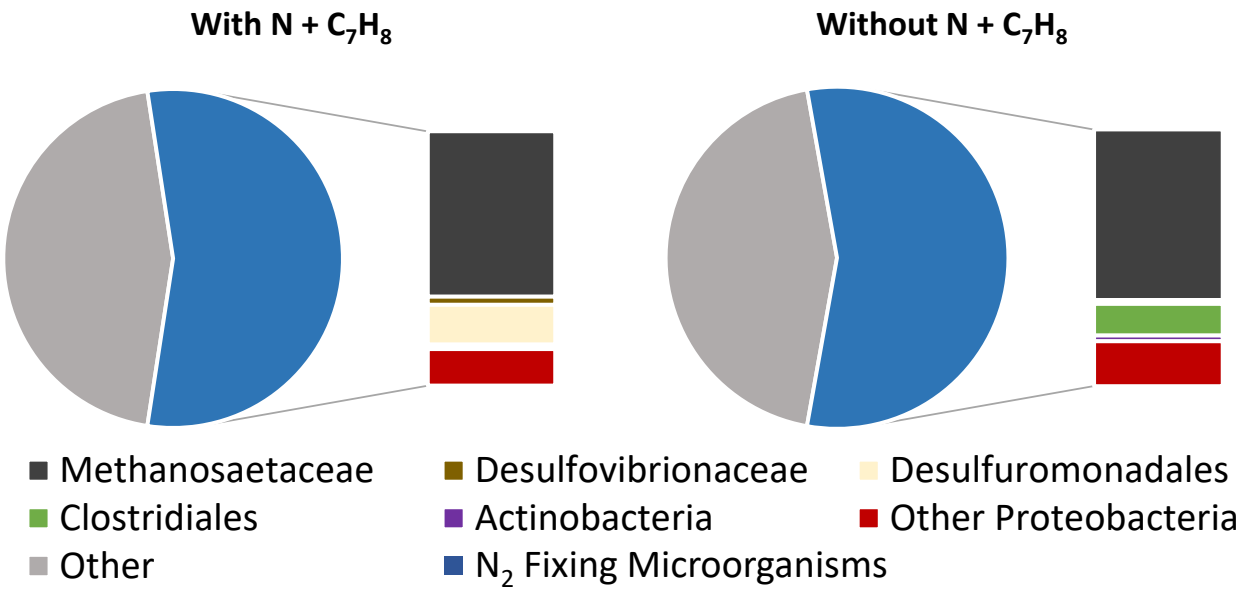

Figure 2-11: Microbial community composition of potential N_2-fixing microorganisms from toluene amended CNRL cultures at day 189 using Illumina sequencing of the 16S rRNA gene. Prokaryotes where *nifH* genes were not detected were grouped as "Other".

Nitrogen-fixation in CNRL cultures without N was supported by the presence of *nifH* gene transcription in these cultures (**Figure 2-12**). Similar to *n*-alkane cultures, *nifH* expression was not detected in amended CNRL cultures with N. Gene expression from toluene amended CNRL cultures without N were also dominated by the family *Methanosaetaceae* but also contained a large proportion of the order *Desulfovibrionales* indicating that these microbial groups were primarily responsible for N_2-fixation in this treatment. This is interesting as *Desulfovibrionales* was present in amended CNRL and Albian *n*-alkane cultures without N but not detected in the RNA samples. These data suggest a higher frequency of *nifH* expression in *Desulfovibrionales* when amended with toluene as compared to the *n*-alkanes examined in this study.

While the effects of different hydrocarbons on the *nifH* expression is not documented in the literature, a study by Sun et al. (2012) found the N_2-fixing community changed when amended with different polycyclic aromatic hydrocarbons (PAHs). In this study, the *nifH* containing population was composed of basically the same groups whether amended with *n*-alkanes or toluene, only expression seems to have been affected by this change. The effect of hydrocarbon contamination on gene expression could have widespread implication and merits further study.

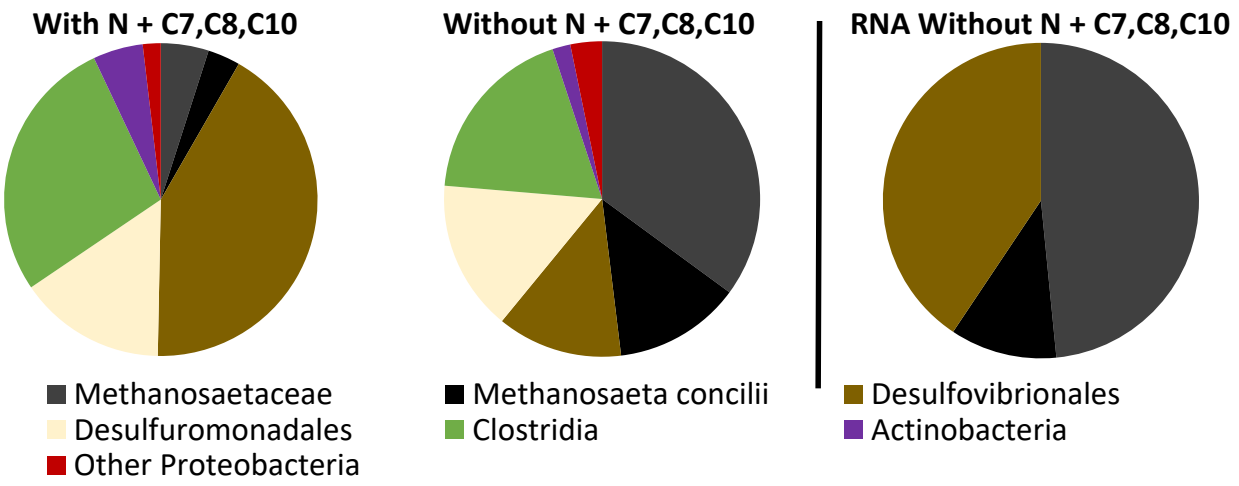

Figure 2-12: *NifH* gene abundance at day 189 of toluene amended CNRL cultures based on Illumina sequencing and BLASTx match of *nifH* gene. "RNA" indicates sequenced cDNA reverse transcribed from extracted RNA. Reverse transcribed RNA sequences that returned fewer than 50 reads were discarded, OTUs with no match or off-target match were discarded. No RNA transcription was detected in cultures with N. The presence and expression of *nifH* and genes coding for other anaerobic N transformations from all live treatments are shown in **Figures S 12 – 15**.

2.4 Conclusions

The results of this study suggest methanogenic activity and hydrocarbon degradation in oil sands tailings may be supported by N_2-fixation in the absence of fixed N. This was evidenced by the presence of nitrogenase activity and transcription of the *nifH* gene in conjunction with the metabolism of both *n*-alkanes and the model aromatic hydrocarbon toluene, under methanogenic conditions. This research built on our previous study, which suggested N_2-fixation may support methanogenic citrate metabolism under N deficient conditions. However, citrate is more labile and less abundant in oil sands tailings ponds than hydrocarbons from naphtha and paraffinic solvent. Therefore, further investigation was needed to determine if N_2-fixation by the tailing's community could support the methanogenic metabolism of hydrocarbons. Continued methanogenesis in the absence of fixed N is relevant to industry as it was generally assumed that methanogenesis would cease in the absence of bioavailable nutrients. Continued microbial metabolism in reclaimed wet landscapes that incorporate tailings material may result in mineral dissolution and methane production, which could create channels releasing residual bitumen and other contaminants to the cap waters where plants and animals may be affected. The results of this study may inform fugitive methane emissions modelling and inform reclamation planning.

2.5 References

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3. Reducing Methane Emissions in Oil Sands Tailings Using Amorphous Iron Concurrent with Hydrocarbon Degradation Under Nitrogen Limited Conditions

3.1 Introduction

The Alberta Oil Sands region contain the third largest oil reserves in the world and contain an estimated 166 billion barrels of extractable oil (Canadian Association of Petroleum Producers, 2016). Bitumen ores are recovered from open pit mining and processed using a modified Clark Hot Water Process. In this process, ores are treated with water, steam, mechanical processing, caustics, and solvents to separate and recover bitumen form the mineral matrix (Kasperski and Mikula, 2011; Mikula et al., 1996). Following solvent recovery, the remaining slurry is generally combined with consolidating agents and pumped into setting basins known as tailings ponds (Masliyah et al., 2004). These tailings, consisting primarily of sand, silt, clay, and residual solvent and bitumen, are left to settle while the density of the material slowly increases (Allen, 2008).

Methane production from oil sands tailings ponds began in the early 1990s from Syncrude's Mildred Lake Settling basin (MLSB). Tailings ponds belonging to Suncor, CNRL, and CNUL (formerly Shell Albian) have all since become a major source of biogenic methane. Field measurements from 2016 reported that three tailings ponds with a combined tailings surface area of 19.7 km^2 (osip.alberta.ca) produced 66.75 kt CH_4 (Kong et al., 2019). If all ponds were equally methanogenic, tailings ponds could theoretically release 956 tCH_4/day based on water surface area data from 2016 (103 km^2; osip.alberta.ca). Canada released 4,300 kt CH_4 in 2014 (Environment Canada, 2014) meaning biogenic methane emissions from tailings ponds could account for 8% of emissions in Canada. With a current atmosphere concentration of 1847.9 ppb (Dlugokencky and NOAA/ESRL, 2017), methane has become a GHG of global concern. Current government mandates are aiming for a 45% decrease in methane emissions below 2012 levels by 2025 (Environment and Climate Change Canada, 2018). Therefore, decreasing methane production from the oil sands tailings ponds will be important in reaching emission reduction targets.

The origin of methane production in tailings is biogenic. The highly stratified layers of the tailings ponds are dominated by diverse communities of both facultative and obligate anaerobes capable of reducing nitrate, sulfur, iron, and CO_2 to metabolize organic and inorganic ligands (Foght et al., 2017; Holowenko et al., 2000; Penner and Foght, 2010; Salloum et al., 2002). Mature fine tailings (MFT) are found in the deeper layers 5-15 m below the water-tailings interface. Most terminal electron acceptors have been

depleted at this depth and methanogens dominate the Archaeal population. This results in substantial methane production, however the addition of more energetically favourable electron acceptors can inhibit methane production. N-oxides (NO_2^-/NO_3^-, NO, N_2O) (Klüber and Conrad, 1998), sulfate (Ma et al., 2017), and amorphous Fe(III) (Van Bodegom et al., 2004), have been found to suppress or entirely inhibit methane production. In theory, one or more of these compounds could be used to treat methanogenic "hot spots" where the rate of methane release is higher in tailings ponds to reduce overall emissions however, the intermediate and end products of these biological reactions would need to be inert, which is not the case for many terminal electron acceptors. Reduction of NO_2^-/NO_3^- results in the production of nitrous oxide (N_2O), a greenhouse gas 298 times more potent than CO_2 (climatechangeconnection.org), whereas sulfate reduction results in the formation of the toxic gas, H_2S. Of these options, trivalent metal ions such as amorphous Fe(III) are an obvious option as the reduction product of amorphous Fe(III) is Fe(II). Fe(III) should also be inexpensive in the form of the waste product known as rust, which is safe to transport. However, hydrocarbon degradation is still desirable to remediate hydrocarbon content in waste tailings.

For microbial metabolism to occur, N and other trace nutrients must be present. Mature fine tailings contain few nutrients and are N deficient in some ponds (Collins et al., 2016; Penner and Foght, 2010), and yet methanogenic metabolism continues to be observed *in-situ*. Our previous study (Collins et al., 2016), found that the methanogenic community might be supported by N fixation under methanogenic conditions. However, electron acceptor availability will shift the population dynamics within the microbial community. The objective of this current study was to determine if the addition of amorphous Fe(III) could inhibit methanogenesis while maintaining hydrocarbon degrading activity under N depleted conditions.

3.2 Methods and Materials

3.2.1 Chemicals, gasses, and materials

The following hydrocarbons were purchased from Fisher Scientific (Ontario, Canada); *n*-pentane, *n*-octane, *n*-heptane, toluene (>99% purity) and *n*-hexane (>96% purity). *n*-decane (>99% purity) was purchased from Sigma-Aldrich (Ontario, Canada). All chemicals were analytical reagent grade or higher and purchased from Fisher Scientific. All gasses including 70% N_2/30% CO_2 and acetylene (scientific grade) were supplied by Praxair. Amorphous iron oxide minerals ($Fe(OH)_3$) were made using an 11% wt/vol solution of $FeCl_3$ in deionized H_2O adjusted to pH ~7.1 using NaOH. Solids precipitated and settled overnight. Solids were then washed 5 times with deionized water by centrifuging and removing the

supernatant. Solids were then resuspended, sealed in a serum bottle under anaerobic conditions and autoclaved.

CNRL and Syncrude MFT sourced for primary enrichment cultures were collected by site operators using a wireline fluid sampler in July 2013 from ETF at a depth of 16 M and MLSB at a depth of 20.75 M, respectively. Tailings were sealed without a headspace to maintain anaerobic conditions at 4˚C for four months before use. Albian MFT was described previously (Collins et al., 2016; Mohamad Shahimin, 2016).

3.2.2 Primary enrichment cultures from Syncrude and CNRL tailings

As previously described, primary enrichment cultures (**2.2.2 Primary enrichment cultures**) were setup to enrich for toluene and octane degrading bacteria from Syncrude and CNRL tailings under methanogenic conditions to study methanogenic hydrocarbon degradation in the absence of fixed N. The experiment was unsuccessful due to the abundance of fixed N endogenous to the tailings materials and cultures were salvaged for use as inoculums in the current study (**Table S 1**). These cultures were established in 158 ml serum bottles using 50 ml MFT and 50 ml methanogenic media either without added N (without N) or containing NH_4^+ (with N; Collins et al., 2016). Blue butyl stoppers and foil crimp caps were used to seal serum bottles under an N_2/CO_2 headspace as described previously (Collins et al., 2016). Cultures were incubated in the dark for two weeks prior to hydrocarbon amendment at day 0. Syncrude and CNRL MFT were amended with 200 mg L^{-1} toluene or octane and incubated for 140 days with periodic methane monitoring (**Figure S 1**, **Figure S 2**). All primary enrichment cultures and subsequent culture sets were established in triplicate if live and amended with a carbon source, and in duplicate if sterile or unamended. Albian primary enrichment cultures were described previously (Collins et al., 2016).

3.2.3 Transfer cultures from Albian methanogenic enrichments

Previously described Albian cultures (Collins et al., 2016; 5 ml) were transferred into 25 ml fresh media and sealed with butyl stoppers and crimp caps in 60 ml bottles. Cultures were amended with *n*-hexanes, a component of Albian's C_5,C_6 paraffinic solvent, and incubated for 55 days in an attempt to study hydrocarbon degradation in the absence of fixed N under methanogenic conditions. Methanogenesis was not observed during this time suggesting the microbial population was incapable of hydrocarbon metabolism (data not shown). Albian transfer cultures were inoculated with *n*-hexane/*n*-pentane degrading cultures enriched from the same Albian MFT retrieved in 2008 as previously described (Mohamad Shahimin, 2016). The liquid phase was removed from 3 ml culture volume under anaerobic conditions (**2.2.3 Transfer cultures** from Albian enrichments) and resuspended in N deficient methanogenic media before addition to Albian transfer cultures used in this study. Following 4 months of

incubation, methane production was detected in amended cultures and cultures were subsequently used as inoculum in the current study.

3.2.4 n-Alkane cultures under iron reducing conditions

Under anaerobic conditions, culture inoculum (2 ml) from Syncrude and CNRL *n*-alkane amended primary enrichment cultures or Albian transfer cultures were centrifuged to remove the aqueous phase containing residual fixed N from primary cultures, and resuspended in 2 ml methanogenic media (**2.2.2 Primary enrichment cultures**). The resuspended volume was then used as culture inoculum in *n*-alkane cultures. Amorphous $Fe(OH)_3$ in solution (3 ml, 0.05 g; generated as described in Chemicals, gases, and materials) was added to all cultures to a final concentration of 0.053% wt/vol. Cultures were incubated in the dark for two weeks before hydrocarbon amendments at day 0. Syncrude and CNRL amendments consisted of a 1:1:1 by volume mixture of *n*-heptane, *n*-octane, and *n*-decane (final concentrations 114, 117, and 122 mg L^{-1} respectively). Albian amendments were prepared in a 1:1 ratio by volume of hydrocarbon solution for *n*-pentane and *n*-hexane (final concentrations 157 and 165 mg L^{-1} respectively). Samples (5 ml) were taken at day 0 and periodically throughout the incubation for ferrozine and DNA and mRNA extraction and analysis, and at day 518 for hydrocarbon analysis. Two samples for ferrozine, and samples for DNA and mRNA analyses were stored at -20˚C. An additional 3 ml of $Fe(OH)_3$ was added at day 149 following ferrozine analyses.

3.2.5 Toluene cultures under iron reducing conditions

At day 316, 20 ml from each *n*-alkane culture were transferred to sealed, sterile, anaerobic 60 ml serum bottles using a needle and syringe. Cultures were flushed for 10 minutes with N_2/CO_2 to volatilize any remaining *n*-alkanes and were then amended with 200 mg L^{-1} toluene. Cultures were incubated at room temperature but were moved to an incubator set to 30˚C at day 80 to promote microbial metabolism. Cultures were returned to room temperature at day 140. Day 0 hydrocarbon samples (0.5 ml) taken after 24 hours of culture amendments were preserved with 1 ml methanol in a 2 ml autosampler vial (Agilent Technologies, #51855820), and filled to the brink with methanol such that no headspace remained. Vial threads were wrapped 3 times with Teflon tape to ensure vials were gas-tight. These samples were stored at 4˚C and analyzed alongside samples taken at day 189. Sterile matrix standards for quantification where prepared alongside cultures and were stored at the same time for later analysis.

3.2.6 Ferrozine analysis

Ferrozine iron (II) analysis was conducted on samples taken from *n*-alkane culture sets at day 0, 149, 250, 330, and 565, however data points 250 and 330 were from frozen samples and provided inconsistent data due to delays in analysis. Day 0 values were subtracted from later timepoints to show the changes in Fe(II) concentrations over time. The toluene culture set was analyzed at time point 0 and 188. A 0.1% wt/vol solution of ferrozine in 0.05 M HEPES at pH 7 solution (1.67 ml) was added to 0.333 ml culture sample in a 2 ml microfuge tube. Tubes were vortexed for 15 s and centrifuged on maximum (21,130 x g) for 3 min to remove fine particulates. Sample absorbance was measured on a spectrophotometer at 562 nm. Standards were created by preparing a 100 mg L^{-1} stock solution of $Fe(NH_4)_2(SO_4)_2*6H_2O$ in deionized water and acidified by adding 1 ml L^{-1} of 6 M HCl. Standard solutions were prepared by diluting the stock solution.

3.2.7 Acetylene reduction in iron reducing cultures

In the *n*-alkane culture experimental set, acetylene reduction cultures were established at day 316 by transferring 20 ml of all amended cultures including sterile cultures to sealed, sterile, and anaerobic 60 ml serum bottles. Culture headspaces were flushed for 5 min with N_2/CO_2 and amended with 200 mg L^{-1} hydrocarbons to replace the volatilized hydrocarbons lost during flushing. The acetylene reduction assay for the toluene experiment culture set was prepared by adding acetylene directly to cultures at day 190. Positive controls run alongside *n*-alkane cultures were setup as previously described (**2.2.6 Acetylene reduction cultures**; Collins et al., 2016). Acetylene was added at 3% of the headspace volume (99.2 µmol) via syringe. The culture sets for *n*-alkane and toluene were incubated with periodic monitoring for 27 and 48 days, respectively.

3.2.8 Chemical analysis

Methane and pressure measurements were taken periodically as previously described to determine the µmoles of methane produced over the course of the incubation (**2.2.7 Chemical analysis**; Collins et al., 2016). Culture headspace pressure was assessed using a digital pressure gauge (DPG1000B ± 15.00PSIG-5, MOD-TRONIC Instruments Limited, Brampton, ON) with a luer-lok fitting and needle. Methane concentrations were determined by direct injection of headspace gas (0.1 ml) into a gas chromatographer equipped with a flame ionization detector (GC-FID; Trace 1300 GC-FID, ThermoFisher, column: TG-Bond (30mx0.32mm), oven temperature 40°C, and helium flow 3 ml min^{-1}), and quantified using external standards (0.16%, 1%, 4%, 8%, and 15% methane). Theoretical maximum methane production was calculated using the Symons and Buswell equation (Roberts, 2002). Ethylene produced from acetylene

reduction was also measured with GC-FID and quantified using external standards (0.00316%, 0.0158%, 0.0379%, 0.0569%, and 0.0758% ethylene).

Hydrocarbons were determined in both the culture headspace gas and liquid culture volume. The headspace from the Syncrude and CNRL *n*-alkane experimental sets was analyzed via direct injection of 0.1 ml culture headspace into a GC-MS (TRACE 1300 gas column: TraceGold TG-5MS, 30 m by 0.25 mm internal diameter, flow rate 1.2 mL min^{-1}; Thermo Scientific) as previously described (**2.2.7 Chemical analysis**). Headspace gas from both Albian *n*-alkane cultures and all toluene cultures were analyzed using a CTC PAL autosampler by injecting 0.5 ml in a 20 ml headspace vial. Hydrocarbons were quantified using external standards except for Albian *n*-alkane cultures where 1,1,3-trimethylcyclohexane was added as an internal standard to ensure consistent injections in autosampler vials. External standards (matrix standards) for hydrocarbons were prepared similarly to cultures containing 92 ml water and 3 ml tailings. The standard curve consisted of 5 standards ranging from 15 mg L^{-1} to 350 mg L^{-1} for hexane and pentane, 17 mg L^{-1} to 400 mg L^{-1} for heptane, octane, and decane, and 17 mg L^{-1} to 215 mg L^{-1} for toluene.

Hydrocarbons from the liquid phase were prepared for analysis by extracting 0.5 ml culture volume with 1 ml methanol. Samples were vortexed for 10 seconds and centrifuged at max (21,130 RCF) for 3 min to remove solids. Supernatant (1 ml) was transferred to a 44 mL EPA glass vial containing MilliQ H_2O and filled with MilliQ H_2O such that no headspace remained once sealed. External standards were prepared using the same standards prepared at day 0. Liquid phase hydrocarbons were analyzed on a GC-FID (Hewlett-Packard 6890) equipped with a purge and trap system.

Total carbon analyses (Non-purgeable organic carbon; NPOC, total inorganic carbon; TIC, total organic carbon; TOC) were performed by the Natural Resources Analytical Laboratory (NRAL) at the University of Alberta. Liquid fraction NPOC and TIC were analyzed using the Persulfate Oxidation Method on an OI Aurora 1030W, and liquid TOC was analyzed using a Shimadzu TOC-V/TN instrument (Shimadzu Corporation, 2001; Williams, 2000). Solid phase TOC was determined using a Costech Elemental Analyzer (Sparks, 1996).

To correct for varying culture and headspace volume between *n*-alkane treatments at the time of analysis, hydrocarbon concentration data were normalized using the equation previously described (**2.2.8 Hydrocarbon normalization**).

Unless otherwise noted, statistical significance was determined in Excel using a two tailed, paired T-Test.

3.2.9 DNA/RNA sequencing and analysis

DNA and RNA extractions were performed on samples taken at day 334 from the Albian *n*-alkane experiment cultures, and day 189 from Albian, CNRL, and Syncrude cultures from the toluene experimental sets. Extractions were executed in duplicate for each culture replicate using beadbeating. RNA purification, and cDNA generation were prepared using methods previously described (**2.2.9 DNA/RNA sequencing and analysis**). PCR amplification for 16S rRNA and functional genes *nifH, nosZ, nirS*, and *nrfA* were amplified using custom primers containing Illumina forward or reverse overhangs (Illumina, 2013). Primers and PCR conditions were as outlined in **Table 2-1**. Reactions were prepared in duplicate and pooled, reaction contents were as described (**2.2.9 DNA/RNA sequencing and analysis**). Amplicon samples were sequenced using MiSeq Illumina sequencing (TAGC, University of Alberta). Following library preparation for each gene, samples were pooled by treatment for sequencing. The concentration for 16S rRNA amplicons were normalized across all samples.

Raw Illumina data was processed using MetaAmp 2.0 (Dong et al., 2017) using default MetaAmp workflow, paired end analyses, a similarity cutoff of 0.97, and a maximum expected error of 1. Specific settings for each gene were as previously described (**2.2.9 DNA/RNA sequencing and analysis**). The 16S rRNA gene was identified using the SILVA 132 database, functional genes were processed using BLASTx and MegaBLAST (blast.ncbi.nlm.nih.gov/), excluding uncultured/environmental sample sequences. All functional gene sequences were manually curated to remove non-target sequences, and all treatments that returned fewer than 30 reads were discarded (**Table S 4**).

3.3 Results and Discussion

3.3.1 Inhibition of methanogenesis

3.3.1.1 n-alkane experimental cultures

Methane concentrations were monitored in Albian, Syncrude, and CNRL cultures from the *n*-alkane experimental setup for 499 days (**Figure 3-1**). All treatments with and without N, amended and unamended, live and sterile from Albian, Syncrude, and CNRL cultures produced very little methane (< 5 µmol) when compared to methanogenic cultures established in parallel under similar conditions as detailed in **Chapter 2**, which produced as much as 572 µmol methane. This was due to the change in redox conditions, which promoted the more energetically favourable iron reducing activity over the less energy efficient methanogenic metabolism through direct or indirect inhibition of the methanogenic community (Chidthaisong and Conrad, 2000; Lovley and Phillips, 1986; Roden and Wetzel, 1996; Van Bodegom et al., 2004).

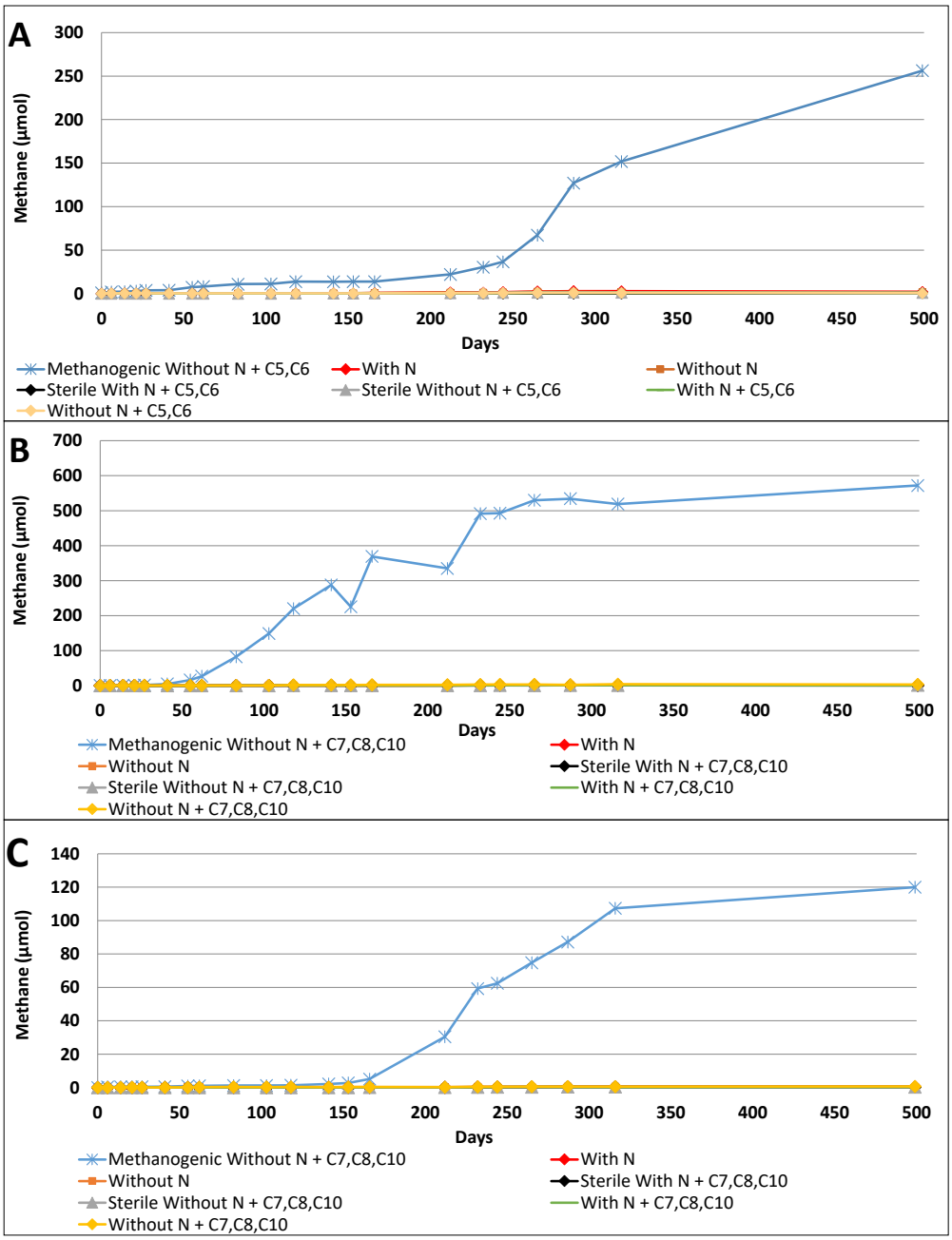

Figure 3-1: Inhibition of methanogenesis from the addition of amorphous Fe(III) in n-alkane amended and baseline cultures (**A**) Albian, (**B**) Syncrude, and (**C**) CNRL. All treatments received $Fe(OH)_3$ except for the blue dataset. The blue dataset is displayed for comparison and represents methane production in equivalent methanogenic cultures without $Fe(OH)_3$ as described in **Chapter 2**. Values represent the mean µmol for triplicate amended cultures and duplicate sterile and baseline cultures. Error bars indicate ± standard deviation.

3.3.1.2 Toluene experimental cultures

Methane production under iron reducing conditions in toluene amended cultures was similar to *n*-alkane cultures (**Figure 3-2**). Methane yield did not exceed 3.5 µmol under any treatment and methane production was negligible as compared to the methane produced under methanogenic conditions, which

could theoretically result in a maximum methane yield of 176 µmol (Symons and Buswell, 1933) in toluene amended cultures. Cultures established in parallel under methanogenic conditions (**Chapter 2**) produced as much as 82 µmol methane after 185 days of incubation.

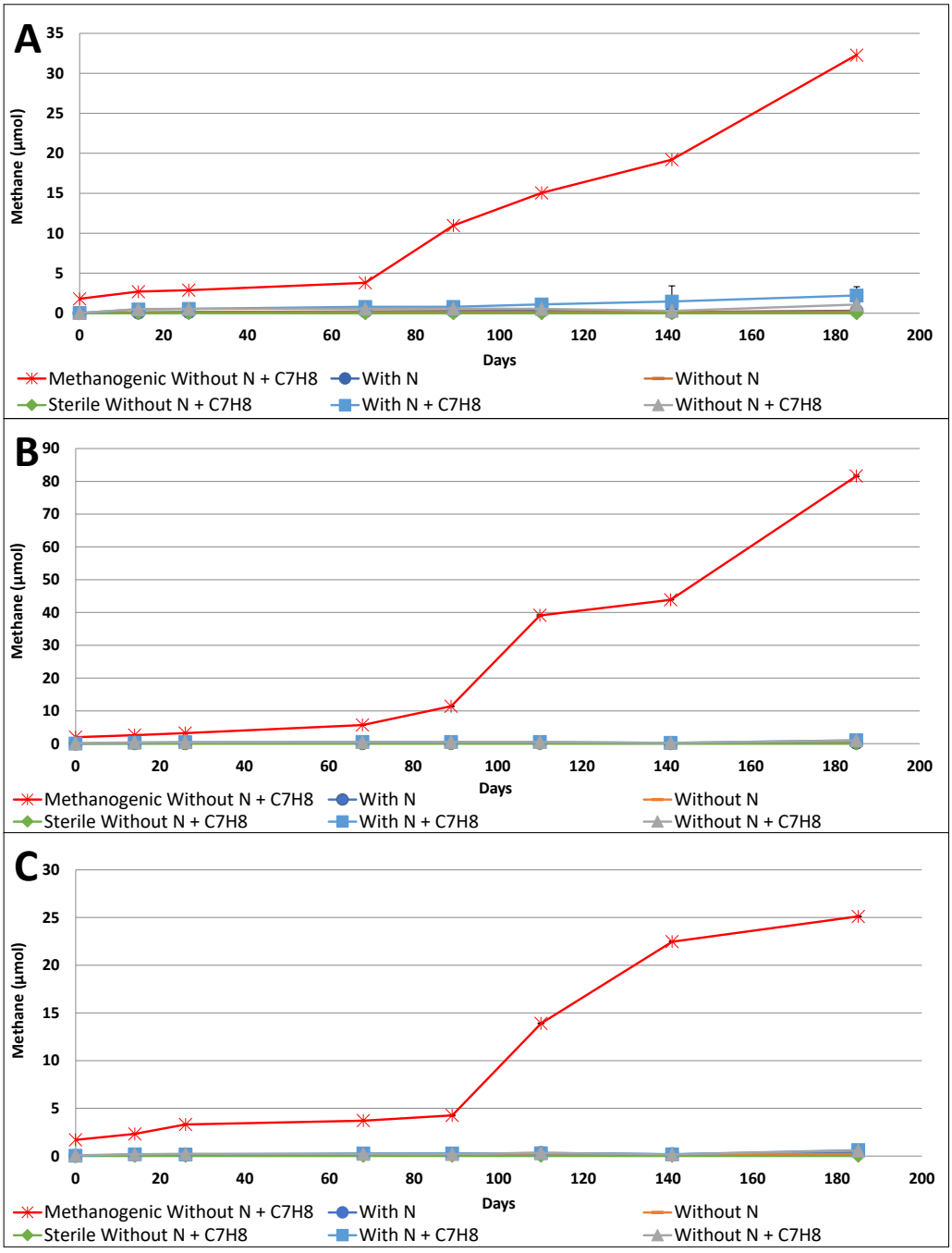

Figure 3-2: Inhibition of methanogenesis from the addition of amorphous Fe(III) in toluene amended and baseline cultures (**A**) Albian, (**B**) Syncrude, and (**C**) CNRL. All treatments received $Fe(OH)_3$ except for the red dataset. The red dataset is displayed for comparison and represents methane production in equivalent methanogenic cultures without $Fe(OH)_3$ as described in **Chapter 2**. Values represent the mean µmol for triplicate amended cultures and duplicate sterile and baseline cultures. Error bars indicate ± standard deviation.

3.3.2 Hydrocarbon degradation under Fe(III) reducing conditions

3.3.2.1 n-alkane experimental cultures

Hydrocarbon concentration was determined in *n*-alkane amended cultures at day 518 (**Table 3-1**). There was no significant decrease in *n*-alkane concentration in living cultures as compared to sterile cultures by day 518. The persistence of hydrocarbons in these cultures as compared to the hydrocarbon degradation observed in methanogenic *n*-alkane cultures (**2.3.2 Hydrocarbon degradation;** Gieg et al., 2008; Jones et al., 2008; Siddique et al., 2011, 2006; Zengler et al., 1999) is likely due to induced iron reducing conditions as *n*-alkanes are not biodegraded under iron reducing conditions (Mbadinga et al., 2011; Zwolinski et al., 2001).

Table 3-1: Hydrocarbon concentration in *n*-alkane amended cultures with $Fe(OH)_3$. Cultures were prepared in triplicate amended cultures and duplicate sterile cultures.

Treatment	***n*-Pentane**		***n*-Hexane**	
Albian Liquid	mg L^{-1}	StDev	mg L^{-1}	StDev
With N + C_5,C_6	164.87	0.03	44.79	8.80
Without N + C_5,C_6	155. 50	3.60	37.57	5.92
Sterile With N + C_5,C_6	151.95	24.59	32.09	1.92
Sterile Without N + C_5,C_6	87.48	27.63	27.95	1.25
Albian Headspace	mg L^{-1}	StDev	mg L^{-1}	StDev
With N + C_5,C_6	125.41	71.77	116.36	34.25
Without N + C_5,C_6	128.32	74.67	102.84	34.29
Sterile With N + C_5,C_6	123.74	12.51	68.00	4.58
Sterile Without N + C_5,C_6	104.63	9.40	88.02	5.94

Treatment	***n*-Heptane**		***n*-Octane**		***n*-Decane**	
Syncrude	mg L^{-1}	StDev	mg L^{-1}	StDev	mg L^{-1}	StDev
With N + C_7,C_8,C_{10}	128.50	3.53	73.16	2.17	67.73	6.01
Without N + C_7,C_8,C_{10}	105.20	17.92	61.03	1.05	58.83	6.83
Sterile With N + C_7,C_8,C_{10}	107.78	40.59	60.51	25.09	59.14	25.42
Sterile Without N + C_7,C_8,C_{10}	92.44	25.94	56.13	10.03	55.68	3.30
CNRL	mg L^{-1}	StDev	mg L^{-1}	StDev	mg L^{-1}	StDev
With N + C_7,C_8,C_{10}	88.04	23.80	50.63	13.88	53.94	22.43
Without N + C_7,C_8,C_{10}	78.70	17.74	43.05	8.63	52.20	12.16
Sterile With N + C_7,C_8,C_{10}	75.06	27.55	41.55	10.74	52.34	11.36
Sterile Without N + C_7,C_8,C_{10}	67.06	23.88	38.36	15.40	44.99	19.77

Studying methanogenic cultures in **Chapter 2**, we observed significant *n*-alkane degradation in conjunction with methane production in both Albian and Syncrude amended cultures without N. Alkane degradation has been well reported under methanogenic conditions (Gieg et al., 2008; Jones et al., 2008; Mohamad Shahimin et al., 2016; Siddique et al., 2015, 2011, 2006; Zengler et al., 1999). The inhibition of

methanogenesis and *n*-alkane degradation in this experiment were the result of induced iron reducing conditions resulting from the presence of amorphous Fe(III). This is supported by Mbadinga et al. (2011) and Zwolinski et al. (2001) who confirm no studies to date have demonstrated alkane degradation under iron reducing conditions. Inhibition of methanogenesis under iron reducing conditions has previously been attributed to substrate competition between methanogens and iron reducing organisms (Lovley and Phillips, 1986; Roden and Wetzel, 1996), or by direct inhibition through changes in metabolic activity from methanogenesis to iron reduction and Fe(III) interactions with proteins or co-factors on the cell membrane (Van Bodegom et al., 2004). Like Van Bodegom et al. (2004), we have demonstrated the total inhibition of methanogenesis with the addition of amorphous $Fe(OH)_3$. However, Bodegom demonstrated this inhibition in pure culture, whereas this study demonstrates inhibition of methanogenesis using amorphous Fe(III) in a complex culture.

3.3.2.2 Toluene experimental cultures

The liquid phase of the cultures were sampled after 24 hours and preserved in methanol as outlined in the methods section until samples could be analyzed (**Table 3-2**). Headspace hydrocarbon recovery was initially very low due to immediate analysis following toluene amendment to culture volume without allowing sufficient time to reach equilibrium with the headspace gas that was sampled (**Figure S 16**). Toluene recovery varied between Albian, Syncrude, and CNRL culture sets, this variance may be due to sorption of hydrocarbons on bitumen and humic substances present in varying quantities amongst tailings sourced from different operators.

Table 3-2: Toluene concentration in toluene amended cultures with $Fe(OH)_3$. Values represent the mg L^{-1} toluene for triplicate amended cultures and duplicate sterile and baseline cultures.

Treatment	With N + C7H8	StDev	Without N + C7H8	StDev	Sterile Without N + C7H8	StDev
Day 0						
Albian	159.83	27.97	157.60	3.68	171.81	22.79
Syncrude	149.94	27.13	164.59	9.63	157.26	1.14
CNRL	164.37	7.49	155.07	13.02	159.72	1.19
Day 189						
Albian	134.11	50.99	**124.22**	1.88	149.37	6.60
Syncrude	117.26	22.76	116.64	52.58	142.87	6.31
CNRL	149.49	9.85	148.50	21.82	170.92	15.01

In Albian toluene amended liquid cultures, toluene degradation in cultures without N was significant between the two time points, and lower than sterile cultures at 189. This decrease, however, was not substantial considering the labile nature of toluene. No significant difference in average toluene concentrations were observed in liquid samples taken from CNRL or Syncrude cultures, however one

replicate from the amended Syncrude cultures without N (**Culture 2**) did show a substantial decrease in toluene (**Figure 3-3**). This culture replicate appeared to be more active and will be discussed throughout this chapter.

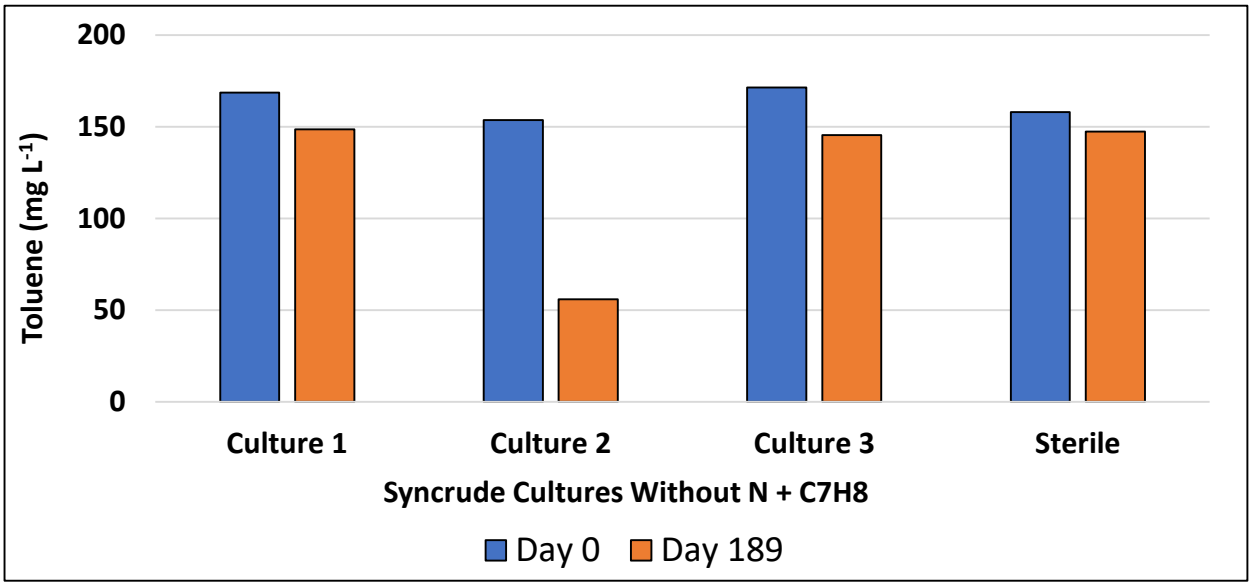

Figure 3-3: Toluene concentrations from individual Syncrude replicates and sterile reference culture under Fe(III) reducing conditions.

Like *n*-alkanes, toluene cultures produced very little methane due to inhibition by Fe(III) (Van Bodegom et al., 2004). However, unlike *n*-alkanes there is some evidence from headspace gas analyses that toluene may have begun to degrade in Albian cultures without N, Syncrude amended cultures, and in CNRL with N cultures. Despite erratic data, degradation is apparent in one Syncrude replicate culture. While *n*-alkane degradation has not been reported under iron reducing conditions, toluene degradation has been well studied under a broad range of conditions including methanogenic, sulfate, iron, manganese, and nitrate reducing conditions (Chakraborty and Coates, 2004 and references therein).

3.3.3 Fe(III) reduction in cultures

3.3.3.1 n-alkane experimental cultures

In **Figure 3-4**, we observed an increase in Fe(II) in living cultures as compared to sterile cultures between day 149 and day 565. The concentration of Fe(II) in amended Albian without N rose significantly from 5.6 ±0.9 mg L^{-1} to 23.2 ±5.5 mg L^{-1}, whereas there was no significant difference in amended Albian cultures with N which rose from 7.6 ±2.2 mg L^{-1} at day 149 to 12.5 ±6.7 mg L^{-1} at day 565. The concentration of Fe(II) also changed in amended Syncrude cultures without N from 7.0 ±1.1 mg L^{-1} to 11.3 ±5.9 mg L^{-1}, however the increase was only significant in amended cultures with N, which rose from 7.9 ±0.1 mg L^{-1} to 24.5 ±2.8 mg L^{-1}. In CNRL cultures, both amended cultures showed a significant increase in Fe(II) with time.

Amended cultures with N increased from 4.9 ±1.3 mg L^{-1} to 22.4 ±0.4 mg L^{-1} and amended cultures without N increased from 6.1 ±1.7 mg L^{-1} to 17.6 ±4.1 mg L^{-1} by day 565. There was no significant differences in the Fe(II) concentrations between timepoints in live, unamended, Albian, Syncrude, or CNRL cultures between the two timepoints.

While no detectable *n*-alkanes degradation occurred, Fe(III) was reduced to Fe(II) in many culture treatments. Sulfur compounds such as Na_2S (added as a reducing agent) could have contributed to Fe(III) reduction. Addition of Na_2S will reduce $Fe(OH)_3$, this is suggested by the following equation: $2Fe(OH)_3 + Na_2S = 2Fe(OH)_2 + 2NaOH + S^0$. This abiotic reaction likely explained the presence of Fe(II) in sterile cultures, however this did not explain the continued increase in Fe(II) over time in living cultures. As cultures were established from previous enrichments, residual metabolites or organics were likely present in small concentrations. This would have resulted in H_2 production as these labile carbon sources were metabolized. The observed increase in Fe(II) concentration in live cultures was therefore likely due to electron transfer from residual organics, and available H_2 coupled to Fe(III) reduction. Several organisms including *Pseudomonas*, *Shewanella,* and *Desulfovibrio* species are all capable of this metabolic activity (Lovley, 1993 and references therein).

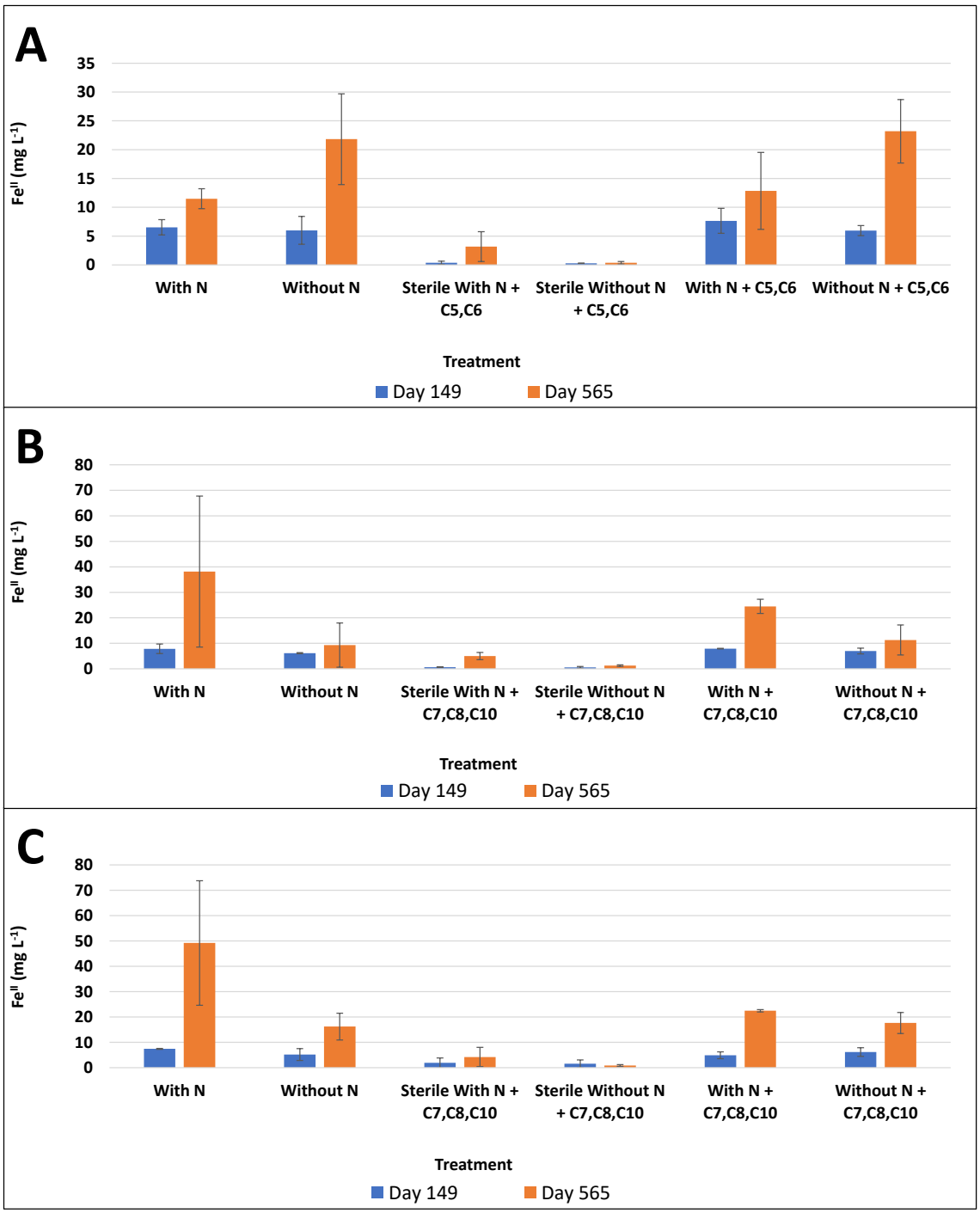

Figure 3-4: Fe(II) production from *n*-alkane amended and baseline cultures (**A**) Albian, (**B**) Syncrude, and (**C**) CNRL. Values represent the mean µmol for triplicate amended cultures and duplicate sterile and baseline cultures. Day 0 values subtracted from day 149 and 565. Error bars indicate ± standard deviation. Measurements from all timepoints in **Figure S 17**.

3.3.3.2 Toluene experimental cultures

Cultures were analyzed for reduced iron (Fe(II)) at day 0 and day 188, and the increase in reduced iron over the course of incubation was determined (**Figure 3-5**). In general, a higher concentration of Fe(II) was

detected in all living cultures as compared to sterile cultures. As discussed by Bazylinski et al. (2013), Fe(III) will oxidize the anaerobic indicator, resazurin, causing it to turn pink under anaerobic conditions. Reduced iron present in sterile cultures is therefore attributed to additional Na_2S added as compared to live cultures, which metabolically reduced Fe(III).

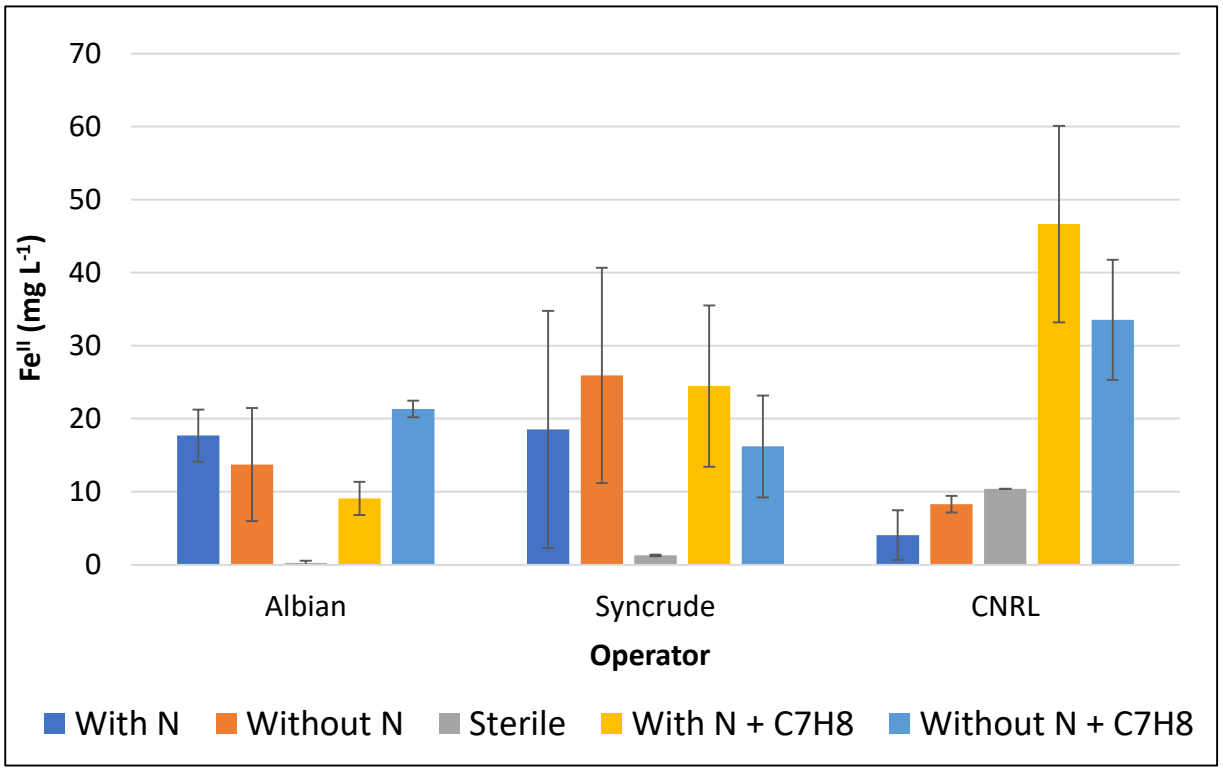

Figure 3-5: Fe(II) production from toluene amended and baseline cultures. Values represent the mean µmol for triplicate amended cultures and duplicate sterile and baseline cultures. Day 0 values subtracted from day 188. Error bars indicate ± standard deviation.

Toluene amended Albian cultures without N produced significantly more Fe(II) than amended cultures with N, however there was no significant difference between amended and unamended cultures. Sterile cultures produced significantly less Fe(II) than living culture treatments. Amended CNRL cultures reduced significantly more Fe(III) to Fe(II) than unamended cultures, however there was no significant difference between live and sterile CNRL cultures. Fe(II) values in CNRL sterile cultures are accounted for by the addition of extra Na_2S. Additional Na_2S was added to these cultures to determine if a larger volume of reducing agent would keep the anaerobic indicator from turning pink before it was known that Fe(III) and not oxygen was causing the colour change, or that Na_2S may affect Fe(II) readings. No other cultures received this volume of reducing agent (500 µl).

No significant difference was observed between Syncrude treatments, however when examined individually, one replicate (Culture 2) from the amended Syncrude cultures without N reduced more Fe(III)

to Fe(II) than the other replicates resulting in a higher standard deviation (**Figure 3-6**). This replicate culture also contained the lowest concentration of toluene at day 189. While Fe(II) concentrations were not tied to hydrocarbon degradation in this study, the relationship between reduced toluene concentrations and increased Fe(II) concentrations in the Syncrude replicate suggest toluene biodegradation under Fe(III) reducing conditions in this culture.

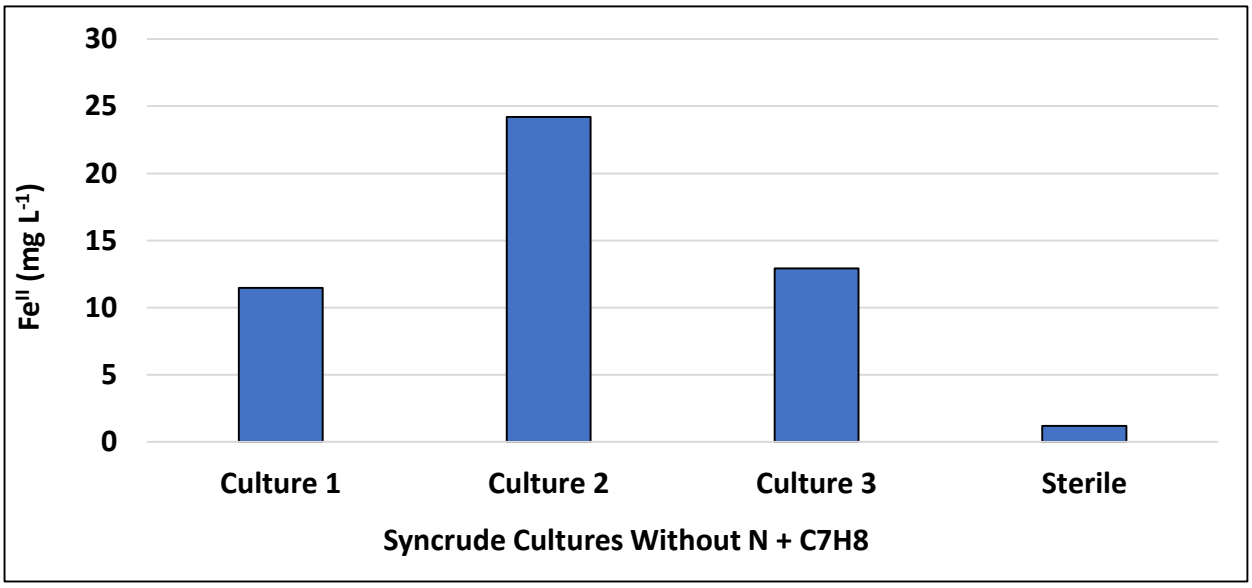

Figure 3-6: Fe(II) concentrations from individual Syncrude replicates under Fe(III) reducing conditions amended with toluene and sterile reference culture. Day 0 values subtracted from day 188.

3.3.4 Acetylene reduction in culture

3.3.4.1 n-alkane experimental cultures

Despite the lack of *n*-alkane degradation, acetylene reduction still occurred indicating N_2-fixation was present. The acetylene reduction assay was conducted over the course of 27 days due to low culture activity (**Figure 3-7**). Albian and CNRL *n*-alkane cultures produced 0.12 ±0.07 and 0.01 ±0.02 µmol of ethylene, respectively. Unlike methanogenic, *n*-alkane amended Syncrude cultures established in **Chapter 2**, which degraded acetylene, iron reducing Syncrude cultures without N reduced acetylene (0.17 ±0.02 µmol). Positive control replicates were substantially more active due the aerobic metabolism of readily degradable carbon in the media but were highly variable (17.7 and 3.4 µmol) producing a total of 10.6 ±10.1 µmol of ethylene. These data suggest the presence of N demand resulting from microbial metabolism in *n*-alkane amended cultures without N even in the absence of hydrocarbon degradation and support our hypothesis that Fe(III) was being used as an electron acceptor for the oxidation of H_2, or

residual organic compounds in the cultures. No ethylene was detected in sterile cultures or cultures with N.

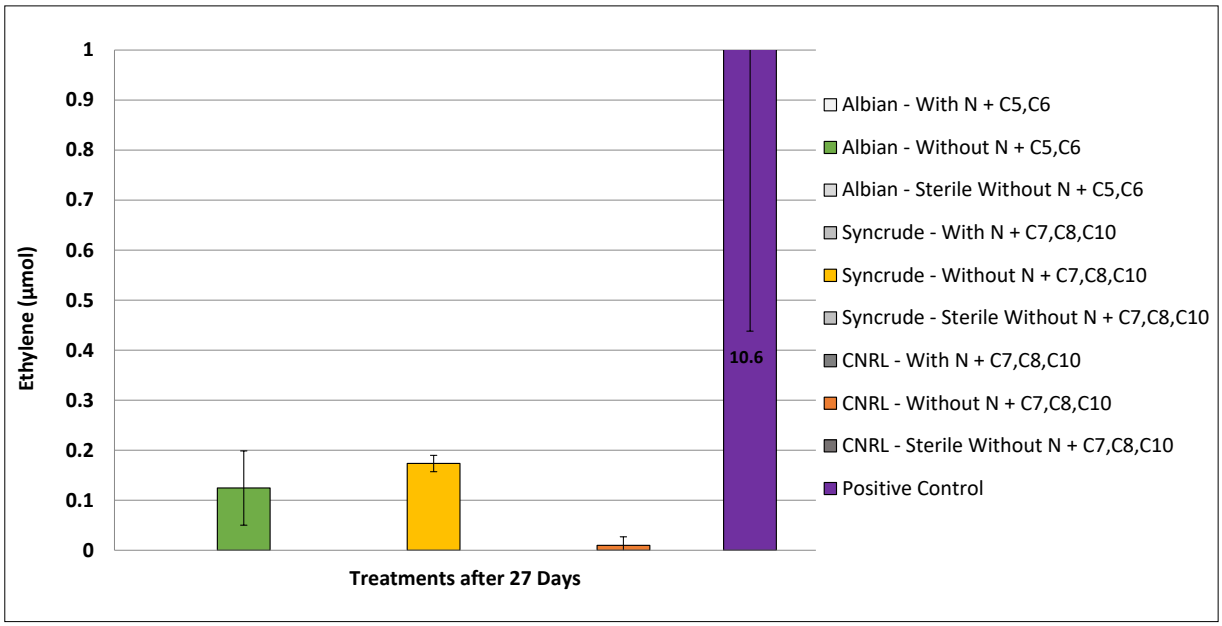

Figure 3-7: Ethylene production under Fe(III) reducing conditions resulting from acetylene reduction in *n*-alkane amended cultures. Values represent the mean µmol in triplicate for each treatment, and error bars indicate ± standard deviation.

Microbial metabolism and transformation of organic material likely supported Fe(III) reduction and N_2-fixing activity. Albian and CNRL *n*-alkane culture samples were analyzed for total inorganic carbon (TIC) and non-purgeable organic carbon (NPOC or TOC) (**Table S 3**). It was found that TIC increased significantly over time in both live unamended and amended Albian cultures with N, and NPOC also increased in *n*-alkane amended Albian cultures with N. This may suggest an increase in dissolved CO_2 or carbonates in these cultures along with an increase in biomass or metabolites. In contrast, CNRL cultures experienced a significant decrease in TIC in all unamended cultures and *n*-alkane amended cultures with N. NPOC also increased significantly in these cultures. CO_2 is required for the growth of some strict anaerobes (Reilly, 1980), therefore it is not unreasonable to suggest dissolved CO_2 was being used through some mechanism in these cultures to promote biomass or metabolite production.

3.3.4.2 Toluene experimental cultures

An acetylene reduction assay was conducted to determine if cultures were fixing N_2 to support microbial metabolism. Ethylene was detected at low concentrations by day 11 (<0.08 µmol) and increased with further incubation (**Figure 3-8**). By day 41, ethylene in Albian cultures without N had increased to 0.13 ±0.01 µmol. Ethylene in Syncrude cultures reached 0.15 ±0.08 µmol and the quantity of ethylene in CNRL

cultures increased significantly to 0.28 ±0.007 µmol. These values exceeded those previously observed during hydrocarbon degradation under methanogenic conditions (**Chapter 2**) suggesting iron, a key component of the molybdenum nitrogenase (Rubio and Ludden, 2008, and references therein), may be a limiting factor in N_2-fixing methanogenic media based cultures. Previously (Collins et al., 2016), we established that N_2-fixation in oil sands tailings cultures under methanogenic conditions was capable of supporting methane production from citrate degradation. These results suggest nitrogen fixation was occurring at a sufficient rate to support toluene degradation and iron reduction in amended cultures without N under iron reducing conditions.

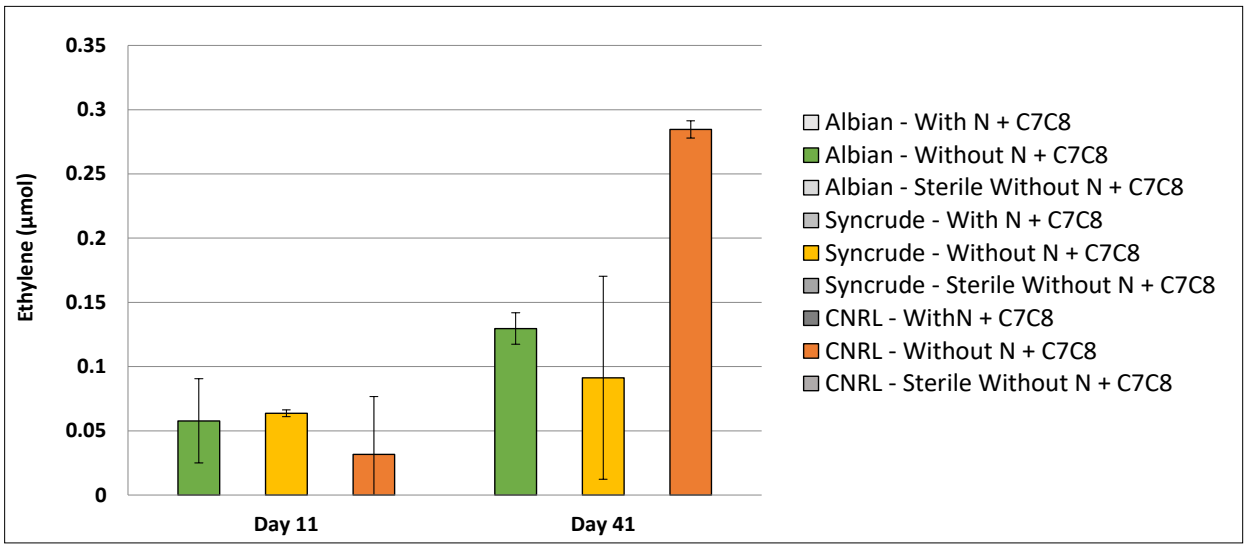

Figure 3-8: Ethylene production under Fe(III) reducing conditions resulting from acetylene reduction in toluene amended cultures. Values represent the mean µmol in triplicate for each treatment except Syncrude, in which a single culture did not reduce acetylene and was omitted. Error bars indicate ± standard deviation.

The high standard deviation in Syncrude data resulted from variance between the replicate cultures (**Figure 3-9**). One replicate did not reduce acetylene and was omitted from the dataset and from Illumina sequencing. Of the remaining two cultures, one culture (Culture 2) exhibited a higher rate of acetylene reduction to ethylene. This was the same culture that exhibited toluene degradation and a higher rate of Fe(III) reduction. These data suggest this culture was more active than the other replicates and suggested that the other Syncrude replicates may have reached similar rates of toluene degradation, Fe(III) reduction and acetylene reduction with time.

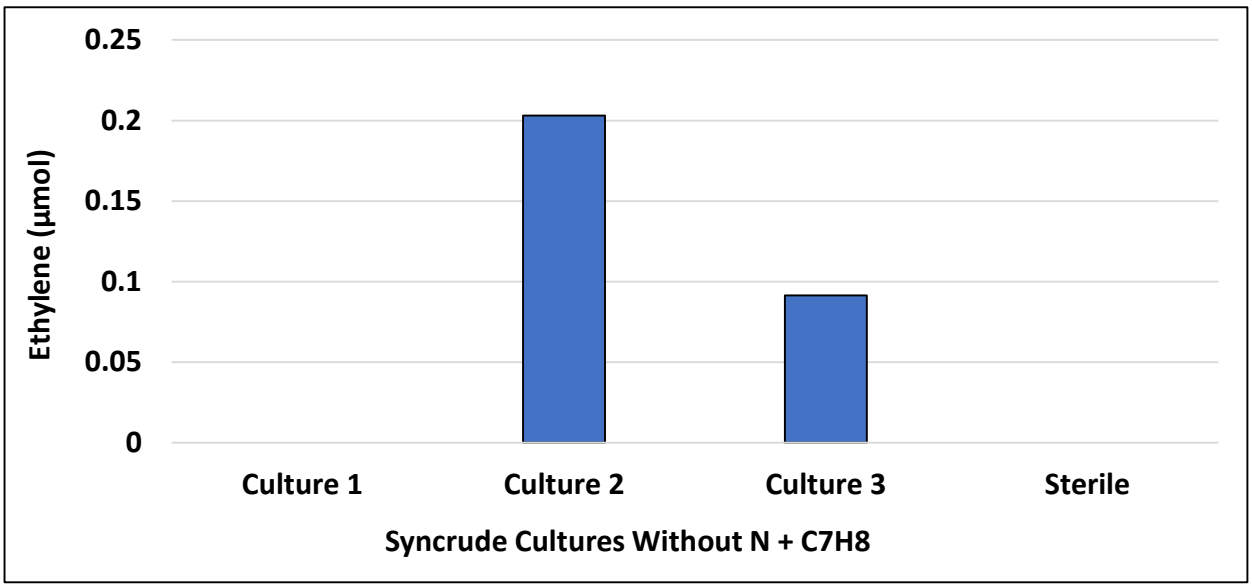

Figure 3-9: Ethylene production under Fe(III) reducing conditions at day 41 of the acetylene reduction assay from individual Syncrude replicates amended with toluene and sterile reference culture. No ethylene was produced in the first replicate, this culture was omitted from the average acetylene reduction in **Figure 3-8** and was not included in the composite DNA and RNA samples set for sequencing.

3.3.5 Microbial community and function

3.3.5.1 n-alkane experimental cultures

The low rate of methanogenesis was reflected in the proportion of methanogenic archaea, as exemplified by 16S rRNA Illumina sequencing data from Albian cultures (**Figure 3-10**). The unamended cultures with N had a substantially higher proportion of Archaea, which was likely due to sampling error as methane concentrations remained low in these cultures. The dominance of bacteria (> 95%) across all treatments regardless of amendment or N content under iron reducing conditions contrasts previous observations under methanogenic conditions where hydrocarbon amendment resulted in an increase in the archaeal population by increasing the availability of methanogenic substrates (Gray et al., 2010; Mohamad Shahimin et al., 2016; Siddique et al., 2015). Using a similar culture setup under methanogenic conditions (**Chapter 2**), we observed a higher proportion of Archaea in hydrocarbon amended cultures as compared to unamended cultures. In methanogenic communities, hydrocarbons are degraded to H_2 and acetate, which can then be oxidized by CO_2 reducing methanogens. However, the reduction of CO_2 is a less energetically favourable metabolic strategy than iron reduction (Chidthaisong and Conrad, 2000, and references therein). In the presence of amorphous ferric iron, iron reduction is expected to inhibit methanogenesis as observed in this chapter. These unfavorable conditions including direct inhibition of methanogenesis by amorphous Fe(III) (Van Bodegom et al., 2004), competition for substrates such as hydrogen by *Desulfovibrio* (Lovley, 1993 and references therein), and a lack of *n*-alkane degradation

products such as acetate, likely suppressed the growth of methanogenic archaea, which otherwise represent the bulk of Archaea in oil sands tailings (Foght, 2010).

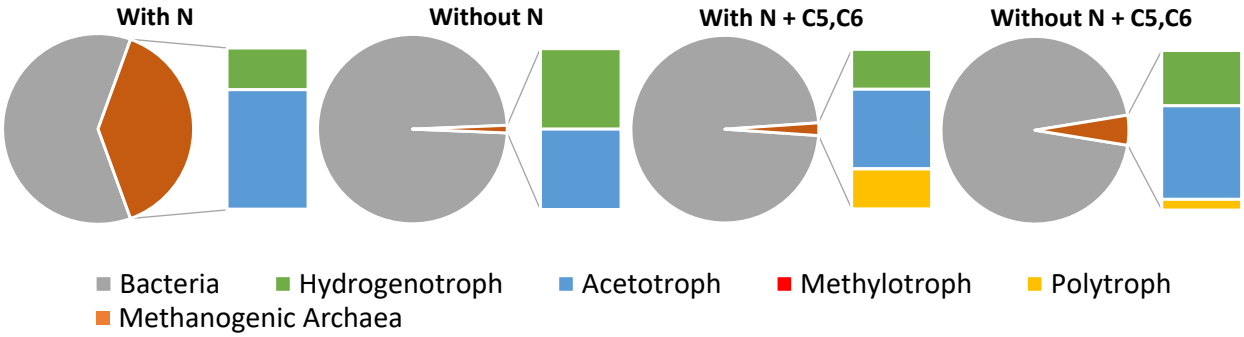

Figure 3-10: Distribution of Bacteria and methanogens in *n*-alkane amended and baseline Albian cultures under Fe(III) reducing conditions based on Illumina sequencing of the 16S rRNA gene at day 334. Polytroph indicates methanogens capable of metabolizing hydrogen, acetate, and methanol to methane. Full Archaea dataset in **Figure S 18A.**

The addition of amorphous $Fe(OH)_3$ caused a shift in the population of hydrocarbon degrading bacteria as exemplified by 16S rRNA sequencing results in Albian cultures (**Figure 3-11**). Methanogenic Albian cultures exhibiting *n*-alkane degradation in **Chapter 2** contained 37% bacteria capable of hydrocarbon degradation, within this group, families within the order *Clostridia* made up 15% of the bacterial population. In Albian cultures with Fe(III), only amended cultures with N contained a substantial proportion (37%) of bacteria capable of hydrocarbon degradation. This population, however, consisted of less than 3% *Clostridia* suggesting *Clostridia* were inhibited in the presence of amorphous $Fe(OH)_3$. Hydrocarbon data also suggested that bacteria capable of fermentation or hydrocarbon degradation under anaerobic conditions such as *Desulfovibrionaceae*, *Geobacteraceae*, and *Peptococcaceae* (Kunapuli et al., 2010; Meyer et al., 2013), were not capable of degrading *n*-alkanes under iron reducing conditions as no *n*-alkane degradation was observed in *n*-alkane amended cultures. Despite this, the abundance of *Geobacteraceae* and *Peptococcaceae* suggested that these families were responsible for the observed iron reduction in living cultures.

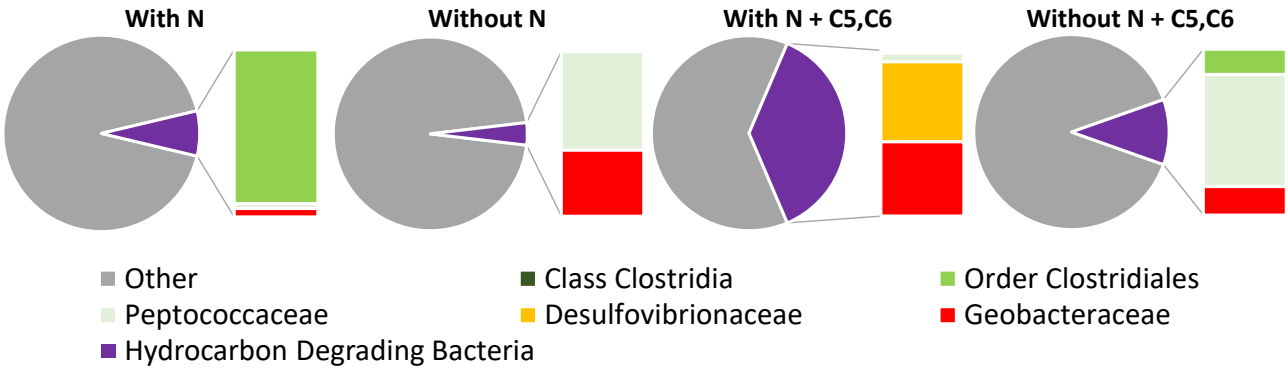

Figure 3-11: Bacterial community composition of potential hydrocarbon degrading bacteria under Fe(III) reducing conditions based on Illumina sequencing of the 16S rRNA gene at day 334 from *n*-alkane amended and baseline Albian cultures. Bacteria not known to degrade hydrocarbons and sequences with less than 2% abundance were grouped as "Other". Full Bacteria dataset in **Figure S 19A**.

Acetylene was reduced to ethylene by the nitrogen fixing microbial community (**Figure 3-12**). Unlike methanogenic cultures in **Chapter 2**, *nifH* gene expression was dominated by Proteobacteria rather than *Methanosaetaceae* suggesting that the N_2-fixing activity of *Rhodocyclaceae* (95-99% ident), *Rhizobiaceae* (88-99% ident.), and *Pseudomonas* (*Pseudomonas stutzeri*, 100% ident.) was enhanced in the presence of amorphous iron and in the absence of competition from *Methanosaetaceae* (**Figure 3-13**). Moreover, *nifH* gene expression was detected despite the absence of ethylene in amended Albian cultures with N. This may have been due to iron mediated gene regulation as *nifH* gene expression may increase in response to the availability of iron (Rosconi et al., 2006). However, the detected cDNA may also be misrepresented. While we've already shown that gene expression can be disproportionate to the presence of genes (**Chapter 2**), the expression of *Methanosaetaceae* is exceptionally high (32%) considering *nifH* genes attributed to this family were only present at 0.06% in DNA samples. In combination with the lack of nitrogenase activity, this observation suggests very few sequences were transcribed and the presence of substantial cDNA reads (**Table S 4**) may also have been due to the attempts to normalize DNA and cDNA concentrations between samples during the library preparation phase prior to sequencing.

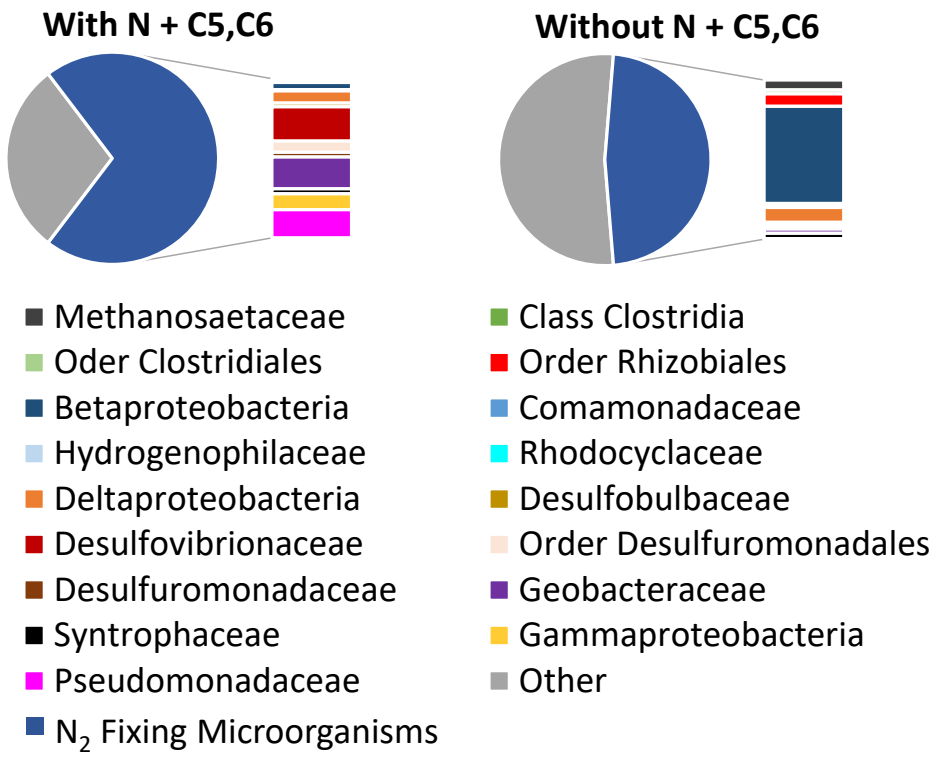

Figure 3-12: Microbial community composition of potential N_2-fixing microorganisms under Fe(III) reducing conditions based on Illumina sequencing of the 16S rRNA gene at day 334 from *n*-alkane amended and baseline Albian cultures. Prokaryotes where *nifH* genes were not detected were grouped as "Other".

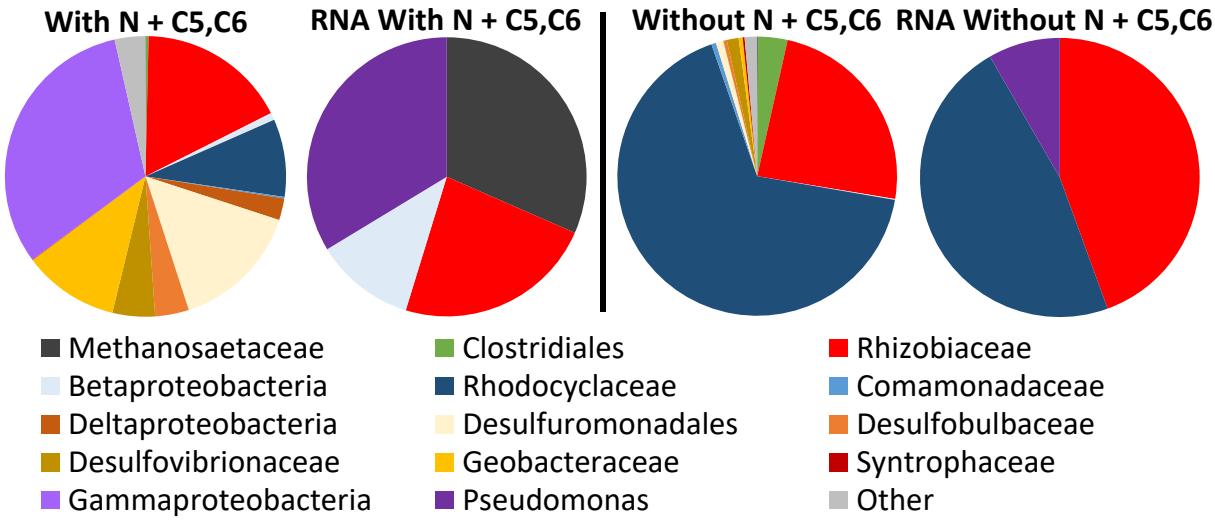

Figure 3-13: *NifH* gene abundance in n-alkane amended Albian cultures under Fe(III) reducing conditions based on Illumina sequencing and BLASTx match of *nifH* gene at day 334. "RNA" indicates sequenced cDNA reverse transcribed from extracted RNA. Sequences (OTUs) that returned fewer than 30 reads were discarded, OTUs with no match or off-target match were discarded. The presence and expression of *nifH* and genes coding for other anaerobic N transformations from all live treatments are shown in **Figures S 20A – 23A**.

3.3.5.2 Toluene experimental cultures

The presence of amorphous Fe(III) caused a shift in the methanogenic population as compared to methanogenic cultures evaluated in **Chapter 2** (**Figure 3-14**). While the population of methanogens was equivalent at 19% between methanogenic toluene amended Albian cultures with N in **Chapter 2** and the iron reducing Albian cultures with N in this study, the population shifted from the acetoclastic methanogen *Methanosaeta,* to the polytrophic methanogen *Methanomethylovorans*. In general, the proportion of *Methanosarcinaceae* was higher across $Fe(OH)_3$ amended treatments as compared to methanogenic treatments. Some species within *Methanosarcinaceae* are capable of shifting from methanogenesis to iron reducing metabolism (Van Bodegom et al., 2004), and while this activity is not well supported in this study, it is possible that other species within this family are better adapted to survive in the presence of amorphous Fe(III) than *Methanosaetaceae.*

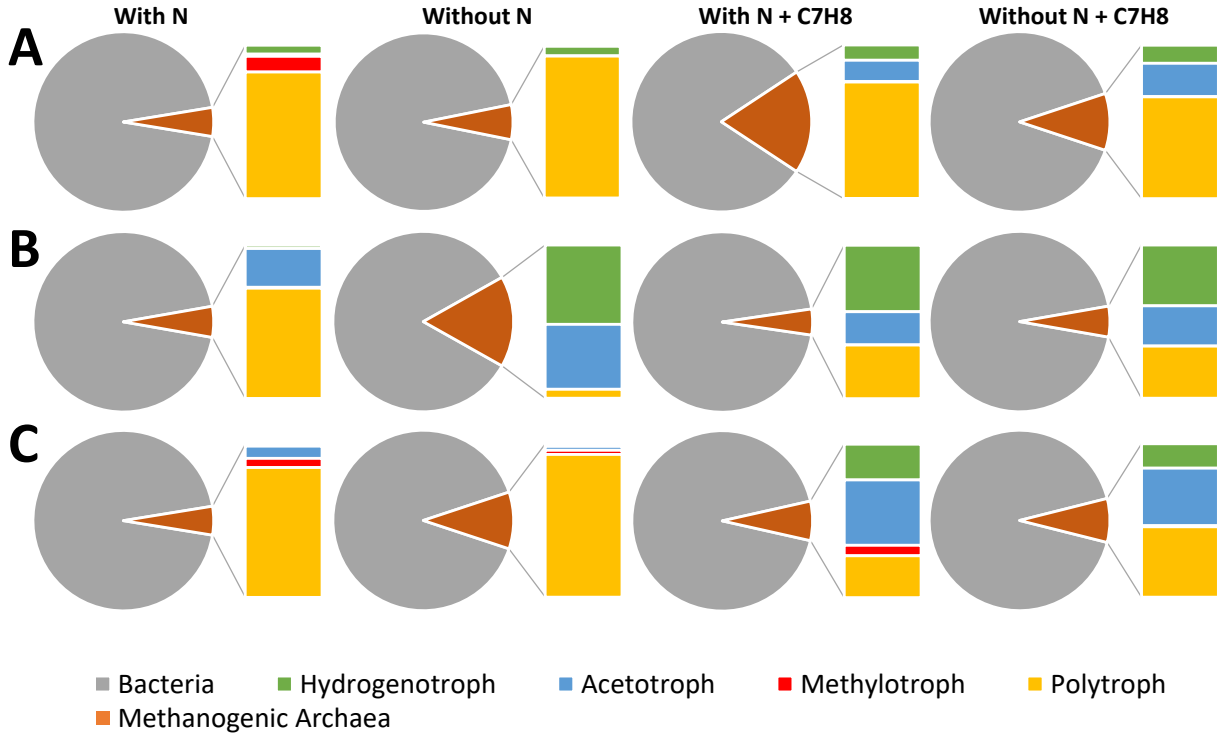

Figure 3-14: Distribution of Bacteria and methanogens in toluene amended and baseline cultures under Fe(III) reducing conditions based on Illumina sequencing of the 16S rRNA gene at day 189 from **(A)** Albian, **(B)** Syncrude, and **(C)** CNRL cultures. Polytroph indicates methanogens capable of metabolizing hydrogen, acetate, and methanol to methane. Full Archaea dataset in **Figure S 18B-D**.

Unlike *n*-alkane treatments in this chapter under Fe(III) reducing conditions, the microbial community in toluene amended cultures was dominated by *Clostridia* suggesting that toluene degradation observed in the headspace data may have been apparent in liquid samples with additional time. Headspace gas data suggested significant degradation in Albian cultures without N, Syncrude cultures with and without N, and

CNRL cultures with N. These data suggest that potential toluene degradation was carried out primarily by unidentified members of the order *Clostridia* in Albian and CNRL cultures (**Figure 3-15**). Syncrude cultures were dominated by *Peptococcaceae*, a family known to contain toluene degrading species capable of dissimilatory iron reduction (Kunapuli et al., 2010). The abundance of *Peptococcaceae* in amended Syncrude cultures as compared to unamended cultures strongly suggests this family played a role in hydrocarbon degradation. Several bacterial groups have been evaluated for toluene degradation under iron reducing conditions in pure culture including *Geobacter* (Coates et al., 2001; Kunapuli et al., 2010; Lovley et al., 1993), and a member of the class *Clostridia* (Kunapuli et al., 2010), as well as in sediment (H. R. Beller et al., 1992). In this study, *Clostridia* were the most likely hydrocarbon degraders. Moreover, the abundance of *Geobacteraceae* in Albian and CNRL cultures likely performed much of the observed iron reduction in live treatments amended with *n*-alkanes or toluene. Syncrude cultures contained higher proportions of *Firmicutes* (predominantly *Peptococcaceae* in toluene amended cultures), which likely reduced iron in these cultures. Many of these iron reducing bacteria can utilize H_2 and acetate, which may have contributed to the inhibition of methanogenesis through substrate competition.

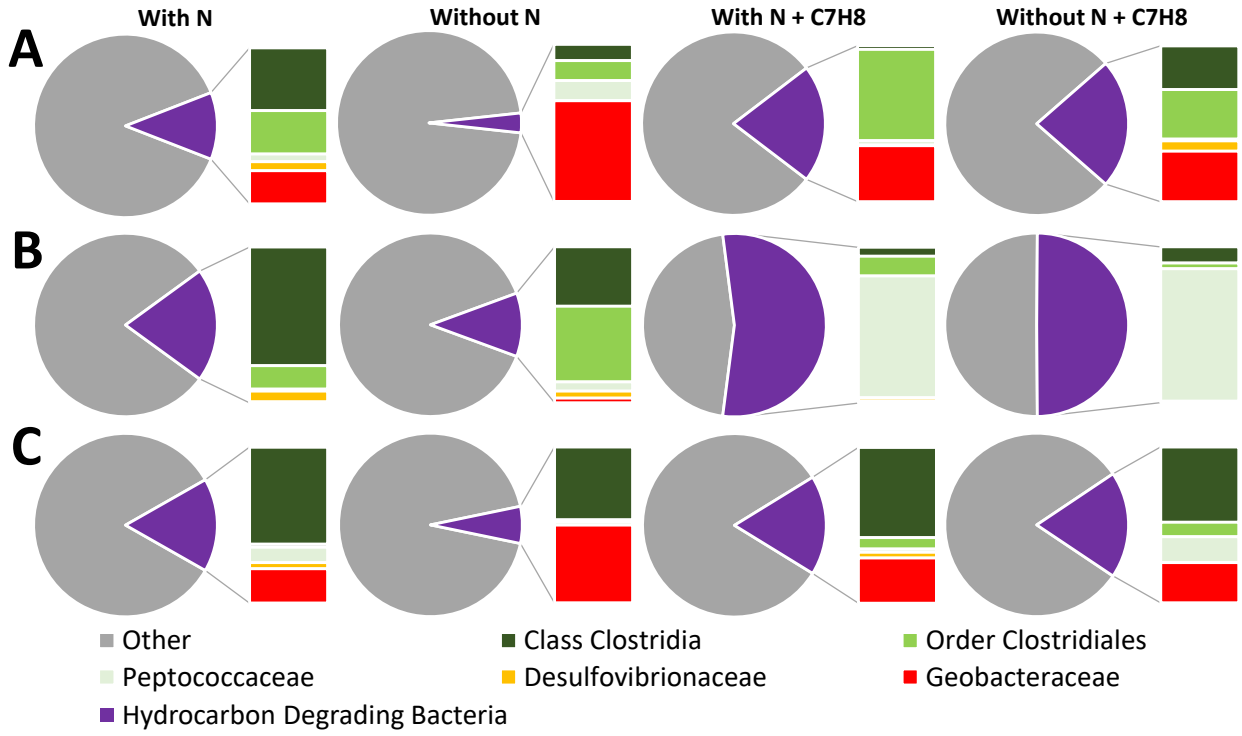

Figure 3-15: Bacterial community composition of hydrocarbon degrading bacteria under Fe(III) reducing conditions from toluene amended and baseline cultures based on Illumina sequencing of the 16S rRNA gene at day 189 from **(A)** Albian, **(B)** Syncrude, and **(C)** CNRL cultures. Bacteria not known to degrade hydrocarbons and sequences with less than 2% abundance were grouped as "Other". Full Bacteria dataset in **Figure S 19B-D**.

The slow metabolic rate observed in these cultures was likely because the microbial community was accustomed to surviving on clay surfaces in cultures with ~12.5% solids and were transferred to cultures with low solids content (~0.3%) as discussed in **Chapter 2**. The lack of solid surfaces would have left the community directly exposed to toluene and other environmental factors resulting in an inhospitable environment. The low volume of inoculum also would have resulted in a lower initial number of microorganisms in cultures. Toluene degradation may have been observed in liquid samples given enough time for the microbial community to grow in number and create biofilms.

Acetylene reduction data was supported by the microbial community (**Figure 3-16**) as evidenced by transcription of the *nifH* gene (**Figure 3-17**). Like *n-al*kane *nifH* expression results, gene expression was detected in toluene amended cultures with N in both Albian and CNRL cultures. Expressed *nifH* genes from Albian cultures were also dominated by *Methanosaetaceae* (87%) despite the low abundance of *Methanosaetaceae* sequences detected in DNA samples (< 1%). Taking into consideration the lack of acetylene reduction, it is likely that little transcription occurred despite the detection of transcribed genes. Transcription in Albian cultures without N were dominated by the same Proteobacteria groups as found in *n*-alkane amended cultures without N indicating that *Rhodocyclaceae*, *Rhizobiaceae*, and *Pseudomonas* were the dominant N_2-fixers in amended iron reducing Albian cultures without N.

Similar *nifH* gene distribution was detected in both amended Syncrude cultures with and without N except for *Comamonadaceae* (97-100% ident.), which were more abundant in amended cultures without N. These sequences were dominant alongside unidentified Betaproteobacteria in expressed genes suggesting these bacteria were responsible for the observed acetylene reduction in Syncrude cultures. Gene expression was more diverse in CNRL cultures. Amended CNRL cultures with N expressed a similar transcription profile to the amended cultures without N, however the absence of acetylene reduction activity suggested these cultures were not fixing N_2. Like what was observed in Albian *n*-alkane cultures, transcription may have been due to upregulation of gene expression in response to the abundance of iron or may have been due to DNA and cDNA concentration normalization. Extracted RNA from amended CNRL cultures without N were also composed predominantly of sequences with low abundance in the DNA samples, however the higher rate of acetylene reduction supports *nifH* gene transcription. It is therefore probable *Methanosaetaceae* and the Proteobacteria *Rhodocyclaceae*, unidentified Betaproteobacteria, *Rhizobiaceae*, and *Pseudomonas* were responsible for N_2-fixing activity in these cultures. It is currently unclear what role methanogens such as *Methanosaeta* played in these cultures in the absence of substantial methane production.

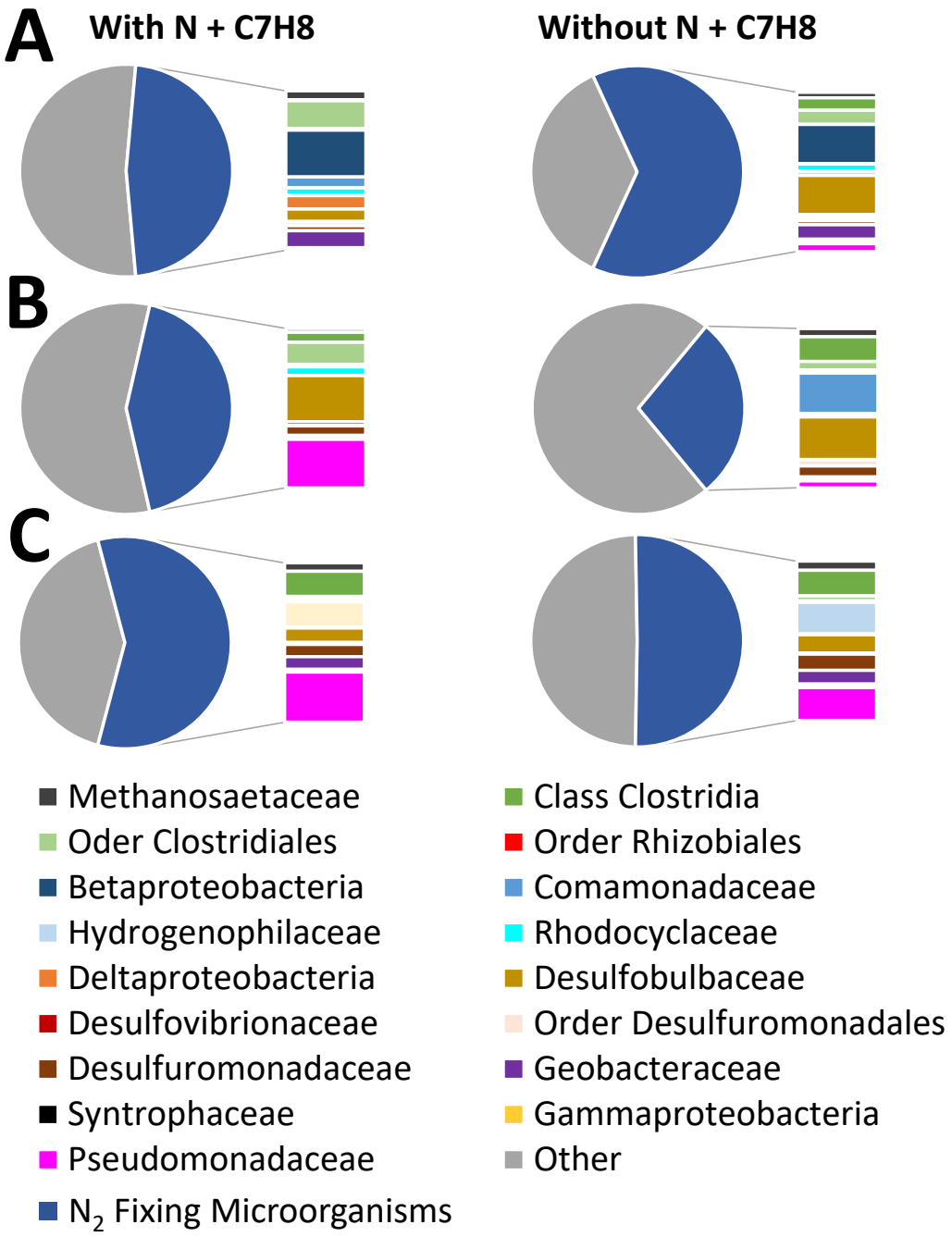

Figure 3-16: Microbial community composition of potential N_2-fixing microorganisms under Fe(III) reducing conditions from toluene amended and baseline cultures based on Illumina sequencing of the 16S rRNA gene at day 189 from **(A)** Albian, **(B)** Syncrude, and **(C)** CNRL cultures. Prokaryotes where *nifH* genes were not detected were grouped as "Other".

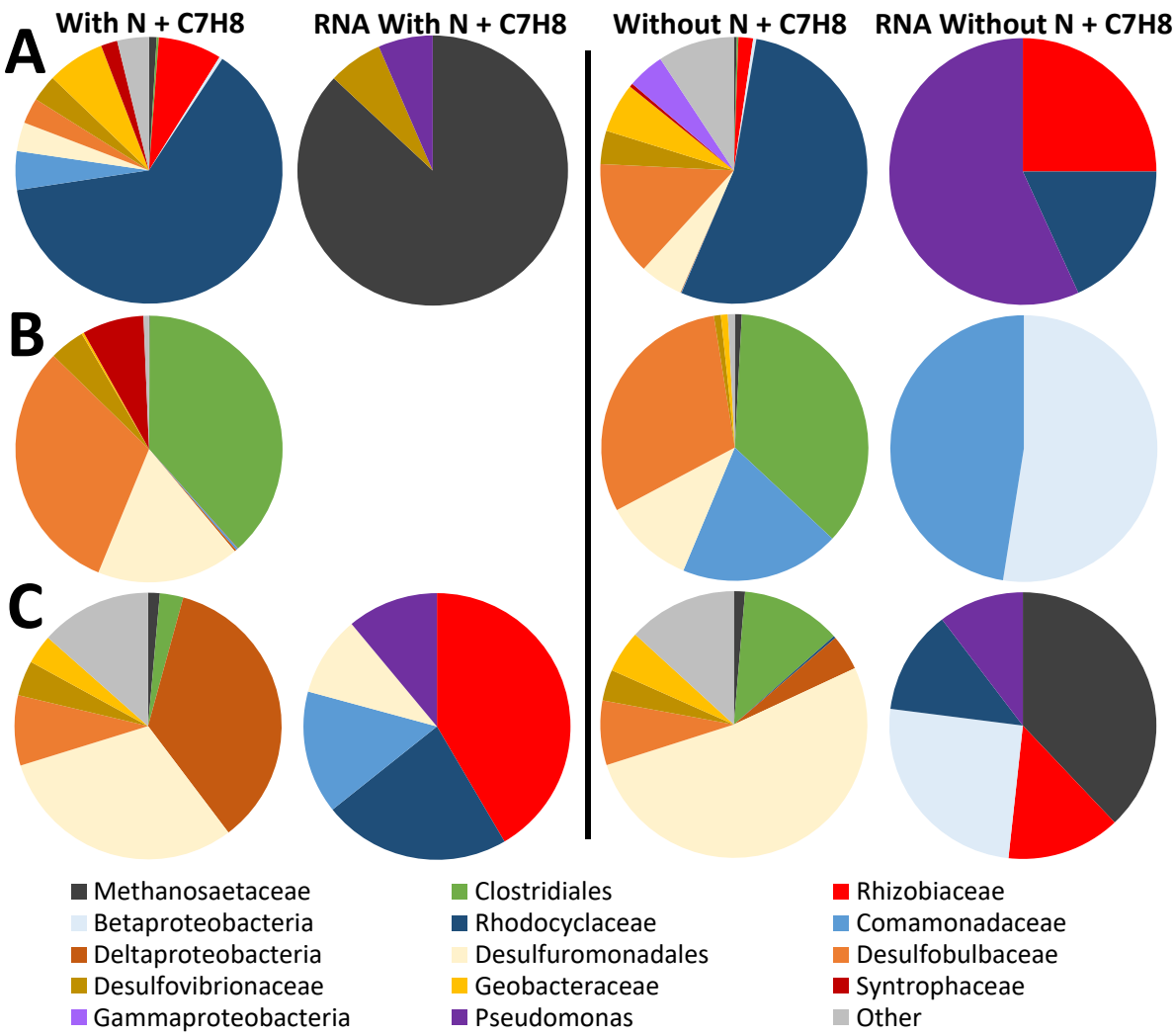

Figure 3-17: *NifH* gene abundance in toluene amended cultures under Fe(III) reducing conditions based on Illumina sequencing and BLASTx match of *nifH* gene at day 189 from **(A)** Albian, **(B)** Syncrude, and **(C)** CNRL cultures. "RNA" indicates sequenced cDNA reverse transcribed from extracted RNA. Sequences (OTUs) that returned fewer than 30 reads were discarded, OTUs with no match or off-target match were discarded. The presence and expression of *nifH* genes coding for other anaerobic N transformations from all live treatments are shown in **Figures S 20B-D – 23B-D**.

3.4. Conclusions

Methanogenesis was inhibited in both *n*-alkane and toluene amended cultures in the presence of amorphous Fe(III) ($Fe(OH)_3$). Some toluene degradation was observed in toluene amended cultures, this was likely supported by the N_2-fixing community in the absence of bioavailable N. Nitrogenase activity was higher in cultures without N as compared to methanogenic cultures in Chapter 2. In the absence of nitrogenase activity, *nifH* gene expression was also apparent in iron reducing cultures with N. The increase in N_2-fixing activity in cultures without N and the observed gene transcription in cultures with N may have been due to the abundance of iron, which has been found to play a role in regulating *nifH* gene

transcription. Proteobacteria also dominated the *nifH* gene transcription indicating N_2-fixing bacteria were able to compensate for the lack of N_2-fixing methanogens in the microbial community. The results from this study suggest it is possible that aromatic hydrocarbon degradation may be sustained by the N_2-fixing microbial community under iron reducing conditions. While cultures do not replicate *in-situ* conditions, the key microbial groups are present in tailings materials. This suggests that amorphous Fe(III) in the form of $Fe(OH)_3$ would be effective at inhibiting methane production in tailings ponds and potentially reclaimed wet landscapes that incorporate tailings materials. Moreover, using amorphous Fe(III) as a tailings amendment may help oil sands producers and government regulators achieve methane reduction targets by 2025.

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3.5 References

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4. Citrate Contributes to Methane Emissions in Oil Sands Tailings Under Methanogenic and Iron Reducing Conditions

4.1 Introduction

Bitumen extraction from Alberta's Oil Sands accounts for 60% of oil extraction in Canada (Canada's Oil Sands, 2016). To separate and recover bitumen from the mined ores, a process known as Clarks hot water extraction is used (Mikula et al., 1996). This process employs hot water, dispersants, and diluents, and ultimately results in a composite waste stream known as tailings. This slurry is largely composed of water, sand, silt, and clay, and contains unrecovered diluent and solvent, and residual bitumen (Allen, 2008). Tailings are pumped into mined-out pits known as tailings ponds where they settle for years and eventually become mature fine tailings (MFT). In the 1990s, bubbling was observed on the surface of a tailings pond known as Mildred Lake Settling Basin, the produced gas was methane and CO_2 (MLSB; Holowenko et al., 2000). Since then, all tailings ponds in operation have become methanogenic.

Tailings ponds are highly stratified below the oxic cap water and contain a diverse array of microorganisms capable of oxidizing organic compounds. These include nitrate-, iron-, and sulfate reducers as well as methanogens (Penner and Foght, 2010). Nitrate, iron, and sulfate reduction are more energetically favourable than CO_2 reduction to methane and occur in the higher layers of the tailings ponds. Methane production is generally inhibited in the presence of these electron acceptors (Chidthaisong and Conrad, 2000; Choi and Rim, 1991; Holowenko et al., 2000; Lovley and Phillips, 1987; Van Bodegom et al., 2004). In the absence of other electron acceptors deep below the surface (5-15 m), sulfate-reducing bacteria, fermenters, syntrophs and methanogens are abundant (Ramos-Padrón et al., 2011). While sulfate-reducing bacteria will outcompete methanogens for substrates such as acetate and H_2 at higher concentrations of SO_4^{2-}, methanogenesis will occur below 20 mg L^{-1} SO_4^{2-}.

Approximately 66.75 kt CH_4 was released from a 19.7 km^2 area of tailings ponds in 2016 (Kong et al., 2019). With a total tailings pond surface area of 103 km^2 (osip.alberta.ca, 2016), 344.2 kt CH_4 may have been generated in the oil sands region during 2016. These values suggest that methane emissions from tailings ponds could account for 8% of methane emissions in Canada (Environment Canada, 2014). Methane is a powerful greenhouse gas and reducing methane emissions is a national priority. Current government mandates require the reduction of methane emissions by 45% below 2012 levels before 2025 (Environment and Climate Change Canada, 2018), suggesting that reducing fugitive methane emission

from tailings ponds would be highly beneficial in achieving this mandate. The addition of amorphous Fe(III) such as $Fe(OH)_3$ has been found to inhibit methanogenesis in previous studies (Lovley and Phillips, 1986; Roden and Wetzel, 1996; Sivan et al., 2016a; Van Bodegom et al., 2004), and the degradation of aromatic hydrocarbons have been previously observed under these conditions (Foght, 2008 and references therin). In **Chapter 3**, we examined amorphous Fe(III) as a potential tailings amendment to mitigate methane emissions under N limited conditions as found in some ponds. We observed near total inhibition of methanogenesis in all treatments alongside iron reducing activity. *NifH* gene expression and acetylene reduction experiments to test for nitrogenase activity suggested nitrogen fixation was occurring and was likely supporting the microbial community in the absence of endogenous N. We also found evidence of aromatic hydrocarbon degradation under these conditions; however *n*-alkanes were not degraded. This observation has been previously discussed in the literature but the mechanism by which alkane degradation was inhibited is not well described (Mbadinga et al., 2011; Zwolinski et al., 2001). We concluded the iron reduction observed in these cultures was likely due to oxidation of endogenous organic compounds and H_2.

Based on the literature, the inhibition of methane production occurs either due to substrate competition (Achtnich et al., 1995; Bodegom and Stams, 1999; Lovley and Phillips, 1987; Roden and Wetzel, 2003), methanogenic archaea converting to iron reducing metabolism (Bond and Lovley, 2002; Liu et al., 2011; Sivan et al., 2016a; Van Bodegom et al., 2004; Zhang et al., 2013, 2012), and direct interaction with the cell membranes of methanogens (Van Bodegom et al., 2004). In **Chapter 3**, we observed both a decrease in the methanogenic population suggesting competitive strain, and a shift to *Methanosarcina* sp. potentially capable of Fe(III) reduction. In all cases, methanogenic activity was effectively absent. However, methane production is still possible in the presence of Fe(III). In a study by Van Bodegom et al. (2004), *Methanosarcina barkeri* and *Methanosaeta concilii* were found to produce methane under Fe(III) reducing conditions in the presence of acetate while methane production from another methanogen, *Methanospirillum hungatei*, was completely inhibited. Methane production has also been reported in the presence of chelated iron such as ferric citrate complexes (Hales et al., 2018; Wu et al., 2016). These observations suggest that amorphous iron may not be an effective treatment for methanogenesis under certain conditions. In addition to diluents and solvents, citrate is an organic molecule that is used in some bitumen recovery processes. These compounds then end up in tailings ponds where they are metabolized by the microbial community. In addition to being fermented to acetate, citrate is a siderophore for Fe(III) and can solubilize $Fe(OH)_3$ at neutral pH (pH 7.4) thereby forming ferric citrate complexes (Silva et al., 2009). These processes have been shown to decrease the reduction potential of Fe(III) thereby promoting

the growth of iron reducing bacteria capable of direct interspecies electrons transfer (DIET) with methanogens and facilitating methanogenesis (Li et al., 2019). Therefore the presence of citrate may negate the effects of an Fe(III) based treatment for the inhibition of methanogenesis in tailings.

4.2 Methods and Materials

4.2.1 Chemicals, gasses, and materials

Chemicals, including trisodium citrate (analytical reagent grade or higher) were purchased from Fisher Scientific. Gasses 70% N_2/30% CO_2 and acetylene (scientific grade), and all other gasses (certified standard) were purchased from Praxair (Alberta, Canada). Enrichment cultures used to inoculate transfer cultures used in this study were previously described (Collins et al., 2016).

4.2.2 Transfer cultures

Methanogenic cultures were established by transferring 5 ml inoculum from previously described primary cultures established from tailings originating from the former Albian sands (hereafter Albian, Collins et al., 2016) to 90 ml methanogenic media with or without inorganic N (NH_4^+) in the media (hereafter, referred to as "with N" and "without N"). Blue butyl stoppers and foil crimp caps were used to anaerobically seal cultures in 158 ml serum bottles as previously described (Collins et al., 2016). Iron reducing cultures contained 2 ml primary cultures and 3 ml $Fe(OH)_3$ in solution (0.05 g) to a final starting concentration of 0.053% wt/vol. Medium with N contained $CaCl_2$, NaCl, NH_4Cl, $MgCl_2$, $(NH_4)_6Mo_7O_{24}$, $ZnSO_4$, H_3BO_3, $FeCl_2$, $CoCl_2$, $MnCl_2$, $NiCl_2$, $AlK(SO_4)_2$, $NaHCO_3$, KH_2PO_4, Resazurin (anaerobic indicator), and Na_2S as the reducing agent (**Chapter 2**). Ammonium containing salts were omitted from N deficient media and Na_2MoO_4 was used in place of $(NH_4)_6Mo_7O_{24}$ (Collins et al., 2016). This was done to ensure the concentration of molybdenum was consistent between the two types of media as molybdenum is a key element required for the formation of the nitrogenase enzyme and N_2-fixing activity. Cultures were established under an N_2/CO_2 headspace. Cultures were stored for two weeks in the dark before trisodium citrate amendments were added for a final concentration of 550 mg L^{-1} citrate. Samples (5 ml) were taken at day 0 and additional 2 ml samples were taken at day 149, day 250, and day 330 for Fe(II) and microbial analysis, and stored at -20˚C. An additional 3 ml of $Fe(OH)_3$ was added following ferrozine analysis at day 149.

4.2.3 Ferrozine analysis

Ferrozine analysis was conducted on time point samples taken at day 0, 149, 250, 330 and an additional sample taken at day 565 (**Figure S 24**). Analyses performed at day 0, 149, and 565 were from fresh samples whereas samples time points 250 and 330 were previously frozen. The ferrozine solution (0.1% wt/vol)

was prepared in 0.05 M HEPES (pH 7). Culture samples (0.333 ml) were combined with 1.67 ml ferrozine solution in a 2 ml microfuge tube, vortexed for 15 s and centrifuged for 3 min on maximum speed (21,130 x g) to remove fine particulates. Absorbance was measured on a spectrophotometer at 562 nm and quantified using a standard curve. Standard stock solution used to prepare standards contained 100 mg L^{-1} $Fe(NH_4)_2(SO_4)_2*6H_2O$ in DI H_2O acidified by adding 1 ml L^{-1} of 6 M HCl.

4.2.4 Acetylene reduction cultures

Acetylene reduction cultures were established at day 316. Secondary cultures were needed to avoid altering the microbial activity in the larger cultures as acetylene is known to inhibit methanogenic metabolism (Sprott et al., 1982). Cultures consisted of 20 ml of citrate amended transfer cultures in sealed and sterile 60 ml serum bottles under a N_2/CO_2 headspace. Acetylene reduction cultures were flushed for 5 min with N_2/CO_2 and reamended with 500 mg L^{-1} citrate. Acetylene was added via syringe to achieve 3% vol headspace (99.2 µmol) and cultures were monitored periodically until ethylene was observed at day 27.

4.2.5 Chemical analysis

Periodic methane and pressure measurements were taken as previously described to quantify methane production over the course of the incubation (Collins et al., 2016). Methane and ethylene were measured through direct injection of the headspace gas (0.1 ml) using a Trace 1300 GC-FID (ThermoFisher, column: TG-Bond (30mx0.32mm), oven temperature 40°C, and helium flow 3 ml min^{-1}), and quantified employing a standard curve using external standards. Culture pressure was determined using a digital pressure gauge (DPG1000B ± 15.00PSIG-5, MOD-TRONIC Instruments Limited, Brampton, ON) with a luer-lok metal fitting and disposable needle. Prior to each injection, cultures were shaken by hand. Headspace gas was directly injected into the GC-FID. Theoretical maximum methane production was calculated using the Symons and Buswell equation (Roberts, 2002). Unless stated otherwise, statistical significance was determined in Excel using a two tailed, paired T-Test.

4.2.6 DNA/RNA sequencing and analysis

DNA and RNA extractions were performed on samples taken at day 334. Duplicate extractions were performed for each sample using methods previously described (**2.2.9 DNA/RNA sequencing and analysis**). Functional genes *nifH, nosZ, nirS*, and *nrfA*, and 16S rRNA, were PCR amplified using custom primers containing Illumina forward or reverse overhangs (Illumina, 2013). Primers and PCR conditions were as outlined in **Table 2-1**. Reactions were prepared in duplicate and pooled for analysis as described

(**2.2.9 DNA/RNA sequencing and analysis**). The DNA concentration for 16S rRNA amplicons were normalized across all samples, and DNA and cDNA concentrations were normalized for functional genes when possible. Amplicon samples were sequenced using MiSeq Illumina sequencing (TAGC, University of Alberta). Samples were pooled by treatment for sequencing following library preparation.

Raw Illumina data was processed using MetaAmp 2.0 (Dong et al., 2017). MetaAmp workflow used default settings for paired end analyses, a similarity cutoff of 0.97, and a maximum expected error of 1. Specific settings for each gene were as previously described (**2.2.9 DNA/RNA sequencing and analysis**). The SILVA 132 database was used to process 16S rRNA samples and functional genes were processed using BLASTx and MegaBLAST (blast.ncbi.nlm.nih.gov/), excluding uncultured/environmental sample sequences. All functional gene sequences were manually curated to remove non-target sequences, and all treatments that returned fewer than 60 reads were discarded (**Table S 5**).

4.3 Results and Discussion

4.3.1 Methanogenesis

Methane production from the biodegradation of citrate was monitored for 316 days. **Figure 4-1** shows the methane data from methanogenic cultures. Theoretical maximums based on the complete degradation of citrate were determined using the Symons and Buswell (1933) stoichiometric equation. Theoretical maximums represent the cumulative total of background methane generated from baseline cultures and citrate conversion to methane, which increased with subsequent citrate additions. In chronological order, cumulative maximum methane yields are as follows: 526 µmol (550 mg L^{-1} citrate at day 0), 1151 µmol (660 mg L^{-1} citrate at day 153), and 1656 µmol methane (505 mg L^{-1} citrate at day 250).

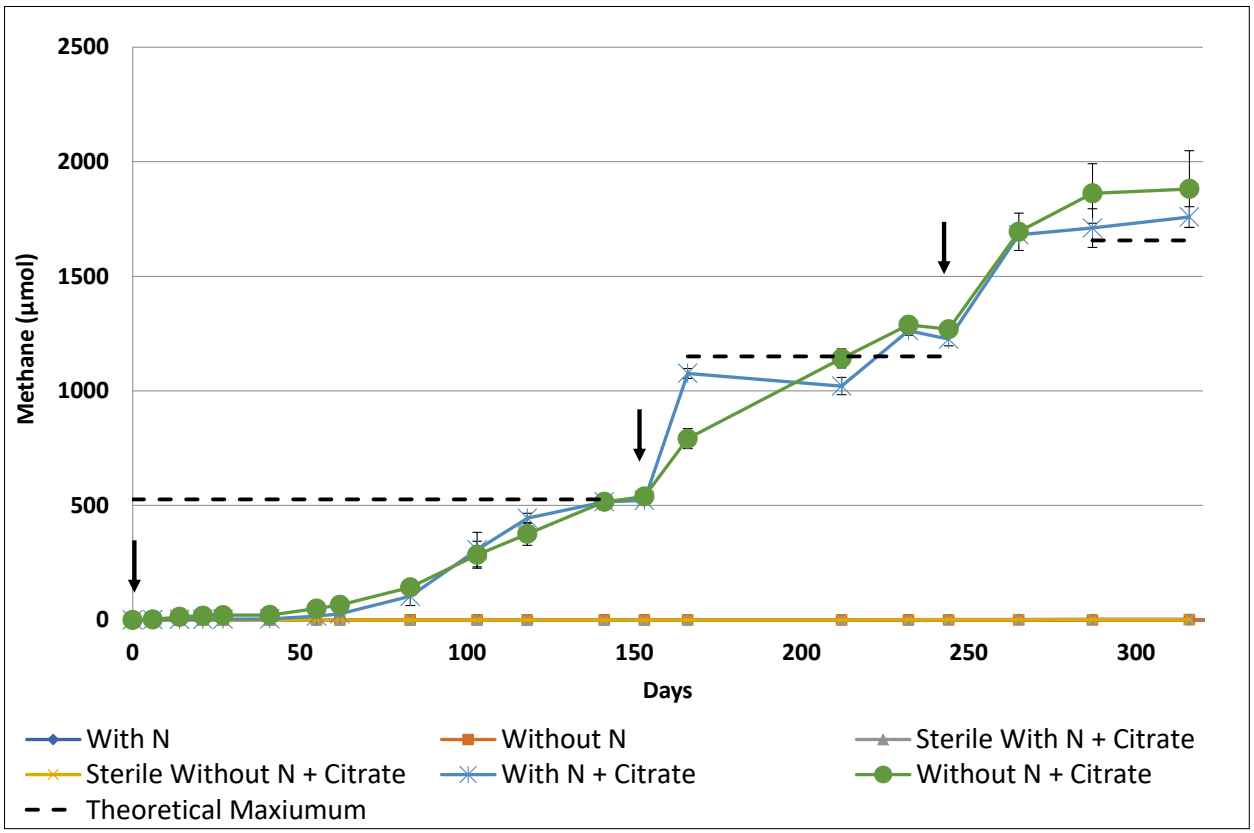

Figure 4-1: Methane production in citrate amended Albian cultures. Baseline cultures with and without N sourced from **2.3.1 Methanogenesis** for comparison. Values represent the mean µmol for triplicate amended cultures and duplicate sterile cultures. Error bars indicate ± standard deviation. Arrows indicate citrate additions.

Effectively no methane was produced by day 316 in sterile controls or unamended cultures (< 3.2 µmol). Citrate amended cultures with N produced 1758.7 ±45.5 µmol methane and amended cultures without N produced 1881.1 ±166.7 µmol methane by day 316. The rate of methane production was significantly higher than previously observed in hydrocarbon amended cultures (**Chapter 2**) due to the labile nature of citrate.

Figure 4-2 shows methane production in cultures established with amorphous Fe(III). Citrate was added to cultures twice, once at day 0 (550 mg L^{-1}) and once at day 153 (660 mg L^{-1}) with a cumulative theoretical maximum methane yield of 526 µmol and 1151 µmol, respectively. Sterile and unamended cultures with Fe(III) produced effectively no methane (< 2.9 µmol) however, methane production varied greatly between Fe(III) treated cultures and the microbial population produced methane at a slower rate than cultures without Fe(III). Amended cultures with N approached the first theoretical maximum from the initial citrate amendment at day 166 (513.9 ±628.9 µmol), however amended cultures without N produced only 188.4 ±102.3 µmol methane. Neither treatment reached the second theoretical maximum methane

concentration. Methane production in amended cultures with N reached 835.5 ±237.7 µmol and amended cultures without N reached 415.8 ±91.6 µmol by day 316, suggesting $Fe(OH)_3$ resulted in partial inhibition of methanogenesis rather than near total inhibition as previously observed in this thesis.

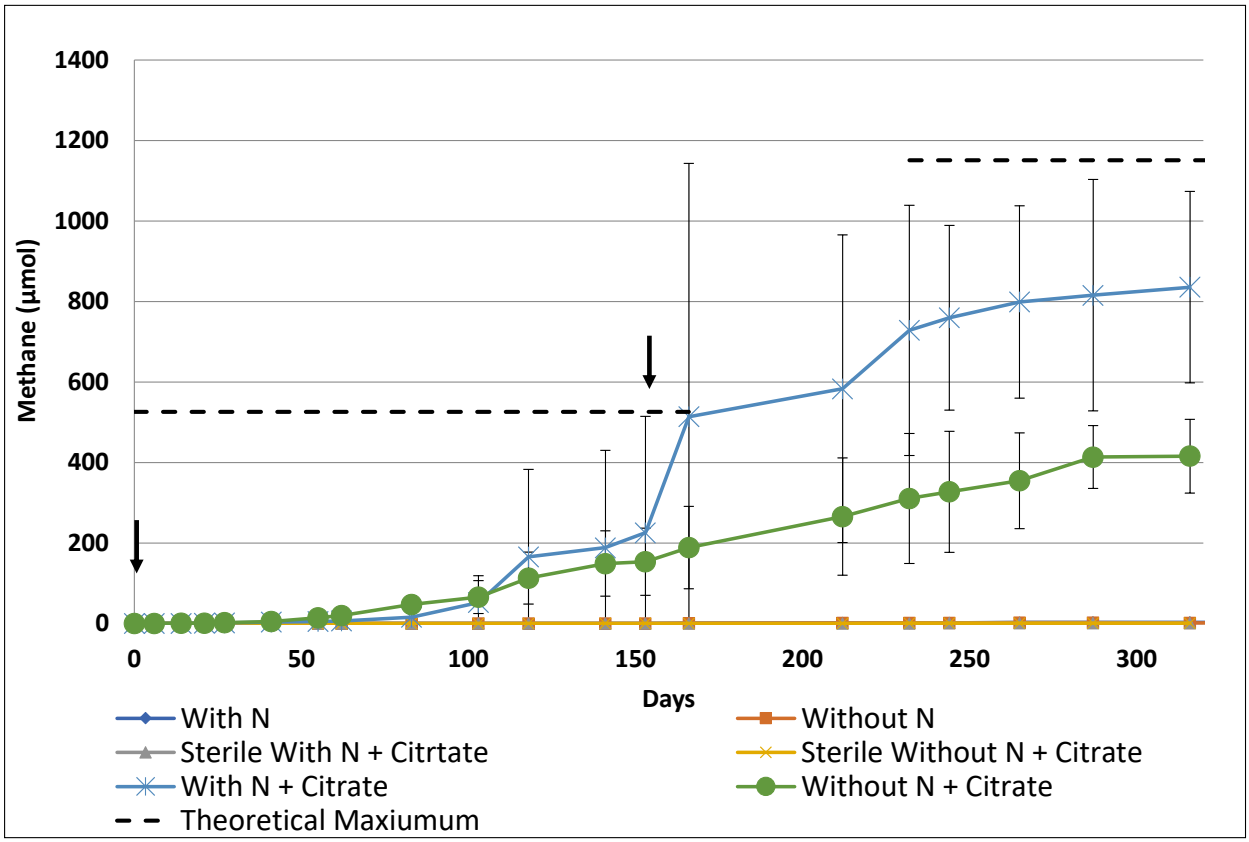

Figure 4-2: Methane production in citrate amended Albian cultures with amorphous Fe(III). Baseline cultures with and without N sourced from **3.3.1 of methanogenesis** for comparison**.** Values represent the mean µmol for triplicate amended cultures and duplicate sterile cultures. Error bars indicate ± standard deviation. Arrows indicate citrate additions, an additional (0.05 g) $Fe(OH)_3$ was added at day 149.

Methane production from citrate metabolism has been observed in our previous study (Collins et al., 2016). However, inhibition of methanogenesis in the presence of amorphous Fe(III) such as $Fe(OH)_3$ is well documented (Lovley and Phillips, 1986; Roden and Wetzel, 1996; Van Bodegom et al., 2004). In **Chapter 3**, we observed significant inhibition of biogenic methane production in hydrocarbon amended cultures in contrast to similarly established methanogenic cultures in **Chapter 2**. Citrate amended cultures were established under nearly identical conditions and contained the same amorphous $Fe(OH)_3$ solution at the same concentration (0.053% wt/vol.). Citrate can also solubilize $Fe(OH)_3$ at neutral pH (pH 7.4) by forming ferric citrate complexes (Silva et al., 2009). Citrate was added to these cultures at a nearly 1:1 ratio (550 mg L^{-1} citrate, 530 mg $^{-1}$ $Fe(OH)_3$) at day 0, which could have substantially reduced the concentration of amorphous Fe(III) in favour of ferric citrate complex formation in these cultures. Ferric citrate has not

been found to inhibit methanogenesis in cultures (Hales et al., 2018; Wu et al., 2016). Citrate is also easily fermented into acetate, and certain methanogens such as *Methanosaeta* have been found to produce methane in the presence of acetate even under iron reducing conditions (Van Bodegom et al., 2004). A recent paper by Li et al., (2019) reported that phenol was methanogenically degraded up to 1.3 times faster in the presence of $Fe(OH)_3$ and citrate in combination as compared to iron reducing conditions with $Fe(OH)_3$ alone where methanogenesis was partially inhibited. They suggested iron reducing bacteria were degrading the phenol while engaged in syntrophic metabolism using direct interspecies electron transfer (DIET) with methanogenic archaea (Li et al., 2019). Their data supported increased solubility and decreased reduction potential of $Fe(OH)_3$ in the presence of citrate as a mechanism facilitating this process. While the microbial community data discussed later in this chapter does not support citrate oxidation by iron reducing bacteria, the mechanism proposed by Li et al., (2019) may have facilitated metabolite oxidation and DIET with methanogens to support methanogenesis due to the lower electron accepting affinity of the ferric citrate complex. Likely a combination of these factors enabled methanogenesis to continue in live, amended treatments.

4.3.3 Fe(III) reduction in cultures

Iron reduction was also observed in live cultures containing amorphous $Fe(OH)_3$ (**Figure 4-3**). By day 330, sterile cultures contained <1.7 mg L^{-1} Fe(II), whereas unamended cultures did not exceed 6.5 mg L^{-1} Fe(II) over the course of the incubation. At day 330, amended cultures with and without N had produced 9.3 ±4.9 mg L^{-1} and 15.4 ±4.4 mg L^{-1} Fe(II), respectively. While the difference in Fe(II) concentrations between amended and unamended cultures were not significant, the differences between amended and sterile cultures were significant indicating that Fe(III) reduction to Fe(II) was a microbially mediated process. Amended cultures over all reduced significantly more Fe(III) to Fe(II) than unamended cultures by day 565 (**Figure S 24**) suggesting that iron reduction may have played a role in the degradation of citrate or the resulting metabolites (Lovley, 1993 and references therein).

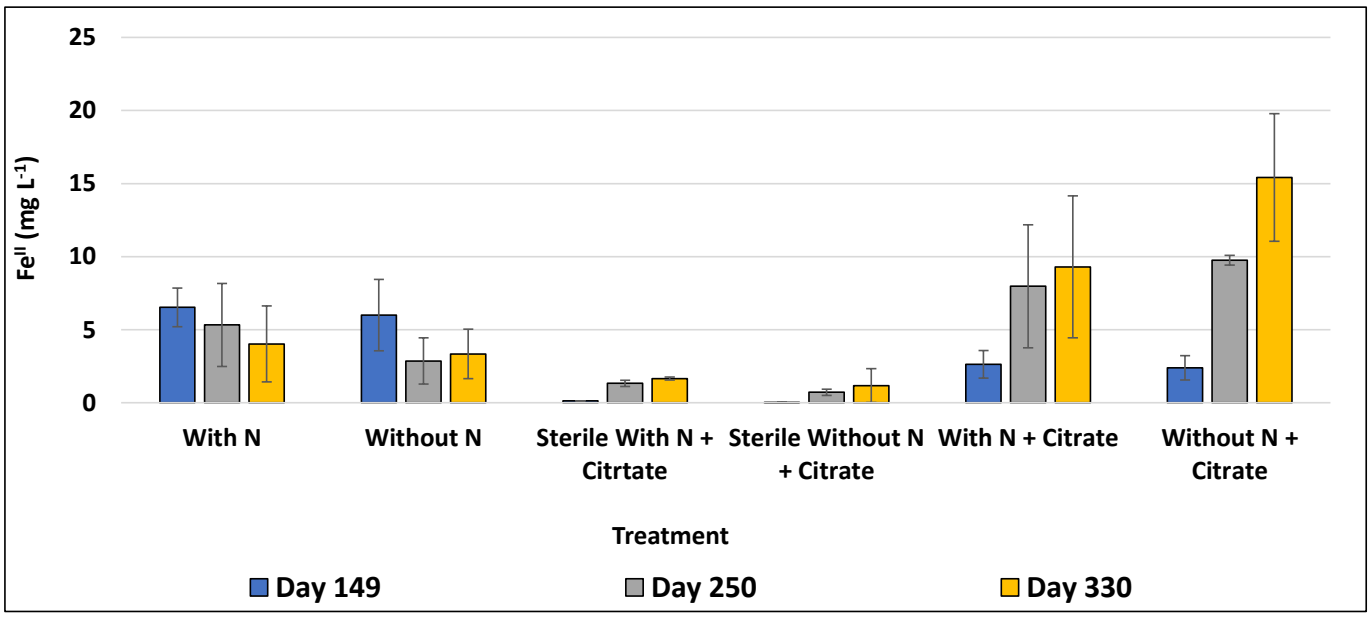

Figure 4-3: Fe(II) production in citrate amended Albian cultures with amorphous $Fe(OH)_3$. Values represent the mean µmol for triplicate amended cultures and duplicate sterile cultures. Baseline cultures with and without N sourced from **3.3.3 Fe(III) reduction** for comparison**.** Day 0 values subtracted from all timepoint measurements. Error bars indicate ± standard deviation. Data from Day 565 in **Figure S 24**.

Fe(III) reduction was concurrent with methanogenesis in this study. Typically, methanogenesis begins following the reduction of bioavailable Fe(III), however Reiche et al. (2008) have previously observed overlap in these activities. Following the addition of amorphous $Fe(OH)_3$ to peat microcosms, they observed both methane and Fe(II) production in parallel but methane production was inhibited to 54% as compared to controls without added $Fe(OH)_3$. When BES was added to selectively inhibit methanogenesis, the rate of Fe(II) formation dropped to 45% suggesting methanogens played a role in Fe(III) reduction. Their results suggested that fermenters performed most of the iron reduction while these parallel processes were at play and likely reduced Fe(III) while oxidizing H_2 or non-competitive substrates such as ethanol (Reiche et al. 2008). Another paper by Siddique et al., (2014) suggested that electrons may be inadvertently transferred to reduce Fe(III) during methanogenic substrate oxidation. Because citrate is an iron chelator and chelated Fe(III) stimulates iron reduction due to increased solubility (Lovley et al., 1996), in this study Fe(III) reduction was likely coupled to H_2, methane, or acetate oxidation in citrate amended cultures (Reiche et al., 2008; Segarra, 2012; Silva et al., 2009) and H_2 in unamended cultures (Lovley, 1993 and references therein).

4.3.2 Acetylene reduction in culture

An acetylene reduction assay was conducted to determine if N_2-fixation was occurring, the experiment was run over 27 days. Acetylene reduction to ethylene was only detected in live, N deficient cultures

(**Figure 4-4**). Cultures without N produced 0.15 ±0.02 µmol ethylene and cultures without N with added $Fe(OH)_3$ produced 0.18 ±0.03 µmol ethylene. Aerobic positive controls amended with labile carbon produced 10.6 ±10.1 µmol ethylene (**2.3.3 Acetylene reduction in culture**). In our previous study (Collins et al., 2016), we demonstrated N_2-fixation in citrate amended methanogenic cultures. Ethylene was produced more rapidly in that study; however, cultures were also more metabolically active. This was due to the culture composition, which contained 50% MFT (~ solids content of 12.5%) as compared to this study where cultures contained less than 0.7% solids. Microorganisms preferentially grow on surfaces (Dunne, 2002), therefore the low solids content in these cultures likely resulted in slower growth and lower metabolic rates. Nevertheless, the rate of acetylene reduction was similar to those observed previously in the presence of *n*-alkanes (**Chapter 2**, **Chapter 3**). This is interesting as the rate of methanogenesis was much higher in this study suggesting more N would be needed to support culture growth. However, it is possible most of the carbon is converted to CO_2 and methane rather than biomass.

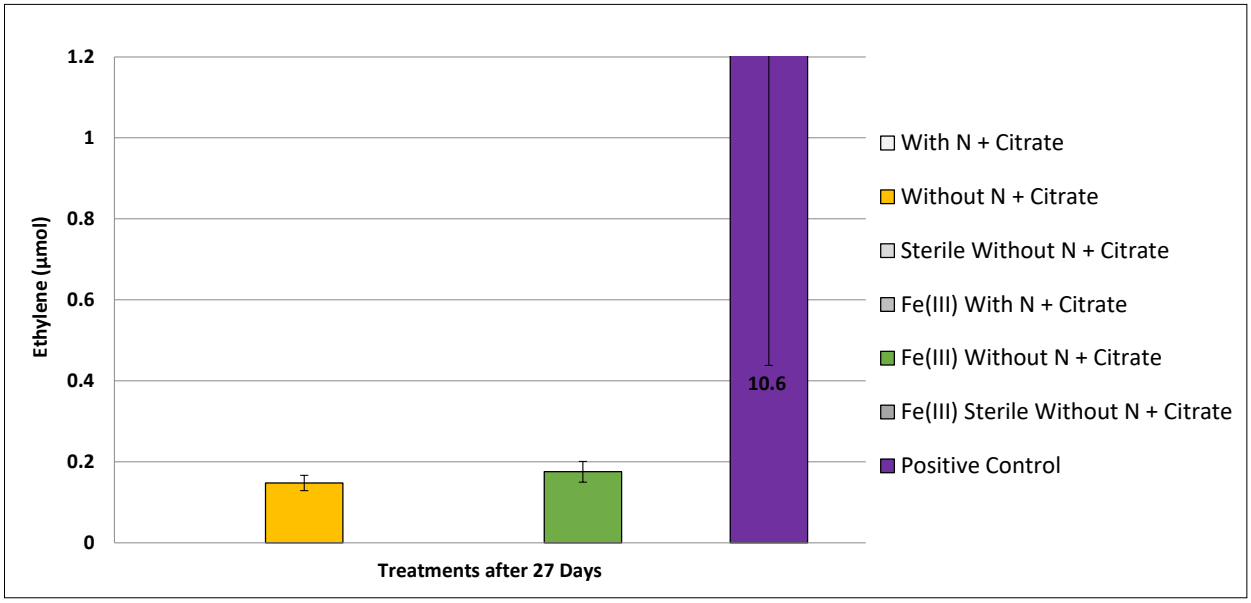

Figure 4-4: Ethylene production resulting from acetylene reduction in citrate amended Albian cultures. Values represent the mean µmol in triplicate for each treatment, and error bars indicate ± standard deviation.

4.3.4 Microbial community and function

Methanogenesis was predominantly supported by the acetoclastic population (*Methanosaeta*), as determined by 16S rRNA Illumina sequencing data (**Figure 4-5**). In general, unamended cultures were comprised of < 1.4% methanogenic archaea with the exception of $Fe(OH)_3$ treated unamended cultures with N, however these cultures did not produce methane over the course of the incubation suggesting sampling bias (**Chapter 3**). Methanogens made up 26% of cultures with N and 34% of cultures without N established under methanogenic conditions. A large proportion of citrate amended $Fe(OH)_3$ treated

cultures without N was also comprised of methanogenic archaea (42%) however, methanogens only made up 7% of the population in amended $Fe(OH)_3$ treated cultures with N. This was unexpected as amended iron cultures with N exhibited a higher rate of methanogenesis than amended iron cultures without N over the course of the incubation. This discrepancy may have been due to the potentially large number of *Trichococcus* in this treatment, which made up 64% of the total microbial population as compared to 6% in amended iron cultures without N.

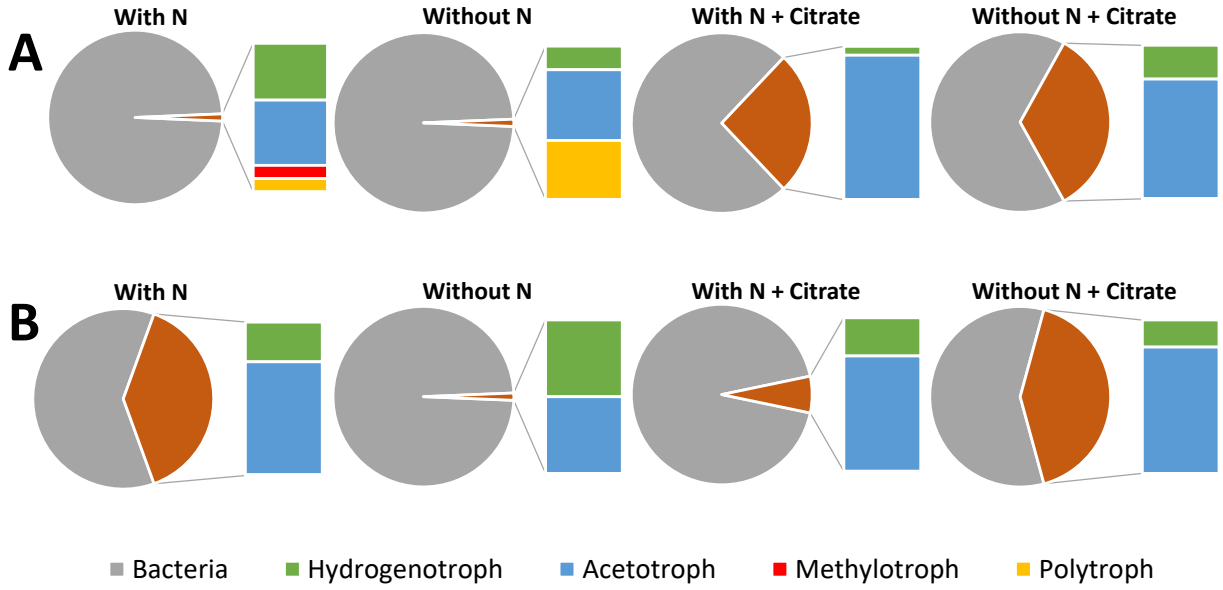

Figure 4-5: Distribution of Bacteria and methanogens in citrate amended Albian cultures **(A)** without $Fe(OH)_3$, **(B)** with $Fe(OH)_3$, based on Illumina sequencing of the 16S rRNA gene at day 334. Polytroph indicates methanogens capable of metabolizing hydrogen, acetate, and methanol to methane. Full Archaea dataset in **Figure S 25**. Baseline cultures with and without N sourced from **2.3.1 Methanogenesis** and **3.3.1 of methanogenesis** for comparison**.**

Citrate amended cultures with N in the presence and absence of $Fe(OH)_3$ were both dominated by *Trichococcus* (**Figure 4-6**), similar to previous observations (Collins et al., 2016; Li et al., 2019). This genus contains species capable of fermenting citrate to acetate (Stams et al., 2009), and likely supported the acetoclastic methanogens in these cultures. Amended methanogenic cultures with N also supported a higher population of *Clostridiaceae* than were present in $Fe(OH)_3$ treated cultures. These fermentative bacteria were dominant in the amended cultures without N where amorphous Fe(III) was not added. Amended $Fe(OH)_3$ treated cultures without N only contained a small proportion of bacteria capable of fermenting citrate (*Trichococcus* and *Clostridiaceae*), and a large population of *Rhodocyclaceae*. Species within this family have been found to reduce amorphous $Fe(OH)_3$ in conjunction with acetate and H_2 oxidation (Cummings et al., 1999; Thrash et al., 2010), research also suggests *Rhodocyclaceae* may also

be capable of DIET with methanogens (Inaba et al., 2019). The increased solubility and decreased reduction potential of the added $Fe(OH)_3$ in the presence of citrate may have promoted the growth of iron reducing bacteria, and facilitated DIET between *Rhodocyclaceae* and acetoclastic methanogens (Li et al., 2019). It is therefore likely the *Rhodocyclaceae* were oxidizing citrate degradation products such as acetate and producing Fe(II) in $Fe(OH)_3$ amended cultures. The low population of citrate fermenters and high population of acetate and H_2 oxidizing bacteria also likely resulted in substrate competition between methanogens and *Rhodocyclaceae*, thereby contributing to the lower rate of methane production observed in these cultures.

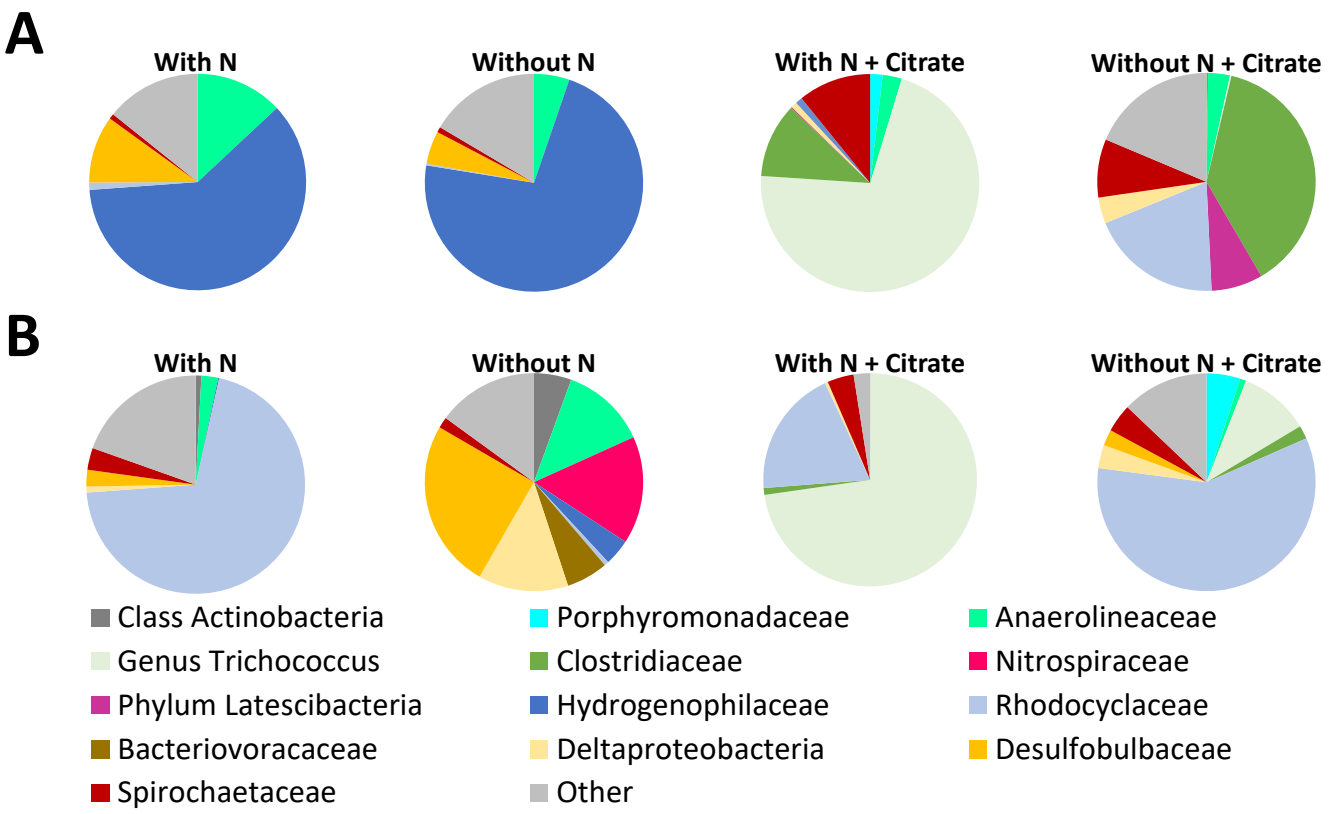

Figure 4-6: Distribution of Bacteria in citrate amended and unamended Albian cultures **(A)** without $Fe(OH)_3$, **(B)** with $Fe(OH)_3$, based on Illumina sequencing of the 16S rRNA gene at day 334. Baseline cultures with and without N sourced from **2.3.4 Microbial community and function** and **3.3.5 Microbial community and function** for comparison**.**

Acetylene reduction was performed by the N_2-fixng community (**Figure 4-7**). In citrate amended cultures that did not contain $Fe(OH)_3$, *Methanosaetaceae* (89-100% ident.) was present in high proportions dominating cultures with N and comprising over a third of the *nifH* genes present in cultures without N. Cultures with N contained a small proportion of *Clostridiaceae* (90-99% ident.), the majority of which were a match for *Youngiibacter* (99% ident.). Cultures without N contained small proportion of *Rhodocyclaceae*

(*Propionivibrio*; 99% ident.) and a third of the community were Betaproteobacteria. While this Betaproteobacteria group most closely matched *Burkholderiaceae* and *Hydrogenophilales* (98% ident.), the absence of these bacteria in 16S rRNA gene data suggests this Betaproteobacteria group likely belonged to another species of *Rhodocyclaceae.* Most transcription products in cultures without N were of the family *Methanosaetaceae*. In our 2016 study (Collins et al., 2016), we hypothesized that the archaeal population was not responsible for N_2-fixation. This was because the proportion of archaea were similar between amended treatments however, we've shown here that the transcription and community function can operate independently of gene abundance in oil sands tailings communities. This observation was reinforced in **Chapter 2** where Albian *n*-alkane cultures contained primarily *nifH* genes from *Rhodocyclaceae* but transcription products were primarily from *Methanosaetaceae*.

In iron treatments, both cultures with and without N contained predominantly genes from *Rhodocyclaceae*. A larger proportion of the *nifH* genes in cultures with N were *Methanosaetaceae* as compared to cultures without N, which contained a higher percentage of *nifH* genes from Deltaproteobacteria. Despite this, transcribed genes in iron cultures without N were predominantly *Methanosaetaceae* similar to citrate amended cultures without $Fe(OH)_3$ and hydrocarbon amended cultures discussed in **Chapter 2**. This contrasts with hydrocarbon amended iron reducing cultures in **Chapter 3**, where expression was dominated by Proteobacteria. These data indicate that the N_2-fixing community is largely the same in citrate amended Albian cultures regardless of added $Fe(OH)_3$ and supports the argument that citrate chelated the Fe(III) in these cultures allowing the development of a methanogenic community with the capacity for N_2-fixation. No transcription was detected in treatments with N, which supported the absence of acetylene reduction in these cultures.

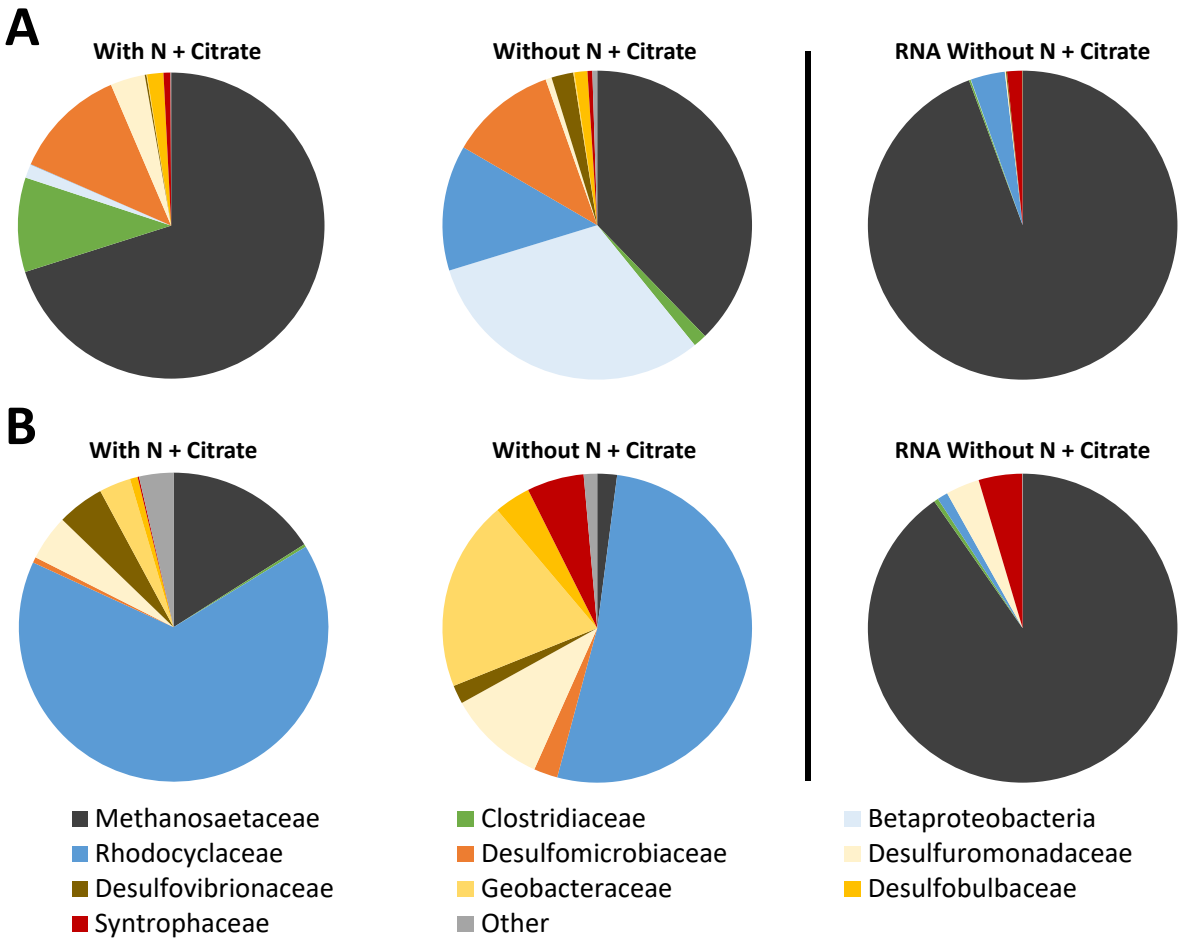

Figure 4-7: *NifH* gene abundance in citrate amended Albian cultures **(A)** without $Fe(OH)_3$, **(B)** with $Fe(OH)_3$, based on Illumina sequencing and BLASTx match of *nifH* gene at day 334. "RNA" indicates sequenced cDNA reverse transcribed from extracted RNA. Sequences (OTUs) that returned fewer than 100 reads were discarded, OTUs with no match or off-target match were discarded. The presence and expression of *nifH* and genes coding for other anaerobic N transformations from all live treatments are shown in **Figure S 26-29.**

4.4 Conclusions

This results of this study suggested that methanogenesis could be supported by the presence of N_2-fixing activity, and that methane production is not inhibited in the presence of $Fe(OH)_3$ in combination with citrate. This was likely due to the chelating effect of citrate, which decreased the reducing potential of $Fe(OH)_3$ and the potential to accept electrons. This change state likely allowed iron reducing bacteria to engage in DIET with methanogens while oxidizing H_2 and acetate, thereby promoting methanogenesis. N_2-fixing activity was performed by methanogenic archaea as observed in Chapter 2 suggesting the effect of $Fe(OH)_3$ on the key players in the microbial community had been largely negated by citrate. The results of this study indicate that iron amendments will not be effective at treating methanogenic sites in tailings ponds if tailings streams contain citrate-based treatments, even under N limited conditions.

4.5 References

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5. Summary, Conclusions, and Future Research

Oil sands tailings contain a diverse microbial community (Penner and Foght, 2010). This community is capable of degrading hydrocarbons and organic molecules to produce methane. Currently, all oil sands tailings ponds are methanogenic. Some ponds, such as Albian's Muskeg River tailings pond are capable of producing methane in the absence of readily available N in the tailings slurry indicating N requirements are being met through other means (Collins et al., 2016; Li, 2010). This poses a challenge for reclamation where tailings materials are used to establish wetlands and end-pit lakes. The microbial community is transferred to these wet landscapes where metabolic activity continues, even under nutrient poor conditions. This may cause mineral dissolution and the release of salts, heavy metals, and other environmentally hazardous contaminants into the cap water. In addition to reclamation challenges, methane is also a potent greenhouse gas currently rated at 1:25 CO_2e (canada.ca). To combat climate change, the Canadian government has a mandate to reduce methane emissions by 45% before the year 2025 (capp.ca). We previously observed N_2-fixation concurrent with methane production when Albian tailings cultures were amended with citrate, a labile organic dispersant used in bitumen extraction (Collins et al., 2016), however hydrocarbons from unrecovered diluent and bitumen are the primary organic ligands in tailings (Small et al., 2015). The predisposition to N_2-fixation in our previous study also suggested that N_2 was present in oil sands tailings despite the small amount of mixing between atmosphere and the lower layers of mature fine tailings (MFT).

The goal of this research was to investigate biogenic methane emissions from hydrocarbon degradation in oil sands tailings pond bacteria in the absence of organic and inorganic N such as NO_2^-/NO_3^- and NH_4^+, and to investigate if biogenic methane emissions could be mitigated. This research includes an examination of the microbial community using both 16S rRNA genes as well as functional genes for anaerobic N-cycling processes and their transcription products. To date, no publications exist examining the genetic potential and expression of N transformation genes in oil sands tailings.

5.1 Research Summary

5.1.1 Methanogenesis

Methane production was monitored in culture headspaces to assess the effect of organic amendments, hydrocarbons and citrate, and the addition of amorphous $Fe(OH)_3$ on methanogenesis. Results indicated that a higher rate of methane production was present in amended cultures as opposed to unamended cultures supporting previous findings where metabolism of organic amendments supported

methanogenesis (Collins et al., 2016; Gieg et al., 2008; Jones et al., 2008; Mohamad Shahimin et al., 2016; Siddique et al., 2015, 2011, 2006; Zengler et al., 1999). However, the addition of amorphous iron inhibited methanogenesis in hydrocarbon amended cultures in Chapter 3. This was likely due to a combination of direct inhibition through interactions between iron and proteins in the cell membranes of methanogenic archaea (Van Bodegom et al., 2004), and indirect inhibition through substrate competition with iron reducing bacteria such as *Geobacteraceae* and *Peptococcaceae*. The addition of citrate in Chapter 4 likely chelated the $Fe(OH)_3$ and negated the inhibitory effects of the Fe(III) by decreasing the reducing potential (electron accepting potential) of the iron such that it was more energetically favourable for bacteria such as *Rhodocyclaceae* to engage in direct interspecies electron transfer (DIET) with methanogens than to reduce Fe(III) (Inaba et al., 2019). This is supported by the absence of *Geobacteraceae* and abundance of *Rhodocyclaceae* in the microbial community of cultures with both $Fe(OH)_3$ and citrate.

5.1.2 Hydrocarbon degradation

Hydrocarbon concentrations were measured in amended cultures to determine if degradation was occurring. We found that both *n*-alkanes and toluene degradation occurred under methanogenic conditions in Chapter 2 however, only toluene degradation was observed under iron reducing conditions in Chapter 3. Toluene degradation in cultures with $Fe(OH)_3$ also occurred at a slower rate as compared to Chapter 2, and was only observed in some cultures. This was due to iron induced changes in the microbial community.

5.1.3 Nitrogen Fixation

Nitrogen fixation was evaluated using an acetylene reduction assay whereby acetylene is reduced to ethylene by the nitrogenase enzyme. Genes coding for the nitrogenase enzyme (*nifH*) and *nifH* expression products were also sequenced to determine which member of the microbial community were capable of N_2-fixation, and which microorganisms were responsible for the observed activity. We found evidence of nitrogenase activity and *nifH* gene expression in cultures deficient in bioavailable N (NO_2^-/NO_3^- and NH_4^+) in conjunction with sustained microbial metabolism in the form of methanogenesis, hydrocarbon degradation or iron reduction. Interestingly, *nifH* expression was detected in Albian and CNRL cultures with N where $Fe(OH)_3$ was added and methanogenesis was inhibited. This may have been due to attempts to normalize DNA concentrations at the sequencing facility, which may have resulted in a decrease in DNA concentration and sequence reads from cultures without N and a comparative increase in cultures with N. However, this unanticipated phenomenon was only present in Chapter 3 where $Fe(OH)_3$ was added

and no chelator was present. Iron is an important component of the nitrogenase enzyme and has been found to play a role in gene regulation, therefore the presence of unbound iron in these cultures may have increased gene transcription (Rosconi et al., 2006). Despite the presence of gene expression in cultures with N, these cultures did not show evidence of N_2-fixing activity. These data supported our hypothesis that N_2-fixation was supporting the microbial community in N deficient cultures.

Interestingly, the proportions of microorganisms with the *nifH* gene (DNA) was vastly disproportionate to those actively expressing the gene as mRNA. Coding genes (DNA) originated predominantly from proteobacteria in nearly all amended cultures whereas gene expression (RNA) indicated the dominant N_2-fixing microorganisms in methane producing cultures were the methanogenic archaea *Methanosaeta*. Inducing iron reducing conditions changed the key players in the microbial community and proteobacteria took over the role of fixing N_2 however, the community proportions still showed disparity between coding genes and expressed genes. These data contrast our previous hypotheses where we attributed N_2-fixing function to members of the microbial community based on abundance in the 16S rRNA data (Collins et al., 2016), and suggests that regulation of gene expression is affected by factors in the culture environment.

5.1.4 Conclusions

The results of this study suggest that $Fe(OH)_3$ could feasibly be used as an effective treatment to mitigate methane emissions in oil sands tailings ponds while potentially maintaining aromatic hydrocarbon degrading activity under N limited conditions, provided process water does not contain high enough concentrations of citrate to chelate the $Fe(OH)_3$ added to ponds. Based on the low cost of amorphous Fe(III), this approach merits further investigation as a tailings amendment.

5.2 Research Applications

It was generally assumed that microbial metabolism would cease under nutrient deficient conditions however, the results of these studies suggest that the microbial community present in tailings will continue to metabolize hydrocarbons and produce methane in the absence of bioavailable N. This information is relevant to tailings ponds that have become depleted in bioavailable N due to microbial metabolism, and wet reclamation landscapes such as end pit lakes and wetlands that incorporate N deficient tailings materials. The reasoning for this is twofold:

1. Methane a powerful greenhouse gas and current government regulations call for a 45% reduction in greenhouse gas below 2012 levels by 2025.
2. Continued microbial metabolism can cause mineral dissolution, thereby releasing contaminants and residual bitumen to the cap water where plants and animals can be affected.

Results from the iron addition studies suggest that $Fe(OH)_3$ and likely other iron oxides found in rust can be added to tailings to inhibit methanogenesis. Following further study in a more field applicable setting, rust may prove to be a cost-effective tailings treatment to reduce methane emissions where bubbling is observed in legacy tailings ponds, and in reclaimed landscapes that incorporate tailings materials. However, application limitations must be considered before use in the field. Iron (III) is reduced to Fe(II) in this process, and Fe(II) can auto oxidation in the presence of oxygen and generate free radicals that can cleave polyacrylamide (PAM) into acrylamide. Therefore Fe(III) should not be added to tailings containing PAM. In the case of wetlands, Fe(II) can become toxic to plants and animals at high concentrations, therefore the dosage must be carefully considered and Fe(II) concentrations in the water should be monitored with treatment. Moreover, all hydrocarbons were not degraded in the presence of $Fe(OH)_3$. This suggests $Fe(OH)_3$ should not be used as a permanent solution since the Fe(III) will eventually all be reduced to Fe(II) and methanogenesis will resume and persist until all the remaining hydrocarbons are degraded. For permanent inhibition of methanogenesis, other forms of amorphous Fe(III) should be investigated to determine if complete hydrocarbon degradation can be achieved.

Should iron be selected as an amendment for the treatment of tailings materials, the results from these studies suggest that Fe(III) should not be used on tailings containing citrate. Citrate is capable of chelating Fe(III) and can potentially promote methanogenesis in the presence of other organics such as hydrocarbons. As such, tailings material should be analyzed before Fe(III) is utilized.

5.3 Research Limitations

The primary limitation to this research was that all experiments were conducted in cultures at the bench scale. By conducting all experiments under ideal conditions (room temperature, adding nutrients through media, small volumes, etc.), the data produced will not be directly applicable at scale. The intention of these studies was to provide preliminary and proof of concept data to test our hypotheses and not to directly inform industry practices. Studies must first be conducted outdoors, at larger scales, and without addition of nutrients before applications such as addition of $Fe(OH)_3$ can be trialed at pilot scale.

Cultures were also highly dilute containing less than 1% solids. This is not representative of *in-situ* conditions and the low solids content caused some bias in the culture data. In this case, we saw increased metabolic activity in cultures without N, likely due to the presence of aggregate forming N_2-fixing microorganisms. While we were not expecting higher metabolic activity in N deficient cultures, it did not interfere with our ability to test our primary hypotheses; 1) N_2-fixation could support methanogenesis and hydrocarbon degradation, 2) $Fe(OH)_3$ could inhibit methanogenesis, and 3) the chelating effect of citrate may change the interactions between $Fe(OH)_3$ and methanogens.

Other limitations included number of replicates and tailings storage. Only 2 or 3 replicates were used in culture studies depending on the treatment. This cased the data from some treatments to have high variability. Use of 5 replicates would have been ideal as data from outliers could have been removed. Regardless, statistical significance was still achieved between many treatments and allowed for meaningful conclusions. Tailings storage was also non-optimal in this study as tailings were stored at 4˚C for several months before use however, this is common for research labs as tailings can be difficult to obtain and can only be collected fresh during the field season (May-September) due to operational and climate based limitations. Moreover, tailings are a dynamic environment for the microbial population, which is continually changing due to pond influx streams and changes in temperature. As such, the effect from this storage period was likely minimal and would not have interfered with a proof of concept study where the microbial population would change as a result of culture conditions regardless.

5.4 Future Research

This thesis demonstrated that N cycling processes occur in oil sands tailings and that N_2-fixation can support methane production and hydrocarbon degradation. Moreover, we demonstrated that amorphous Fe(III) can be used to inhibit methanogenesis by shifting the microbial community. However, these processes are not sufficiently well understood to apply to practice. Therefore, we suggest further examination of iron as a methane inhibiting treatment, to determine if a complete N cycle is possible in bulk MFT, and to further examine the inhibition of methanogenesis under iron reducing conditions in tailings.

Future research should include a culture study examining and comparing the three major constituents of rust, Fe_2O_3, FeOOH, and $Fe(OH)_3$, both independently and the three in combination as iron oxide. Because different microbial groups may respond differently to each oxide, we may find that one oxide is more effective at inhibiting methanogenesis than another. Examining the oxides independently would allow individual evaluation of the efficacy of inhibition of methanogenesis. This process would need to be

conducted using a range of concentrations on bulk MFT to determine the minimum quantity of iron oxide to effectively inhibit methanogenesis. Microbial community sequencing would also give more insight into how the process is mediated in oil sands tailings communities under the effect of different iron oxides. Because systems can behave unexpectedly in combination, rust as a complete iron oxide should also be evaluated. This would account for unpredicted abiotic or biotic reactions that may cause undesirable changes in the community or unforeseen increases in methane production. Maintaining hydrocarbon degradation under these conditions is also desirable and should be examined alongside methane production. From this proposed study, we would gain better understanding of how rust can be utilized to inhibit methanogenesis as a cost-effective tailings amendment.

In this study, we discuss the presence of N_2-fixation and evaluated the genetic the potential for other anaerobic N transformations as a source for N_2 in-situ (**S. Supplementary Materials**). An abiotic process known as Feammox may also play a role driving the regeneration of NO_2^-/NO_3^- and completing the anaerobic N cycle. In this process, ammonium is oxidized by Fe(III) under anaerobic conditions to form N_2, nitrate, or nitrite (Sawayama, 2006). This process is mediated by Actinobacteria (*Acidimicrobiaceae*; Huang and Jaffé, 2015), which may be present in these cultures. However, we have not confirmed that bacteria capable of this process exist in our system. Current research on Feammox and the bacteria responsible for those reactions is limited and new developments are published regularly. Current and future primers should be used to determine if the known Feammox bacteria, *Acidimicrobiaceae* A6, are present in oil sands tailings. If found, stable isotope incorporation into enrichment cultures would allow tracking of $^{15}NH_4$ to $^{15}NO_2^-/NO_3^-$, $^{15}N_2O$, and $^{15}N_2$ in the tailings community to assess the potential for a complete anaerobic N cycle in oil sands tailings. The potential for anaerobic N cycling in oil sands tailings communities may explain the presumed origin of N_2 in tailings ponds and therefore, the predisposition to N_2-fixation. This is also important for better understanding of GHG emissions from tailings. For example, some oil sands operators add ammonia-based treatments to the bitumen processing streams, the resulting tailings ponds contain high concentrations of NH_4^+. The presence of Feammox reactions in these ponds could result in higher concentrations of NO_2^-/NO_3^- in these tailings, which could in turn be reduced to N_2O, a GHG 298 times more potent than CO_2 (canada.ca). Therefore, this knowledge can directly inform current practice with the end goal of reducing GHGs to curve climate change.

Finally, determining the mechanism by which methane is inhibited by iron would be beneficial to our understanding of iron as a tailings treatment and iron cycling in tailings. To do this, I propose a study whereby bulk MFT cultures are established containing toluene and amorphous Fe(III). Similar to the study by Van Bodegom et al. (2004), treatments would include acetate and H_2, but the experiment would

examine the tailings communities from several operators rather than pure cultures. Monitoring methane, Fe(II), toluene and acetate, and sequencing the microbial community would provide insight into the type of inhibition observed in tailings cultures. For example, if methane is produced in the presence of acetate, substrate competition may be responsible for the inhibition whereas inhibition and an increase in *Methanosarcina* suggests conversion from methanogenesis to iron reduction. Obtaining a better understanding of the mechanism of inhibition in oil sands tailings communities would benefit the field of study and help inform potential decisions regarding iron as a tailings amendment.

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S. Supplementary Materials

Table S 1: Nitrogen content in sterile "without N" primary enrichment cultures containing 50:50 culture media and MFT. Liquid represents liquid fraction, solids represent whole culture containing both liquid and solid phase.

Primary Enrichment Cultures "Without N"				
Liquid (mg/L)	**NH_4^+**	**NO_3^-**	**Total N**	**Total N Solids (%wt)**
CNRL	0	0	1.05 ±0.006	0.055 ±0.002
Syncrude	2.76 ±0.081	0	10.19 ±0.07	0.056 ±0.003
A2013	2.09 ±0.008	0	88.97 ±0.47	0.163 ±0.004

Table S 2: Number of reads used to calculate community composition following data processing in Chapter 2 as outlined in the methods section.

	Total Good Reads *n*-alkane Culture Experiment				
Albian	***nifH***	***nirS***	***nosZ***	***nrfA***	**16S rRNA**
With N	1522	8154	2324	311	2591
Without N	2729	7581	425	1046	2027
With N + C5,C6	4215	6525	538	8975	2949
RNA With N + C5,C6	-	-	-	-	-
Without N + C5,C6	1768	5737	3895	9463	2142
RNA Without N + C5,C6	1750	69	-	-	-
CNRL Day 0					
With N	2202	312	1118	838	2268
Without N	2113	3390	2344	606	1508
With N + C7,C8,C10	1333	-	-	-	4695
Without N + C7,C8,C10	3731	577	229	496	5196
CNRL					
With N	2623	3310	1625	320	1962
Without N	1728	3720	904	204	1676
With N + C7,C8,C10	3019	4595	2334	6614	3402
RNA With N + C7,C8,C10	-	240	-	-	-
Without N + C7,C8,C10	4418	4593	1262	3556	3488
RNA Without N + C7,C8,C10	107	208	-	-	-
	Total Good Reads Toluene Culture Experiment				
CNRL	***nifH***	***nirS***	***nosZ***	***nrfA***	**16S rRNA**
With N	1887	970	261	722	1610
Without N	3124	2008	1757	1015	3659
With N + C7H8	2192	3378	2088	3437	2007
RNA With N + C7H8	-	-	-	-	-
Without N + C7H8	2262	3311	1760	400	4698
RNA Without N + C7H8	57	141	-	-	-

Table S 3: Total inorganic carbon (TIC) and non-purgeable organic carbon (NPOC) in Albian and CNRL iron amended n-alkane cultures from Chapter 3. Sterile CNRL culture without N sample was lost.

Treatment	Total Inorganic carbon (mg L^{-1})				Non-Purgeable Organic Carbon (mg L^{-1})			
Albian	Day 0	Stdev	Day 520	Stdev	Day 0	Stdev	Day 520	Stdev
With N	60.77	0.69	64.12	0.28	1.44	0.08	1.60	0.08
Without N	63.67	2.09	69.12	1.60	1.63	0.10	1.85	0.04
Sterile C_5C_6 With N	55.55	7.15	55.72	14.32	1.33	0.27	1.45	0.34
Sterile C_5C_6 Without N	59.13	1.52	53.53	5.67	2.74	1.47	1.77	0.06
C_5C_6 With N	57.82	1.29	66.61	1.46	1.25	0.17	1.95	0.15
C_5C_6 Without N	62.75	2.85	67.95	2.67	1.77	0.48	2.24	0.60
CNRL								
With N	67.26	0.76	53.97	1.45	1.57	0.04	2.83	0.24
Without N	65.48	2.12	56.67	0.41	1.81	0.01	2.62	0.00
Sterile $C_7C_8C_{10}$ Without N	59.33	6.99	63.14	4.19	1.96	0.02	2.05	0.05
$C_7C_8C_{10}$ With N	68.45	1.38	53.90	2.29	1.62	0.05	2.47	0.05
$C_7C_8C_{10}$ Without N	64.94	4.54	53.79	2.83	1.97	0.06	5.07	2.66

Table S 4: Number of reads used to calculate community composition following data processing in Chapter 3 as outlined in the methods section.

	Total Good Reads *n*-alkane Culture Experiment				
Albian	***nifH***	***nirS***	***nosZ***	***nrfA***	**16S rRNA**
With N	1050	2071	959	353	3123
Without N	1170	447	856	468	312
With N + C5,C6	1675	1741	913	2655	184
RNA With N + C5,C6	95	73	-	-	-
Without N + C5,C6	2770	159	4429	703	1456
RNA Without N + C5,C6	36	-	-	-	-
	Total Good Reads Toluene Culture Experiment				
Albian	***nifH***	***nirS***	***nosZ***	***nrfA***	**16S rRNA**
With N	717	37		108	1333
Without N	1583	-	-	-	1487
With N + C7H8	1512	108	78	100	3698
RNA With N + C7H8	46	68	-	-	-
Without N + C7H8	4174	1323	336	1151	3258
RNA Without N + C7H8	44	-	-	-	-
Syncrude					
With N	164	74	196	-	4192
Without N	252	59	94	-	8454
With N + C7H8	3139	2331	392	-	10078
RNA With N + C7H8	-	-	-	-	-
Without N + C7H8	119	-	34	333	3836
RNA Without N + C7H8	40	-	-	-	-
CNRL					
With N	184	153	39	116	3290
Without N	10	-	-	-	2508
With N + C7H8	141	534	1967	245	5734
RNA With N + C7H8	154	55	-	-	-
Without N + C7H8	371	52	-	-	4771
RNA Without N + C7H8	87	146	-	-	-

Table S 5: Number of reads used to calculate community composition following data processing in Chapter 4 as outlined in the methods section.

	Total Good Reads Citrate Culture Experiment				
No Fe(III)	***nifH***	***nirS***	***nosZ***	***nrfA***	**16S rRNA**
With N + Citrate	4488	363	60	823	6630
RNA With N + Citrate	-	-	-	-	-
Without N + Citrate	3752	3455	76	2298	4526
RNA Without N + Citrate	6564	-	-	-	-
	Total Good Reads Citrate with Fe(III) Culture Experiment				
With Fe(III)	***nifH***	***nirS***	***nosZ***	***nrfA***	**16S rRNA**
With N + Citrate	1363	2830	956	4716	2890
RNA With N + Citrate	-	-	-	-	-
Without N + Citrate	923	1750	1464	463	2088
RNA Without N + Citrate	2244	-	-	-	-

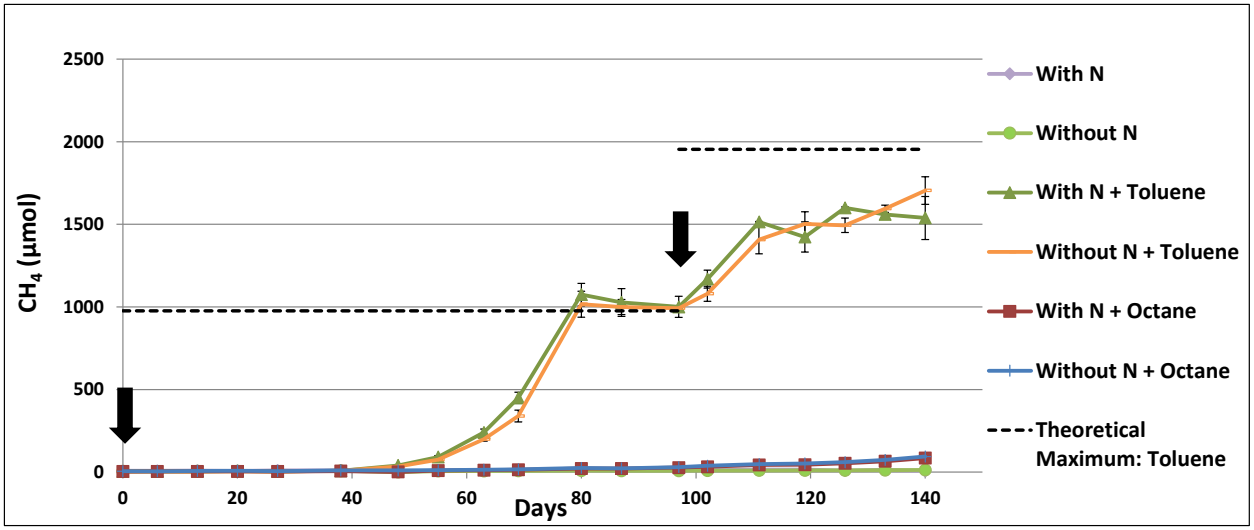

Figure S 1: Methane production in primary enrichment cultures. Error bars represent standard deviation from three culture replicates in live amended cultures and two culture replicates in sterile and unamended baseline controls. Arrows represent repeat amendments with 200 mg L^{-1} toluene or n-octane.

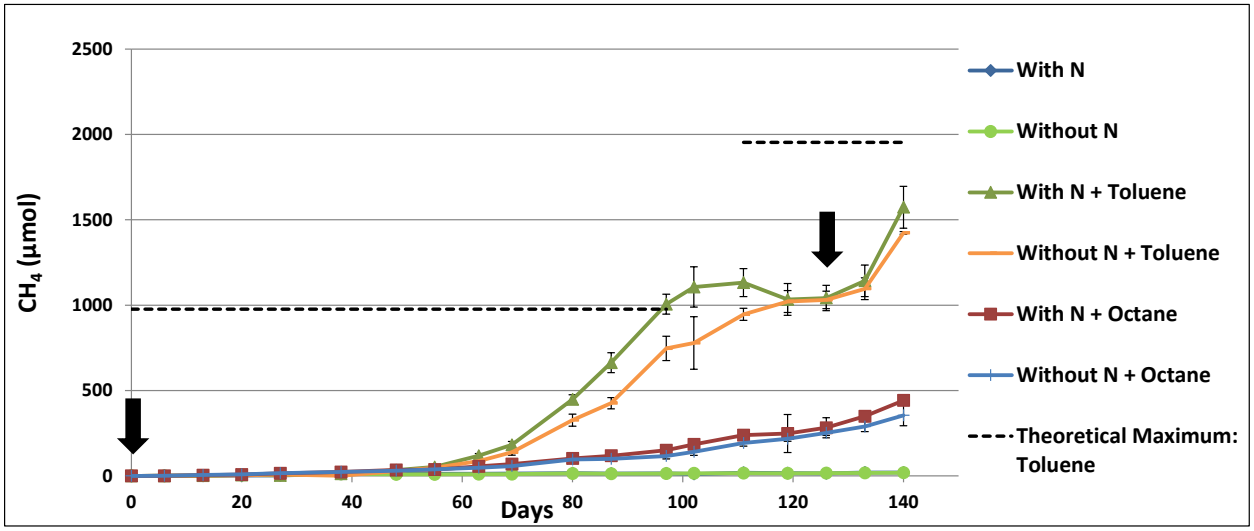

Figure S 2: Methane production in primary enrichment cultures. Error bars represent standard deviation from three culture replicates in live amended cultures and two culture replicates in sterile and unamended baseline controls. Arrows represent repeat amendments with 200 mg L^{-1} toluene or n-octane.

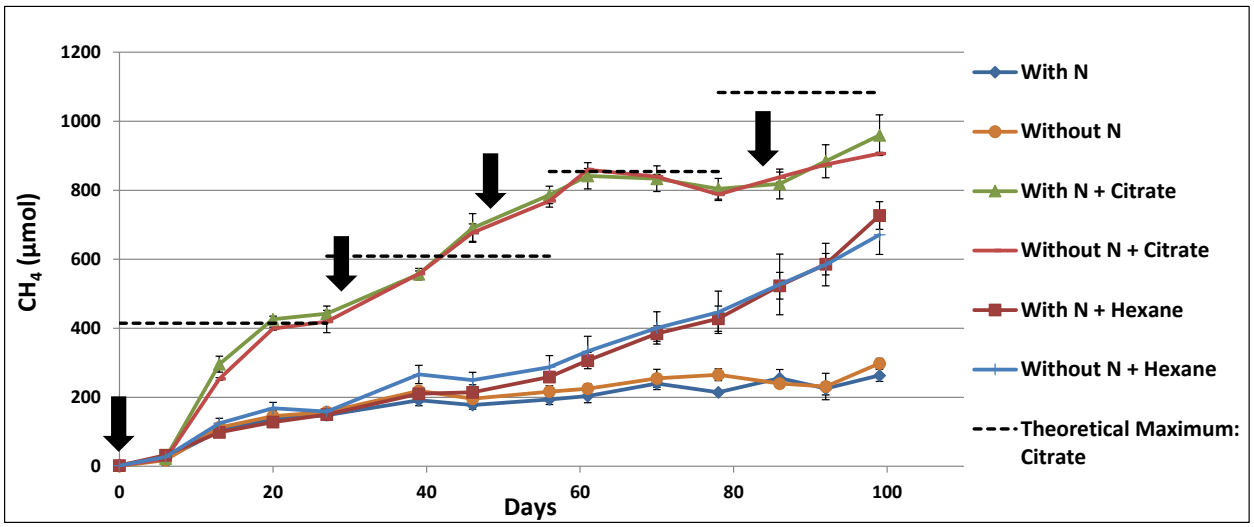

Figure S 3: Methane production in primary enrichment cultures. Error bars represent standard deviation from three culture replicates in live amended cultures and two culture replicates in sterile and unamended baseline controls. Arrows represent repeat amendments with 200 mg L^{-1} n-hexane or citrate.

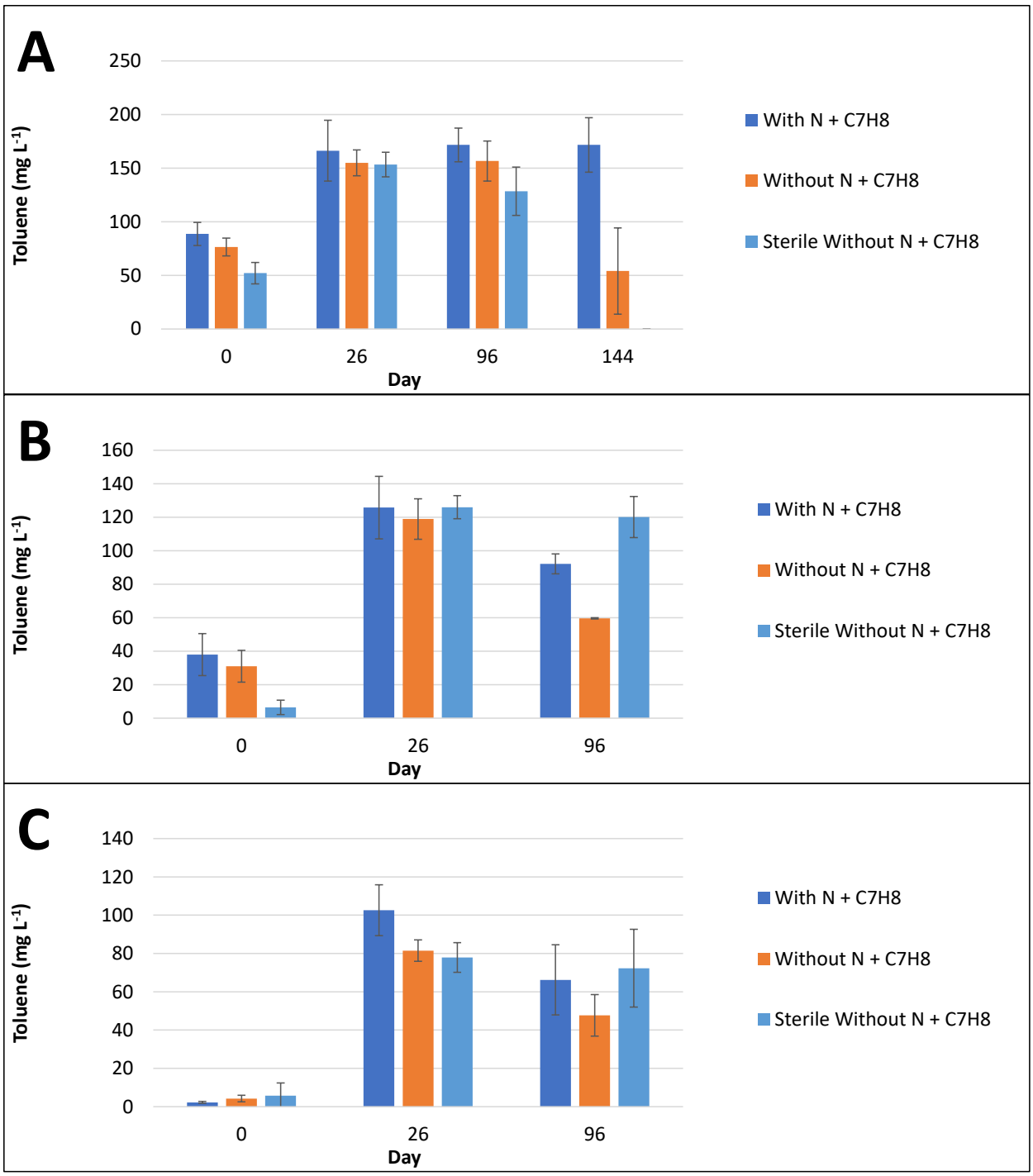

Figure S 4: Headspace toluene concentration in toluene amended cultures as measured in **A**) Albian, **B**) Syncrude, and **C**) CNRL. Day 0 results were low in all headspace analyses, this was due to performing headspace analysis on day 0 following amendments before phase partitioning equilibrium could be reached, as such, only a very low concentration of toluene could volatilize into the headspace. Values represent the mean mg L^{-1} for triplicate amended cultures and duplicate sterile and baseline cultures. Error bars indicate ± standard deviation.

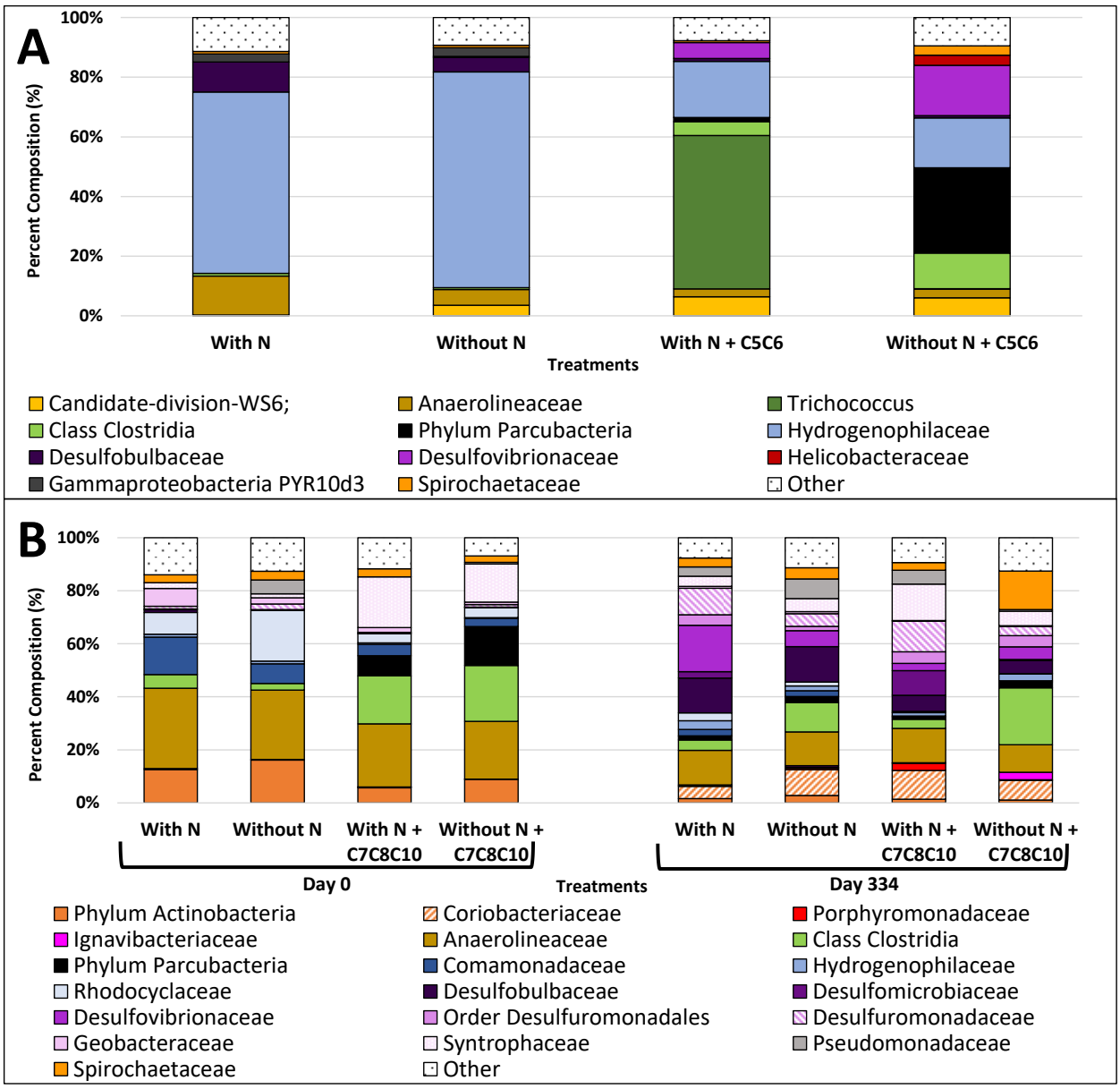

Figure S 5: Bacterial community composition of n-alkane amended culture experiment based on Illumina sequencing of the 16S rRNA gene at (**A**) day 334 from Albian cultures, and (**B**) CNRL cultures day 0 and day 334. Unidentified Bacteria sequences and sequences with less than 2% abundance are grouped as "Other".

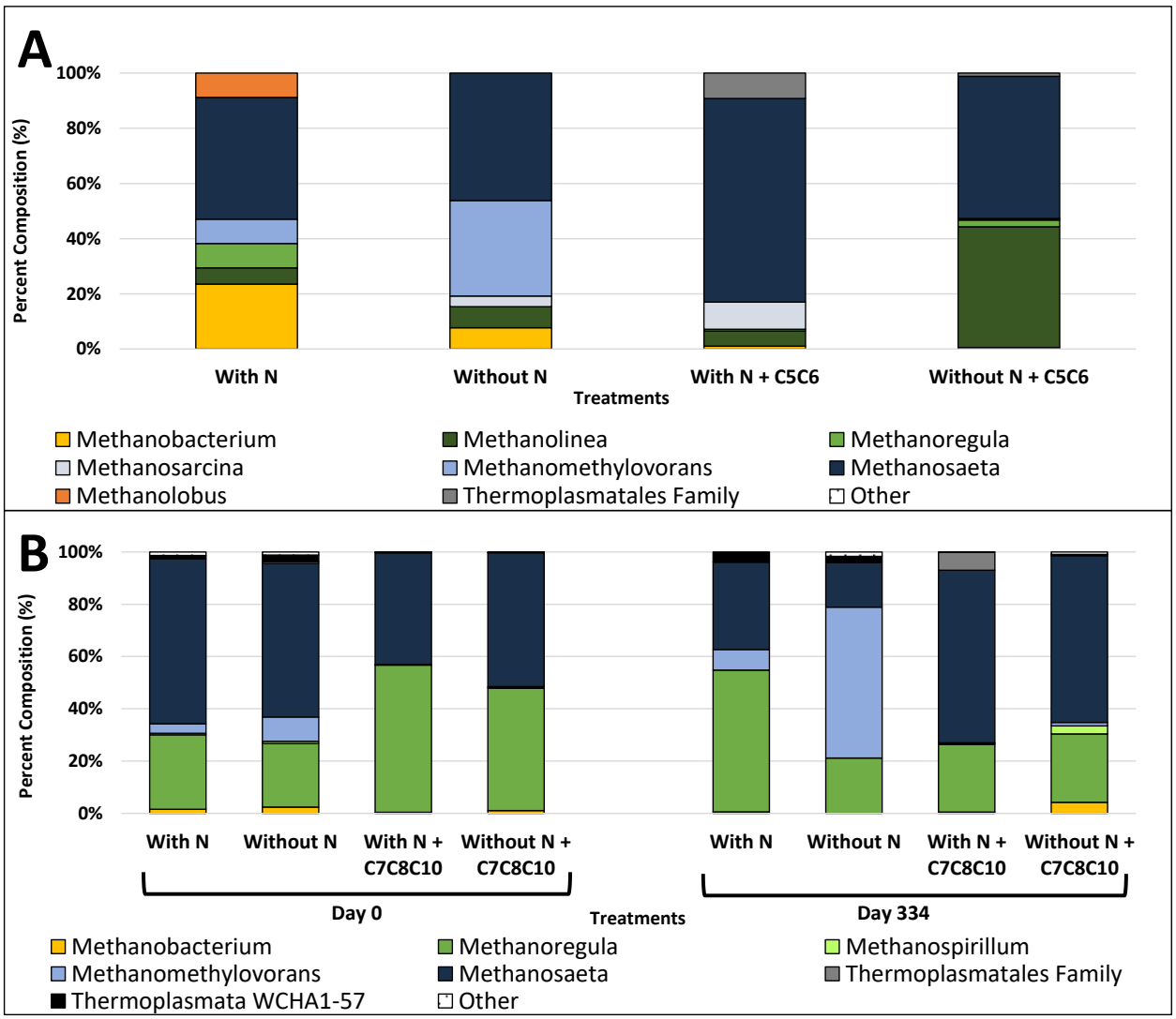

Figure S 6: Archaeal community composition of n-alkane amended culture experiment based on Illumina sequencing of the 16S rRNA gene at (**A**) day 334 from Albian cultures, and (**B**) CNRL cultures day 0 and day 334. Unidentified Archaea sequences and sequences with less than 2% abundance are grouped as "Other".

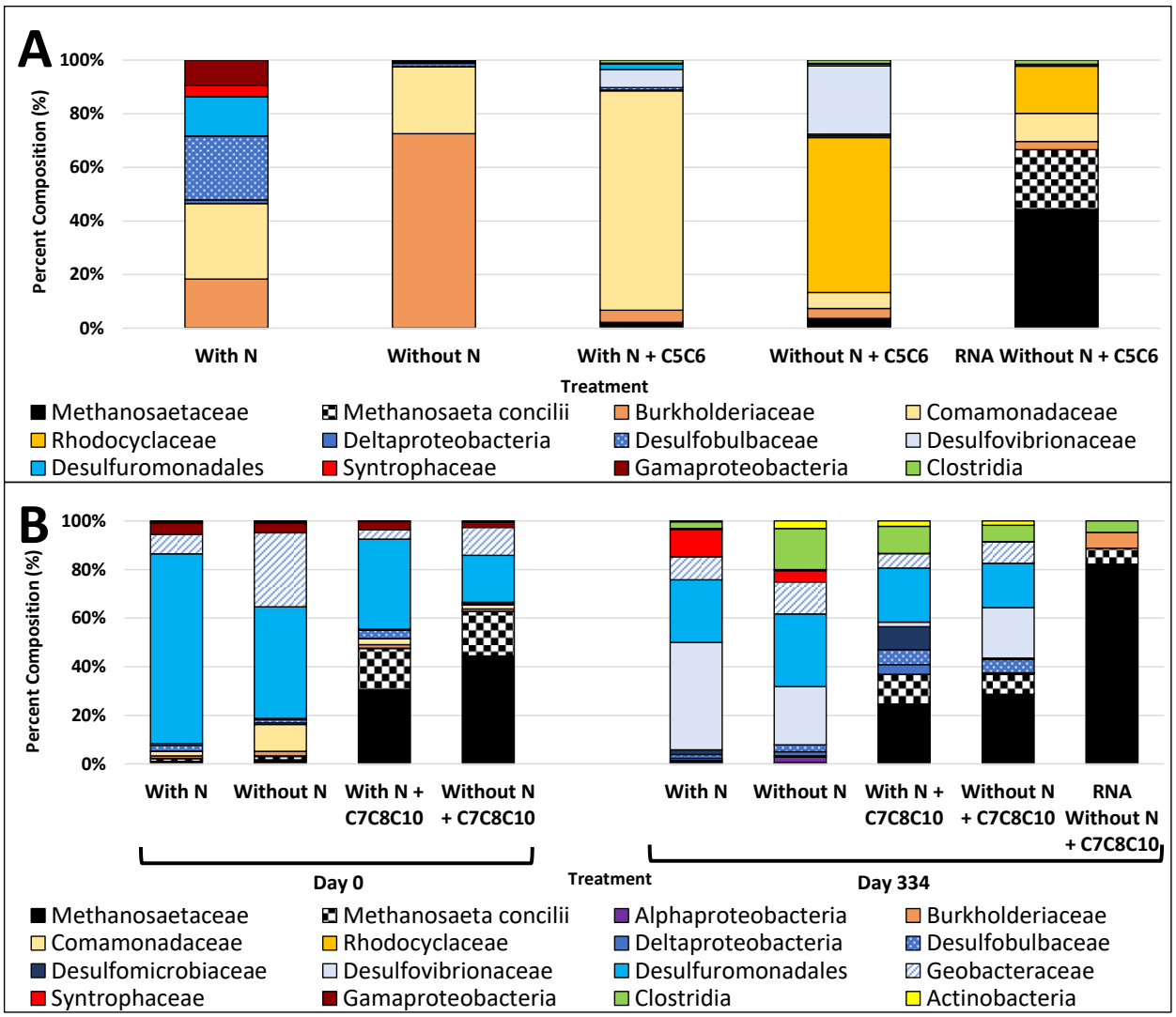

Figure S 7*: NifH* gene abundance of *n*-alkane amended culture experiment based on Illumina sequencing and BLASTx match of *nifH* gene at (**A**) day 334 from Albian cultures, and (**B**) day 0 and day 334 from CNRL cultures. "RNA" indicates sequenced cDNA reverse transcribed from extracted RNA from the respective treatment. Treatments or reverse transcribed RNA that returned no reads or less than 50 reads were discarded, OTUs with no match or off-target match were discarded.

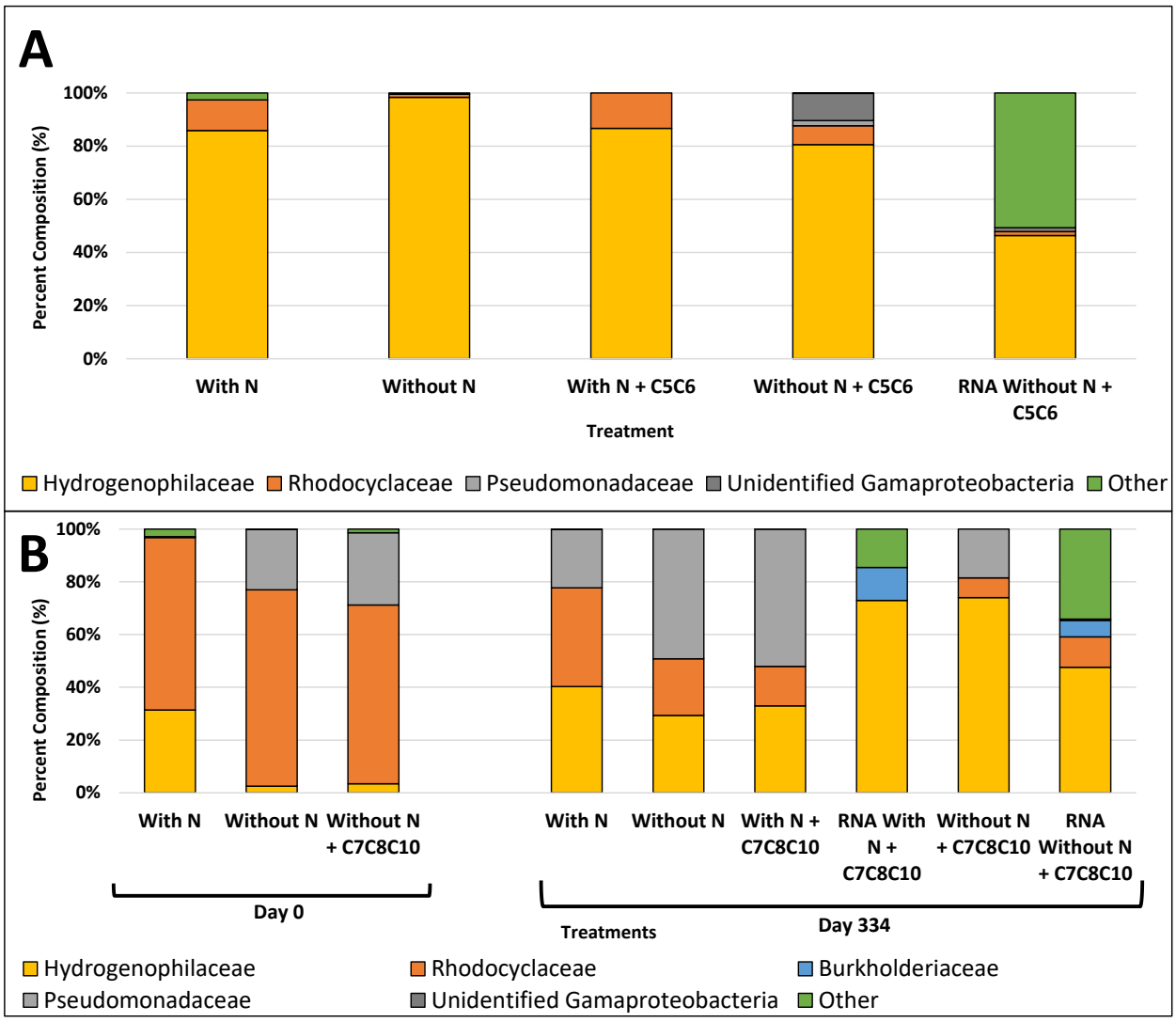

Figure S 8: *NirS* gene abundance of *n*-alkane amended culture experiment based on Illumina sequencing and BLASTx match of *nirS* gene at (**A**) day 334 from Albian cultures, and (**B**) day 0 and day 334 from CNRL cultures. "RNA" indicates sequenced cDNA reverse transcribed from extracted RNA from the respective treatment. "Other" represents bacteria with ambiguous identity, less than 90% ident. match, or groups present at less than 2%. Treatments or reverse transcribed RNA that returned no reads or less than 50 reads were discarded, OTUs with no match or off-target match were discarded.

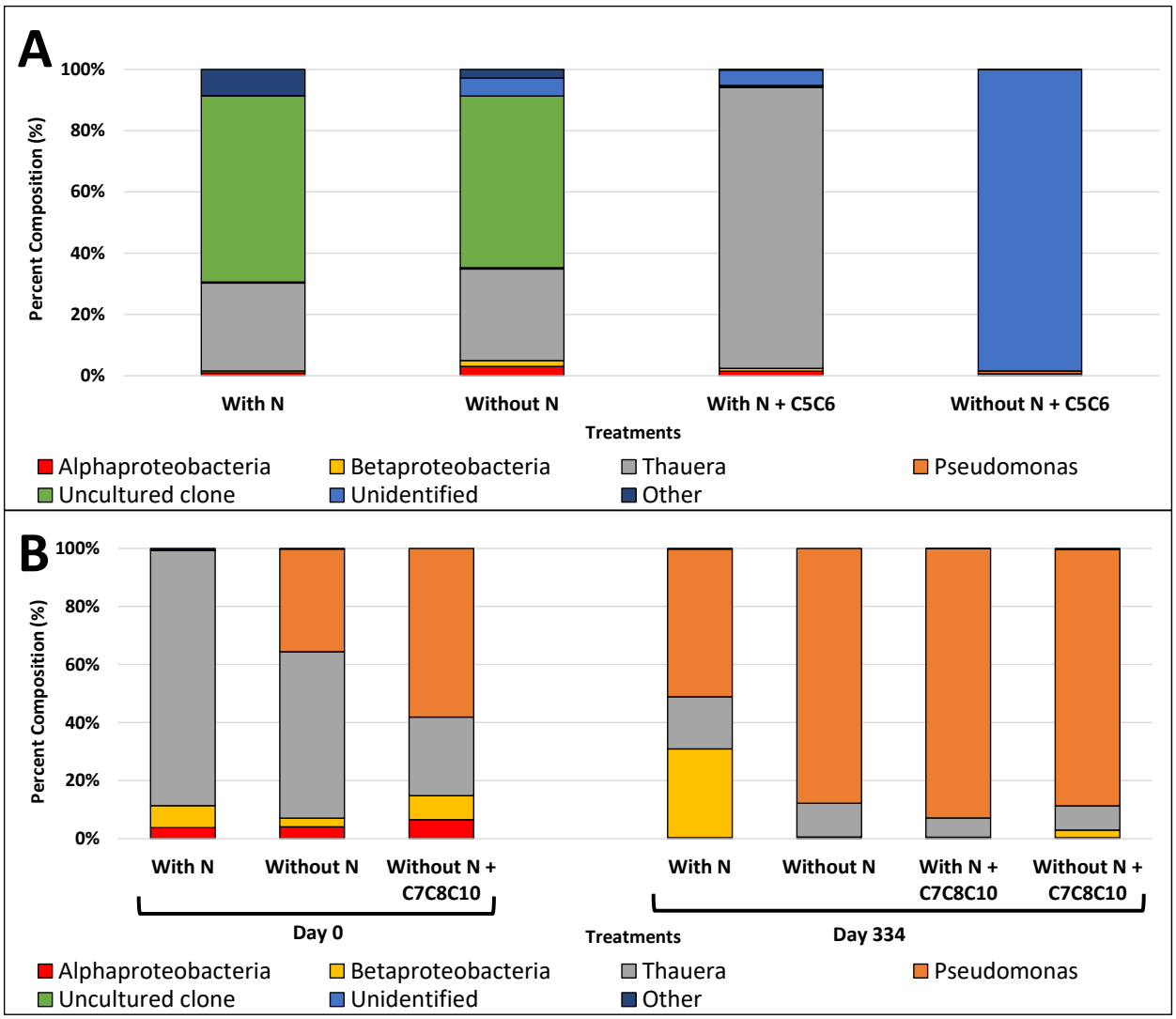

Figure S 9: *NosZ* gene abundance of *n*-alkane amended culture experiment based on Illumina sequencing and BLASTx match of *nosZ* gene at (**A**) day 334 from Albian cultures, and (**B**) day 0 and day 334 from CNRL cultures. "RNA" indicates sequenced cDNA reverse transcribed from extracted RNA from the respective treatment. "Other" represents bacteria with ambiguous identity, less than 95% ident. match, or groups present at less than 2%. Treatments or reverse transcribed RNA that returned no reads or less than 50 reads were discarded, OTUs with no match or off-target match were discarded.

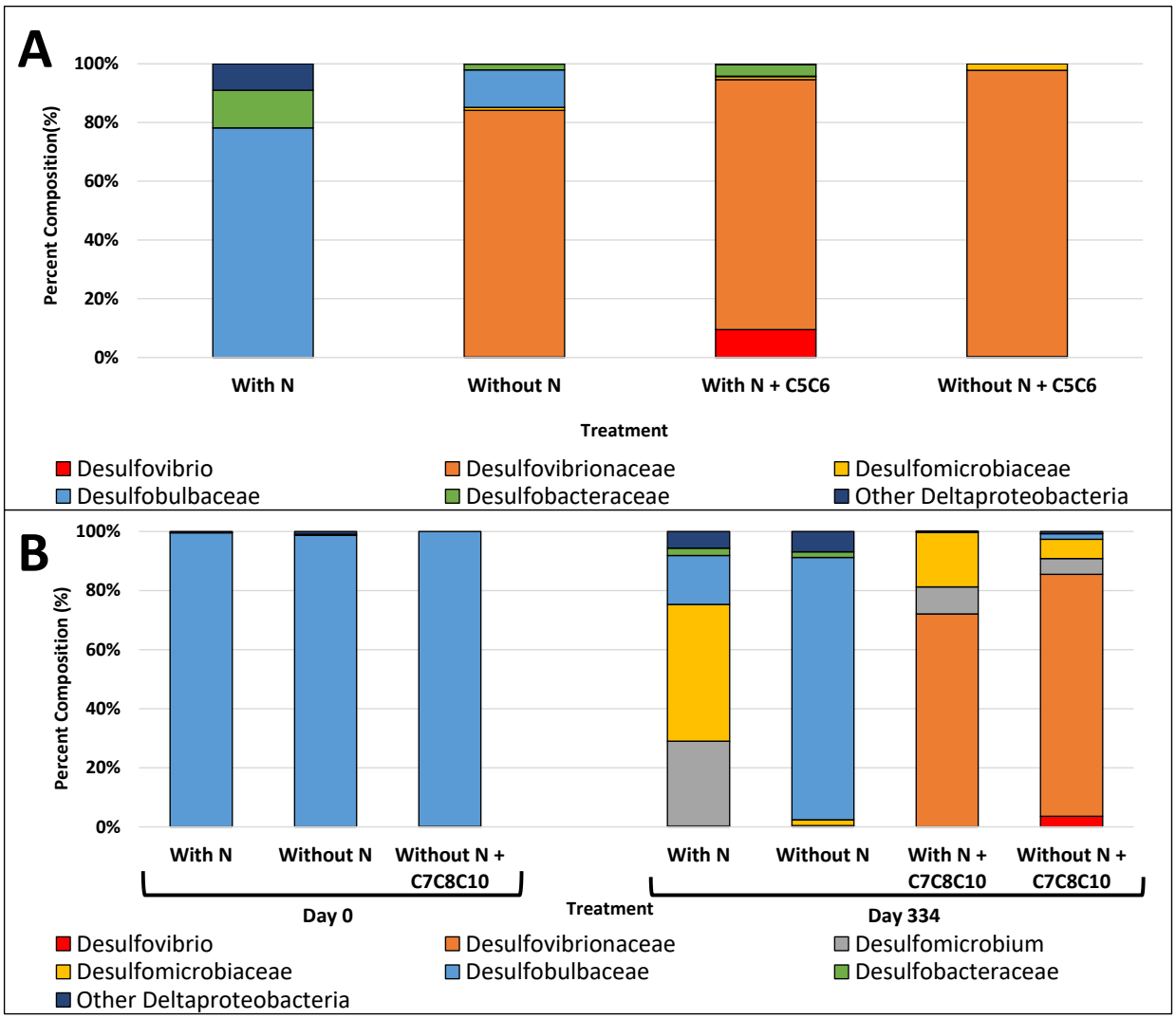

Figure S 10: *NrfA* gene abundance of *n*-alkane amended culture experiment based on Illumina sequencing and BLASTx match of *nrfA* gene at (**A**) day 334 from Albian cultures, and (**B**) day 0 and day 334 from CNRL cultures. "Other Deltaproteobacteria" represents Deltaproteobacteria bacteria with ambiguous identity, less than 74% ident. match, or Deltaproteobacteria OTUs present at less than 2%. Treatments or reverse transcribed RNA that returned no reads or less than 50 reads were discarded, OTUs with no match, or off-target match were discarded.

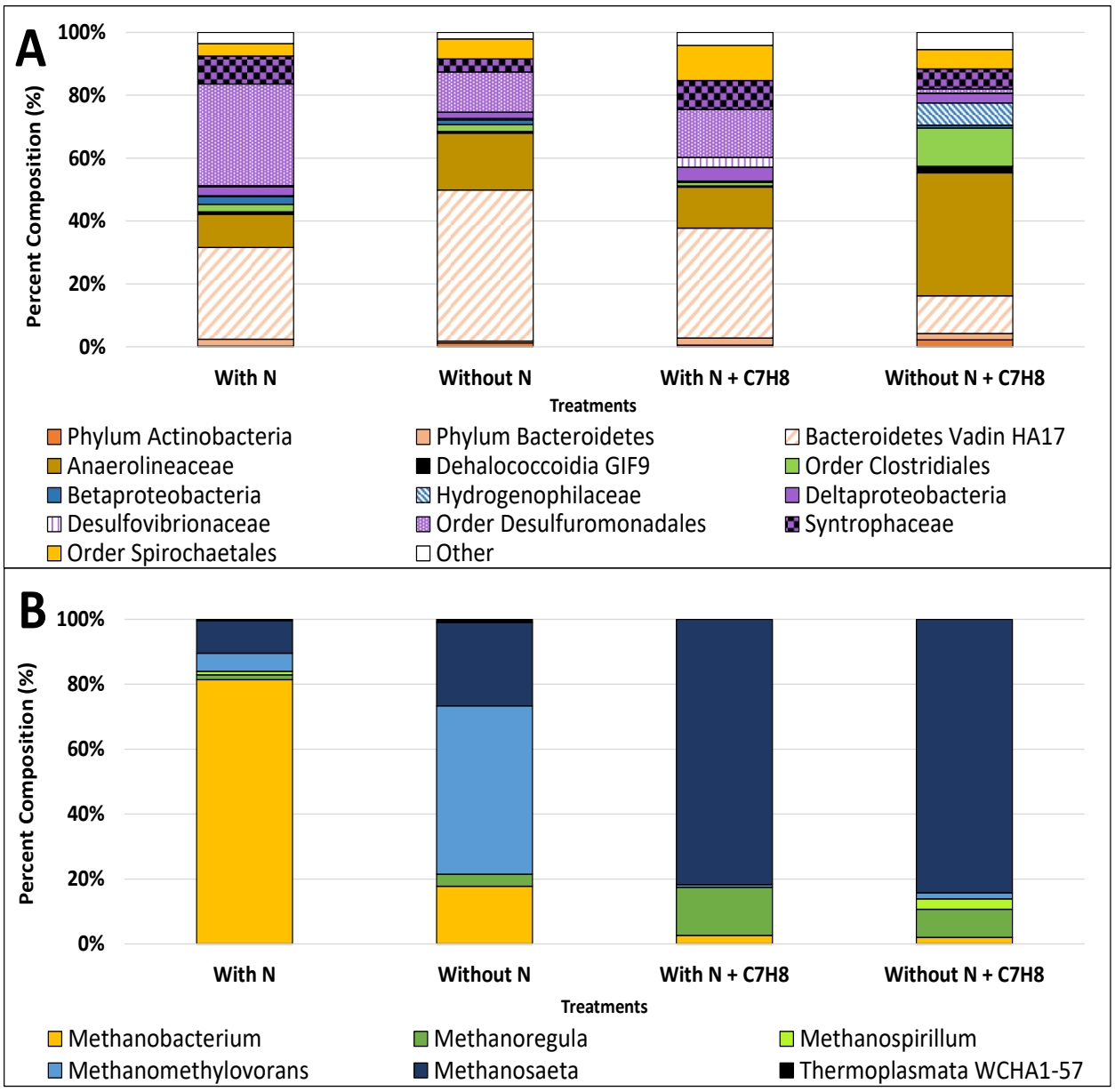

Figure S 11: (**A**) Bacterial and (**B**) Archaeal community composition of toluene amended CNRL culture experiment based on Illumina sequencing of the 16S rRNA gene at day 189. Unidentified Bacteria or Archaea sequences and sequences with less than 2% abundance are grouped as "Other".

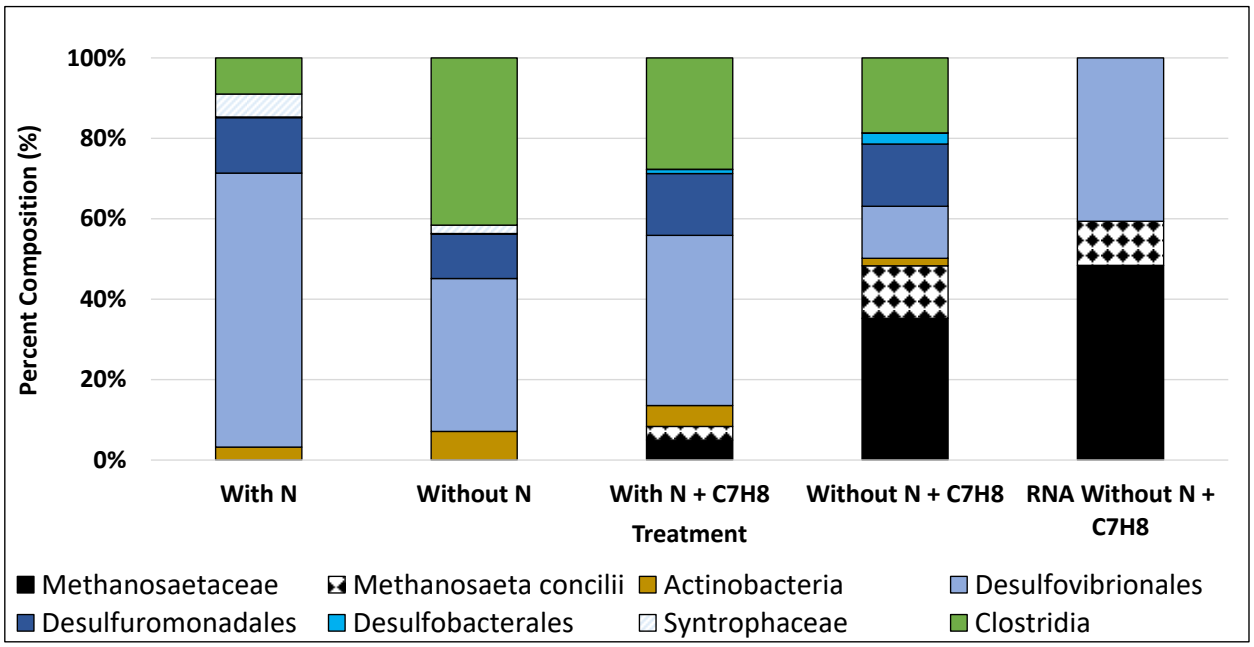

Figure S 12: *NifH* gene abundance of toluene amended CNRL culture experiment based on Illumina sequencing and BLASTx match of *nifH* gene at day 189. "RNA" indicates sequenced cDNA reverse transcribed from extracted RNA from the respective treatment. Treatments or reverse transcribed RNA that returned no reads or less than 50 reads were discarded, OTUs with no match or off-target match were discarded.

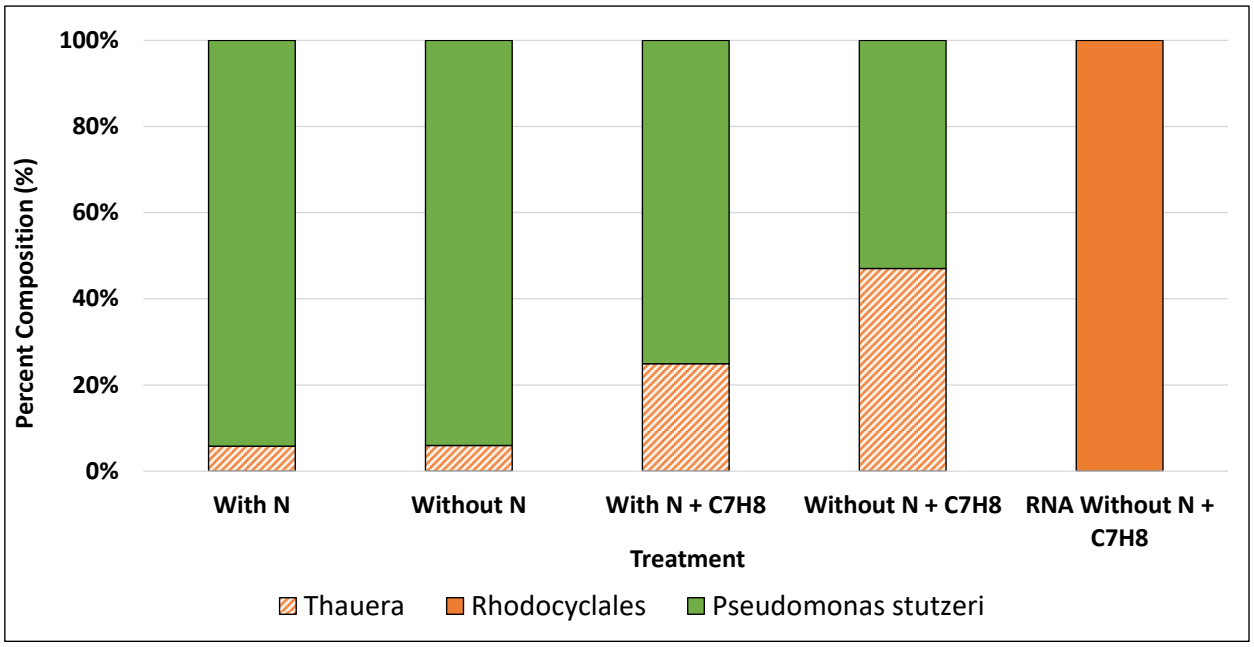

Figure S 13: *NirS* gene abundance of toluene amended CNRL culture experiments based on Illumina sequencing and BLASTx match of *nirS* gene at day 189. "RNA" indicates sequenced cDNA reverse transcribed from extracted RNA from the respective treatment. Treatments or reverse transcribed RNA that returned no reads or less than 50 reads were discarded, OTUs with no match or off-target match were discarded.

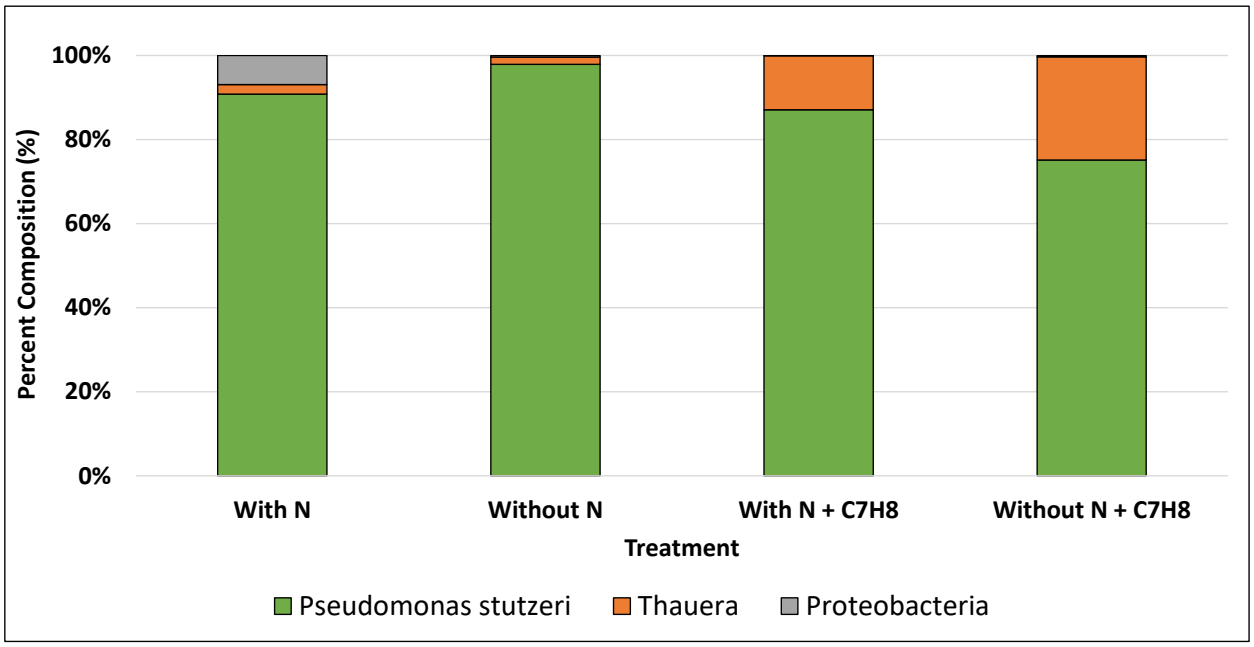

Figure S 14*: NosZ* gene abundance of toluene amended CNRL culture experiment based on Illumina sequencing and BLASTx match of *nosZ* gene at day 189. Treatments or reverse transcribed RNA that returned no reads or less than 50 reads were discarded, OTUs with no match or off-target match were discarded.

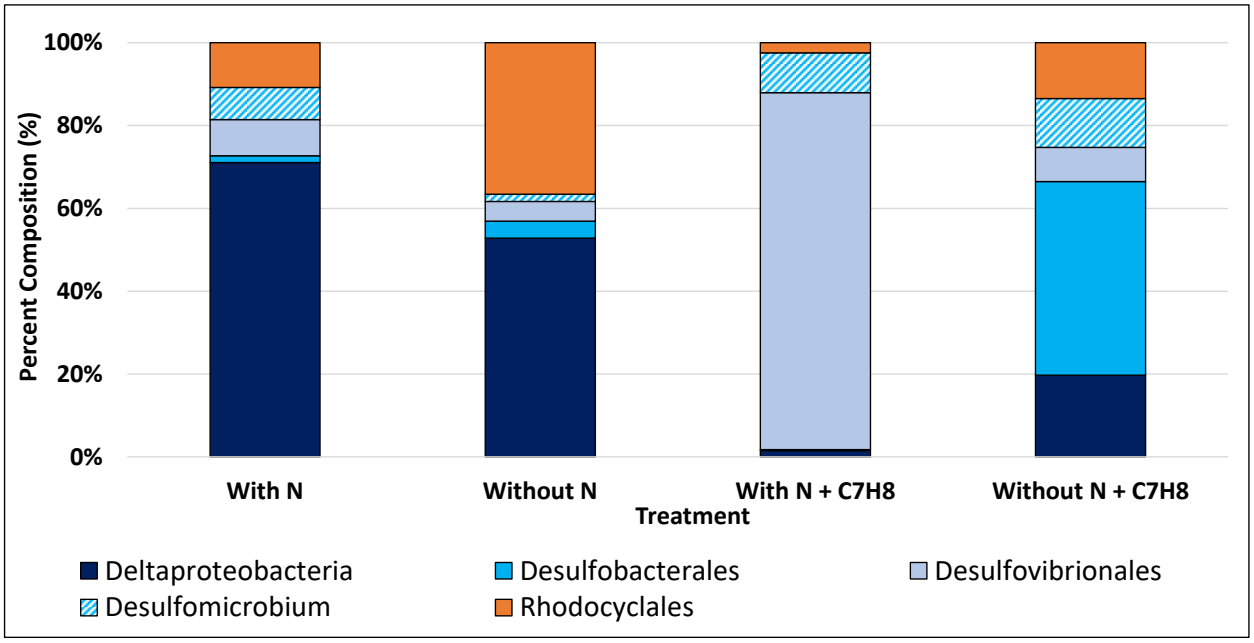

Figure S 15: *NrfA* gene abundance of toluene amended CNRL culture experiment based on Illumina sequencing and BLASTx match of *nrfA* gene at day 189. Deltaproteobacteria consists of all ambiguous Deltaproteobacteria OTUs and OTUs with less than 80% ident. Deltaproteobacteria. Treatments or reverse transcribed RNA that returned no reads or less than 50 reads were discarded, OTUs with no match or off-target match were discarded.

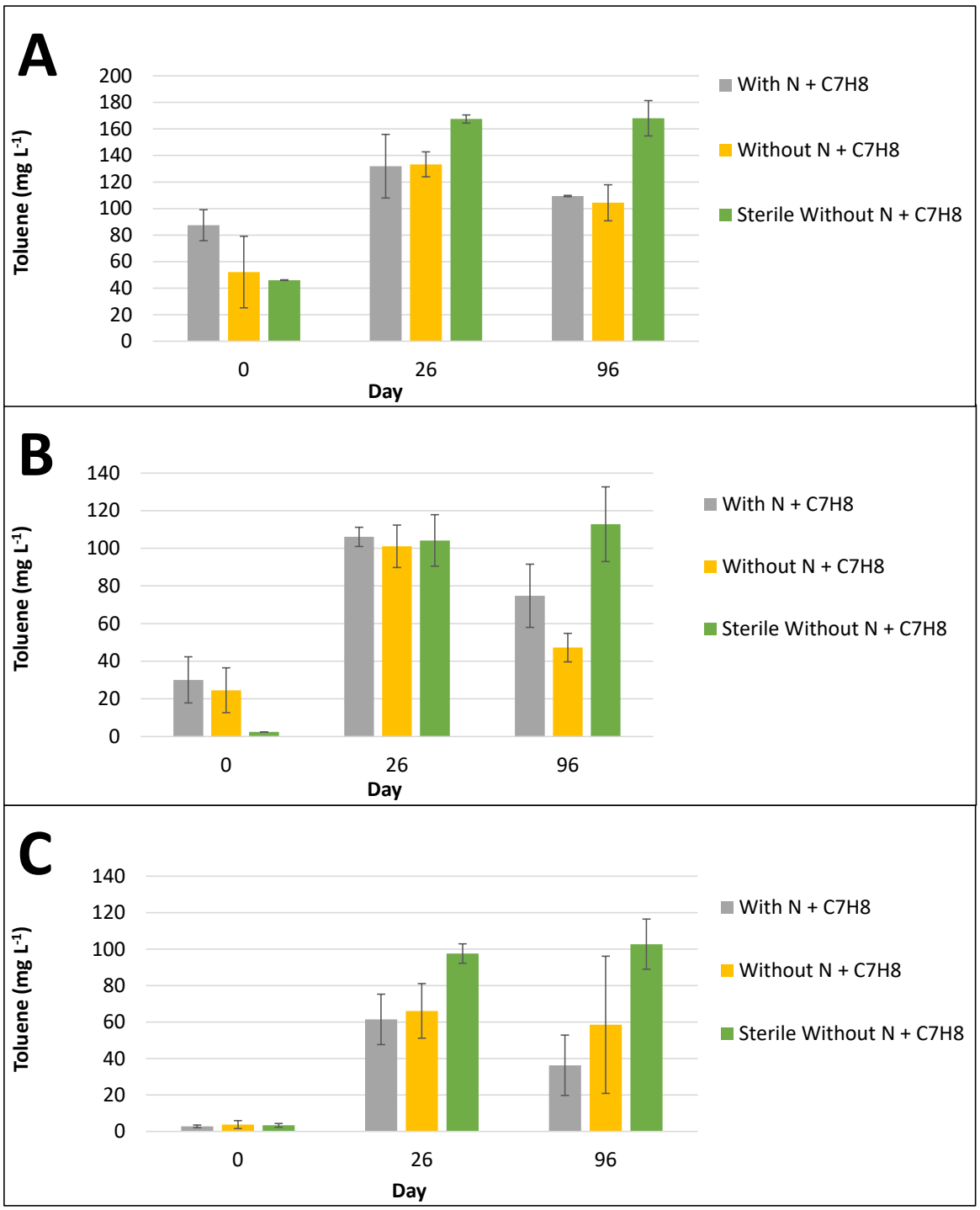

Figure S 16: Headspace toluene concentration in toluene amended cultures with $Fe(OH)_3$ as measured in **A**) Albian, **B**) Syncrude, and **C**) CNRL. Day 0 results were low in all headspace analyses, this was due to performing headspace analysis on day 0 following amendments before phase partitioning equilibrium could be reached, as such, only a very low concentration of toluene could volatilize into the headspace. Values represent the mean mg L^{-1} for triplicate amended cultures and duplicate sterile and baseline cultures. Error bars indicate ± standard deviation.

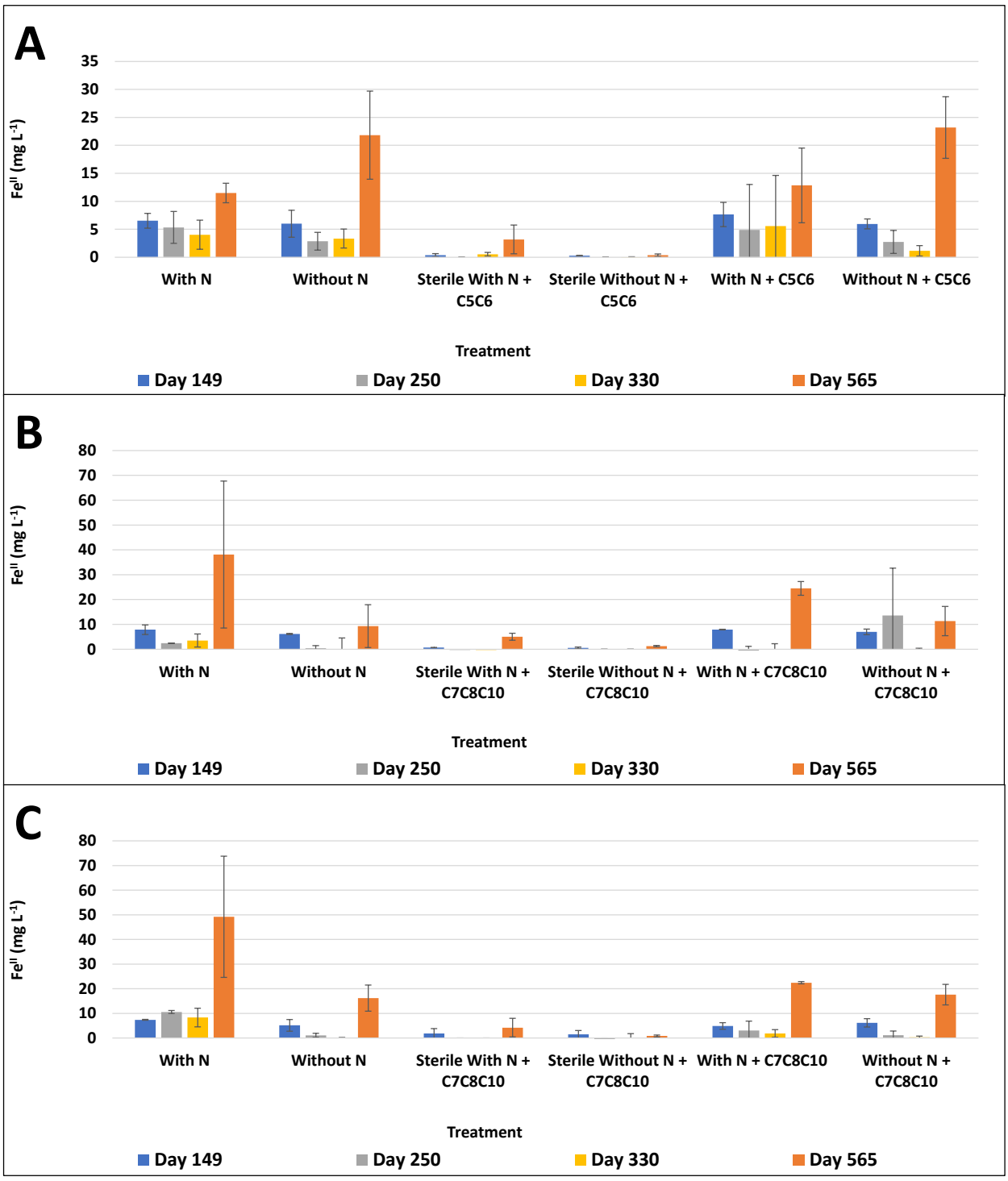

Figure S 17: Fe(II) production in I-alkane cultures from Chapter 3, **A**) Albian, **B**) Syncrude, and **C**) CNRL. Values represent the mean µmol for triplicate amended cultures and duplicate sterile and baseline cultures. Day 0 values subtracted from all timepoint measurements. Error bars indicate ± standard deviation.

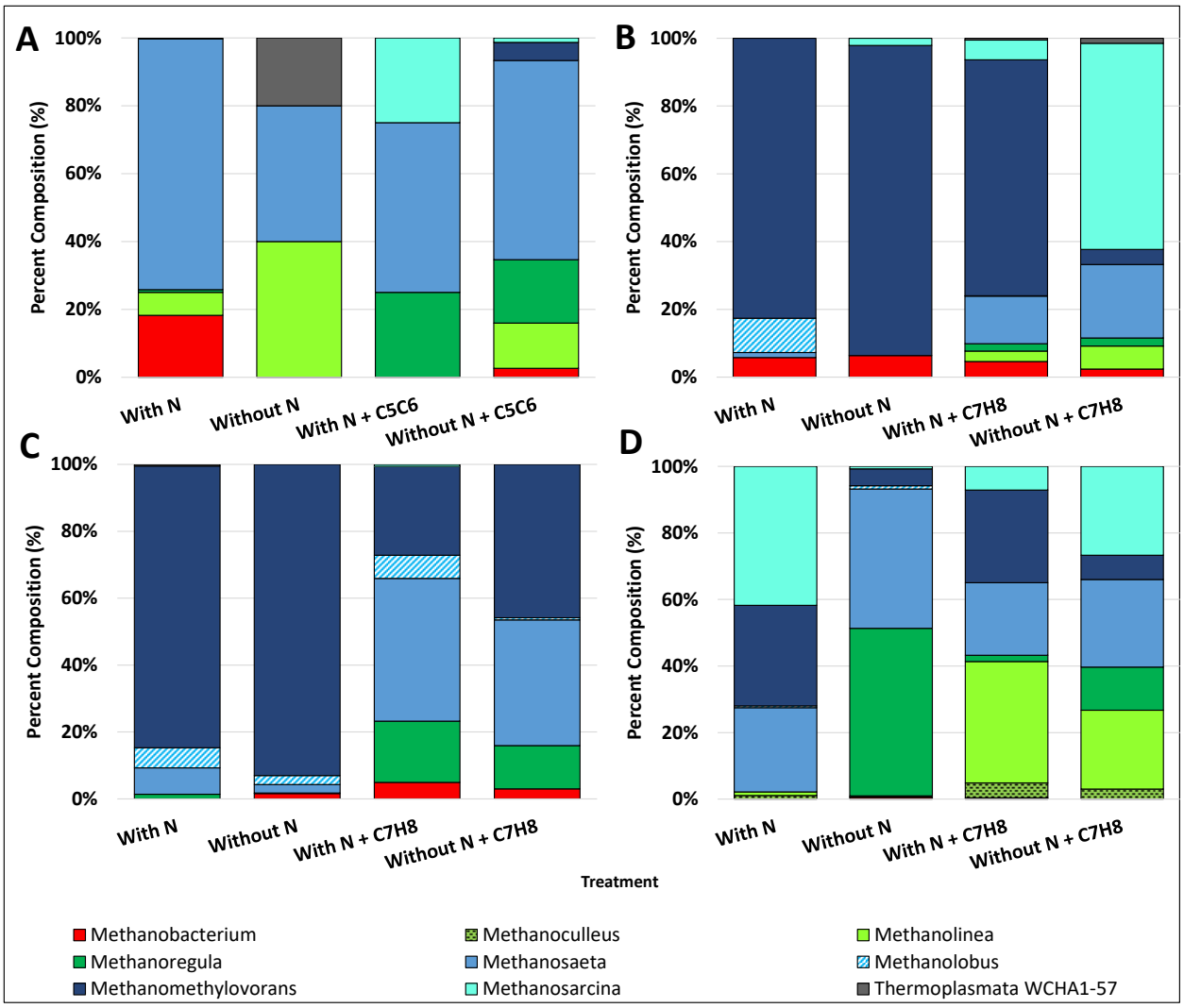

Figure S 18: Archaeal community composition of cultures based on Illumina sequencing of the 16S rRNA gene at (**A**) day 334 from the *n*-alkane Albian culture set, and day 189 in toluene experimental sets, (**B**) Albian, (**C**) CNRL, and (**D**) Syncrude.

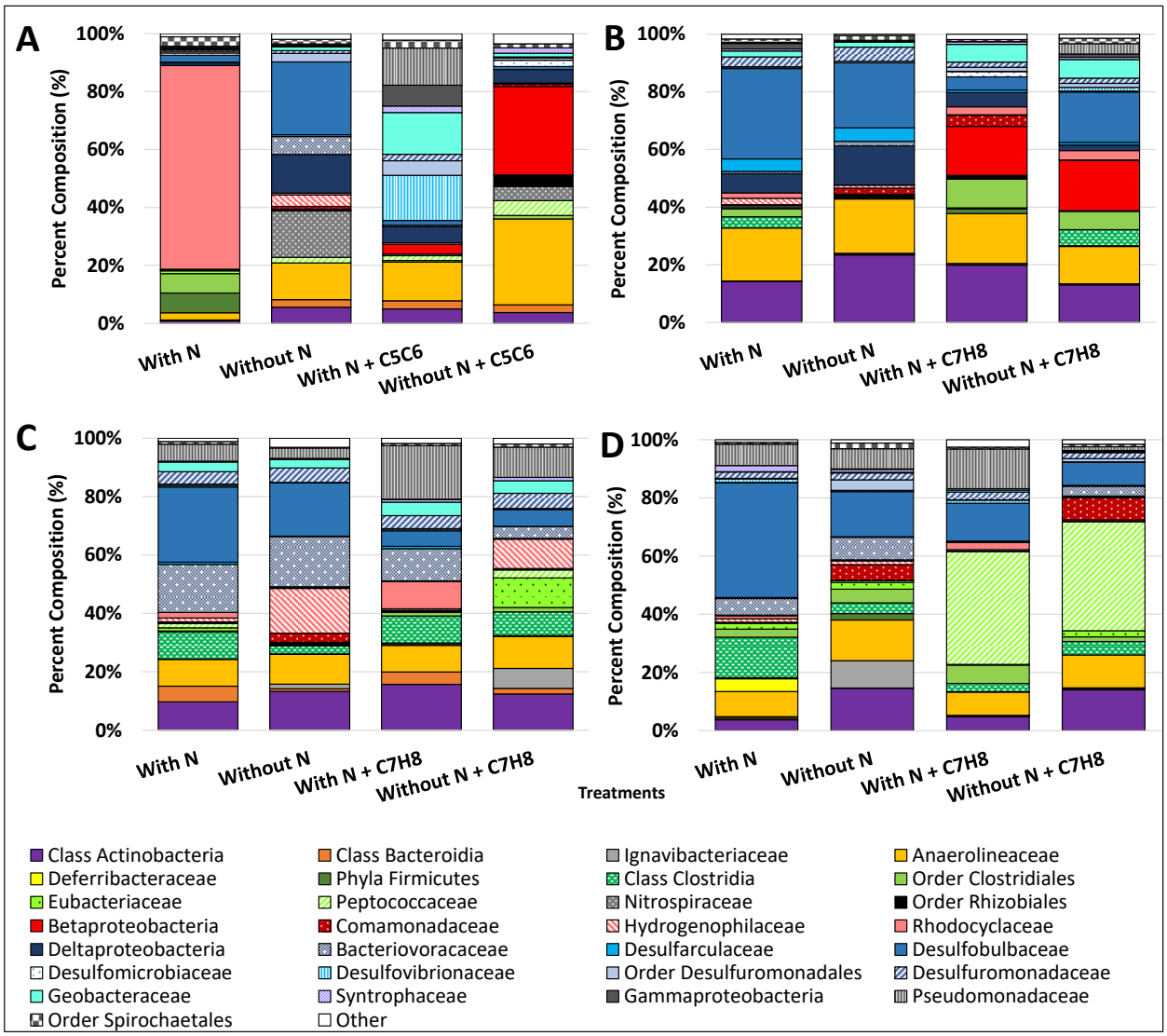

Figure S 19: Bacterial community composition of cultures based on Illumina sequencing of the 16S rRNA gene at (**A**) day 334 from the *n*-alkane Albian culture set, and day 189 in toluene experimental sets, (**B**) Albian, (**C**) CNRL, and (**D**) Syncrude. Unidentified Bacteria sequences and sequences with less than 2% abundance are grouped as "Other".

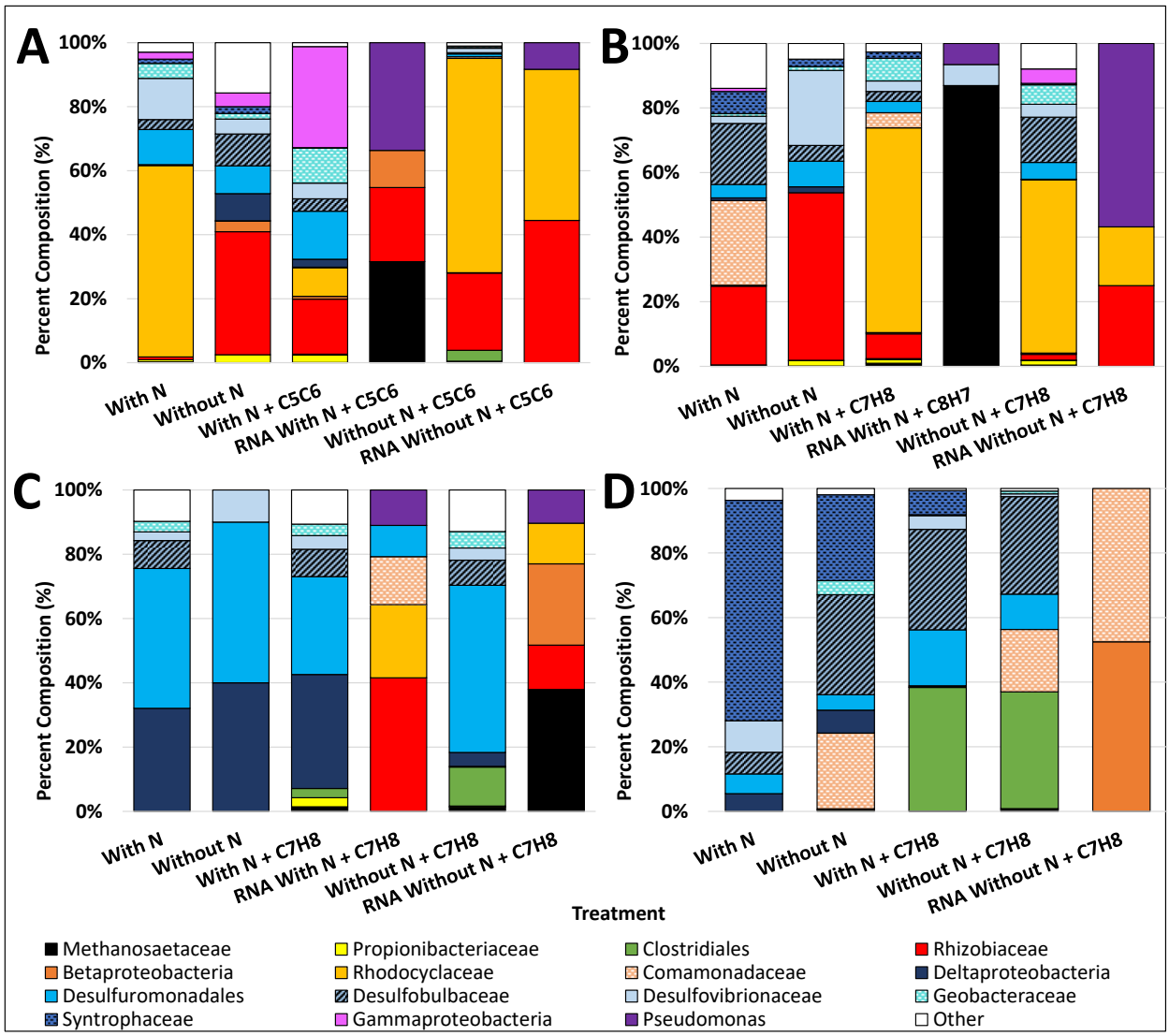

Figure S 20*: NifH* gene abundance from cultures based on Illumina sequencing and BLASTx match of the *nifH* gene at (**A**) day 334 from the *n*-alkane Albian culture set, and day 189 in toluene experimental sets, (**B**) Albian, (**C**) CNRL, and (**D**) Syncrude. "RNA" indicates sequenced cDNA reverse transcribed from extracted RNA from the respective treatment. Unidentified Bacteria or Archaea sequences and sequences with less than 2% abundance are grouped as "Other". Treatments or reverse transcribed RNA that returned no reads or less than 30 good reads were discarded, OTUs with no match or off-target match were discarded.

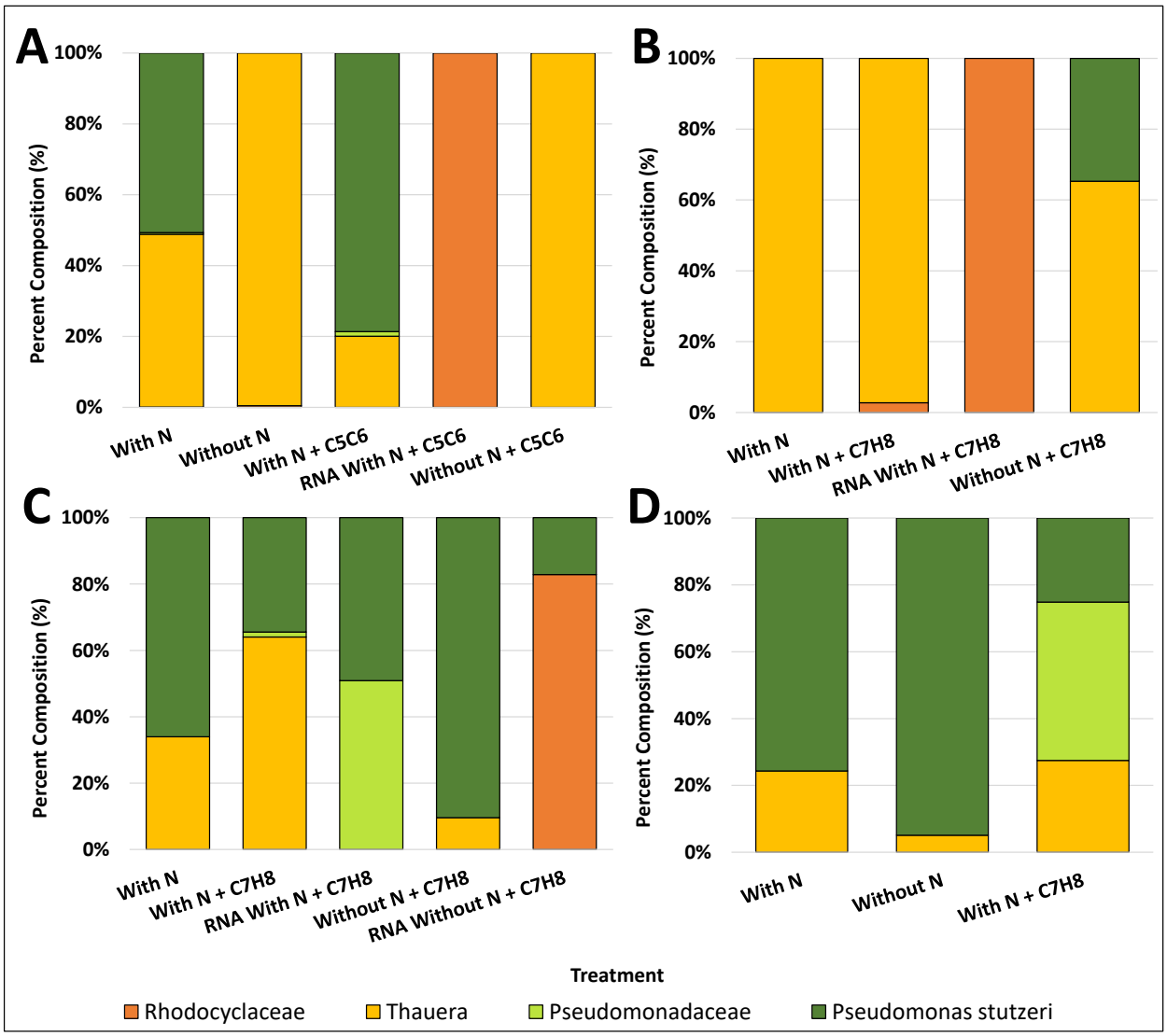

Figure S 21: *NirS* gene abundance from cultures based on Illumina sequencing and BLASTx match of the *nirS* gene at (**A**) day 334 from the *n*-alkane Albian culture set, and day 189 in toluene experimental sets, (**B**) Albian, (**C**) CNRL, and (**D**) Syncrude. "RNA" indicates sequenced cDNA reverse transcribed from extracted RNA from the respective treatment. Treatments or reverse transcribed RNA that returned no reads or less than 30 good reads were discarded, OTUs with no match or off-target match were discarded.

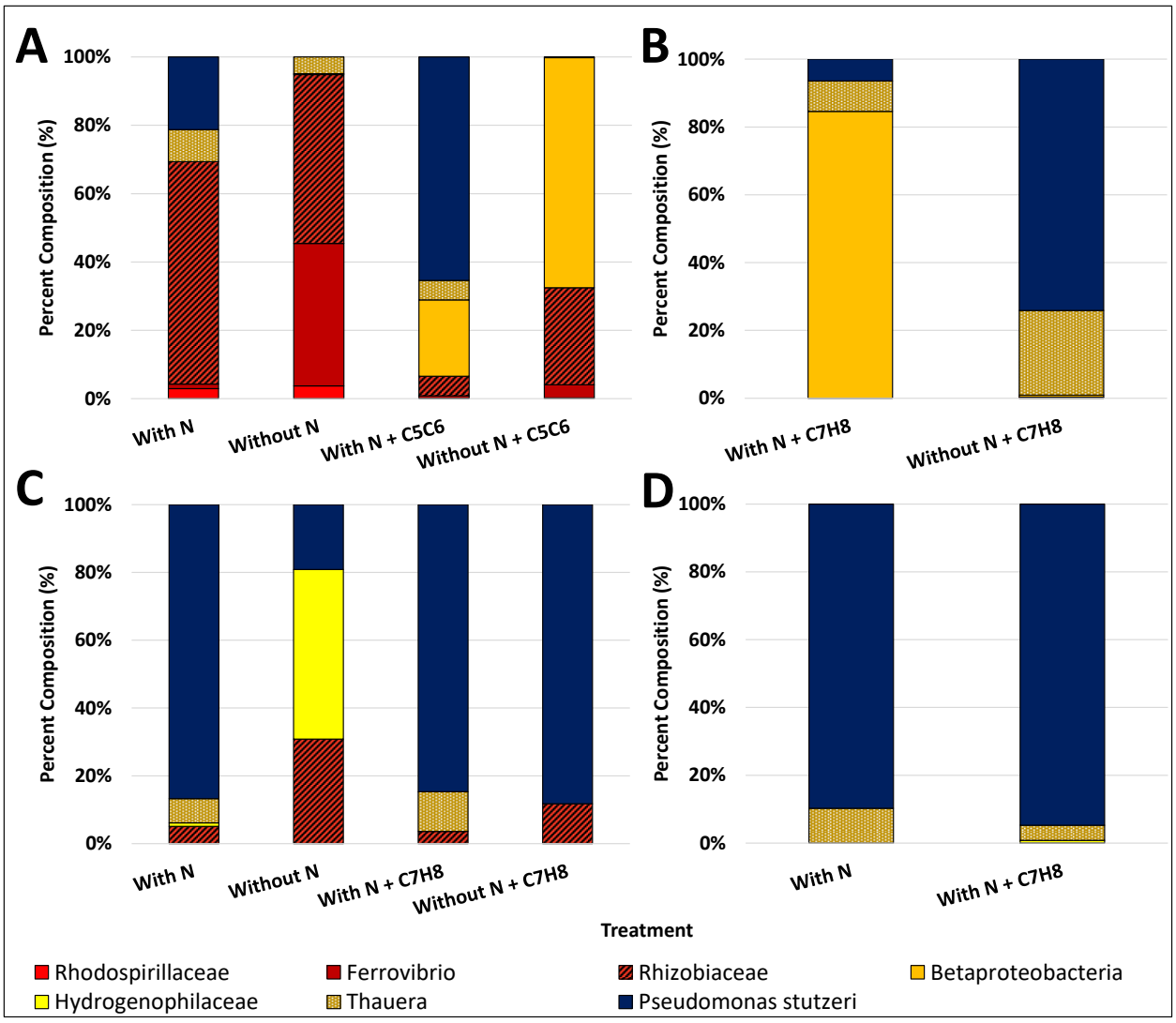

Figure S 22: *NosZ* gene abundance from cultures based on Illumina sequencing and BLASTx match of the *nosZ* gene at (**A**) day 334 from the *n*-alkane Albian culture set, and day 189 in toluene experimental sets, (**B**) Albian, (**C**) CNRL, and (**D**) Syncrude. Treatments or reverse transcribed RNA that returned no reads or less than 30 good reads were discarded, OTUs with no match or off-target match were discarded.

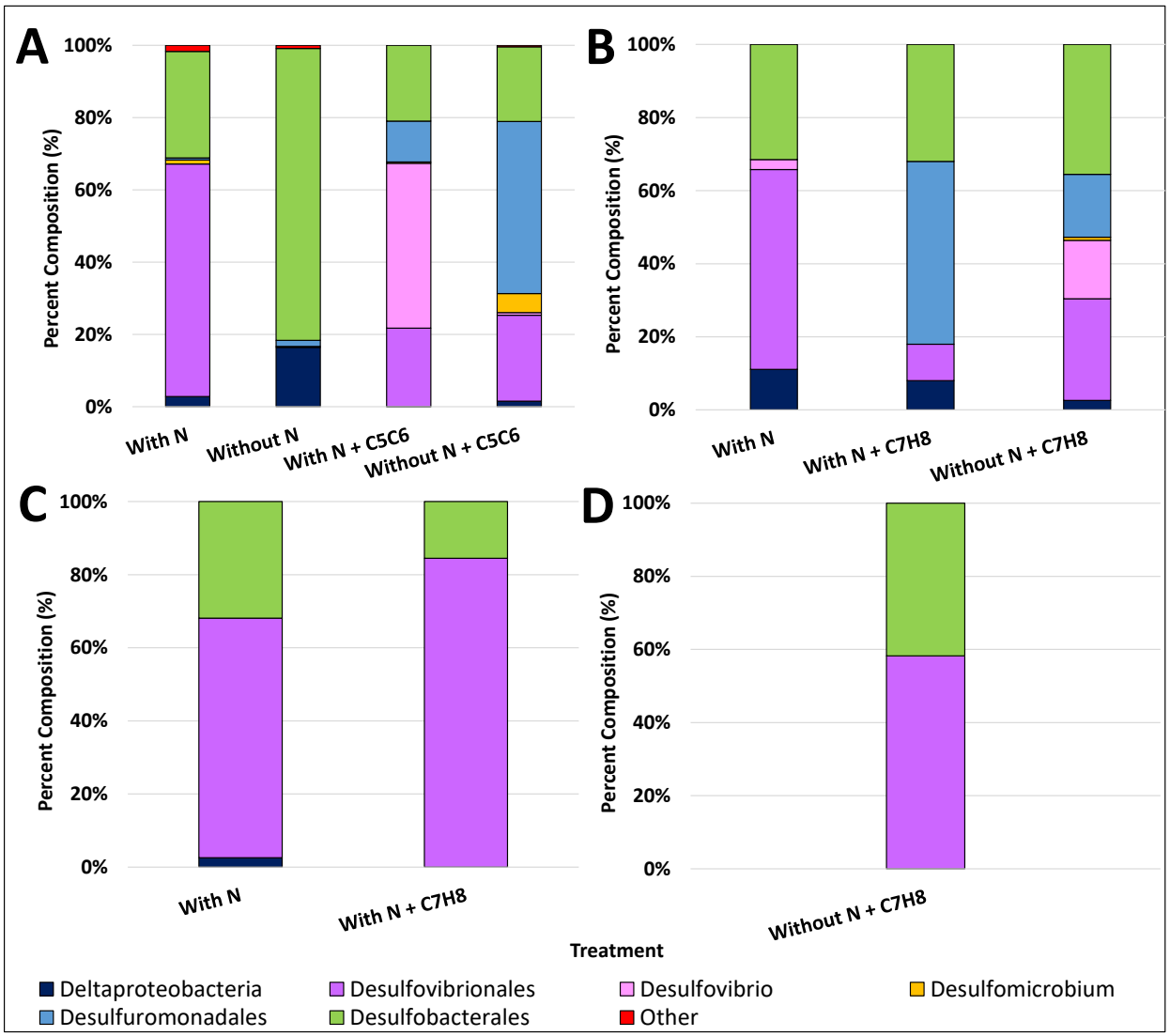

Figure S 23: *NrfA* gene abundance from cultures based on Illumina sequencing and BLASTx match of the *nrfA* gene at (**A**) day 334 from the *n*-alkane Albian culture set, and day 189 in toluene experimental sets, (**B**) Albian, (**C**) CNRL, and (**D**) Syncrude. Unidentified Bacteria or Archaea sequences and sequences with less than 2% abundance are grouped as "Other". Treatments or reverse transcribed RNA that returned no reads or less than 30 good reads were discarded, OTUs with no match or off-target match were discarded.

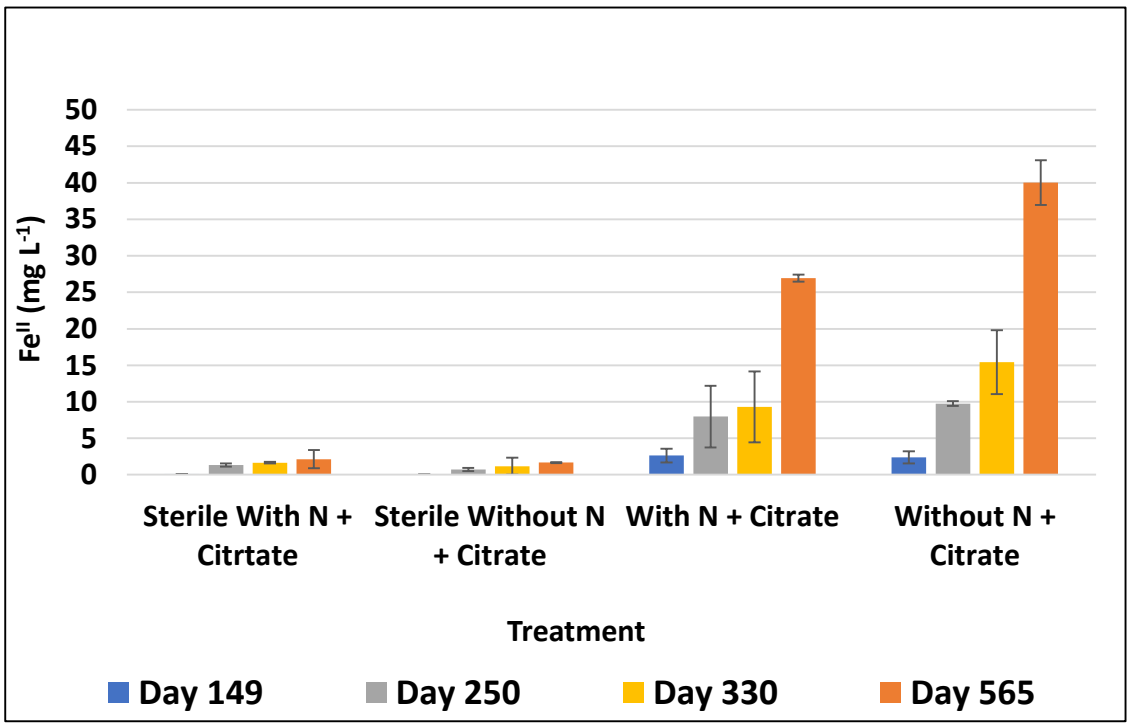

Figure S 24: Fe(II) production in citrate amended Albian cultures with amorphous $Fe(OH)_3$ from Chapter 4. Values represent the mean µmol for triplicate amended cultures and duplicate sterile cultures. Analysis performed at day 565, 250 days after the end of the experiment. Day 0 values subtracted from all timepoint measurements. Error bars indicate ± standard deviation.

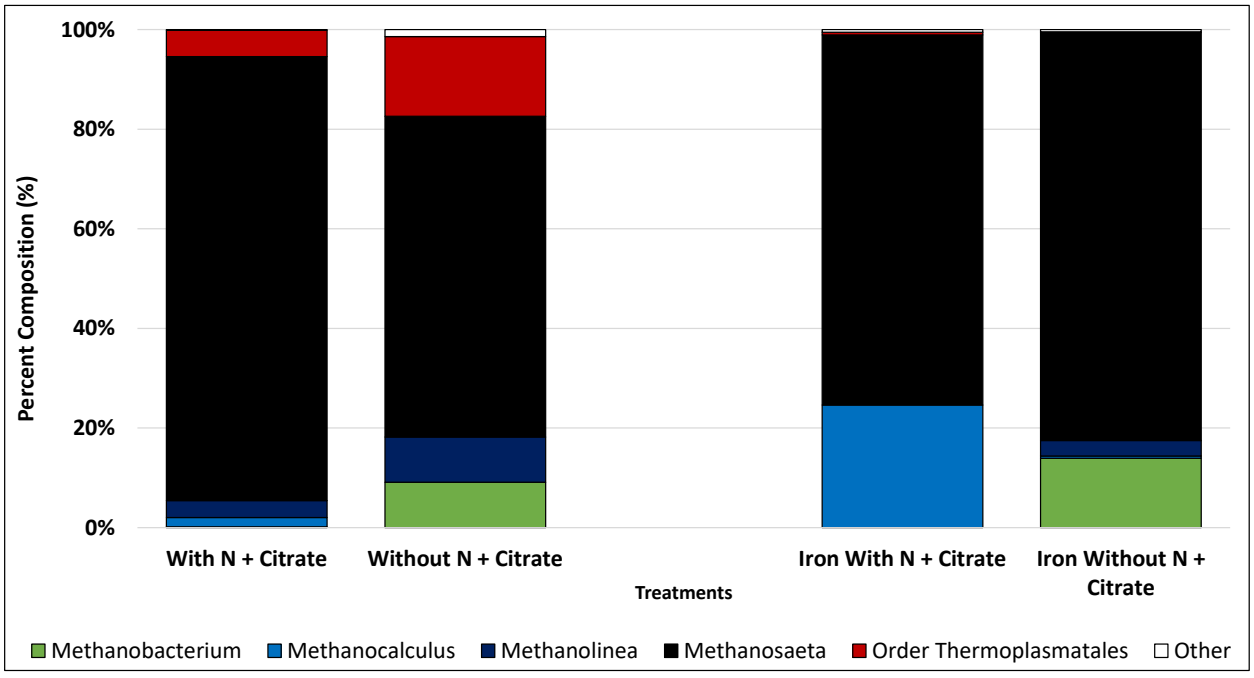

Figure S 25: Community composition of Archaea based on Illumina sequencing of the 16S rRNA gene at day 334 "Iron" indicates cultures containing $Fe(OH)_3$. Unidentified Archaea sequences and sequences with less than 2% abundance are grouped as "Other".

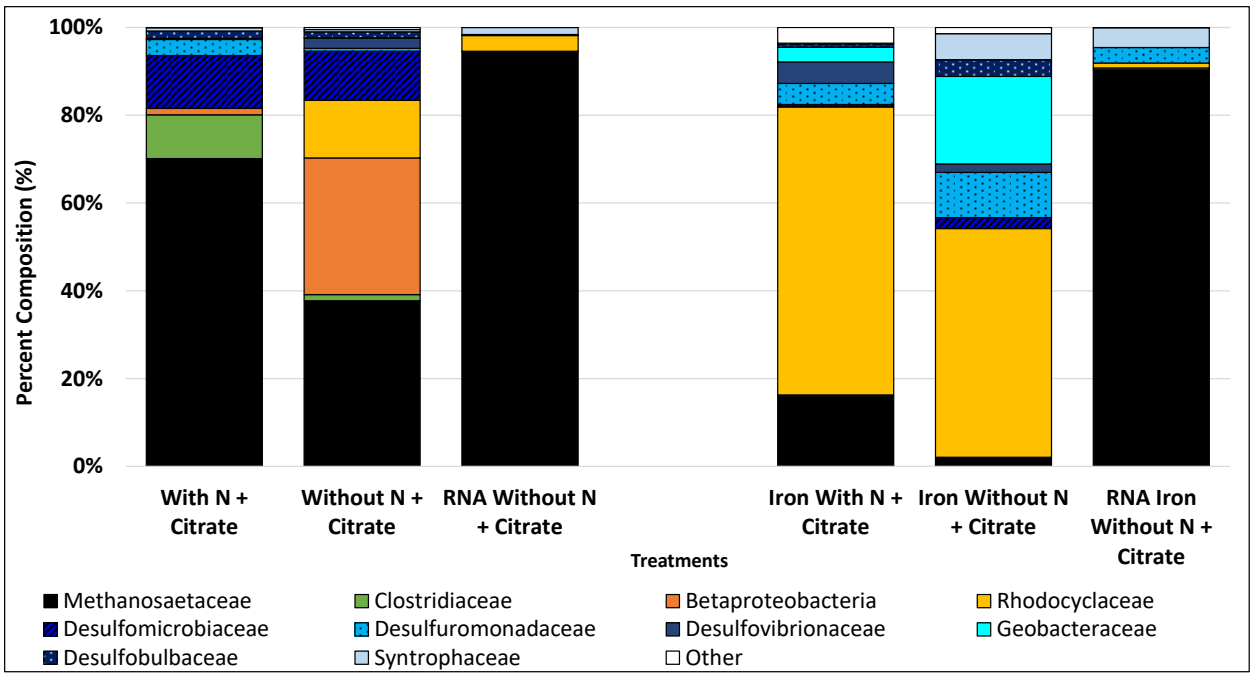

Figure S 26: *NifH* gene abundance of living cultures based on Illumina sequencing and BLASTx match of the *nifH* gene at day 334. "Iron" indicates cultures containing $Fe(OH)_3$, "RNA" indicates sequenced cDNA reverse transcribed from extracted RNA from the respective treatment. Unidentified Bacteria or Archaea sequences and sequences with less than 2% abundance are grouped as "Other". Treatment or reverse transcribed RNA with no sequence matches or less than 60 reads were discarded.

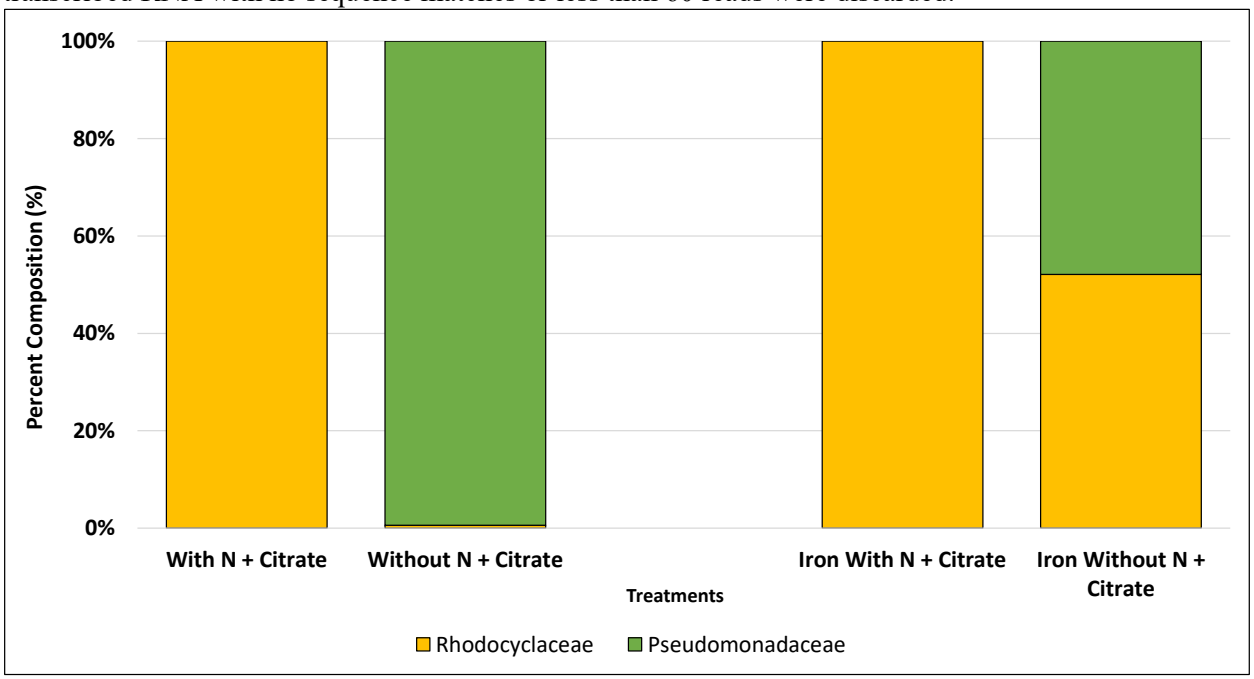

Figure S 27*: NirS* gene abundance of living cultures based on Illumina sequencing and BLASTx match of the *nirS* gene at day 334. "Iron" indicates cultures containing $Fe(OH)_3$. Unidentified Bacteria sequences and sequences with less than 2% abundance are grouped as "Other". Treatment or reverse transcribed RNA with no sequence matches or less than 60 reads were discarded.

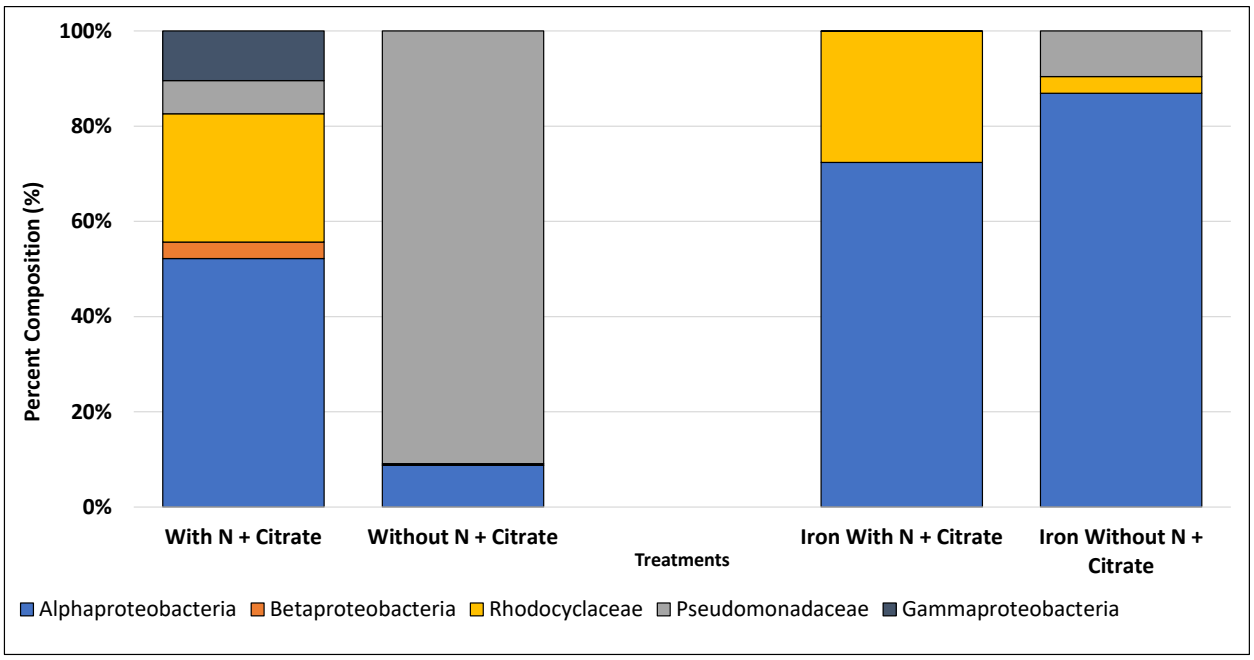

Figure S 28: *NosZ* gene abundance of living cultures based on Illumina sequencing and BLASTx match of the *nosZ* gene at day 334. "Iron" indicates cultures containing $Fe(OH)_3$. Treatment or reverse transcribed RNA with no sequence matches or less than 60 reads were discarded.

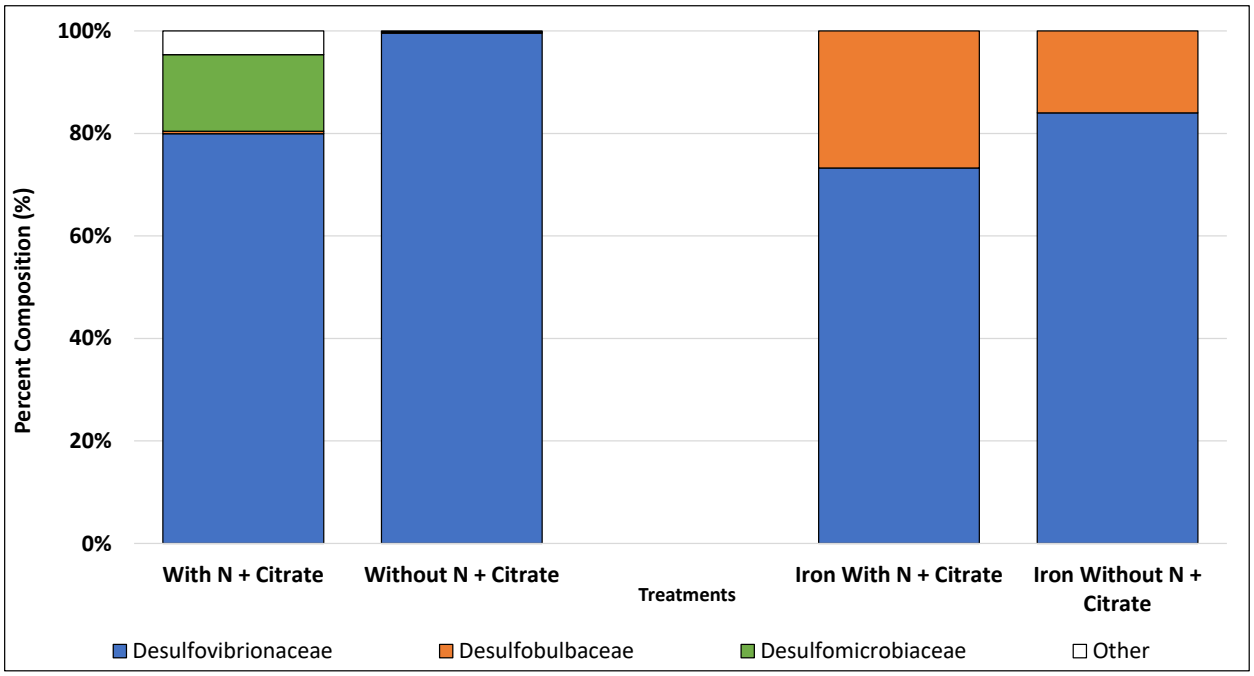

Figure S 29: *NrfA* gene abundance of living cultures based on Illumina sequencing and BLASTx match of the *nrfA* gene at day 334. "Iron" indicates cultures containing $Fe(OH)_3$. Treatments or reverse transcribed RNA with no sequence matches or less than 60 reads were discarded.