Biodegradation of Cycloalkanes Under Different Redox Conditions

by

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Abstract

Current bitumen extraction technologies used in surface mined oil sands in Alberta, Canada require large volumes of fresh water, which in turn generate large volumes of fluid fine tailings (FFT). Managing these tailings is a major challenge for oil sands operators. Non-aqueous extraction (NAE) is an alternative method which uses hydrocarbon solvents such as cyclohexane, producing smaller volumes of dry tailings with most of the solvent being recoverable post-extraction. However, residual cyclohexane remains in the non-aqueous extraction solids; therefore, developing technology for cycloalkane biodegradation is the aim of this study.

Microcosm experiments involve setting up sealed bottles in which a microbial source such as soil or FFT is mixed with nutrient media and/or NAE dry tailings. These microcosms were amended with distinct electron acceptors and cycloalkane NAE solvents to create aerobic, nitrate-reducing, sulfate-reducing, iron-reducing, or methanogenic conditions for cycloalkane biodegradation. These conditions simulated dry tailings management under either upland and wetland reclamation scenarios. Gas chromatography was used to measure cycloalkane concentrations in the microcosm. Electron acceptor depletion and gas production resulting from biodegradation were also being monitored over the course of the experiments.

Microcosms containing active microbial communities capable of cycloalkane degradation were found to have three elements in common: maintaining aerobic conditions via oxygen addition, sufficient concentrations of nitrogen and phosphorus, and FFT inoculum. In all other treatments, including anaerobic conditions or other inoculates such as soil or oil sands process affected waters, no significant degradation was observed over the allotted 2 year incubation despite other indications of microbial activity. Therefore, future cycloalkane biodegradation technologies will likely require oxygen and nutrients for adequate cycloalkane removal.

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1.1 The Oil Sands

The Canadian oil sands is located in northeastern Alberta and is estimated to contain the third largest reserve of crude oil in the world, exceeded only by deposits in Saudi Arabia and Venezuela (Alberta Government, 2017; Honarvar, Rozhon, & Millington, 2011; Small, Cho, Hashisho, & Ulrich, 2015). Distributed across the Peace River, Athabasca, and Cold Lake oil sands areas, the total oil sands area totals approximately 142,200 square kilometres (Alberta Government, 2017). Recoverable bitumen reserves have been projected to be 165.4 billion barrels, with production averaging at 2.8 million barrels per day in 2017 (Alberta Government, 2017; Brown & Ulrich, 2015; Clothier & Gieg, 2016). Additionally, oil sands development is a significant contributor to both Alberta and Canada's economy, with provincial resource revenue from bitumen equalling $1.48 billion dollars and employing an estimated 140,300 people in mining, oil, and gas extraction (Alberta Energy, 2015; Alberta Energy Regulator (AER), 2018).

1.1.1 Oil Sands Extraction Processes

Multiple types of extraction and recovery techniques are regularly used in industry, and process selection usually depends on the depth of the oil sands (Alberta Government, 2017; Oil Sands Discovery Centre, 2016). The two primary types of recovery methods are surface-mining and in situ technology, wherein in-situ methods are used for deeper oil sands reserves located deeper than 75 metres from the surface and are applied in a variety of ways, the most prevalent being steam assisted gravity drainage (SAGD) (Oil Sands Discovery Centre, 2016). This technology was developed to recover high viscosity heavy oil that is effectively immobile and therefore not accessible through conventional techniques (Butler, 1994). After drilling two parallel horizontal wells through the reservoir, steam is injected via the upper well and introduces high temperatures (Butler, 1994; Oil Sands Discovery Centre, 2016). This heat

reduces the viscosity and mobilizes the crude oil, allowing it to flow down to the lower production well. Since the heated oil is slightly thinner than the original bitumen, it can be pumped to the surface and recovery can range between 25 and 75%. Though physical and geological characteristics can vary across large areas, the overall higher vertical permeability of reservoirs in countries like Canada and Venezuela provide suitable conditions for in situ methods to be used (Al-Bahlani & Babadagli, 2009; Oil Sands Discovery Centre, 2016). Alternatively, surface-mining involves the use of heavy machinery such as scrapers and backhoes to remove the overburden covering the oil sands (Grant, Dyer, & Woynillowicz, 2008; Oil Sands Discovery Centre, 2016). The exposed oil sands are then crushed and transported to an extraction plant to separate the sands and bitumen. Surface mining is reserved for shallower deposits that are less than 75 metres from the surface and the Athabasca area is the only reserve that is sufficiently shallow to be surface mined (Alberta Government, 2017; Grant et al., 2008). Although only a small percentage of total oil sands reserves are accessible via surface mining, it remains the foremost process for oil sands development, responsible for more than half of production in 2015 (Alberta Government, 2017; Grant et al., 2008; Hooshiar, Uhlik, Liu, Etsell, & Ivey, 2012).

1.1.2 Hot Water Bitumen Extraction

Surface mined ores that have been crushed and transported to an extraction plant require further processing to separate the mixture of bitumen and sands, as well as treat the separated bitumen to reduce its viscosity and allow it to eventually be pumped to upgraders and refineries (Hamza, Xu, Masliyah, Czarnecki, & Zhou, 2010). The collective process is referred to as the Clark Hot Water Extraction process (CHWE), beginning with mixing the crushed oil sands with high temperature process water and transferring via hydrotransport pipeline, with the resulting slurry being sheared to reduce the size of the remaining oil sands lumps (Hamza et al., 2010;

Hooshiar et al., 2012). The slurry is then treated with chemical additives such as caustic soda, then aerated and heated in gravity separation vessels, ventilating the slurry and letting the aerated bitumen float to the top of the slurry and be skimmed off (Giesy, Anderson, & Wiseman, 2010; Small et al., 2015). Along with clay and sand, the bitumen is also separated from other slurry constituents like dissolved metals, or organic compounds such as polycyclic aromatic hydrocarbons (PAHs) and naphthenic acids (NAs). During the primary separation process, a middle layer containing sand, clay, water, and bitumen is formed; consequently, a secondary separation is necessary and involves injecting air into this middle layer to create additional bitumen froth (Oil Sands Discovery Centre, 2016). This can also be achieved via vortex separation in hydrocyclones or cyclo-separators (Hamza et al., 2010). Slurry temperatures can vary from 35-75°C to increase the degree of bitumen liberation from sand grains (Hamza et al., 2010). The recovered bitumen froth is de-aerated, mixed with solvents such as naphtha or paraffinic diluents, and sufficiently upgraded so it can be transported to upgraders and refineries for further processing into products such as conventional gasoline or heating oil.

1.1.3 Environmental Impacts of Oil Sands Extraction

The production of bitumen in Alberta's oil sands has increased significantly over time and is projected to continue to expand from 2.8 million barrels of oil per day in 2017 to 4 million barrels per day in 2030 (Alberta Government, 2017; Jordaan, Keith, & Stelfox, 2009). Multiple steps have been taken by industry to mitigate the environmental impact of oil sands development and expansion, including recycling 80-95% of the water used in extraction and recovery (Alberta Energy, 2015). This industry-wide shift towards sustainability extends to almost all steps of the oil sands recovery process: in steam assisted gravity drainage, water is injected into bitumen-drained areas to maintain deposit stability, while in surface-mining, much of the overburden

overlying the ore deposit is returned after the ores are depleted along with soil replacement and revegetation to facilitate land reclamation (Grant et al., 2008; Oil Sands Discovery Centre, 2016). Nevertheless, oil sands processes remain a substantial source of physical environmental disturbance as well as a source of significant volumes of waste materials and chemical by-products (Grant, Angen, & Dyer, 2013). Furthermore, current recovery methods have been criticized for the considerable energy required and the large volumes of fresh water consumed in surface mining, in situ technologies, and the hot water extraction process.

One of the most visually apparent environmental impacts of oil sands mining is the disturbance of natural terrestrial and aquatic ecosystems. Research performed at the Pembina Institute by Grant et al. (2013) estimated and compared the per-barrel statistics regarding land disturbance of surface mining and in situ technologies, as well as other factors such as water use, air quality, and greenhouse gas emissions (Grant et al., 2013). For water use intensity, in situ technologies use only 0.45 barrels of fresh water per barrel of bitumen produced, while surface mining requires 2.41 barrels of fresh water per barrel of bitumen despite efforts to recycle water (Grant et al., 2013). When compared to conventional oil production, oil sands mining requires three times as much fresh water. For greenhouse gas emissions, in situ process generate 0.082 tonnes of carbon dioxide per barrel of oil, while surface mining produces 0.073 tonnes of carbon dioxide per barrel. However, in terms of air emissions produced, surface mining produces higher quantities of nitrogen oxides, sulphur dioxide, and particulate matter when compared to in situ processes. A similar pattern can be found for land disturbance: based on available data, Grant et al. (2013) estimate that in situ extraction requires approximately 1.6×10^{-6} hectares of land per barrel of oil, while surface mining requires a significantly higher area totalling 9.4×10^{-6} hectares per barrel of oil. While land use for surface mining is easily recognizable by the complete

clearing and draining of forested and wetland habitats, land use intensity for in situ technology is somewhat less noticeable but still significant (Giesy et al., 2010; Grant et al., 2008; Grant et al., 2013). In situ extraction necessitates the development of networks of pipelines, transport roads, and processing facilities, causing habitat fragmentation of the surrounding boreal forest (Grant et al., 2013). Consequently, the spatial footprint is more widely distributed across the landscape, producing a long term impact on biodiversity (Jordaan et al., 2009). Fragmentation often proves beneficial to generalist species, using anthropogenic landscape features for accessing prey habitats; for specialist species occupying more specific niches, fragmentation is typically much more detrimental, and has led to extirpation and even extinction in extreme cases (Fahrig, 2003; Jordaan et al., 2009). Nevertheless, one of the most detrimental environmental effects originates in the hot water separation process performed after surface mining, wherein significant amounts of waste by-products are generated and have since become difficult to manage.

1.2 Tailings

The Clark Hot Water Extraction method produces an effluent stream during the separation process collectively referred to as tailings. After bitumen is removed from the slurry during primary and secondary separation, the remaining mixture is composed of water, sand particles, dispersed fines, dissolved salts, minerals, organic compounds, and residual bitumen (Allen, 2008; Chalaturnyk, Scott, & Özüm, 2002; Hamza et al., 2010). Although the overall proportion of tailings varies based on the extraction process used and the age of the tailings, they typically contain ~70 to 80 wt% water, ~20 to 30 wt% solids, and ~1-3 wt% residual bitumen (Allen, 2008). Tailings are then pumped to settling basins, also referred to as tailings ponds, which are used for solid-liquid separation wherein clarified cap water can be recycled back to the extraction plant. When the tailings are initially discharged, coarse particles tend to separate

quickly, while the remaining fines within fluid fine tailings (FFT) accumulate within the pond, requiring 2-4 years to settle from ~10 wt% to ~30 wt% (Chalaturnyk et al., 2002; Kuznetsova, Kuznetsov, Foght, Arkell, & Siddique, 2014). After this settling period, this new slurry is referred to as mature fine tailings (MFT). MFT has a very slow consolidation rate, causing it to remain in a fluid state for decades (Chalaturnyk et al., 2002). This difficulty originates from the initial conditions within the slurry stimulating bitumen recovery while also promoting clay dispersion within the water, resulting in slower flocculation and consolidation within the tailings ponds. One possible explanation for this observed trend is water-soluble asphaltic acids derived from bitumen which reduce the interfacial tension of the water and thereby dispersing clay particles (Chalaturnyk et al., 2002). Another potential cause is that ultrafine clay particles (less than 0.2 μm) create a gel-like structure within the aqueous media that can contain high volumes of water, thereby preventing the tailings from dewatering (Ripmeester, Kotlyar, & Sparks, 1993). Other research has found that pH influences the clay structure within the slurry, allowing organic material to adhere to the clay surface, stimulating bitumen emulsions within the water and reducing dewatering of the tailings (Chalaturnyk et al., 2002). Therefore, treatments performed by industry to improve consolidation and reduce freshwater demand includes pH treatment as well as other chemical additives (ex. gypsum) serving as densification agents to speed up the process (Chalaturnyk et al., 2002; Ramos-Padrón et al., 2011). Nevertheless, the production of tailings has become a source of concern for industry, with increasing global demand for oil production also increasing the production and total volume of tailings.

Recent statistics state that the total volume of tailings produced by oil sands production is approximately 1.21 billion cubic metres (OSIP, 2016). It is also estimated that for each barrel of bitumen produced, between 1.5 and 4 barrels of tailings are produced (Grant et al., 2013; Ramos-

Padrón et al., 2011). These tailings are restricted on site due to a zero discharge policy adhered to by oil sands mining companies in the interest of limiting any potential spread of environmental impact (Alberta Government, 2017; Brown & Ulrich, 2015; Quagraine, Peterson, & Headley, 2005). Current research has investigated the effectiveness of this strategy, discovering multiple undesirable and deleterious effects (Foght, Julia M., Gieg, & Siddique, 2017; Penner & Foght, 2010). The total volume of tailings continues to grow, with slow consolidation and porewater recovery deterring reclamation and water recycling for reuse in bitumen extraction processes. Tailings also contain residual hydrocarbons and other chemical contaminants such as naphthenic acids and toxic metals, which may potentially impact surface and groundwater systems. Additionally, tailings ponds are a significant source of greenhouse gas emissions. Therefore, management and wet/dry reclamation of tailings has become a long-term and difficult process. Reclamation efforts have determined that microbial communities within tailings ponds play a key role in these issues, either by assisting in remediation or further contributing to contamination. In certain circumstances, microbial metabolic activity can incorporate and break down hydrocarbon contaminants; however, the biological endpoints of these metabolic pathways are diverse, and may result in the generation of more toxic metabolites or the generation of greenhouse gases such as methane and carbon dioxide. Furthermore, microbiological activity also influences tailings consolidation via biodensification, wherein the ebullition of biogenic gases such as carbon dioxide and methane penetrate FFT which thereby provides potential pathways for porewater migration (Holowenko, MacKinnon, & Fedorak, 2000; Siddique, Stasik, Mohamad Shahimin, & Wendt-Potthoff, 2018). The next section will explore the origins and diversity of these microbes, as well as previous research into the capability of these unique communities to biodegrade oil sands hydrocarbon contaminants in different conditions.

1.2.1 Diversity and Degradative Proficiency of Microbial Communities in Tailings Ponds

Oil sands related microbial communities have been the focus of various investigations, which have determined that the long-term heterogeneous environment generated by tailings deposition and consolidation in mature ponds produces heterogeneous and diverse microbial communities with spatial variation (Foght, Julia M. et al., 2017; Siddique, Fedorak, Mackinnon, & Foght, 2007; Tan, Semple, & Foght, 2015). Within mature ponds, the depth tends to correspond to specific physical, chemical, and biological factors; at lower depths, there is increased solids content due to dewatering and tends to contain older solids that were deposited earlier than those at shallower depths. Similarly, ore quality and processing methods may vary over time and will have an impact on the contents of the stratified layers (Ramos-Padrón et al., 2011). Temperature also tends to increase with depth due to tailings being deposited relatively quickly after the hot water extraction process, thereby retaining heat (Foght, Julia M. et al., 2017; Penner & Foght, 2010). Another possible explanation is that the density of the mature tailings may retain heat as a by-product of microbial metabolism (Siddique, Penner, Klassen, Nesbø, & Foght, 2012a). A depth-dependent chemical gradient is also observable in which residual hydrocarbons and diluent from bitumen extraction have higher concentrations near the surface where they are replenished by newly deposited tailings. A similar trend is seen for electron acceptors, where oxygen and sulfate availability tends to decrease with depth (Dompierre, Lindsay, Cruz-Hernández, & Halferdahl, 2016; Foght, Julia M. et al., 2017; Penner & Foght, 2010). This may either be due to oil sands operators changing processing methods over time, or possibly due to microbial communities using both residual hydrocarbons and electron acceptors for consumption and metabolism. This creates an opportunity for a diverse set of microbes that proliferate using different redox metabolic pathways to coexist within tailings ponds in different

conditions. Similarly, most probable number and 16S analyses found multiple anaerobic species such as methanogens as well as nitrate, sulfate, and iron reducers (Foght, Julia M. et al., 2017; Penner & Foght, 2010; Siddique, Fedorak, & Foght, 2006; Stasik & Wendt-Potthoff, 2014).

The exact source of these complex microbial communities in tailings ponds has not yet been determined, but a variety of possibilities has been suggested to explain where they may have originated. One possible source is indigenous microbial species that have subsisted within pore waters after getting buried with oil sands ores, which has been elucidated by metagenomic analysis of communities residing deep oil sands cores (An et al., 2013). This study found that although anaerobic hydrocarbon degraders are expected to dominate in deeper resident communities, a significant number of aerobic hydrocarbon degrading bacteria were also discovered in the metagenome, suggesting that concurrent aerobic and anaerobic degradation may be taking place. These communities are able to slowly utilize heavier molecular weight oil hydrocarbons to survive in situ for long periods and may be a potential source of hydrocarbon-tolerant inoculate within tailings ponds. Another potential source of microbial communities is fresh water taken from the nearby Athabasca River and used for extraction processes (Foght, Julia M. et al., 2017). Although marine microbial communities would undergo an extreme amount of physical and chemical stress during the froth treatment step of hot water extraction, some members may be capable of survival in these strenuous conditions. One laboratory study performed by Wong et al. 2015 discovered thermophilic bacteria and fungi in an oil sands outcropping capable of degrading residual bitumen at a temperature of 55°C, suggesting potential survivability in higher temperature conditions (Wong et al., 2015). In either case, tailings ponds have been found to possess diverse microbial communities that have been exposed to hydrocarbons derived from both residual bitumen and diluents. Numerous studies have

investigated the biological mechanisms behind their ability to proliferate in these circumstances, finding that tailings possess microbial species capable of incorporating hydrocarbons into their metabolic pathway, thereby biodegrading chemical contaminants similar to hydrocarbon-degrading microbes in oil sands ores. Therefore, this diverse, hydrocarbon-tolerant consortium is currently utilized in research as a source of microbial inoculum in simulated hydrocarbon contaminated environments to examine their potential as a remediation biotechnology.

Within the deeper layers of mature fine tailings, oxygen and nitrate concentrations are extremely low and are therefore home to primarily methanogenic and sulfate-reducing bacteria (Stasik & Wendt-Potthoff, 2016). After the Mildred Lake Settling Basin (MLSB), a tailings pond used by Syncrude, was discerned to be a source of methane gas production, researchers found that a particular area of the pond which had previously received tailings enriched with diluent (Siddique et al., 2006). It was suggested that the diluent, naphtha, was responsible for sustaining methanogenesis by providing hydrocarbons to be used in methanogenic metabolic pathways. This study then examined MFT in closed systems using microcosm experiments, finding that amending microcosms with linear, short-chain alkanes resulted in methane production corresponding to complete mineralization of these compounds (Siddique et al., 2006). These *n*-alkanes were chosen specifically because they constitute a significant portion of the naphtha used in processing as a diluent in hot water extraction, thereby supporting the idea that residual diluent in the tailings as the primary source of methane. Another study looking at the microbial communities within MLSB found that acetoclastic methanogens are prominent in all depths of MFT, suggesting that acetoclastic methanogenesis is the most probable metabolic pathway in which methane was produced (Penner & Foght, 2010). However, a follow-up research study found that hydrogenotrophic methanogens were selected for and consequently enriched in

microcosms amended with benzene, toluene, ethylbenzene, and xylenes, which are also components of naphtha and also quite toxic (Siddique, Penner, Klassen, Nesbø, & Foght, 2012b). They also found that microcosms amended with total naphtha enrich a mixture of both types of methanogens, suggesting that syntrophy between the two pathways is necessary for in situ degradation of naphtha in methanogenic conditions. Using microcosm experiments, methanogenic communities in MFT have also been proven to be capable of degrading other hydrocarbons such as iso-alkanes, cycloalkanes, and paraffinic diluents (Abu Laban, Dao, Semple, & Foght, 2015; Mohamad Shahimin & Siddique, 2017; Tan et al., 2015).

Biodegradation of hydrocarbons has also been tested and examined under other redox conditions as well. Sulfate reducing bacteria (SRB) have been observed to co-exist with and sometimes suppress methanogenesis in tailings, depending on the availability of the electron acceptor and depth of the MFT (Stasik & Wendt-Potthoff, 2014). Although both sulfate reducers and acetoclastic methanogens utilize and compete for acetate in their metabolic pathways, younger tailings were capable of sustaining a syntrophic relationship between SRB and methanogens (Stasik & Wendt-Potthoff, 2016). SRB oxidize acetate to carbon dioxide, which is used by hydrogenotrophic methanogens and converted to methane. Sulfate reducers have been determined to be involved in degrading multiple hydrocarbon species, including longer chain alkanes (C_{12} to C_{17}) within crude oil, surrogate naphthenic acids in controlled microcosms, as well as other labile hydrocarbons (Clothier & Gieg, 2016; Laban, Dao, & Foght, 2015; Maguire et al., 2013; Tan et al., 2015). Fewer studies are available for nitrate and iron reducing conditions because although nitrate reducers have been found in tailings, nitrate reduction is not significant in situ due to low concentrations of nitrate and nitrite (Foght, Julia M. et al., 2017; Stasik, Loick, Knöller, Weisener, & Wendt-Potthoff, 2014). For iron reduction, reactive iron species and iron

reducing microbes are present within tailings with iron reduction rates tending to increase with depth below the mudline (Stasik et al., 2014). These species have a close relationship with the sulfur cycle within the tailings pond, generating amorphous iron sulfide minerals and preventing the release of toxic hydrogen sulfide gas. Interestingly, one research paper found that both nitrate and iron reducers in tailings were capable of degrading simple surrogate naphthenic acids (Clothier & Gieg, 2016). Research into aerobic hydrocarbon degradation thus far has centered around oil sands process affected waters (OSPW) which are found above the mud line in tailings ponds (Foght, Julia M. et al., 2017). These waters are in direct contact with atmospheric air, which can aerate the water and allows for in situ aerobic biodegradation of hydrocarbons. Initial studies have found that OSPW-dwelling microbes may be aerobically degrading hydrocarbons such as naphthalene and benzene (Foght, Julia M. et al., 2017). Further research is required in all redox conditions to examine the full hydrocarbon range that microbes are capable of degrading.

1.3 Non-Aqueous Extraction

The growing volume of oil sands related tailings has become difficult for industry to manage on-site in accordance with the zero discharge policy; coupled with the substantial demand for thermal energy and fresh water in the hot water extraction process, surface mining operators are seeking alternative methods for bitumen recovery from oil sands ores. Non-aqueous extraction (NAE) is one such method involving the use of organic solvents rather than hot water to release bitumen from oil sands ore after surface mining. Unlike hot water extraction, NAE uses significantly less fresh water and does not require large amounts of thermal energy to induce separation of bitumen from the water-ore slurry during the extraction process. Consequently, NAE produces significantly smaller volumes of “dry” solid tailings that do not require long-term storage and reclamation like the current extraction method. In addition to these

environmental advantages, NAE also has high bitumen recovery rates, with most solvents tested recovering approximately 95% of the bitumen present (Nikakhtari, Vagi, Choi, Liu, & Gray, 2013). Although the majority of the solvent is recoverable for reuse, a small portion of unrecovered solvent remains incorporated with the NAE solids and may be co-deposited with them during disposal, which is a potential source of environmental impact (Hooshiar et al., 2012). The environmental considerations surrounding solvent co-deposition may differ depending on the solvent used in the NAE process. Different hydrocarbons have been analysed to determine which compound or mixture of compounds provides adequate bitumen recovery with minimal residual solvent, and cyclohexane was found to have the highest recovery and least residual concentration (Nikakhtari et al., 2013). In another study looking at the performance of cyclohexane and cyclopentane mixed with different n-alkanes, cyclopentane was found to have a somewhat lower recovery rate compared to cyclohexane (91.6 ± 0.8% vs. 97.9 ± 0.8%, respectively), but is still an effective solvent for the purposes of NAE (Pal et al., 2015). Although the microbiological fates of other solvents such as naphtha, n-alkanes, and toluene are either well studied or currently being examined, the fate of cycloalkanes in the environment is unknown, especially in the context of NAE solids (Abu Laban et al., 2015; Laban et al., 2015; Siddique et al., 2007). Since the potential environmental impact of these solvents is unknown, NAE has not yet been implemented at a commercial scale.

1.3.1 Environmental Fates of Aliphatic Hydrocarbon Solvents

When released into natural environments, hydrocarbons can have various biological and non-biological fates depending on the circumstances into which they are released. Previous research has demonstrated that solvents could leach into groundwater, volatilize into the atmosphere, or persist and accumulate in deposits. Microbiological fates may include complete

mineralization, which involves complete oxidation of the hydrocarbon to produce water, carbon dioxide, and/or methane, making these compounds a potential source of greenhouse gas emissions. Another possibility is biotransformation, in which microbial communities partially oxidize compounds to create polar compounds of unknown structure or mobility in groundwater. In some particular cases, these microbially produced compounds can be more toxic than the original compound and may cause further environmental contamination. Furthermore, if the compound is recalcitrant and unable to be degraded by microbial activity, cumulative toxicity within the environment after repeated deposition may occur.

There are currently three possible scenarios for solid reclamation after NAE in which NAE solids are mixed with soils or other microbial inoculate and disposed of in the natural environment: in-pit deposition where NAE solids are mixed with pristine soils, upland deposition where NAE solids are once again mixed with soils, and wetland deposition in which the NAE solids are mixed with wetland material. These scenarios generate a multitude of redox conditions in which cycloalkanes may or may not be biodegradable. On the surface and margins of the NAE deposit, aerobic or sub-oxic conditions will likely permit aerobic microbes using oxygen as an electron acceptor in their metabolic pathway to degrade NAE solvents. Within the dry solids deposit, however, oxygen concentrations will likely be limiting and anaerobic pathways would be dominant. This would be especially prominent in the wetland scenario, where soil pore space would be saturated with water, making even less oxygen readily available. The specific method anaerobic degradation in these scenarios (nitrate-reducing, sulfate-reducing, iron-reducing, methanogenic) depends on availability of electron acceptors and composition of local microbial communities. If local communities are incapable of cycloalkane degradation, bioaugmentation using hydrocarbon-tolerant communities from FFT may be necessary.

1.3.2 Potential Metabolic Pathways for Degradation of Cyclohexane

In different redox conditions, cyclohexane undergoes drastically different microbially induced chemical changes to eventually be mineralized or biotransformed to a subsequent compound. Although these pathways have not been confirmed to occur in respect to NAE solids specifically, other studies have proposed aerobic and anaerobic pathways that may be relevant. In one paper examining the microbial degradation of mineral oil, researchers proposed a linear aerobic pathway for cyclohexane biodegradation performed by mixed cultures (Fritsche & Hofrichter, 2008) (Figure 1). Cyclohexane monooxygenase enzymes use oxygen to oxidize cyclohexane to create cyclohexanol, an alcohol which is then further oxidized by cyclohexanol dehydrogenase to create cyclohexanone. This ketone is then oxidized by cyclohexanone monooxygenase to make Ɛ-caprolactone, which is then converted by Ɛ-caprolactone hydrolase to adipic acid, a carboxylic acid that undergoes ß-oxidation to form acetyl-CoA. Acetyl-CoA is used within the cell for a variety of biochemical reactions, including the citric acid cycle.

For anaerobic cyclohexane degradation, the currently proposed pathways are much less straightforward. Cyclohexane is thought to be initially activated by addition to fumarate to yield the metabolite cyclohexylsuccinate (Maguire et al., 2013). In methanogenic pathways, further degradation produces cyclohexanecarboxylic acid, which may undergo further degradation to methane and carbon dioxide (Jaekel, Zedelius, Wilkes, & Musat, 2015). Research examining cyclohexane degradation by SRB isolated from hydrocarbon-contaminated sediments proposed a possible sulfate-reducing pathway in which cyclohexane undergoes fumarate addition to produce cyclohexylsuccinate, which is then metabolized to cyclohexylsuccinyl-CoA (Jaekel et al., 2015). This compound is structurally rearranged to (cyclohexylmethyl)malonyl-COA, which is then decarboxylated to 3-cyclohexylpropionyl-CoA. This compound undergoes ß-oxidation to form

cyclohexanecarboxyl-CoA, which can be further oxidized to acetyl-CoA. Acetyl-CoA can either be used for terminal oxidation or the regeneration of fumarate to continue metabolism.

1.4 Experiment Rationale and Hypothesis

Given the promising results of the NAE process in oil sands extraction and the growing volume of wet tailings produced by current extraction methods becoming difficult to manage, insight into the potential environmental impacts of NAE is absolutely necessary in order to work towards implementing the process on a larger scale. Multiple explanations have been proposed for the fate of a cycloalkane solvent in the environment but would vary based on specific circumstances such as soil conditions and local microbial community composition. This study is necessary to elucidate whether cycloalkane biodegradation is feasible under multiple conditions.

Based on what is known about microbial activity and the current state of oil sands tailings ponds, it is expected that either aerobic or methanogenic microbial communities would be effective cycloalkane degraders, while communities that proliferate in other redox conditions would require longer incubation times in order to eventually break down cycloalkanes.

1.5 Thesis (Chapter Distribution)

The work done during this thesis was divided into four different incubation experiments set up at different time points with experimental monitoring being performed concurrently. The second chapter includes a fluid fine tailings (FFT) microcosm experiment consisting of 35 160 mL microcosms testing the ability of FFT microorganisms to degrade cyclohexane in five different redox conditions. The second chapter also includes the subsequent experiment, which involved 16 500 mL microcosms which contained both FFT and non-aqueous extraction (NAE) tailings, also referred to as dry solids or NAE solids. Cyclohexane and cyclopentane biodegradation was monitored in aerobic and methanogenic redox conditions. The third chapter

includes data from the microcosm incubation experiment established first, which was comprised of forty 160 mL microcosms containing soils under four different redox conditions and amended with either cyclohexane or cyclopentane. Additionally, this experiment also included 8 supplementary FFT microcosms amended with different combinations of cyclohexane, cyclopentane, and n-heptane. The appendix includes data from a currently incomplete experiment involving the set up of aerobic 160 mL microcosms identical to the second chapter that were amended with isotopic carbon-13 cyclohexane used to mark cyclohexane-degrading species. These species will be identified using stable isotope probing techniques in the future.

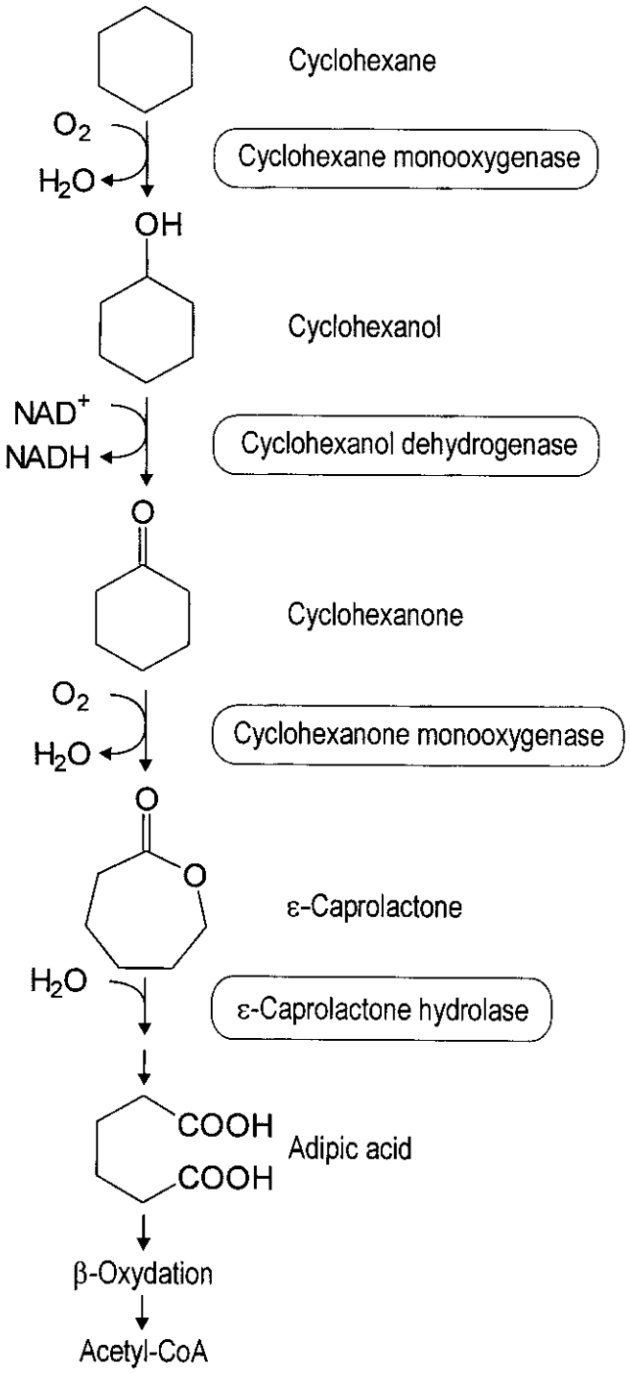


Figure 1-1. Possible cyclohexane biodegradation pathway for aerobic microbial communities. Adapted from (Fritsche & Hofrichter, 2008).

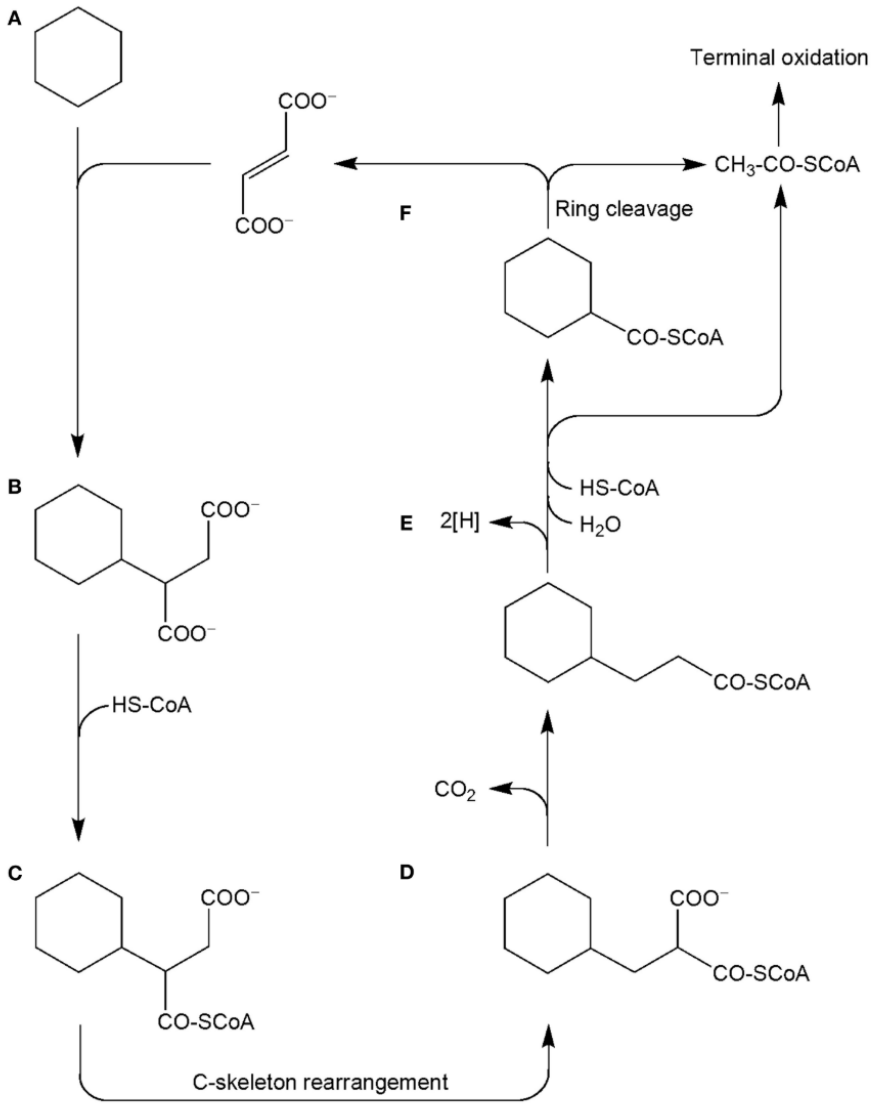


Figure 1-2. Possible anaerobic cyclohexane biodegradation pathway via fumarate addition occurring in either methanogenic or sulfur-reducing redox conditions. Adapted from (Jaekel et al., 2015).

Chapter 2: Incubation of Fluid Tailings, Non-Aqueous Solids, and Cycloalkanes in Multiple Redox Conditions

2.1 Introduction

The Alberta oil sands is located in the northeast region of the province and has been projected to contain 165.4 billion barrels of crude oil (Alberta Government, 2017). Although only approximately 20% of these reserves are accessible by surface mining, it serves as the primary method of oil sands extraction and was responsible for more than half of oil sands production in 2012 (Hooshiar et al., 2012). However, surface mining has several drawbacks, one of the most significant being the production of oil sands tailings. Currently, the total volume of tailings produced by oil sands production totals almost 1.21 billion cubic metres and has become increasingly difficult for oil sands operators to manage (OSIP, 2016). Therefore, new extraction technologies are now being sought out to eliminate tailings production and the need for tailings ponds. One promising candidate is non-aqueous extraction (NAE), which involves replacing the current hot water method with hydrocarbon solvent extraction. Unlike the current method, NAE uses very little freshwater and therefore generates smaller volumes of "dry" solids suitable for immediate disposal as opposed to the long-term aqueous storage and reclamation seen in tailings ponds. Additionally, bitumen recovery is increased, with cycloalkane solvents such as cyclohexane being capable of 94.4% bitumen recovery (Nikakhtari et al., 2013). Although much of the solvent is recoverable from the ores and can be recycled, small quantities are incorporated with the NAE solids and may potentially be co-deposited with them upon disposal, which may have unintended deleterious environmental effects.

Multiple potential pathways in the environment are possible for the deposited NAE solids, including loss to the atmosphere, groundwater contamination, and accumulation in natural deposits. Another possibility is the incorporation of the solvent into microbiological systems, wherein cyclohexane may be completely oxidized (producing water, carbon dioxide, and/or

methane) or partially oxidized, which involves biotransformation into polar compounds of unknown mobility and toxicity. In different deposition scenarios, multiple redox conditions are possible depending on the availability of different electron acceptors, and bench-scale microcosm studies are typically used to simulate hydrocarbon biodegradation in these different scenarios. Previous experiments have found that cyclo-alkanes either remain undegraded or can require months or even years to be degraded in hydrocarbon contaminated environments under anaerobic conditions (Faidz, Shahimin, & Siddique, 2017; Tan et al., 2015). However, cycloalkane degradation in the context of NAE solids is currently unknown and has a paucity of published literature. This project is divided into two experiments, wherein the initial experiment involved the incubation of fluid fine tailings (FFT) from oil sands tailings ponds that have previous exposure to hydrocarbon-enriched conditions and amended with 300 ppm of cyclohexane, a promising NAE solvent. These microcosms also receive media to promote different redox conditions, including aerobic, nitrate-reducing, sulfate-reducing, iron-reducing, and methanogenic conditions, and then sealed to monitor solvent degradation and gas/metabolite production via gas chromatography. Similarly, the second experiment scales up the microcosm size from 158 mL to 500 mL and includes NAE solids in the incubation. FFT and oil sands process affected water (OSPW) were investigated as potential bacterial inoculates, and the influence of nutrient deficiency on biodegrading microbial communities was also examined. Both cyclohexane and cyclopentane amendments were included at concentrations of 200 ppm. These experiments use the establishment of microcosms to replicate environmental conditions, albeit in a controlled scenario where we are capable of monitoring physical, chemical, and biologically induced endpoints for cycloalkanes.

2.2 Materials and Methods

2.2.1 Chemicals and Reagents

All chemicals were purchased from Fisher Scientific, located in Ontario, Canada. For a complete list of reagents and chemicals used in the lab, as well as manufacturers and grade, refer to the List of Reagents located in Appendix A.

2.2.2 Gases

70% N_2/CO_2, N_2 (98% purity), N_2 (95% purity), and pure O_2 canisters were purchased from Praxair, which is located in Edmonton, Alberta, Canada.

2.2.3 Microbial Inoculate Collection

Fluid fine tailings (FFT) were collected in bulk in 2016 from an oil sands tailings pond near Fort McMurray at a depth of 10.5 metres below the surface and stored in air-tight pails in the dark at a temperature of 4°C. After removal from the refrigerator, samples were immediately used in the culture setup in either 158 or 500 mL sealed serum bottles.

2.2.4 Media Preparation

Five different types of nutrient media were prepared for the five different redox conditions that the microcosms are simulating. Nitrate-reducing media was prepared according to the protocol outlined in Rabus and Widdel (1995) (Rabus & Widdel, 1995). Media promoting sulfate-reducing conditions was prepared based on a protocol adapted from Chi Ming So & L.Y. Young by Sara Ebert (So & Young, 1999). Media for iron reducing conditions was prepared according to Lovely and Philips (1986) (Lovley & Phillips, 1986). The recipe for methanogenic media was adapted from Fedorak and Hrudey (1984) as well as Holowenko et al. (2000) (Fedorak & Hrudey, 1984; Holowenko et al., 2000). Aerobic media, also referred to as Bushnell-Haas media, was prepared for aerobic incubations.

For nitrate-reducing media, six stock solutions were prepared and stored at 4°C before use. An EDTA-chelated mixture of trace elements containing sodium EDTA (5.2 g) , $FeSO_4 \cdot 7H_2O$ (2100 mg), H_3BO_3 (30 mg), $MnCl_2 \cdot 4H_2O$ (100 mg) $CoCl_2 \cdot 6H_2O$ (190 mg), $NiCl_2 \cdot 6H_2O$ (24 mg), $CuSO_4 \cdot 5H_2O$ (29 mg), $ZnSO_4 \cdot 7H_2O$ (144 mg), $Na_2MoO_4 \cdot 2H_2O$ (36 mg), and 987 mL of distilled water was dispensed into bottles, sealed, and autoclaved. Preparation of the vitamin mixture included mono and di-basic sodium phosphate as buffers, as well as 4-aminobenzoic acid (4 mg), D(+)-Biotin (1 mg), nicotinic acid (10 mg), calcium D(+)-pantothenate, and pyridoxine dihydrochloride (15 mg). The solution is then filter sterilized (0.2 μm pore size) before storage. The next solution included 100 mL of 25 mM sodium phosphate buffer and 10 mg thiamine chloride dihydrochloride, which was also filter-sterilized and then stored for future use. The Vitamin B_{12} solution contained cyanocobalamin (5 mg) dissolved in 100 mL of water which was then filter sterilized. The selenite-tungsten solution contains NaOH (400 mg), $Na_2SeO_3 \cdot 5H_2O$ (6 mg), and $Na_2WO_4 \cdot 2H_2O$ (8 mg). Finally, an $NaHCO_3$ solution with a concentration of 84 g/L is autoclaved and cooled under CO_2. 500 mL of nitrate-reducing media was then prepared containing KH_2PO_4 (0.25 g), NH_4Cl (0.15 g), $MgSO_4 \cdot 7H_2O$ (0.25 g), $CaCl_2 \cdot 2H_2O$ (0.05 g), 10 mM $NaNO_3$ (0.425 g), and 5 mL of resazurin, a redox indicator that turns pink in the presence of oxygen. This media was bubbled with 70:30 N_2:CO_2 gas for 1 hour, then dispensed in 100 mL aliquots into serum bottles flushed with N_2:CO_2 gas during transfer. These bottles were then autoclaved, and after cooling, each 100 mL aliquot received 4 mL of the $NaHCO_3$ solution and 100 μL of each stock solution before storage.

For sulfate-reducing media, five stock solutions were prepared and stored at 4°C before use. A trace elements solution was prepared that contained $CoCl_2 \cdot 6H_2O$ (3 g), $CuCl_2$ (15 mg), $FeCl_2 \cdot 4H_2O$ (0.15 g), H_3BO_3 (0.57 g), $MnCl_2 \cdot 4H_2O$ (2 g), $Na_2MoO_4 \cdot 2H_2O$ (0.25 g), $NiCl_2 \cdot 6H_2O$

(0.15 g), $ZnCl_2$ (0.21 g), and 0.4165 mL HCl all dissolved in 500 mL of distilled water. This solution was autoclaved and stored at 4°C. A vitamin solution containing Vitamin B12 (1 mg), D(+)Biotin (20 mg), folic acid (20 mg), nicotinic acid (50 mg), p-aminobenzoic acid (50 mg), Ca-D(+)pantothenate (50 mg), pyridoxine HCl (100 mg), riboflavin (50 mg), thiamine (50 mg), and thioctic acid (50 mg) all dissolved in 1L of distilled water. The pH is then adjusted to be neutral (pH=7), filter sterilized, sealed, and the headspace is then replaced with N_2 gas. A bicarbonate solution with a concentration of 84.01 g $NaHCO_3$ per litre of distilled water was sparged with CO_2, then filter sterilized into a sterile, anaerobic (N_2 headspace) serum bottles. Sodium sulfide is a reducing agent used to react with oxygen to promote anaerobicity within the media. A 67 mM $Na_2S{\cdot}9H_2O$ solution (16.1 g/L) was also prepared using boiled distilled water and dispensed into serum bottles, which were then sealed and autoclaved before storage. 500 mL of sulfate media solution was then prepared, which contains NaCl (0.1 g), KCl (0.65 g), $MgCl_2{\cdot}6H_2O$ (0.5 g), $CaCl_2{\cdot}2H_2O$ (0.05 g), NH_4Cl (0.25 g), KH_2PO_4 (0.1 g), Na_2SO_4 (0.71 g), and 5 mL of resazurin. The media was then sparged with 70:30 N_2:CO_2 gas for 1 hour, dispensed in 100 mL aliquots to 158 mL serum bottles, and autoclaved. After autoclaving, the media bottle headspace was flushed with N_2:CO_2 gas to maintain anaerobic conditions, and each bottle received 0.1 mL of the vitamin solution, 0.5 mL of the trace element solution, 3 mL of the bicarbonate solution, and 3 mL of the sodium sulfide solution before storage at 4°C.

Preparation of Coates iron reducing media requires four stock solutions, each with different components required for growth of iron-reducing microbial communities. Wolfes Vitamins solution contains biotin (2 mg), folic acid (2 mg), pyroxidine HCl (10 mg), thiamine HCl (5 mg), riboflavin (5 mg), nicotinic acid (5 mg), Na-pantothenate (5 mg), trace amounts of Vitamin B12, p-aminobenzoate (5 mg), and thioctic acid (5 mg) dissolved in 100 mL of distilled

water, which is then filter sterilized. Wolfes Mineral solution contains nitrilotriacetic acid (1.5 g), $MgSO_4 \cdot 7H_2O$ (3 g), $MnSO_4 \cdot H_2O$ (0.5 g), NaCl (1 g), $FeSO_4 \cdot 7H_2O$ (0.1 g), $CoCl_2 \cdot 6H_2O$ (0.1 g), $CaCl_2$ (0.1 g), $ZnSO_4 \cdot 7H_2O$ (0.1 g), $CuSO_4 \cdot 5H_2O$ (0.01 g), $AlK(SO_4)_2 \cdot 12H_2O$ (0.01 g), H_3BO_3 (0.01 g), $Na_2MoO_4 \cdot 2H_2O$ (0.01 g), and $NiCl_2 \cdot 6H_2O$ (0.025 g) dissolved in 100 mL of distilled water. The pH of the solution is adjusted to 7.4, dispensed into a serum bottle, and autoclaved before storage. The 100x $FeCl_2$ solution (250 mM stock) requires 200 mL of distilled water to be boiled, cooled on ice, and then sparged with 70:30 N_2:CO_2. $FeCl_2$ (4.08 g) is then added to a 158 mL serum bottle, and 100 mL of the cooled, now anoxic water is added. The bottle is flushed for 5 minutes with N_2:CO_2, and was then capped, sealed, and autoclaved before storage. The ferrihydrite ($Fe(OH)_3$) slurry involves dissolving 55.04 g $FeCl_3 \cdot 6H_2O$ in 500 mL of distilled water in a sterile beaker to create a 0.4 M solution. In a separate beaker, 40 g of NaOH was dissolved in 1 L of distilled water to create a 1 M solution. The $FeCl_3$ solution was then titrated with NaOH until reaching a neutral pH (7.0-7.1). The solid $Fe(OH)_3$ generated by the reaction was given time to precipitate, the supernatant is removed, and the solid ferrihydrite was washed 5 times with distilled water and then used to bring the volume to 1 L. The slurry was then well mixed and dispensed into sterile serum bottles which were capped, sealed and flushed with N_2:CO_2 before storage. Finally, Coates media for iron reducing conditions was prepared, which contains NH_4Cl (0.125 g), NaH_2PO_4 (0.3 g), $NaHCO_3$ (1.25 g), KCl (0.05 g), and nitrilotriacetic acid (0.375 g) dissolved in 480 mL of boiled distilled water. 5 mL of Wolfes solution and 15 mL of the ferrihydrite slurry is then added to the media, which is then dispensed in 100 mL aliquots to 158 mL serum bottles. The bottles are capped and sealed, and the headspace is flushed with N_2:CO_2 and then autoclaved. After autoclaving, 1.1 mL of Wolfes vitamin solution and 1.1 mL of the $FeCl_2$ solution is added to each bottle before storage.

Methanogenic medium preparation involves mixing two mineral stock solutions that were then added to the media. The first mineral solution contained NaCl (5 g), $CaCl_2 \cdot 2H_2O$ (1 g), NH_4Cl (5 g), and $MgCl_2 \cdot 6H_2O$ (1 g) all dissolved in 100 mL distilled water. The second mineral solution contained $(NH_4)_6Mo_7O_{24} \cdot 2H_2O$ (1 g), $ZnSO_4 \cdot 7H_2O$ (0.01 g), H_3BO_3 (0.03 g), $FeCl_2 \cdot 4H_2O$ (0.15 g), $CoCl_2 \cdot 6H_2O$ (1 g), $MnCl_2 \cdot 4H_2O$ (0.003 g), $NiCl_2 \cdot 6H_2O$ (0.003 g), and $AlK(SO_4)_2 \cdot 12H_2O$ (0.01 g) dissolved in 100 mL distilled water. The next step in the procedure involves mixing 7 mL of mineral solution 1, 0.7 mL of mineral solution 2, 7 mL of resazurin, 7 mL of a 50 g/L KH_2PO_4 solution, and 5.7 g of $NaHCO_3$. 500 mL of boiled distilled water is added to the mixture, and after the ingredients are fully dissolved, the media is sparged with N_2:CO_2 for 1 hour and then dispensed in 100 mL aliquots into serum bottles which are then sealed, autoclaved, and then flushed with N_2:CO_2 in the media bottle headspace. After sterilization, each bottle receives 1 mL of anaerobic 25 g/L sodium sulfide and 0.5 mL of a sterilized, anaerobic vitamin B solution before storage. This solution contains pyridoxine (0.025 g), thiamine (0.005 g), nicotinic acid (0.01 g), pantotheinic acid (0.0025 g), Vitamin B12 (0.01 g), and p-aminobenzoic acid (0.005 g) in 100 mL of distilled water.

Aerobic MFT cultures were incubated with 1L sterile aerobic Bushnell-Haas media, which contained K_2HPO_4 (1 g) , KH_2PO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $CaCl_2 \cdot 2H_2O$ (0.02 g), $FeCl_3$ (0.05 g), and NH_4NO_3 (1 g). For aerobic cultures containing NAE solids, cultures were calculated to be receiving 514 ppm of carbon in the form of cyclohexane and cyclopentane amendment, and nitrogen and phosphorus were added to nutrient amended treatments such that the microcosms have a 100:10:1 C:N:P ratio. Therefore, 51.4 ppm of nitrogen and 5.14 ppm of phosphorus is equivalent to 785 mg of NH_4Cl and 115.6 mg of K_2HPO_4 in 2 L of distilled water, which was added and then autoclaved before storage. This serves as a minimal nutrient media.

2.2.5 Experimental Setup

The initial experiment to establish cyclohexane-degrading communities within FFT was set up first. An anaerobic chamber containing all requisite lab equipment was flushed with industrial grade N_2 gas for twenty minutes, and then 35 mL of FFT was added to sterile 158 mL bottles previously sterilized via autoclaving. 7 bottles were set aside for aerobic redox conditions and established in ambient atmospheric conditions rather than in the anaerobic chamber. Since FFT tends to be difficult to work with and can adhere to the syringe surface, the FFT density was first determined to be 1.29 g/mL and was then added to microcosms using a scale to ensure consistency. 35 mL of FFT corresponds to ~45 g, so each bottle received 45 g (±1 g), with the exception of four bottles receiving an source of FFT inoculate (5 mL) from previous anaerobic experiments. These bottles received 30 mL of FFT to ensure consistent volumes in all microcosms. The nitrate-reducing microcosm receiving inoculate received FFT from a previous experiment done by Lisa Domreis in the Siddique Lab at the University of Alberta which involved the use of nitrate-reducing microbial communities to degrade BTEX compounds. The iron-reducing inoculate also originated in a degradation experiment done by Lisa Domreis. The methanogenic and sulfate-reducing inoculates came from microcosm biodegradation experiments performed by Mohd Faidz Mohamad Shahimin also in the Siddique Lab at the University of Alberta. No active aerobic microbial inoculate was available for the aerobic incubations, so a third amended replicate without inoculate was included instead. All bottles then received 35 mL each of media; 7 bottles received aerobic media, 7 bottles received nitrate-reducing media, 7 bottles received sulfate-reducing media, 7 bottles received iron-reducing media, and 7 bottles received methanogenic media. Bottles were then sealed with autoclaved butyl stoppers cut to 1/3” thickness and crimped with aluminum caps to ensure all gases produced would remain in

microcosm and measured. Anaerobic bottles were then flushed with 70:30 N_2:CO_2 for 8 minutes and flushed again for 8 minutes before amending to ensure conditions were anoxic.

Before amending the microcosms with cyclohexane, 2 bottles were selected from each redox condition and autoclaved once a day for 4 consecutive days. These bottles served as heat-killed, sterile controls in the experiment that demonstrate the contribution of any chemical changes in the microcosms to associated hydrocarbon concentrations as well as any other monitored parameters. After these sterile controls were established, cyclohexane was added to 5 of the 7 bottles in each redox condition. 2 bottles were left unamended to examine any changes in metabolite production not attributable to the degradation of cyclohexane, such as the degradation of endogenous hydrocarbons and organic acids already present in FFT. The other 5 bottles, 2 being sterile controls, were amended to contain 300 mg/L of cyclohexane. This concentration was selected because it is low enough such that it is unlikely to be toxic to FFT microbes, and the amount of cyclohexane is also sufficiently high enough to be detectable and monitored via gas chromatography analysis. The total volume within the microcosm was 70 mL, and was therefore amended with 27 µL of pure cyclohexane before incubation monitoring began.

The next experiment involved authentic NAE solids produced by the non-aqueous extraction process and provided by the Institute for Oil Sands Innovation (IOSI). The microcosms established for the purposes of this project were scaled up significantly (500 mL vs. the previous 158 mL). In this case, two redox conditions were selected as likely candidates for biodegradation to occur: aerobic and methanogenic. Within each microcosm, 75 g of NAE solids were weighed in, along with 75 mL of microbial inoculate (either FFT or OSPW). FFT was added using a scale to ensure consistency, while OSPW was added on a volume basis. An additional 150 mL of liquid was also added to all bottles; in methanogenic treatments, 150 mL of

methanogenic media was added to all microcosms, while in aerobic incubations, microcosms either received a tailored minimal nutrient media to create a 100:10:1 C:N:P ratio, or sterile water for non-nutrient amended treatments. Similar to the previous experiment, addition and mixing of these microcosm components was done in ambient atmospheric conditions for aerobic bottles and in an anaerobic chamber flushed with N_2 gas for methanogenic bottles. After sealing with butyl stoppers cut to 1/3" and crimping with aluminum caps, methanogenic bottles were then flushed with N_2:CO_2 for 8 minutes to ensure anaerobicity. 2 bottles from each treatment were then set aside and autoclaved once a day for 4 consecutive days to serve as heat-killed control microcosms. Before amending with hydrocarbons, aerobic microcosms were flushed with pure oxygen for 8 minutes, and then adding an additional 4995 μmol of oxygen (120 mL at 1 atm) to meet the calculated stoichiometric oxygen demand for the aerobic biodegradation of the added hydrocarbons. Cyclopentane and cyclohexane were then added to both the sterile controls and amendment bottles, with two bottles from each treatment not receiving any admendment and serving as unamended control bottles. Volumes of 80 μL cyclopentane and 77 μL cyclohexane were added to each bottle to give concentrations of 200 ppm; concentrations were chosen because they are likely non-toxic to FFT microbes and are detectable via gas chromatography. Both cyclopentane and cyclohexane were added in this experiment (as opposed to only cyclohexane) to observe whether one hydrocarbon would be preferentially biodegraded sooner than the other. With the exception of the unamended controls, 50 ppm of 1,1,3-trimethylcyclohexane was added to all amended bottles to serve as an internal standard for hydrocarbon measurements. This hydrocarbon has been found to be recalcitrant in almost all circumstances, and any fluctuations in internal standard measurement can be attributed to human or machine error rather than microbiological activity in the microcosm.

In both experiments, all microcosms were incubated in the dark at ambient room temperature (20°C). Anaerobic microcosms were incubated on benchtop without any agitation, while aerobic incubations were placed on a covered gyrotory shaker shaking at 180 rpm for at least 12 hours per day. Bottles remained sealed for the entire incubation duration, with the exception of bottles requiring additional nitrate or sulfate, as well as bottles containing NAE solids that required microbial sampling after degradation had occurred.

2.2.6 Carbon Dioxide (CO_2) Analysis

Carbon dioxide analysis was performed by injecting 100 µL of headspace gas from the microcosm (after shaking the bottle to release any trapped gas) into a gas chromatograph (GC) equipped with a thermal conductivity detector (TCD) using a 28G 500 µL disposable insulin syringe. Carbon dioxide standards (0.16%, 1%, 2%, 4%, 8%, 15%, and 30%) were prepared in sterile 158 mL serum bottles and used to prepare a standard curve to determine the percentage of carbon dioxide in the microcosm headspace. Additionally, pressure was measured in each microcosm using a 25G needle attached to a pressure meter. The pressure and % CO_2 was then used to calculate the amount of carbon dioxide (µmol) using the Ideal Gas Law (PV=nRT).

2.2.7 Methane (CH_4) Analysis

Similar to carbon dioxide analysis, anaerobic microcosms were shaken to release any gas trapped in the liquid phase, and then 100 µL was extracted from the headspace using a disposable 28G 500 µL insulin syringe. This volume was injected into a GC equipped with a flame ionization detector (FID). Methane standards (0.16%, 1%, 4%, 8%, 15%, 30%) were also prepared in sterile 158 mL serum bottles and then used to generate a standard curve to determine the percentage of methane in headspace. Pressure was measured as above, and the methane percentage and pressure were then used to calculate µmol of methane produced (Ideal Gas Law).

2.2.8 Headspace Hydrocarbon Analysis

Both aerobic and anaerobic microcosms were measured for cyclohexane, cyclopentane, and 1,1,3-trimethylcyclohexane using a gas chromatograph equipped with a mass spectrometer (GC-MS). After shaking the microcosm, 100 µL of headspace is extracted using a disposable 28G 500 µL disposable insulin syringe, with the resulting peak output covering 12 minutes post-injection. The retention time for cyclopentane is ~2 minutes and ~3.2 minutes for cyclohexane. Peak areas were documented and monitored over the course of the incubations to determine whether biodegradation of cyclohexane or cyclopentane had occurred.

2.2.9 Liquid Extraction Hydrocarbon Analysis

For the purposes of these experiments, a new protocol was developed to measure the presence of hydrocarbons within the liquid phase of the microcosms. 1 mL of the FFT/media mixutre is extracted using a 1 mL syringe with an 22G needle, and then added to 5 mL of methanol and shaken for 30 minutes. The samples are then centrifuged at 1500 rpm for 20 minutes to separate the solids associated with FFT. The samples are then stored in the refrigerator at 4°C for 30 minutes to 2 hours to ensure adequate settling. 4.5 mL of an NaCl solution (1 g NaCl/4.5 mL) is added into GC headspace analysis vials, and 0.5 mL of the top methanol layer is extracted from the previous vial and added to the headspace vial (1 per sample). The mixture is then sealed, and analyzed via GC-MS analysis. Additionally, 8 hydrocarbon standards are prepared (0 ppm, 50 ppm, 100 ppm, 150 ppm, 200 ppm, 300 ppm, 400 ppm), wherein FFT is amended with known concentrations of cyclohexane or cyclopentane and is processed as above to generate a standard curve, which is then used to calculate concentration. Concentration values were then compared to headspace hydrocarbon values to verify that any changes were due to biological activity rather than measurement error.

2.2.10 Oxygen Analysis

Unlike the carbon dioxide, methane, and hydrocarbon analyses described above, oxygen analysis was performed using a separate GC-TCD; this particular chromatograph was equipped with a molecular sieve that makes it capable of distinguishing between the dinitrogen and oxygen peaks on the chromatograph output. Aerobic microcosms were actively monitored for oxygen depletion. Similar to other headspace analysis, 100 µL of headspace was extracted from each microcosm and injected into the GC-TCD. A standard curve was generated by injecting ambient atmospheric air, with different volumes correlating with different amounts of oxygen. Standard volumes include 250 µL, 200 µL, 150 µL, 150 µL, 100 µL, 50 µL, and 25 µL of atmospheric air.

2.2.11 Nitrate and Sulfate Analysis

For analysis of nitrate and sulfate concentrations, a 5 mL syringe with an 22G needle was used to extract 4 mL from nitrate and sulfate reducing bottles and transferred to 2 eppendorf tubes (2 mL). These tubes were then centrifuged at 15000 rpm for 10 minutes to separate out the FFT-derived solids, and the supernatant was then removed and syringe filtered (0.45 µm filter pore size). Samples were then analyzed via ion chromatography (barium chloride and cadmium column reduction methods for sulfate-reducing and nitrate-reducing microcosms, respectively) at the Natural Resources Analytical Laboratory (U of A).

2.2.12 Reduced Iron Analysis

The ferrozine analysis assay method was used to quantify soluble reduced iron (Fe^{2+}) in the liquid fraction of the iron reducing microcosms. Iron (II) and ferrozine form a complex when in solution together in acidic conditions (pH range of 3 to 6). Initially, ferrous ammonium sulfate was used to create a standard curve which included a range of iron (II) concentrations: 20 ppm, 10 ppm, 5 ppm, 2.5 ppm, 1 ppm, 0.5 ppm, 0.25 ppm, 0.125 ppm, 0.0625 ppm, 0.03125 ppm, and

0.015625 ppm. These standards were added to a solution containing 50 mM HEPES buffer and 0.1% ferrozine and measured using a UV/Vis-spectrophotometer at a wavelength of 562 nm in order to create a calibration curve. 1 mL of liquid from each microcosm was then added to solution containing ferrozine and HEPES buffer and analyzed using the same spectrophotometer and standard curve to determine Fe^{2+} concentrations.

2.2.13 Nutrient Analysis

Nutrient analyses were performed on aerobic microcosms before and after hydrocarbon degradation had occurred to determine whether nutrients had become depleted due to microbial community growth. Phosphate concentrations were determined using the Molybdenum Blue method (EPA Method 365.1) and ammonium concentrations were determined using the salicylate-hypochlorite method (Bower & Holm-Hansen, 1980). Analyses were performed by the Natural Resources Analytical Laboratory at the University of Alberta.

2.2.14 Microbial Community Sampling and Analysis

FFT samples were extracted from microcosms for the purposes of microbial community analysis when hydrocarbon reduction and associated gas production analysis indicated that biodegradation had occurred or was currently taking place. For the FFT experiment, DNA samples were able to be taken with minimal disturbance to the microcosm, since the liquid phase consisted of a 50:50 proportion of FFT and media, which reduced the viscosity of the original FFT. Therefore, samples could be extracted by shaking the microcosm and injecting a 22G needle with an attached 1 mL syringe (both sterile). 1 mL was extracted and stored at -20°C in a sterile eppendorf tube before DNA extraction was performed.

For the larger microcosms containing NAE solids, the proportion of solids and liquid was altered by the inclusion of the NAE solids, which consists of silts, clay, and sediments typically

found in other soils as well. Although multiple needle gauges were attempted to extract sample from these microcosms, no needle could remove any volume of liquid, which is likely due to sediment clogging. Consequently, 20 mL of liquid volume was taken from actively biodegrading microcosms at the end of the experiment after unsealing the bottles, and then also stored at -20°C in sterile 50 mL falcon tubes (some volume taken for nutrient analysis as well).

DNA was extracted from samples using a modified protocol obtained from Foght et al. (2004), which involves the direct extraction of DNA using bead-mill homogenization and SDS-chloroform treatment (Foght, J. et al., 2004). Samples were removed from -20°C conditions and given time to thaw at 4°C, and then centrifuged at 15000 rpm for 10 minutes. The supernatant was discarded and a sterilized scupula was then used to extract the solid FFT pellet (250 mg), which was weighed into sterile 2 mL screw cap tubes containing 0.5 g of 2.3 mm and 0.5 g of 0.1 mm zirconium-silica beads. 300 µL of phosphate buffer (pH=8) is then added as well, which was prepared previously and contains both monobasic ($NaH_2PO_4 \cdot H_2O$) and dibasic (Na_2HPO_4) phosphate (sterilized via autoclave). Next, 300 µL 10% SDS lysis buffer was added, which was also previously prepared by mixing 48 mL H_2O (millipore), 2 mL of NaCl (5M), 50 mL of 1M Tris (pH=8), and 10 g of sodium dodecyl sulfate (SDS). The mixture is then sterilized via autoclave before use. Finally, 300 µL of 24:1 chloroform:isoamyl alcohol solution is added to the tubes before sealing. Tubes were then shaken via beadbeater using the Powerlyzer 24 for 45 seconds at 3400 reciprocations per minute in order to lyse the cells. The tubes were then centrifuged at 15000 rpm for 5 minutes, and the supernatant was transferred into a sterile eppendorf tube. Some samples underwent an optional step, wherein fresh buffers were added into the same screw cap tube and beadbeaten and centrifuged again to ensure as much DNA was extracted from the sample as possible. Ammonium acetate was then added to the supernatant to

obtain a final concentration of 2.5M, causing a precipitate to form in the tube. The tubes were then hand mixed gently and centrifuged at 15000 rpm for 7 minutes. The supernatant was then transferred into another sterile 2 mL eppendorf tube, and 0.54 volume of isopropanol was added. Samples were then put into the refrigerator (4°C) and incubated over night. The next day, the precipitated DNA was recovered by centrifuging at 15000 rpm for 30 minutes. The supernatant was then drained off, and tubes were then centrifuged for an additional minute at 15000 rpm to collect any remaining isopropanol at the bottom of the tube. The remaining supernatant was then removed using a micropipette, and the DNA pellet was left to dry for 2 hours at ambient room temperature. DNA was then dissolved in either 40 µL of TE buffer or 40 µL of sterile water, and then stored in the freezer (-20°C) until further analysis had taken place. Additionally, DNA was examined and quantified using either agarose gel electrophoresis, Nanodrop, or Qubit. A region of the 16S rRNA gene was amplified via PCR using the Pyrotag Sequencing Protocol and was then submitted to The Applied Genomics Core (TAGC) for Illumina sequencing.

2.3 Results

2.3.1 Carbon Dioxide Production

Carbon dioxide attributed to microbial biodegradation of hydrocarbons was measured in aerobic FFT and NAE microcosm cultures, with a stark difference in the amount of time required to produce significant amounts of CO_2. The experimental schemes for each experiment are outlined in Table 2-1 and 2-2 and the CO_2 production is presented in Figure 2-1 and 2-8.

In the FFT experiment, initial headspace was composed of atmospheric air, and after 75 days of inactivity wherein no biodegradation or carbon dioxide production took place, bottles were inferred to be oxygen depleted. After confirming depletion in all 7 bottles via oxygen measurement, bottles were injected with 2497 µmol of pure oxygen (60 mL, 1 atm) and

subsequent carbon dioxide production and oxygen loss was observed in the days following, with the exception of the sterile controls. Following this drastic increase in carbon dioxide concentration in both the amended microcosms and unamended controls (~200 µmol to ~1000 µmol CO_2), carbon dioxide production plateaued significantly until all 7 bottles were replenished with 2497 µmol of oxygen. After the amended and unamended microcosms experienced a similar spike in carbon dioxide concentrations (increasing to above 2000 µmol), liquid hydrocarbon sampling was performed and found that cyclohexane in the amended bottles had dropped below the gas chromatograph detection limit, while cyclohexane in the heat-killed controls still had a majority of the original 300 ppm remaining (257 ppm average). After microbial community samples were extracted, microcosms received two more oxygen inputs over the next two weeks to maintain aerobicity, and then microcosms were flushed with atmospheric air sterilized via filtration (0.2 µm filter pore size) for 10 minutes to remove carbon dioxide from all bottles, as well as any residual cyclohexane from the sterile controls. Since oxygen demand for 300 ppm of cyclohexane was stoichiometrically calculated to be 1214.3 µmol O_2 (29 mL, 1 atm), all bottles received oxygen in excess to ensure biodegradation (2497 µmol) before reamending with cyclohexane. With the exception of the unamended controls, all bottles received 25.4 µL of cyclohexane to create a concentration of 300 ppm in each microcosm. At this point, the heat-killed controls had an average of 136 µmol of carbon dioxide in their headspace, which slightly increased over the next ~150 days to an average of 402 µmol. In the remaining microcosms (unamended and amended), carbon dioxide values increased from ~580 µmol to ~1730 µmol of CO_2. Although all microcosms were provided with excess oxygen during microcosm reamendment, further oxygen additions (2497 µmol at each timepoint) were necessary due to the significant carbon dioxide production and unexpected oxygen depletion

occurring in all non-sterile microcosms. After cyclohexane was confirmed to be depleted, samples were extracted and the experiment ended, thereby releasing all carbon dioxide produced.

Although the larger 500 mL microcosms containing NAE solids were also in aerobic conditions, the treatments differed in that amended bottles were split between receiving no additional nutrients and those receiving nitrogen and phosphorus such that the C:N:P ratio is 100:10:1. Additionally, these bottles received 200 ppm of cyclohexane and 200 ppm of cyclopentane rather than 300 ppm of cyclohexane as per the previous experiment. Including control microcosms, all replicates had similar amounts of carbon dioxide at the onset of the experiment (average of 455 μmol CO_2). Production of carbon dioxide was significantly more rapid compared to the previous experiment, with nutrient amended bottles increasing to almost 3000 μmol CO_2, while the unamended and amended controls increased to almost 2000 μmol in the span of 16 days (Figure 2-8). The sterile controls were relatively stable in comparison, experiencing an overall increase of only 50 μmol over the 16 day timespan. At Day 28, reduction of both cyclopentane and cyclohexane was determined to have occurred in the nutrient amended bottles and is likely responsible for the dramatic increase in carbon dioxide concentrations.

2.3.2 Oxygen Depletion

In the FFT experiment, oxygen measurements were initially performed on Day 41 to confirm that oxygen depletion was preventing degradation of cyclohexane and concurrent production of carbon dioxide. After analyzing, it was determined that all 7 bottles had very low oxygen (~0.04 μmol for all). Similar results were found on Day 147 after finding that carbon dioxide production had stagnated after the initial increase due to oxygen addition. In this case, both the unamended and amended microcosms were found to contain ~0.07 μmol each, and therefore required further oxygen addition to continue biodegradation. Oxygen levels were also

measured on Day 208 immediately after microcosm reamendment (serving as a time zero value), as well as on Day 227, 334 and 370 to determine oxygen depletion over the course of the incubation. In total, microcosms received 4 additional oxygen inputs (2497 µmol each) over the next 150 days, totalling ~13.05 mmol of O_2 provided over the course of the second incubation. At the end of the incubation, sterile controls contained an average of 2.70 mmol, unamended controls contained an average of 1.91 mmol, and amended controls contained 1.60 mmol of O_2.

In the microcosms containing NAE solids, production of carbon dioxide was rapid and produced noticeable differences between treatments after 16 days. At Day 36, bottles were reamended with oxygen, each receiving 7491 µmol of oxygen (180 mL pure O_2 at 1 atm). On Day 84, oxygen levels were measured and found that sterile controls contained 9225 µmol of oxygen, unamended controls contained 7508 µmol, amended bottles with no additional nutrients contained 7338 µmol, and amended bottles with nutrients contained 52 µmol (Figure 2-10).

2.3.3 Methanogenesis

Methane generation was exclusively measured regularly in anaerobic methanogenic microcosms that were incubated with methanogenic media. For the 158 mL FFT microcosms, there was a small amount of initial methane production in amended bottles, which also caused a very small reduction in cyclohexane concentrations. However, upon further amendment with ~111 ppm cyclohexane on Day 136, all methane production ceased for the remainder of the incubation. In larger methanogenic bottles (500 mL), no methane production was observed.

2.3.4 Nitrate/Sulfate Reduction

Nitrate and sulfate reducing microcosms in the FFT experiment were incubated with media containing high concentrations of each electron acceptor in order to induce the activity of microbes capable of utilizing nitrate and sulfate in their metabolic pathways. Therefore, nitrate

and sulfate were measured periodically over the course of the incubation (Figures 2-5 and 2-6). At time zero, the concentration of nitrate in nitrate-reducing microcosms was 87.39 mg/L, while in sulfate-reducing microcosms, the concentration of sulfate was 624 mg/L. At Day 173, measurements for both nitrate and sulfate concentrations were found to be below the detection limit, with the exception of the sulfate reducing sterile controls, which were found to contain ~275 mg/L of sulfate. Since the electron acceptors were depleted in both cases with no evidence of hydrocarbon degradation, the liquid volume in all microcosms were increased to 100 mL with the additional liquid containing nitrate or sulfate. This addition contained a 50:50 ratio of FFT to media to maintain the 50:50 ratio already in the microcosm; however, the media contained either higher concentrations of nitrate (5 g/L $NaNO_3$) or sulfate (8.35 g/L Na_2SO_4) such that the concentration within each microcosm was equal to the original concentration of nitrate and sulfate in their respective medias (0.85 g/L $NaNO_3$ and 1.42 g/L Na_2SO_4) in the interest of adding electron acceptors at non-toxic concentrations. These solutions were mixed with equal volumes of FFT, and then added to the microcosms. In order to add these solutions, microcosms were unsealed in an anaerobic chamber flushed with N_2 containing the already mixed nitrate and sulfate media and FFT, which were then added based on weight using a scale. Bottles were then resealed, sterile controls were re-autoclaved, headspace was flushed with N_2:CO_2 to minimize any residual cyclohexane, and bottles were reamended with 300 ppm of cyclohexane on Day 185. After hydrocarbon and electron acceptor reamendment, sulfate and nitrate were analyzed on Day 194 and 419. Some degree of nitrate reduction takes place in two of the active replicates, while in sulfate reducing microcosms, all unamended and amended replicates are able to reduce some amount of sulfate; in both cases, sterile control concentrations remain steady with little to no variation in both nitrate and sulfate measurements.

2.3.5 Hydrocarbon Biodegradation

In the 158 mL aerobic FFT microcosms, 300 ppm of cyclohexane was initially added to both active microcosms and sterile controls. On Day 188, a liquid hydrocarbon extraction was performed and found that all three active replicates no longer contained a cyclohexane concentration that was detectable via GC analysis, while sterile controls retained an average of 257 ppm of cyclohexane. After flushing all bottles and reamending with 300 ppm of cyclohexane and sufficient oxygen, microcosms were monitored for the next ~150 days via headspace and liquid hydrocarbon analysis (Figure 2-2). Headspace analysis found cyclohexane was decreasing in both the active and sterile microcosms, while liquid analysis confirmed that sterile controls were relatively stable while cyclohexane in active microcosms was absent after ~150 days.

The anaerobic 158 mL microcosms were primarily monitored via headspace analysis, which were compared to initial values to determine if any cyclohexane degradation had taken place. If significant cyclohexane decline had occurred, liquid hydrocarbon extraction would be used to confirm this decline. However, no such decline was observed in methanogenic, nitrate-reducing, sulfate-reducing, or iron reducing microcosms after reamendment with cyclohexane (Figure 2-4). In all anaerobic cases, there is some degree of decrease in measured headspace cyclohexane; however, this decrease is observable in both the active microcosms as well as the sterile controls, and is therefore unlikely to be attributable to biodegradation.

In the NAE solids microcosms, headspace analyses were compared between Day 1 of the incubation and Day 28, with all showing considerable variation in cyclopentane and cyclohexane levels (Figure 2-9). However, in all three aerobic replicates that received FFT inoculate and minimal nutrient media (C:N:P of 100:10:1), both cyclohexane and cyclopentane were degraded to below a concentration of 1 ppm after 28 days. On Day 88, these three bottles were unsealed

along with a sterile control in order to perform a liquid hydrocarbon extraction. This extraction confirmed that no detectable cyclohexane or cyclopentane concentrations remained in the three replicates, while in the sterile control replicate, cyclohexane was present at a concentration of 300 ppm while cyclopentane was present at a concentration of 232 ppm. However, no evidence of hydrocarbon decline was found in aerobic bottles with OSPW inoculate or methanogenic bottles that received FFT inoculate during the incubation experiment.

Headspace and liquid hydrocarbon GC-MS outputs were also analyzed for the presence of metabolites such as cyclohexanol and cyclohexanone, but the presence of either compound was not found in any of the microcosms at a detectable concentration. The GC-MS retention time for both compounds were determined by headspace analysis of bottles containing pure cyclohexanol and cyclohexanone and searching for the resulting peaks in the output.

2.3.6 Nutrient Analysis

Nutrient analysis examined ammonium, nitrate, and phosphate ion concentrations in both the 158 mL aerobic FFT microcosms as well as the 500 mL aerobic NAE microcosms that received FFT inoculate and minimal nutrient media. In the FFT microcosms, two timepoints were analyzed for nutrients, each with three replicates: Day 0 and Day 413 (Figure 2-3). During incubation, concentrations of all three nutrients became depleted. For the NAE microcosms, nutrients were analyzed on Day 87 after degradation of cyclohexane and cyclopentane was complete. At time zero, the concentration of NH_4^+ in the microcosms is 117.72 mg/L and a PO_4^{2-} concentration of 17.34 mg/L, based on the concentration and volume of minimal nutrient media added. On Day 87, NH_4^{2+} concentrations were found to be 1.533, 1.085, and 1.131 mg/L in the three replicates. For PO_4^{2-}, the concentration in the first replicate was 4.1336 µg/L while the other two replicates were found to be below the detection limit (ie. completely depleted).

2.3.7 Characterization of Microbial Communities by 16S rRNA Gene Analysis

Microbial community composition in the FFT experiment was determined via 16S rRNA Illumina sequencing analysis of aerobic microcosms. Four timepoints were analyzed: Day 0, 183, 209, and 251. Similar results were found for the later three timepoints, wherein the genus *Rhodoferax* is dominant in both the amended microcosms as well as the unamended controls, followed by the genera *Burkholderiaceae*, *Caenimonas*, and *Porticoccaceae* in terms of abundance. Multiple anaerobic genera are also present in unamended and amended microcosms, albeit in very low and likely insignificant quantities. For time zero samples, anaerobic genera were present in higher quantities when compared to later in the incubation.

For the NAE solids, microbial community composition shifted drastically: at time zero when FFT was mixed with NAE solids during preparation of the incubation, and after 200 ppm of cyclohexane and 200 ppm of cyclopentane was almost completely degraded in amended microcosms (which also initially received nitrogen and phosphorus to achieve a C:N:P ratio of 100:10:1). In the initial source FFT, the primary genera with the highest fractions include *Rhodoferax* (~40% of total reads) and *Caenimonas* (~15%). Other main groups include *Acidovorax* and *Porticoccaceae*. After addition of NAE solids, the microbial community experiences a significant shift in which there is a reduced amount of *Rhodoferax*, *Caenimonas*, and *Acidovorax*, while *Hydrogenophaga, Sulfuritalea, Thiobacillus, Gammaproteobacteria*, and *Mollicutes* increased when compared to FFT on Day 0. After the amended cycloalkanes have been biodegraded, the proportions of both *Rhodoferax* and *Caenimonas* has been drastically reduced in the overall community composition when compared to FFT and FFT+NAE on Day 0. Alternatively, fractions of *Hydrogenophaga, Acidovorax, Porticoccaceae, Defluviimonas*, and *Galbitalea* were more common than in the Day 0 treatments.

2.4 Discussion

In the initial FFT experiment, carbon dioxide generation was observed parallel to cyclohexane degradation in active aerobic microcosms and was confirmed to have completely degraded cyclohexane via liquid hydrocarbon extraction analysis after two hydrocarbon amendments over the course of ~350 days. In the sterile control, however, carbon dioxide production was minimal and cyclohexane concentrations remained close to the original 300 ppm of cyclohexane that the microcosm originally received as a hydrocarbon amendment. Therefore, one can attribute this observed cyclohexane degradation and carbon dioxide production to microbial metabolic activity, wherein microbial communities incorporate cyclohexane as a carbon source into their cells where it becomes converted into different metabolites like cyclohexanol and cyclohexanone and eventually mineralized into carbon dioxide (Fritsche & Hofrichter, 2008). This is further supported by the headspace oxygen data taken after reamendment, which determined that the molar oxygen levels in the sterile controls were 2.70 mmol at the end of the incubation while the active microcosms contained 1.60 mmol. This 1.10 mmol difference between the active and sterile microcosms is relatively close to the stoichiometrically determined oxygen demand for 300 ppm of cyclohexane, which was calculated to be 1.214 mmol. However, the timeline for complete biodegradation of cyclohexane was unexpected; in other aerobic experiments involving cyclohexane biodegradation, complete biodegradation can occur within a span of days (Foght, J. et al., 2013). However, this can be explained by the scarcity of oxygen within the microcosms during the first ~75 days; after amending with additional oxygen, carbon dioxide production began to rise dramatically, and bioremediation of cyclohexane is presumed to occur concomitantly. After this steep rise in carbon dioxide, headspace concentrations plateau, which suggest degradation was complete.

Conversely, relatively high oxygen levels were maintained after reamending the microcosms with cyclohexane, but microcosms still required ~150 days to degrade cyclohexane. In this instance, the degradation rate being slower than expected may be explained by insufficient nutrients rather than oxygen. It was assumed that Bushnell-Haas aerobic media was a adequate source of ammonium, nitrate, and phosphate; however, nutrient analysis of samples taken at the beginning and end of the incubation found that ammonium and nitrate were completely depleted while less than half of the original phosphate concentration remained (Figure 2-3). This is a probable justification for a slower degradation rate of cyclohexane. Another possible explanation is the accumulation of metabolites that are produced by microbes when degrading cyclohexane, such as cyclohexanol and cyclohexanone. In some cases, excess concentrations of these metabolites can become toxic to the existing microbial community and inhibit biodegradation rates, preventing cyclohexane concentrations from being quickly reduced as expected in aerobic incubations. However, after determining the retention times of cyclohexanol and cyclohexanone in headspace and liquid extraction analysis, the previous GC-MS data was examined to determine whether either metabolite was potentially impeding the cyclohexane biodegradation rate. In both cases, minimal traces of the metabolites were found in the headspace at the start of the experiment, while in the later portion of the incubation, no hydrocarbons other than cyclohexane were detectable in both the liquid and headspace analyses. This suggests that the cyclohexane metabolic pathway for this particular aerobic microbial community does not generate toxic endpoint metabolites. Therefore, the most likely explanation for the extended time requirement is a scarcity of nutrients necessary for microbial growth.

When supplied with ample nutrients and oxygen, it is therefore expected that biodegradation of cyclohexane would take place over a much shorter period. This was observed

and proven in the following experiment, which included cyclohexane-containing NAE solids mixed with a bacterial inoculate and additional cyclohexane and cyclopentane amendments (200 ppm each) to ensure the feasibility of biodegradation at higher hydrocarbon concentrations. This experiment had multiple purposes: determine whether NAE solids possessed indigenous microbial communities capable of degrading cyclic hydrocarbons, if NAE solids could be successfully bioaugmented by aerobic or methanogenic cycloalkane-degrading microbes in FFT and OSPW, if microbes preferentially degrade cyclohexane over cyclopentane or vice versa, and the effect of nutrient availability on aerobic biodegradation. The results indicate that NAE solids do not contain microbial communities capable of cycloalkane degradation, given that microcosms that receive only NAE solids, sterile water, and additional hydrocarbons do not experience any reduction in either hydrocarbon. Similarly, aerobic microcosms inoculated with OSPW and methanogenic microcosms inoculated with FFT are also incapable of developing microbial communities capable of cycloalkane remediation. This may be due to limitations of OSPW in terms of microbial community populations; unlike FFT, OSPW in oil sands tailings ponds would be more exposed to chemical inputs and seasonal changes compared to the settled consolidated tailings, which tend to be deposited with residual hydrocarbons without further inputs. This allows microbial communities to be subjected to a hydrocarbon rich environment and select for communities capable of survival and biodegradation. Conversely, OSPW tends to harbour aerobic communities that can degrade hydrocarbons quickly, causing communities to change and adapt to other possible food sources, such as external chemical or microbial inputs.

In both the FFT and NAE experiments, active aerobic microcosms produced significant amounts of carbon dioxide when biodegrading amended cycloalkanes (Figures 2-1 and 2-8). For the 160 mL tailings microcosms, active bottles produced an average of 2257 μmol of carbon

dioxide after the first 300 ppm cyclohexane amendment and 1811 µmol after the second amendment. For the 500 mL NAE solids microcosms, active bottles produced an average of 6039 µmol of carbon dioxide after being amended with cyclohexane and cyclopentane. Since the initial concentration of all cycloalkanes and the total carbon dioxide production were both measured, experimental carbon dioxide values can be compared to expected amounts which are determined stoichiometrically to verify that complete biodegradation to carbon dioxide is occurring. When calculated stoichiometrically, it was expected that 70 mL of tailings mixed with media and amended with 300 ppm of cyclohexane would produce 1497 µmol of carbon dioxide after the cyclohexane had been completely degraded. For each amendment, the FFT microcosms exceeded the expected amount of carbon dioxide, and this is most likely due to other carbon sources being present in the initial FFT which were similarly degraded by the microbial community. However, the 500 mL bottles amended with both cyclohexane and cyclopentane did not reach the calculated theoretical maximum of 8557 µmol for carbon dioxide after the amended hydrocarbons were degraded. Multiple explanations may be responsible for these results; for instance, one difference between the NAE and FFT experiments is that the FFT-media slurry was determined to be significantly less viscous than the FFT mixed with water and NAE solids, which may have reduced gas exchange between the liquid and headspace fractions of the microcosm. Another important difference between the two experiments is the separate nutrient medias added to promote and sustain microbial activity. The FFT was provided with Bushnell-Haas media, which contains both monobasic potassium dihydrogen phosphate and dibasic potassium monohydrogen phosphate. These chemicals have a high buffering capacity and attenuate any changes in pH. In contrast, the NAE-FFT mixture was provided only with minimal nutrient media containing only ammonium chloride and dibasic potassium monohydrogen

phosphate. Since this mixture was not buffered, the carbon dioxide generated is much more soluble in the liquid phase and will form carbonic acid, lowering the overall pH and reducing the measured amount of carbon dioxide in the headspace of the microcosm.

In both experiments, no significant anaerobic biodegradation was observed over the course of either incubation. However, some nitrate and sulfate reduction was observed in the FFT experiment in the unamended and amended microcosms. This suggests that alternative carbon sources exist in FFT that may be preferentially degraded over cyclohexane in anaerobic conditions. Similarly, significant carbon dioxide production was detected in unamended controls in aerobic microcosms, which also conforms to the idea that other carbon sources exist within FFT that may be interfering with biodegradation of cyclohexane. Aerobic incubations may have been successful because aerobic metabolic pathways tend to require less specificity in terms of carbon source due to oxygen being a higher energy electron acceptor, which means that aerobic communities may have degraded both cyclohexane and endogenous carbon sources concurrently. Another possible issue that may have interfered with anaerobic biodegradation is the concentration of sodium sulphide added into sulfate-reducing and methanogenic media to ensure that all oxygen was depleted before adding into microcosms. High concentrations can be toxic to microbial communities, and an excess may have been added to the medias. This could potentially justify why no methanogenesis had occurred despite FFT being previously observed to produce methane in situ (Holowenko, Mackinnon, & Fedorak, 2000). Another possible explanation as to why anaerobic degradation was not observed is that cyclohexane is recalcitrant in these redox conditions, and these particular anaerobic tailings do not possess the necessary combination of microbial species and requisite enzymes required to degrade cycloalkanes, despite being provided with the requisite nutrients and electron acceptors.

The aerobic microbial communities in both experiments are responsible for the breakdown of the cycloalkanes that were added into each microcosm, and the genera detected in each treatment are provided in Figures 2-11 and 2-12. Within untreated control FFT at the onset of the experiment, the genera which proliferate include *Rhodoferax*, *Acidovorax*, *Comamonodaceae*, and *Porticoccaceae.* As the incubation progresses under aerobic conditions, the proportion of *Rhodoferax* increases in amended microcosms, indicating that *Rhodoferax* species are capable of survival in cyclohexane-rich conditions and potentially play a role in cyclohexane biodegradation. This is supported by other research into *Rhodoferax*, which has confirmed the ability of different *Rhodoferax* species to biodegrade a variety of environmental contaminants such as propylbenzene, benzene, toluene, and polycyclic aromatic hydrocarbons (PAHs) such as phenanthrene (Aburto & Peimbert, 2011; Eriksson, S., Ankner, Abrahamsson, & Hallbeck, 2005; Martin et al., 2012). Furthermore, *Rhodoferax* species have been isolated in petroleum-contaminated groundwater in both aerobic and anaerobic redox conditions (Ning et al., 2018). *Rhodoferax* is therefore a strong contender for cyclohexane biodegradation in the tailings microcosms, displaying survivability in petroleum-rich conditions as well as metabolic pathways used to break down complex cyclic chemical structures, which may be applicable to cyclohexane degradation. Although the *Caenimonas* genus is also found in relatively high abundance in both the unamended and amended microcosms, little information is available on their role in biodegradation; one species, however, is strictly aerobic (which explains why it was lower in abundance at time zero) and involved in biological phosphorus removal and elemental cycling (Ryu et al., 2008). Unlike the previous genera, *Comamonadaceae* maintains its relative abundance when comparing time zero to later stages of the experiment (possibly suggesting less direct involvement), but has been implicated to be involved in biodegradation of benzene and

nitrobenzene (Atashgahi et al., 2018; Li et al., 2008). Two additional genera that both displayed a smaller but significant increase in relative abundance are *Porticoccaceae* and *Immundisolibacter.* *Porticoccaceae* has been detected in situ in oil-contaminated environments and found to be involved in the biodegradation of PAHs (Brakstad et al., 2018). Using metagenomic analysis of chemically dispersed oils in seawater, researchers in a similar study have also discovered that *Porticoccaceae* contains a variety of aromatic degradation genes such as phenylacetone monooxygenase, cyclopentanol dehydrogenase, and 4-hydroxyphenylacetate 3-monooxygenase (Ribicic et al., 2018). It was concluded that *Porticoccaceae* appears to specialize for the degradation of initial alkane degradation byproducts, which would potentially also be applicable in the latter portion of the aerobic cyclohexane biodegradation pathway after the ring structure has been broken. Similarly, *Immundisolibacter* species have been determined to be associated with PAH biodegrading microbial communities and may also contribute to the breakdown of the cyclohexane ring structure (Corteselli, Aitken, & Singleton, 2017). The primary genera that constitute the FFT bacteria community while cyclohexane biodegradation is occurring likely work together, with different members working in different portions of the metabolic pathway.

When comparing the FFT communities from the first experiment with the bacterial communities found in the later NAE solids microcosms, one would expect similar genera to become dominant during the incubation since the conditions are relatively similar. Like the previous microcosms, FFT from the same source is used and is presumed to be a principal source of microbial communities and aerobic conditions are similarly maintained. However, data from the DNA samples taken after NAE solids and FFT were mixed as well as from active microcosms after the incubation was complete display results different than expected. After mixing the FFT and NAE, the proportion of different genera changes slightly: the relative

abundances of *Rhodoferax*, *Caenimonas*, and *Acidovorax* decrease slightly while genera such as *Hydrogenophaga*, *Sulfuritalea*, and *Thiobacillus* increase somewhat as well. Immediately after the degradation of cyclohexane and cyclopentane, the primary genera that display substantial increase when compared to time zero are *Hydrogenophaga*, *Acidovorax*, *Porticoccaceae*, *Immundisolibacter*, and *Defluviimonas*. Different *Hydrogenophaga* species have been observed in a variety of biodegradation studies such as degradation of benzene, toluene, and PAHs (Aburto & Peimbert, 2011; Martin et al., 2012). Similarly, *Acidovorax* was observed in the same cultures (as well as in other research) which have displayed benzene, toluene, and PAH degrading capability (Aburto & Peimbert, 2011; Eriksson, M., Sodersten, Yu, Dalhammar, & Mohn, 2003; Martin et al., 2012; Singleton, Ramirez, & Aitken, 2009). Furthermore, one particular aerobic *Acidovorax* species was recently discovered to possess a novel enzyme which is referred to as cytochrome P450 monooxygenase, which has determined to catalyze the reaction which converts cyclohexane to cyclohexanol, which is the first step in the cyclohexane aerobic pathway (Fritsche & Hofrichter, 2008; Salamanca, Karande, Schmid, & Dobslaw, 2015). *Porticoccaceae* was apresent in both the previous FFT microcosms and the later NAE microcosms and has been determined to be active and present in both petroleum and PAH contaminated environments (Brakstad et al., 2018; Ribicic et al., 2018). When analyzed via metagenomics, *Porticoccaceae* was also found to possess cyclopentanol dehydrogenase, which is likely to have been utilized in cyclopentane degradation to convert cyclopentanol to cyclopentanone, which is then further broken down. Like *Porticoccaceae*, *Immundisolibacter* was found in both sets of microcosms and has been found in PAH-degrading communities (Corteselli et al., 2017). However, one particular genus that was not found in any of the previous DNA samples but was discovered in the active NAE microcosms is *Defluviimonas*, which has

been found to be capable of PAH degradation and may therefore be involved in the initial breakdown of the cyclic structures of cyclohexane and cyclopentane (Zhang et al., 2018).

When comparing the stark differences between the cycloalkane degrading communities from each experiment, two possible explanations may account for this contrast. One possible reason is the distinct hydrocarbon amendments added to the microcosms: while the FFT microcosms received only 300 ppm of cyclohexane, the NAE microcosms received a combination of both cyclohexane and cyclopentane at a concentration of 200 ppm each. Given the relatively similar structure of cyclohexane and cyclopentane, this seems somewhat unlikely to be the cause of these vastly distinct communities. Another much more likely justification is the absence of any pH buffer system in the later NAE microcosms; while the FFT microcosms received Bushnell-Haas media buffered by monobasic potassium dihydrogen phosphate and dibasic potassium monohydrogen phosphate, the NAE microcosms was not buffered and were therefore much more likely to undergo significant pH change over the course of the incubation. Since carbon dioxide was produced by biodegrading microbes, it is probable that a portion of the gas was dissolved in the liquid phase to produce carbonic acid, thereby decreasing the pH. *Rhodoferax* species have been established in previous research as being neutrophilic, which means that a neutral pH is preferred for ideal growth conditions (Imhoff, 2006). Since the NAE microcosms shifted to non-ideal growth conditions for *Rhodoferax*, this provided an ecological niche that could then be occupied by other genera such as *Hydrogenophaga*, *Acidovorax*, *Porticoccaceae*, and *Defluviimonas*. In more acidic conditions, *Hydrogenophaga* would be expected to proliferate because it can tolerate hydrocarbon-rich conditions and also oxidizes hydrogen in its metabolic pathway, which is now more readily available due to increased acidity. Furthermore, one study examined particular strains of *Acidovorax* and its ability to manage

acidity-related stress at pH of 5.8. It was determined that these species were capable of surviving in these conditions, albeit with altered phenotypes (Shrestha et al., 2013). Correspondingly, another study established that a novel species of *Porticoccaceae* (*Porticoccus litoralis*) is capable of growth at a broad pH range of 5-11 (Oh, Kim, Kim, Min, & Cho, 2010). Some strains of *Defluviimonas* are similar in that it is capable of growth at somewhat lower pH's, including *Defluviimonas indica* and *Defluviimonas aquaemixtae*, which are capable of growing in the pH ranges of 5.0-8.0 and 5.0-8.5, respectively (Jiang, Xu, Shao, & Long, 2014; Jung, Park, Lee, & Yoon, 2014). Since these species are capable of growth at lower pH ranges, this provides a probable explanation as to the drastic community shift observed in the NAE bottles.

2.5 Conclusions

In this chapter, two related microcosm experiments were discussed: one involved sealed 160 mL microcosms which were provided with fluid fine tailings as a microbial inoculate, five different types of media to induce five different redox conditions, and 300 ppm of cyclohexane, a non-polar solvent used in non-aqueous extraction techniques. The second experiment involved larger 500 mL microcosms which received identical tailings as a microbial inoculate, "dry" tailings produced from the NAE process containing small concentrations of residual cyclohexane, and either sterile water, minimal nutrient media containing small concentrations of nitrogen and phosphorus, or media to induce methanogenic conditions in anaerobic microcosms. Additionally, these larger microcosms also received a hydrocarbon amendment which provided each microcosm with a concentration of 200 ppm of cyclohexane and 200 ppm of cyclopentane.

Both experiments exhibited similar microbiological activity in similar conditions, wherein microcosms provided with nutrients and sufficient oxygen were capable of degrading cycloalkane amendments while other anaerobic treatments did not display any signs of activity.

Hydrocarbon degradation in these active aerobic microcosms was associated with carbon dioxide production, but the period required for complete degradation and the detected microbial communities differed between the two experiments in spite of similar microcosm conditions. In the second experiment (which includes dry tailings), biodegradation occurs much more quickly than in the first experiment, and this is likely due to degradation kinetics being slowed by an initial paucity of oxygen and a subsequent paucity of nutrients. This divergence in the microbial communities was determined to be attributable to pH change, in which the NAE tailings experiment received minimal nutrient media as opposed to the first experiment where buffered media was used. Therefore, the unbuffered microcosms produced carbonic acid during carbon dioxide production, thereby introducing a more acidic pH and selecting for different microbial genera. In spite of different conditions, both experiments were able to determine that cycloalkane biodegradation is possible for aerobic communities derived from previously anaerobic FFT.

Table 2-1. Experimental scheme for microcosm experiment using fluid fine tailings (FFT) as a microbial inoculate. Each 160 mL microcosm contained a total liquid volume of 70 mL composed of 35 mL of FFT and 35 mL of 5 different type of media tailored to each redox condition. Aerobic microcosm headspaces were flushed with air and anaerobic headspaces were flushed with 70:30 N_2:CO_2 before beginning the incubation. With the exception of the aerobic microcosms, one bottle from each condition received 5 mL of FFT from previous experiments which have had successful biodegradation in each redox condition as an additional inoculate.

Redox conditions	**Treatment**	**Media (mL)**	**Replicates**	**Monitoring parameters**	**Substrate**
Aerobic	Unamended	35	2	CO_2 production (GC-TCD)	FFT + enrichment culture
	Cyclohexane	35	3		
	Heat-killed Cyclohexane	35	2		
Nitrate-reducing	Unamended	35	2	HC degradation followed by confirmation of nitrate depletion (GC-MS and IC)	
	Cyclohexane	35	2		
	Inoculated Cyclohexane	35	1		
	Heat-killed Cyclohexane	35	2		
Sulfate-reducing	Unamended	35	2	HC degradation followed by confirmation of sulfate depletion (GC-MS and IC)	
	Cyclohexane	35	2		
	Inoculated Cyclohexane	35	1		
	Heat-killed Cyclohexane	35	2		
Iron-reducing	Unamended	35	2	Iron reduction (ferrozine method)	
	Cyclohexane	35	2		
	Inoculated Cyclohexane	35	1		
	Heat-killed Cyclohexane	35	2		
Methanogenic	Unamended	35	2	CH_4 production (GC-FID)	
	Cyclohexane	35	2		
	Inoculated Cyclohexane	35	1		
	Heat-killed Cyclohexane	35	2		

Table 2-2. Experimental scheme for microcosm experiment using fluid fine tailings (FFT) as microbial inoculate added to non-aqueous extraction (NAE) dry solids. Each 500 mL microcosm contained a total solid/liquid volume of 300 mL composed of 75 g of NAE solids, 75 mL FFT, and 150 mL of either sterilized water, minimal nutrient media containing nitrogen and phosphorus to provide additional nutrients, or methanogenic media for anaerobic microcosms. Aerobic microcosm headspaces were flushed with pure oxygen while anaerobic microcosms were flushed with 70:30 N_2:CO_2 before beginning the incubation. Aerobic microcosms using OSPW as the primary microbial inoculate are not included due to no activity being observed.

Reclamation Scenario	**Microbial Source (MS)**	**NAE:MS Ratio**	**Treatment**	**Additional Nutrients**	**Replicates**	**Redox Conditions**
Upland	FFT	2:3	Unamended	--	2	Aerobic
			Cyclopentane + Cyclohexane 200 ppm	--	3	
			Cyclopentane + Cyclohexane 200 ppm	C:N:P (100:10:1)	3	
			Heat-killed Cyclohexane + Cyclopentane 200 ppm	C:N:P (100:10:1)	2	
NAE Control	N/A	1:0	Cyclopentane + Cyclohexane 200 ppm	--	2	
Wetland	FFT	2:3	Unamended	--	2	Methanogenic
			Cyclopentane + Cyclohexane 200 ppm	--	2	
			Heat-killed Cyclohexane + Cyclopentane 200 ppm	--	2	

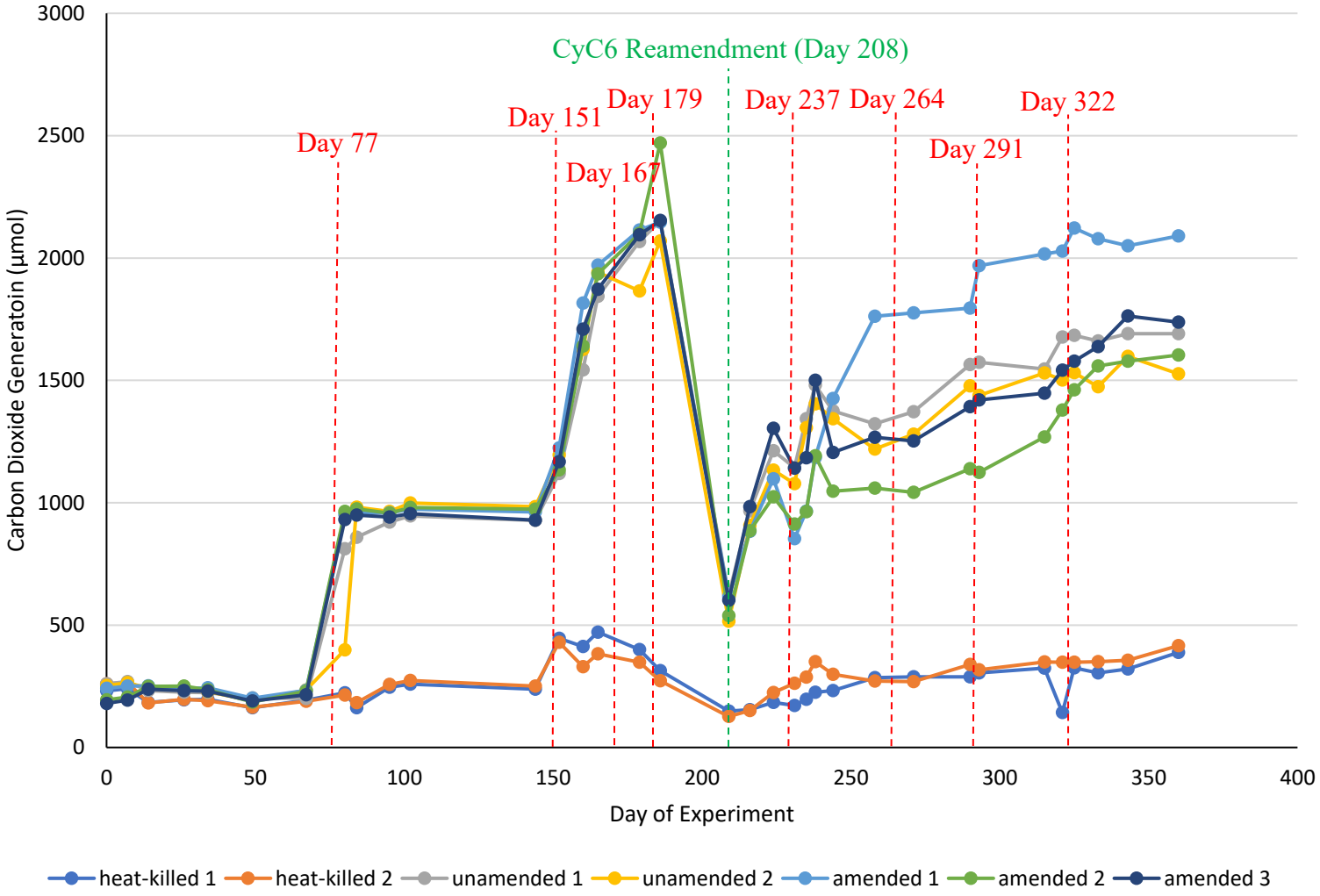


Figure 2-1. Carbon dioxide generation in aerobic FFT microcosms during incubation. Points marked in red were days on which 60 mL of pure oxygen at 1 atm of pressure (2497 µmol) were injected into all bottles to maintain aerobic conditions in the microcosm headspace. After cyclohexane was determined to be depleted after ~160 days, microcosms were flushed with atmospheric air and reamended with cyclohexane on Day 208. After reamendment, microcosms were monitored for carbon dioxide production and received additional oxygen once a month.

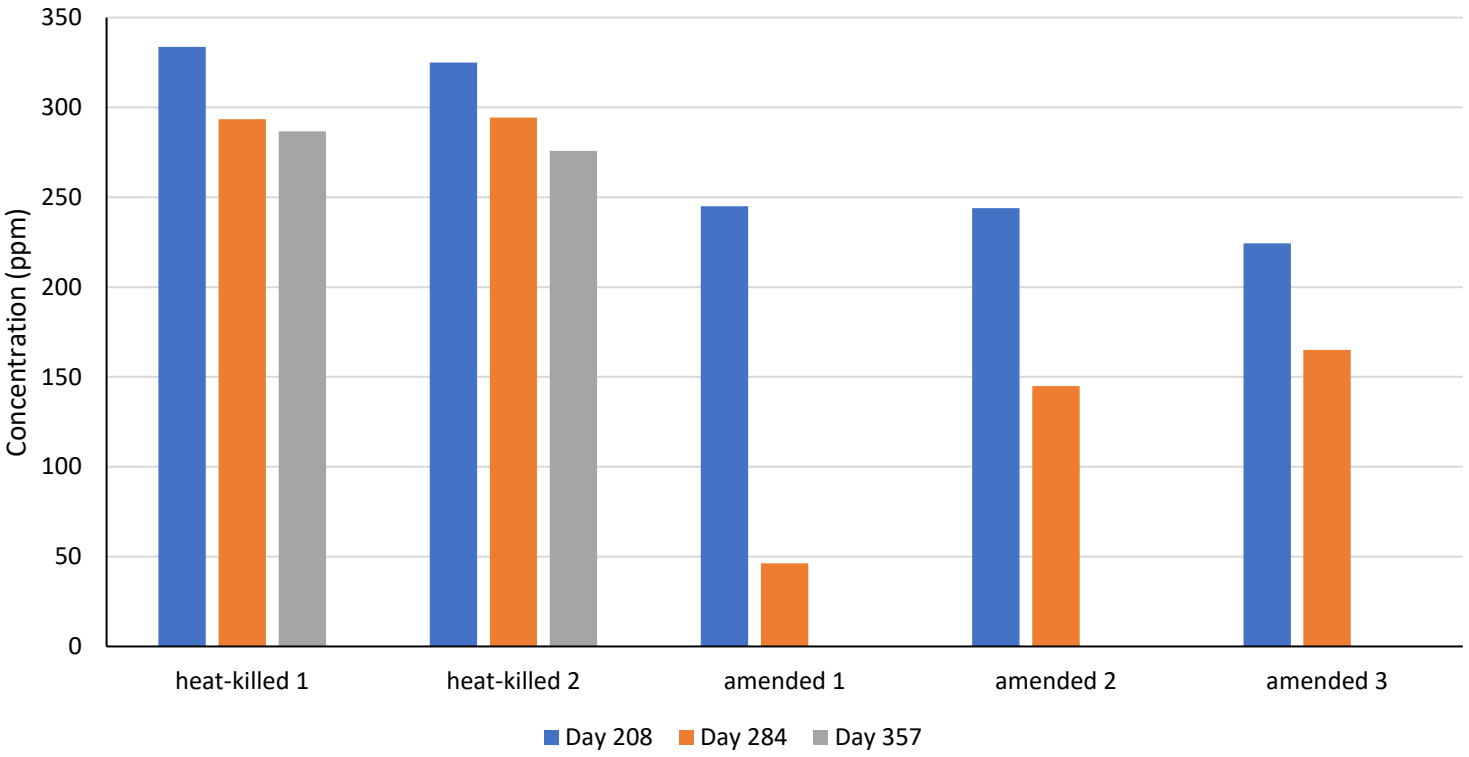


Figure 2-2. Cyclohexane degradation in aerobic FFT microcosms during the latter portion of the incubation after cyclohexane reamendment. The cyclohexane concentration at each time point was measured using liquid extraction and GC-MS analysis.

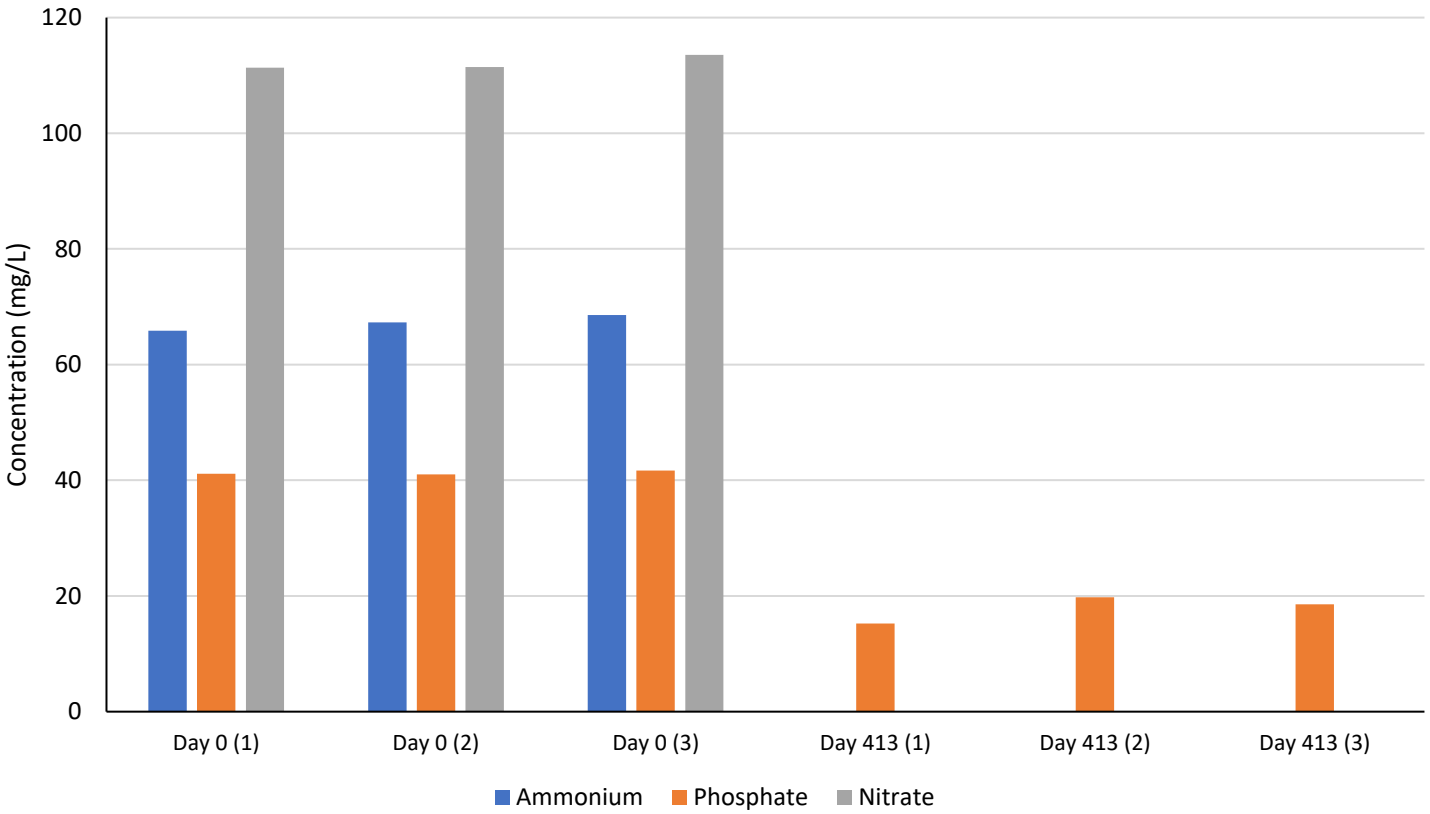
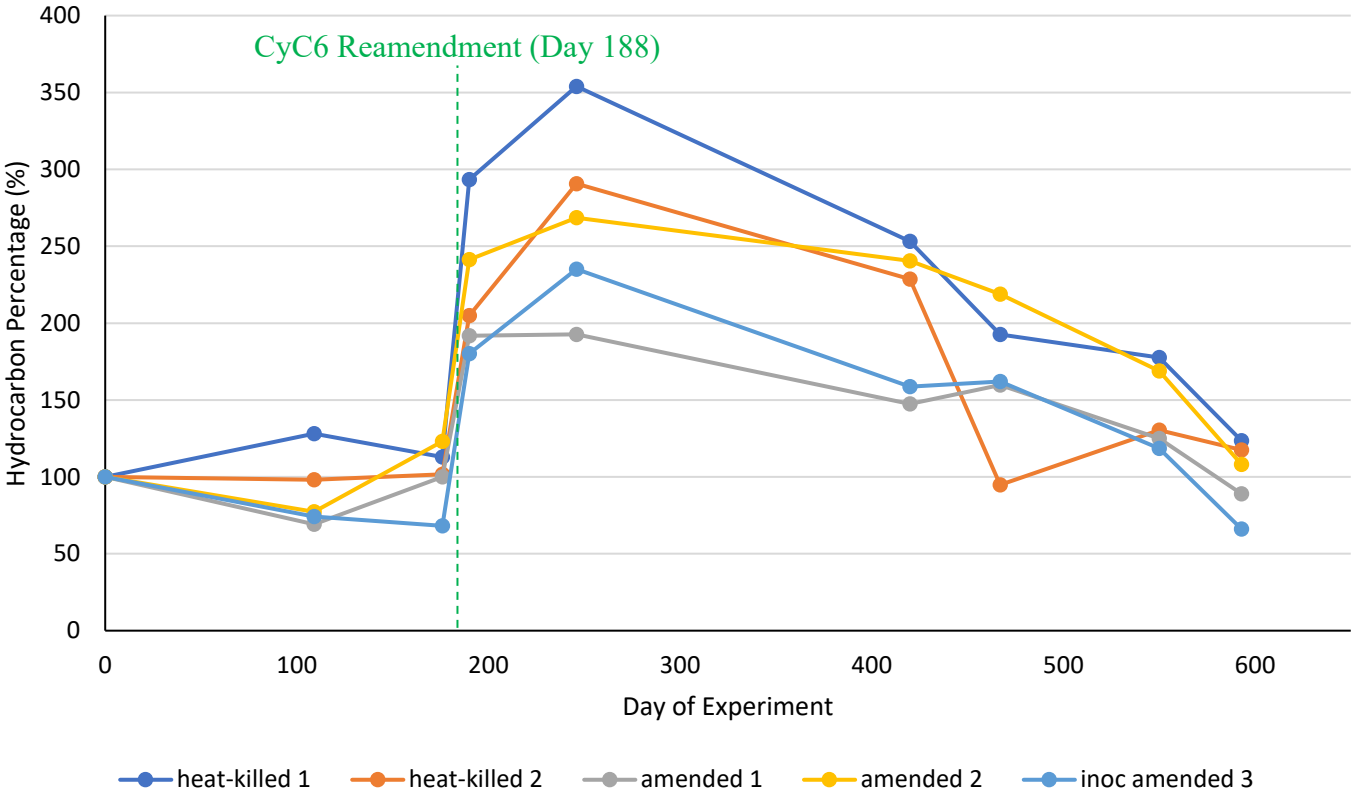
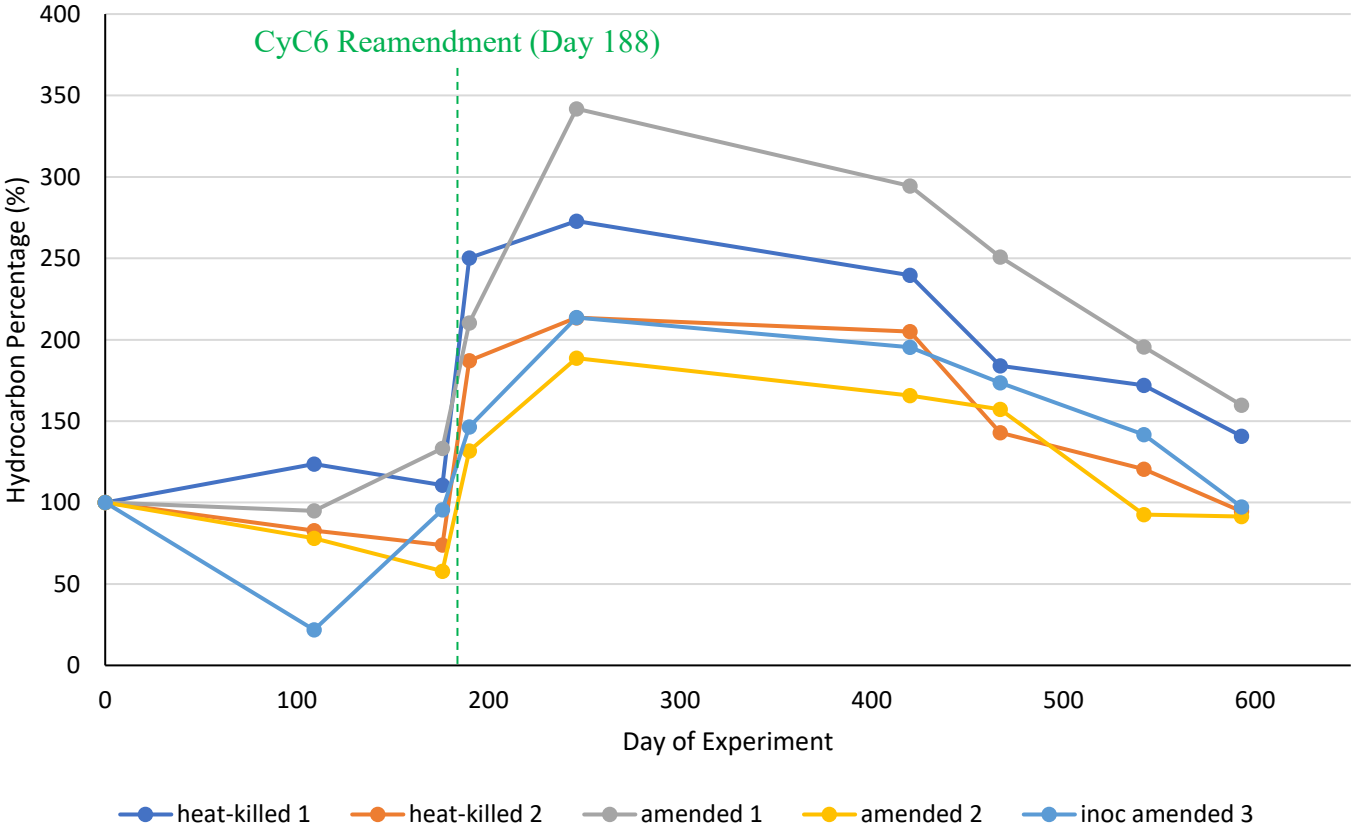


Figure 2-3. Nutrient media depletion in aerobic FFT microcosms after incubating for 413 days. Nutrient concentrations were determined via colorimetric analysis. Analysis was performed using 3 active microcosm replicates for each time point.

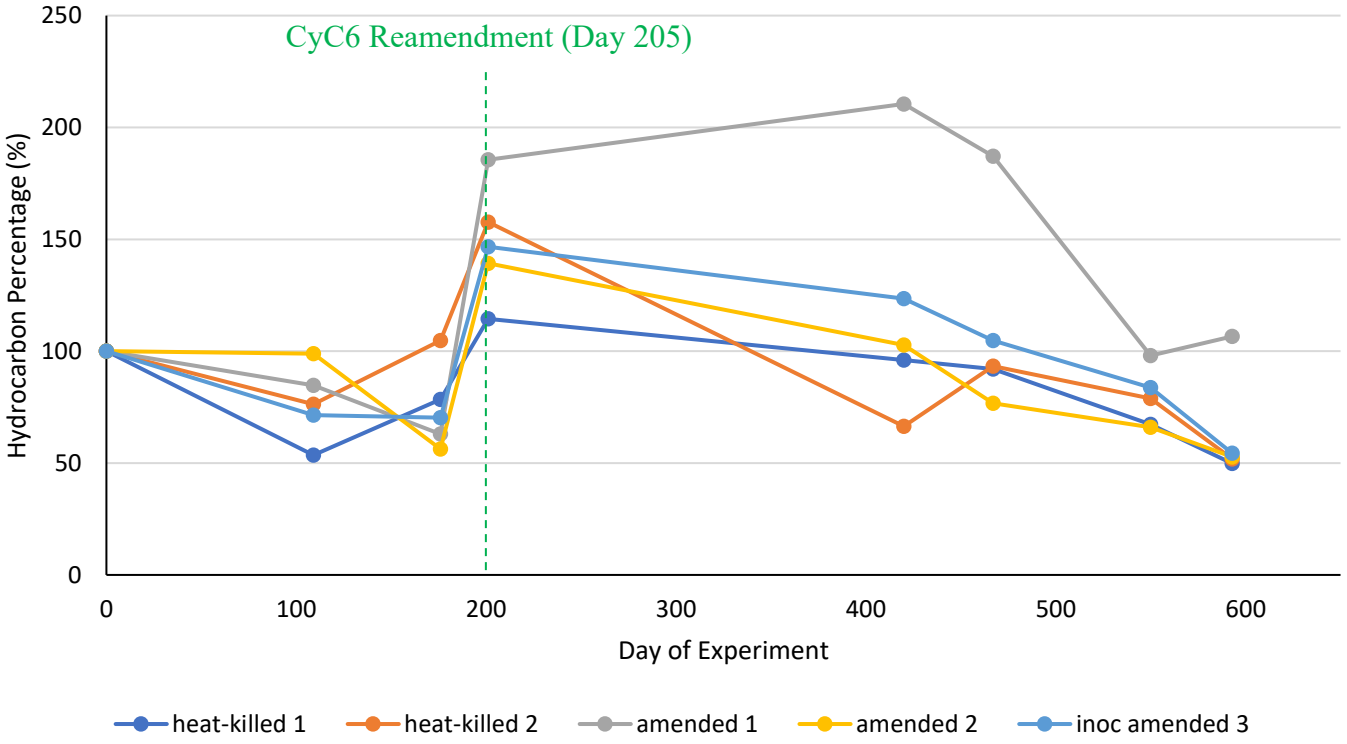
(A)


CyC6 Reamendment (Day 188)
Hydrocarbon Percentage (%)
Day of Experiment
heat-killed 1
heat-killed 2
amended 1
amended 2
inoc amended 3

(B)


CyC6 Reamendment (Day 188)
Hydrocarbon Percentage (%)
Day of Experiment
heat-killed 1
heat-killed 2
amended 1
amended 2
inoc amended 3

(C)



(D)

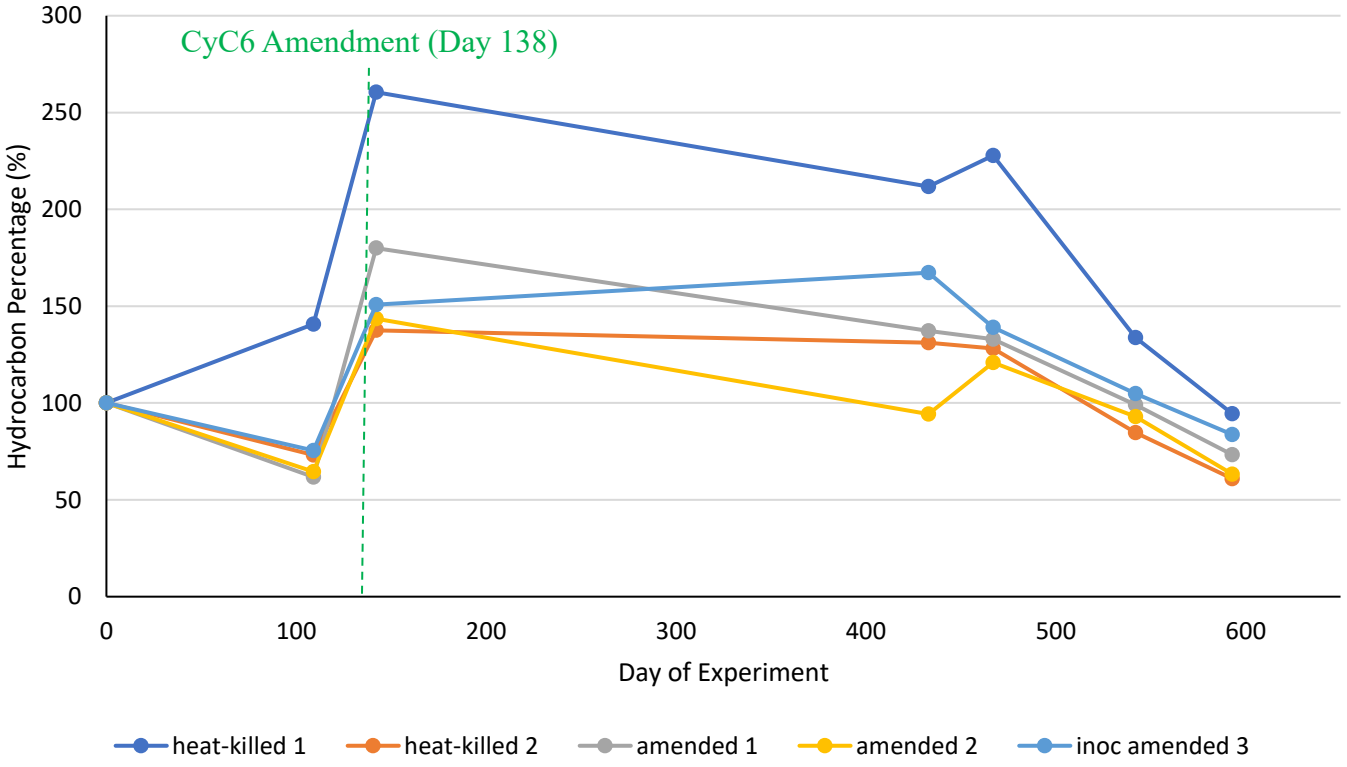


Figure 2-4. GC-MS headspace analysis for cyclohexane in anaerobic FFT microcosms under (A) nitrate-reducing conditions, (B) sulfate-reducing conditions, (C) iron-reducing conditions, and (D) methanogenic conditions over the course of the incubation.

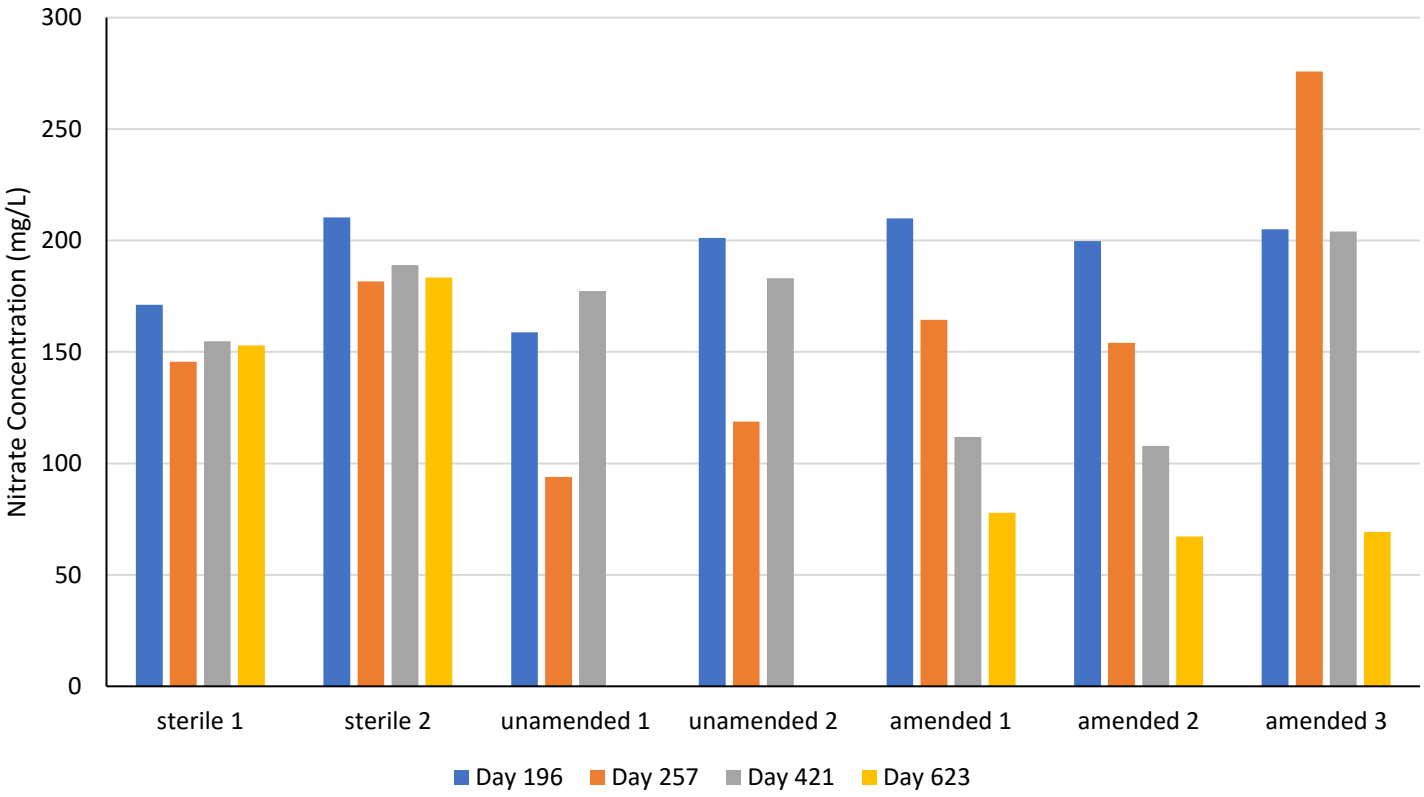


Figure 2-5. Changes in nitrate concentration in anaerobic FFT microcosms subjected to nitrate-reducing conditions. Microcosm samples were analyzed via colorimetry.

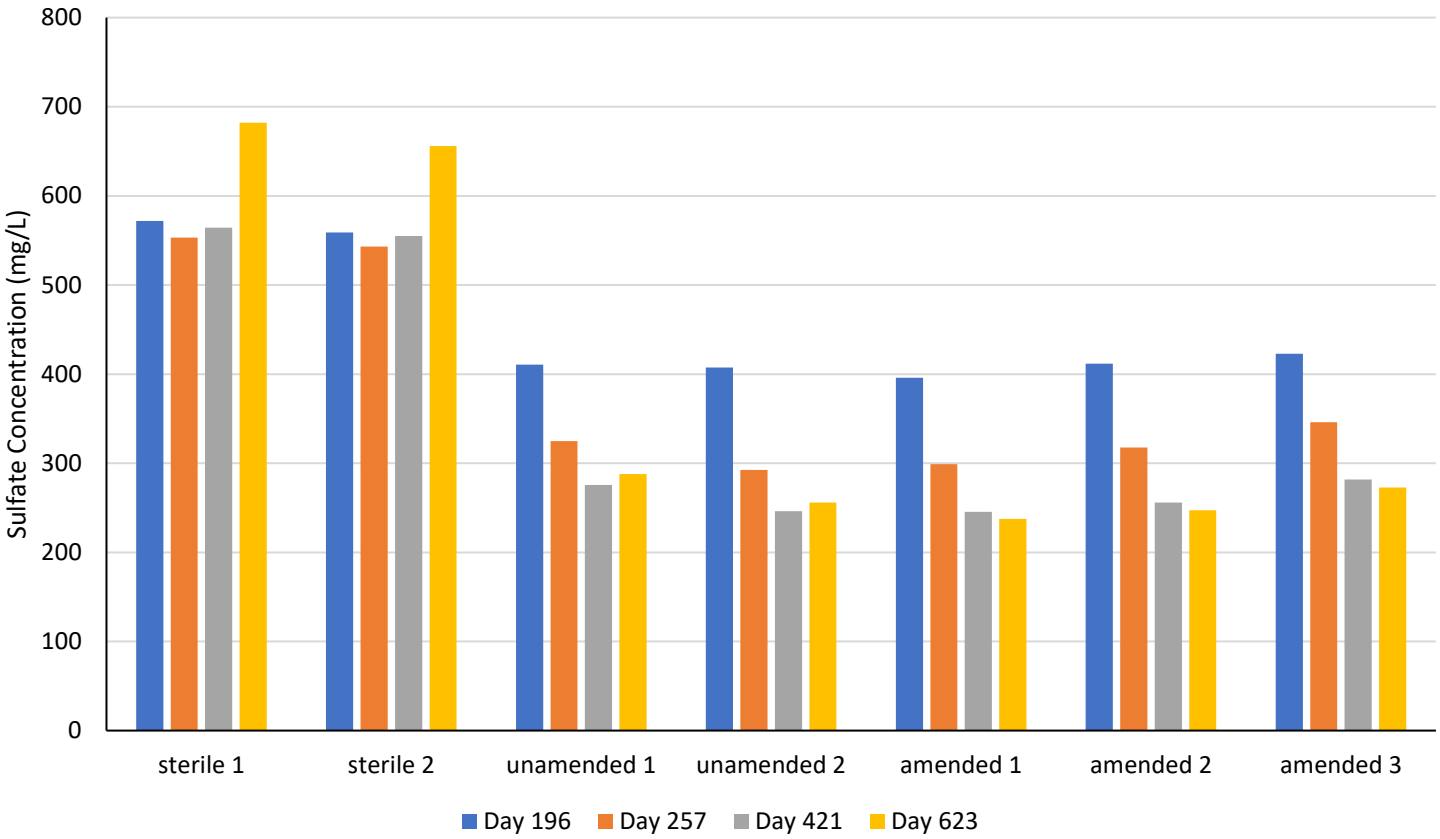


Figure 2-6. Changes in sulfate concentrations in anaerobic FFT microcosms subjected to sulfate-reducing conditions. Microcosm samples were analyzed via colorimetry.

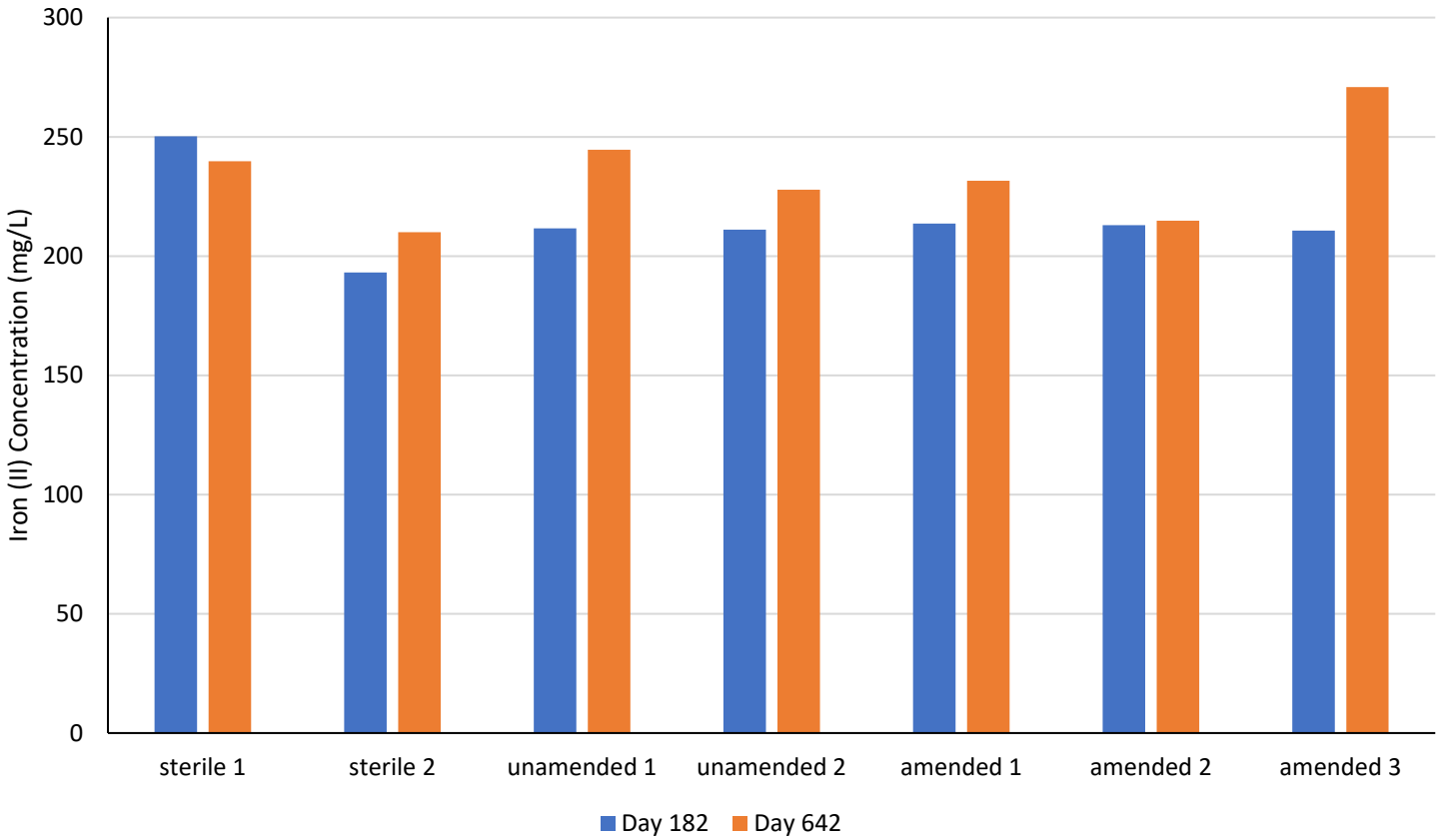


Figure 2-7. Changes in iron (II) concentrations in iron-reducing microcosms over the course of the anaerobic incubation. Concentrations were determined via ferrozine analysis.

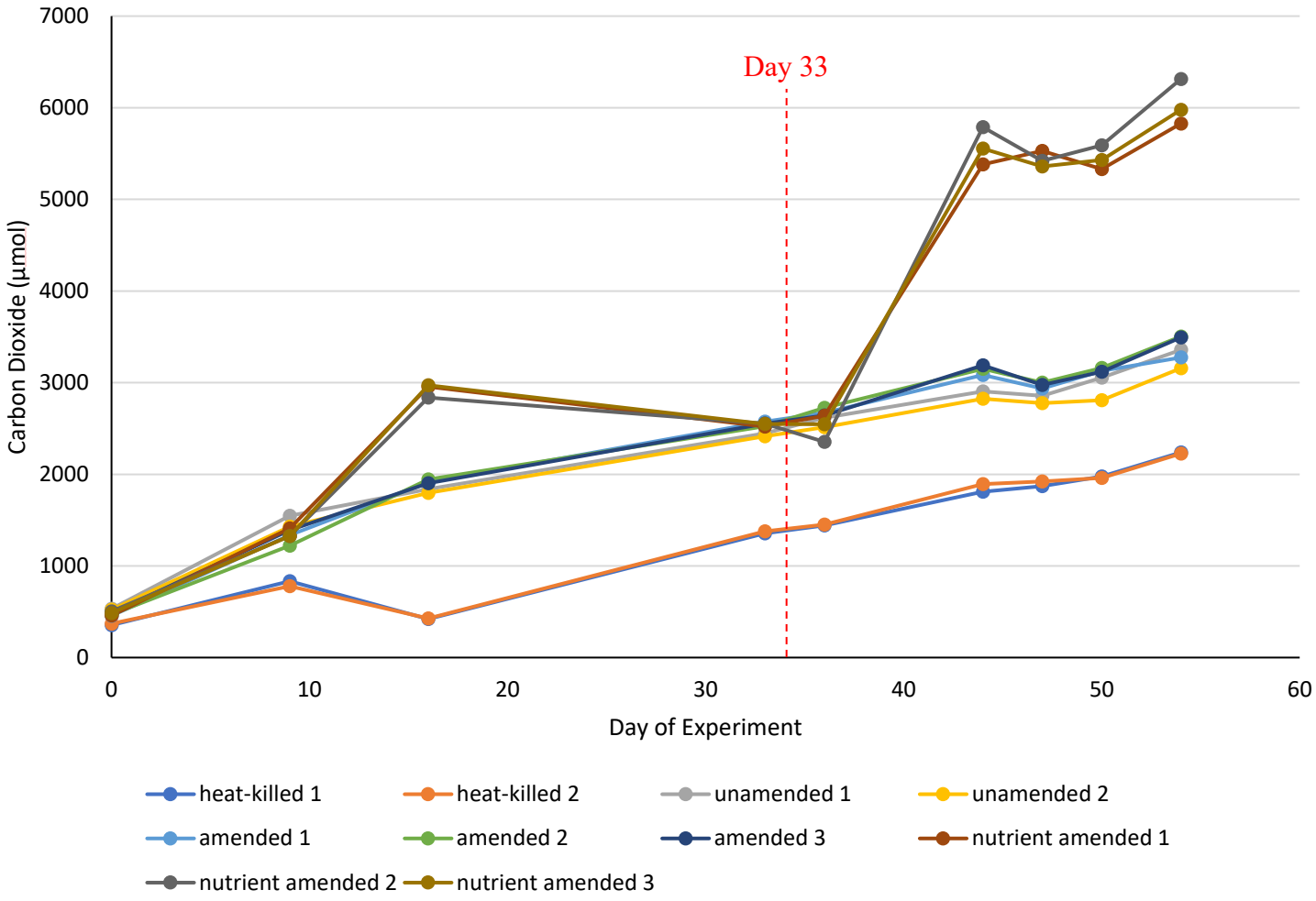
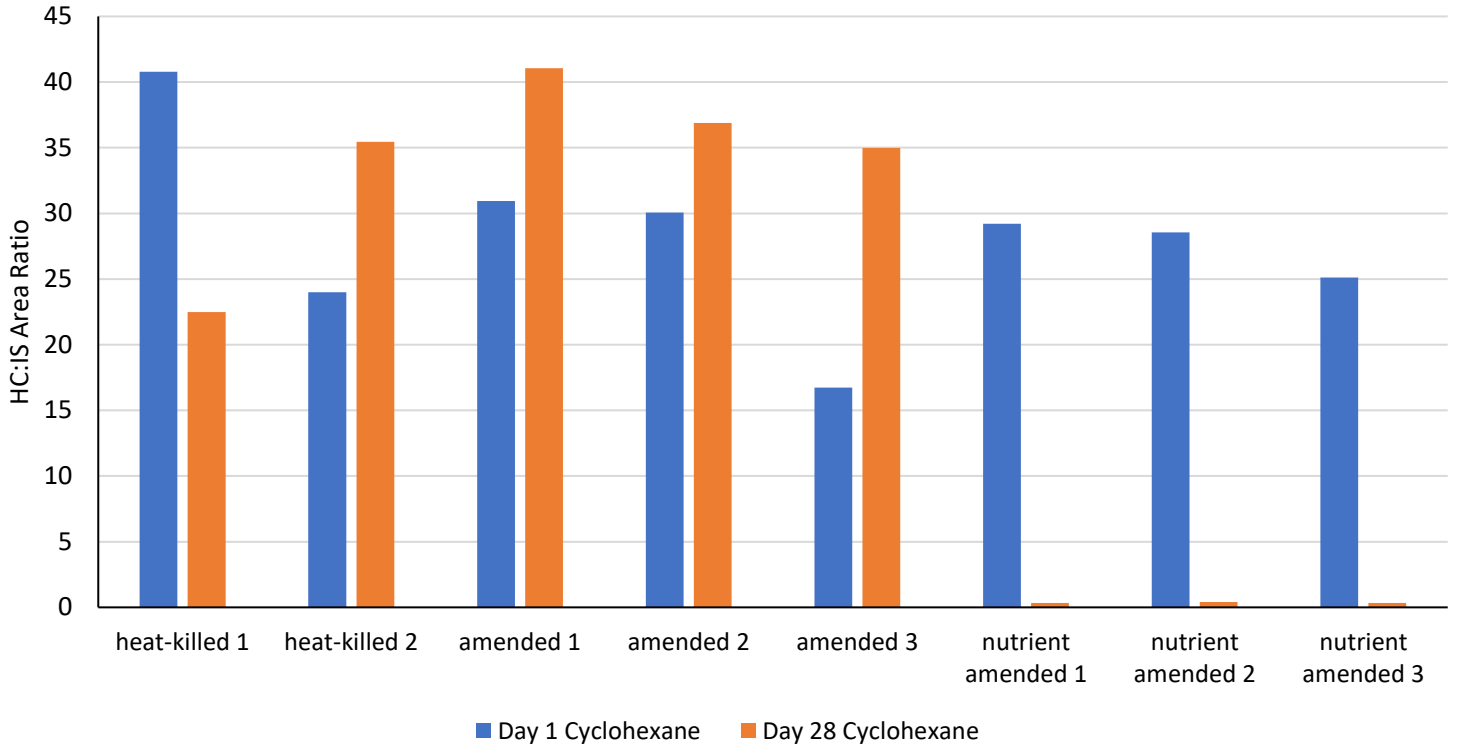


Figure 2-8. Carbon dioxide generation in 500 mL aerobic NAE microcosms which received FFT as a microbial inoculate as determined by GC-FID analysis. On Day 33 of the incubation, 180 mL of pure oxygen at 1 atm of pressure was added into all 10 bottles.



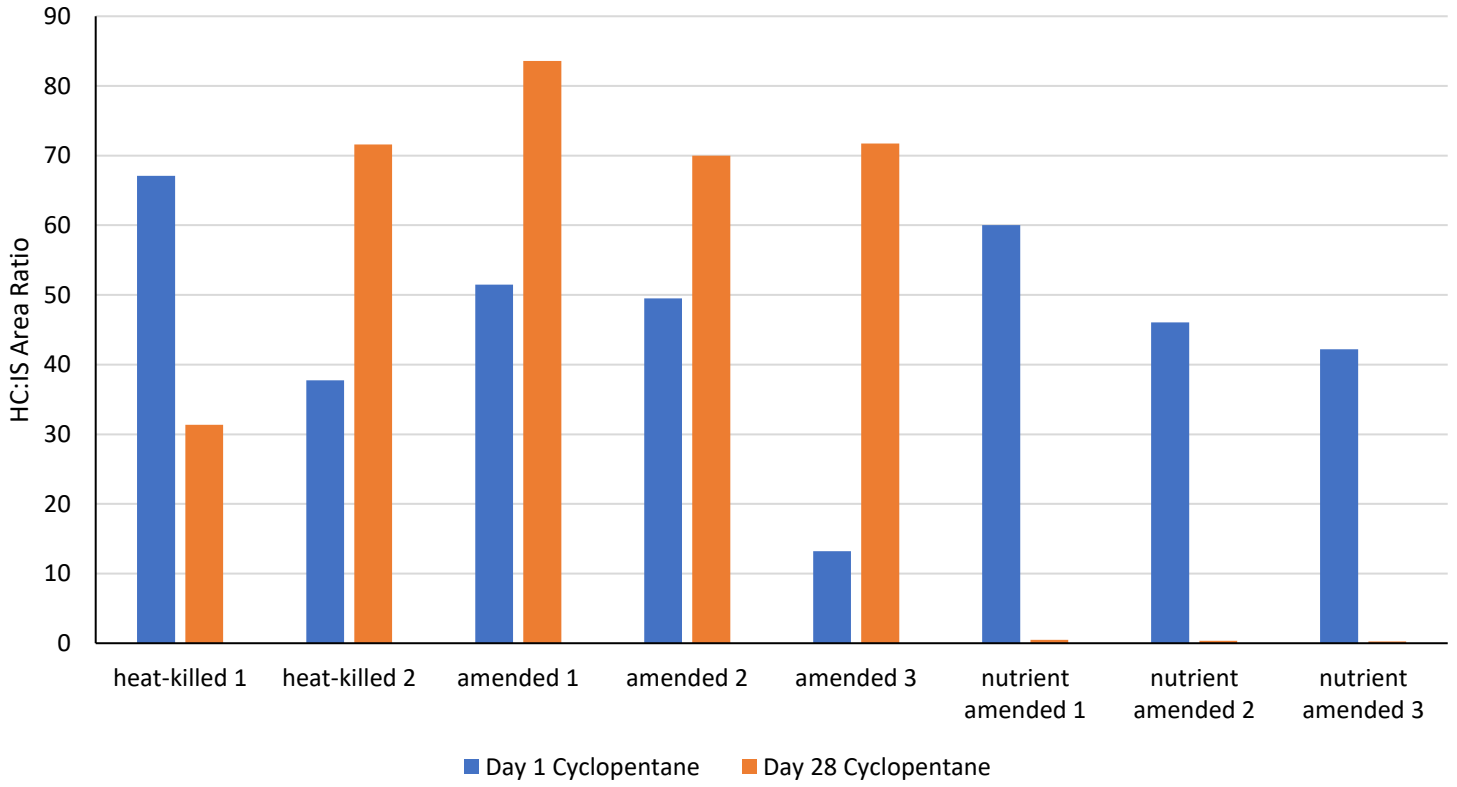


Figure 2-9. Changes in cyclohexane and cyclopentane peak areas in NAE microcosms via headspace GC-MS analysis standardized using 1,1,3-trimethylcyclohexane (internal standard).

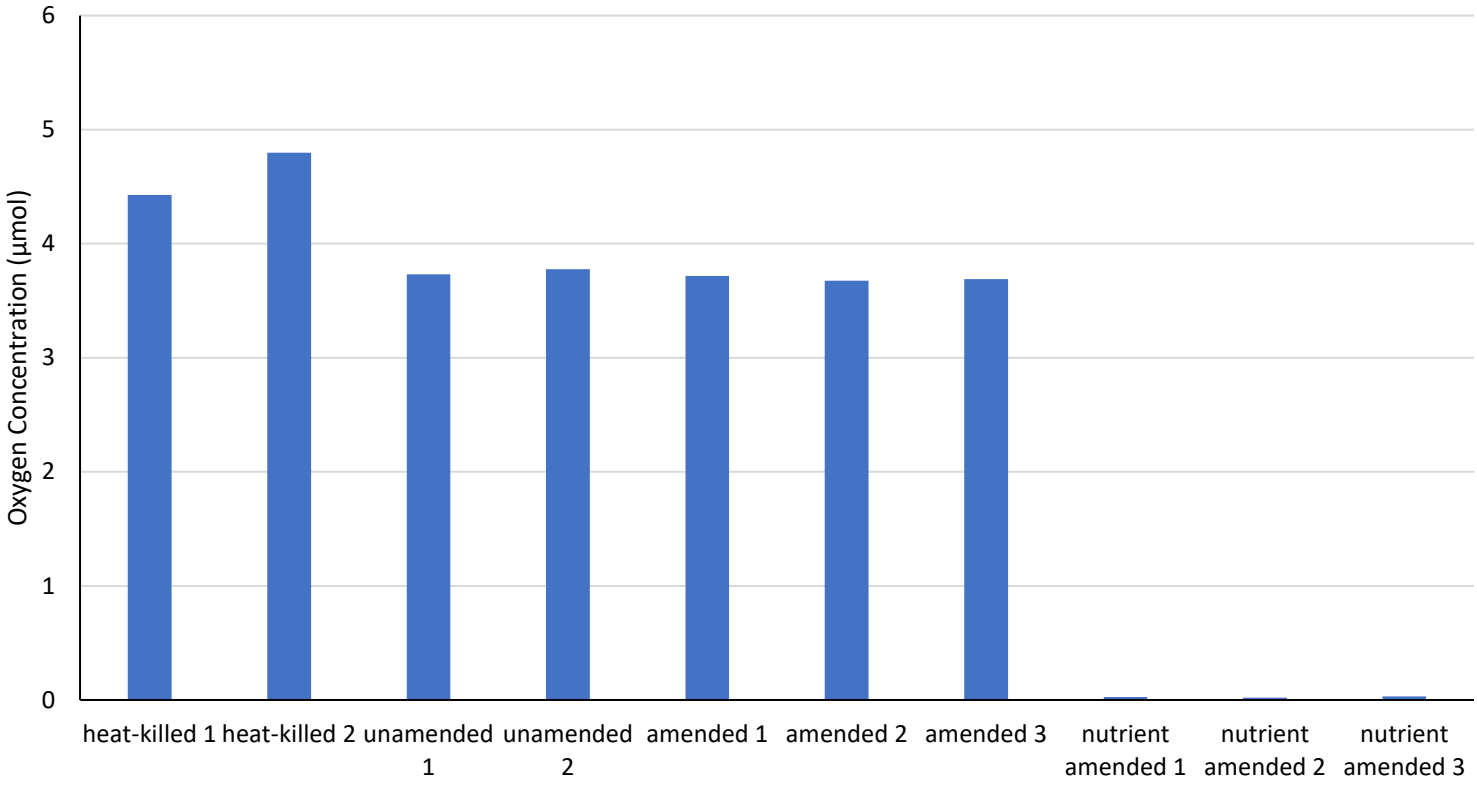


Figure 2-10. Oxygen concentration in the microcosm headspace of the 500 mL FFT microcosms containing NAE solids on Day 73 of the experiment. Measurements were taken using a GC-TCD equipped with a molecular sieve to separate oxygen and dinitrogen peak areas.

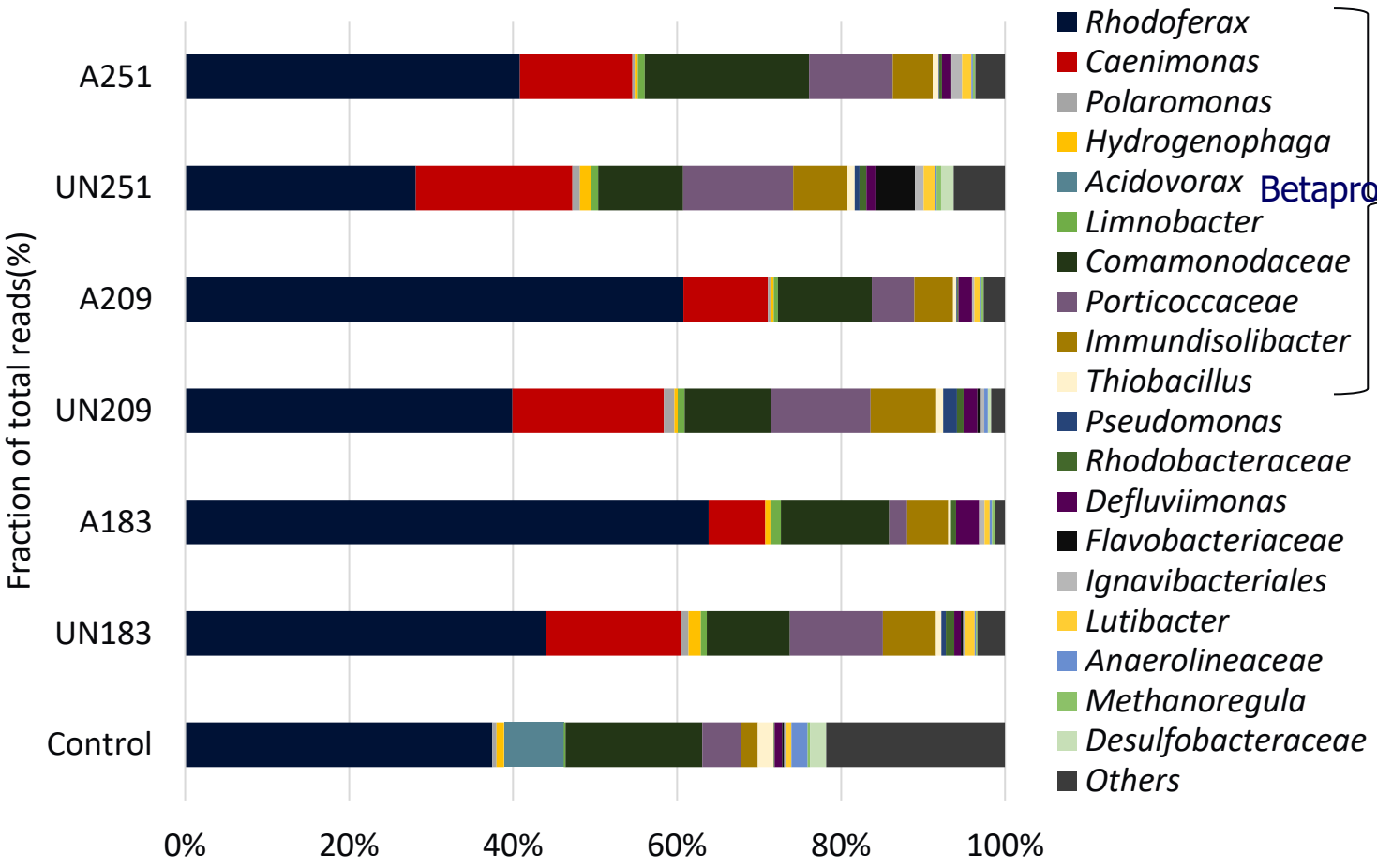


Figure 2-11. Microbial community data for 160 mL aerobic microcosms containing FFT and aerobic media on Day 0 (control), 183, 209, and 251. Control data is based samples taken from the source MFT which was used as microbial inoculate for all FFT microcosms. UN refers to unamended controls while A refers to microcosms which received and degraded cyclohexane.

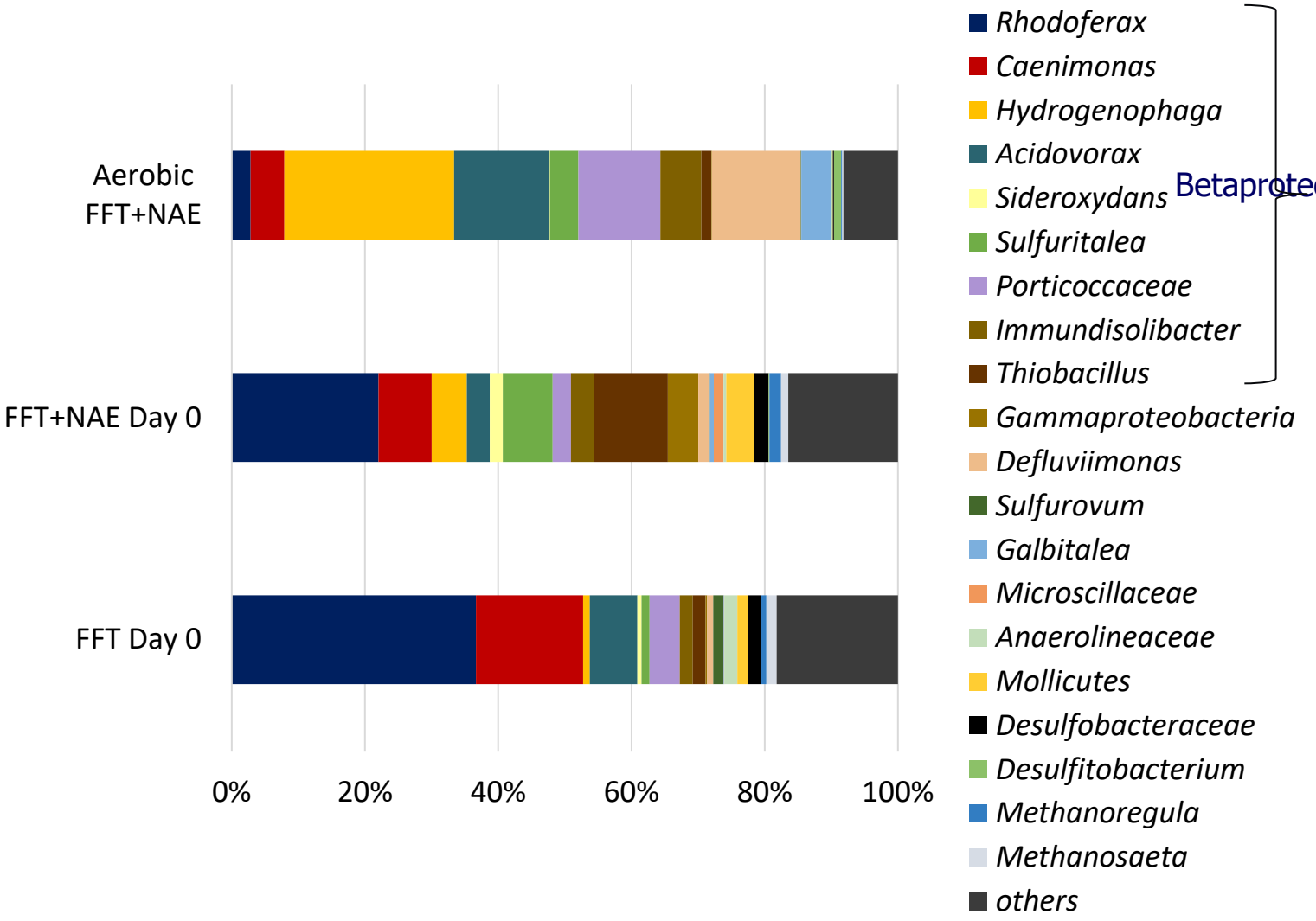


Figure 2-12. Microbial community data for 500 mL aerobic microcosms containing FFT and NAE solids amended with cyclohexane and cyclopentane. FFT Day 0 refers to FFT samples from the source FFT, while FFT+NAE Day 0 refers to samples taken after mixing a 1:1 ratio of NAE solids and FFT. Aerobic FFT+NAE samples came from active and nutrient-amended aerobic microcosms on Day 87 in which cyclohexane and cyclopentane were degraded.

Chapter 3: Examining Biodegradation of Cyclohexane, Cyclopentane, and *n*-Heptane using Soils and FFT

3.1 Introduction

Recent projections of the Alberta oil sands have determined that available ores contain approximately 165.4 billion barrels of crude oil (Alberta Government, 2017). The primary ore removal method is currently surface mining, which involves the use of machinery to expose the oil sands, which are then removed, crushed, and transported to an extraction plant to remove the bitumen (Grant et al., 2008). Separation of the bitumen from the ores requires hot water, generating large volumes of fluid fine tailings (FFT) which have become increasingly difficult for oil sands operators to manage (Foght, Julia M. et al., 2017). Therefore, new separation technologies are now being developed that have fewer environmental drawbacks, such as non-aqueous extraction (NAE). Compared to the existing hot water extraction method, NAE improves extraction and requires less energy, less freshwater, and generates significantly smaller volumes of "dry" tailings that are suitable for in-pit disposal rather than long term storage in tailings ponds. Although a majority of the hydrocarbon solvent used in extraction is recyclable, small amounts may be co-deposited with unknown environmental fates.

Along with the possible physical pathways that may occur, the solvent may also be incorporated into microbiological pathways, resulting in either complete oxidation or partial oxidation to polar compounds of unknown structure or toxicity. One possible source of these hydrocarbon degrading microbial communities are pristine soils located at the deposition site, which could potentially colonize the NAE solids and initiate biodegradation of any remaining hydrocarbon solvent. This experiment involved the establishment of microcosms containing such soils amended with concentrations of cyclohexane and cyclopentane, two solvents which have displayed encouraging results in NAE. Microcosms were provided with different aerobic and anaerobic conditions to examine the biodegradative capability of local communities (Table 3-1).

3.2 Materials and Methods

3.2.1 Chemicals and Reagents

All chemicals were purchased from Fisher Scientific, located in Ontario, Canada. For a complete list of reagents and chemicals used in the lab, as well as manufacturers and grade, refer to the List of Reagents in Appendix A.

3.2.2 Gases

70% N_2/CO_2, N_2 (98% purity), N_2 (95% purity), and pure O_2 canisters were purchased from Praxair, which is located in Edmonton, Alberta, Canada.

3.2.3 Microbial Inoculate Collection

Topsoil from the A_e Luvisol soil horizon was collected from a forested area near Fort McMurray. Soil was collected with a shovel after removal of the active layer and stored in Ziploc bags for transport back to the laboratory at the University of Alberta. Soil was stored at room temperature until the beginning of the experiment. Before adding soil to microcosms, soils were crushed with a mortar and pestle and sifted in order to decrease the particle size and ensure that relatively consistent soil samples were added into each microcosm.

FFT was collected in bulk in 2016 from a tailings pond near Fort McMurray at a depth of 10.5 metres below the surface and stored in pails in the dark at a temperature of 4°C. After removal from the refrigerator, samples were added to autoclaved serum bottles which served as sealed microcosms in the incubation experiment.

3.2.4 Media Preparation

Four different types of media were prepared for the four different redox conditions (aerobic, methanogenic, and nitrate/sulfate reducing) that the microcosms replicate based on the possible deposition scenarios for non-aqueous extraction tailings. Nitrate-reducing media was

prepared according to the protocol outlined in Rabus and Widdel (1995) (Rabus & Widdel, 1995). Media promoting sulfate-reducing conditions was prepared based on a protocol adapted from Chi Ming So & L.Y. Young by Sara Ebert (So & Young, 1999). The recipe for methanogenic media was adapted from Fedorak and Hrudey (1984) as well as Holowenko et al. (2000) (Fedorak & Hrudey, 1984; Holowenko et al., 2000). Aerobic media, also referred to as Bushnell-Haas media, was prepared for aerobic incubations.

For nitrate-reducing media, six stock solutions were prepared and stored at 4°C before use. An EDTA-chelated mixture of trace elements containing sodium EDTA (5.2 g) , $FeSO_4 \cdot 7H_2O$ (2100 mg), H_3BO_3 (30 mg), $MnCl_2 \cdot 4H_2O$ (100 mg) $CoCl_2 \cdot 6H_2O$ (190 mg), $NiCl_2 \cdot 6H_2O$ (24 mg), $CuSO_4 \cdot 5H_2O$ (29 mg), $ZnSO_4 \cdot 7H_2O$ (144 mg), $Na_2MoO_4 \cdot 2H_2O$ (36 mg), and 987 mL of distilled water was dispensed into bottles, sealed, and autoclaved. Preparation of the vitamin mixture included mono and di-basic sodium phosphate as buffers (100 mL), as well as 4-aminobenzoic acid (4 mg), D(+)-Biotin (1 mg), nicotinic acid (10 mg), calcium D(+)-pantothenate, and pyridoxine dihydrochloride (15 mg). The solution was then filter sterilized (0.2 μm pore size) before storage. The next solution included 100 mL of 25 mM sodium phosphate buffer and 10 mg thiamine chloride dihydrochloride, which was also filter-sterilized and then stored for future use. The Vitamin B_{12} solution contained cyanocobalamin (5 mg) dissolved in 100 mL of water which was then filter sterilized. The selenite-tungsten solution contained NaOH (400 mg), $Na_2SeO_3 \cdot 5H_2O$ (6 mg), and $Na_2WO_4 \cdot 2H_2O$ (8 mg). Finally, an $NaHCO_3$ solution with a concentration of 84 g/L was autoclaved and cooled under CO_2. 500 mL of nitrate-reducing media was then prepared containing KH_2PO_4 (0.25 g), NH_4Cl (0.15 g), $MgSO_4 \cdot 7H_2O$ (0.25 g), $CaCl_2 \cdot 2H_2O$ (0.05 g), and 10 mM $NaNO_3$ (0.425 g). The media was sparged with 70:30 N_2:CO_2 gas for 1 hour, then dispensed into serum bottles flushed with N_2:CO_2 gas during transfer in 100

mL aliquots. Media bottles were then sealed with butyl stoppers cut to 1/3" thickness and stored in the fridge at 4°C until addition to microcosms at the beginning of incubation.

For sulfate-reducing media, five stock solutions were prepared and stored at 4°C before use. A trace elements solution was prepared that contained $CoCl_2 \cdot 6H_2O$ (3 g), $CuCl_2$ (15 mg), $FeCl_2 \cdot 4H_2O$ (0.15 g), H_3BO_3 (0.57 g), $MnCl_2 \cdot 4H_2O$ (2 g), $Na_2MoO_4 \cdot 2H_2O$ (0.25 g), $NiCl_2 \cdot 6H_2O$ (0.15 g), $ZnCl_2$ (0.21 g), and 0.4165 mL HCl all dissolved in 500 mL of distilled water. This solution was autoclaved and stored at 4°C. A vitamin solution that contained Vitamin B12 (1 mg), D(+)Biotin (20 mg), folic acid (20 mg), nicotinic acid (50 mg), p-aminobenzoic acid (50 mg), Ca-D(+)pantothenate (50 mg), pyridoxine HCl (100 mg), riboflavin (50 mg), thiamine (50 mg), and thioctic acid (50 mg) all dissolved in 1L of distilled water. The pH was then adjusted to be neutral (pH=7), filter sterilized, sealed, and the headspace was then replaced with N_2 gas. A bicarbonate solution with a concentration of 84.01 g $NaHCO_3$ per litre of distilled water was sparged with CO_2, then filter sterilized into a sterile, anaerobic (N_2 headspace) serum bottles. Sodium sulfide was a reducing agent used to react with oxygen to promote anaerobicity within the media. A 67 mM $Na_2S \cdot 9H_2O$ solution (16.1 g/L) was also prepared using boiled distilled water and dispensed into serum bottles, which were then sealed and autoclaved before storage. 500 mL of sulfate media solution was then prepared, which contains NaCl (0.1 g), KCl (0.65 g), $MgCl_2 \cdot 6H_2O$ (0.5 g), $CaCl_2 \cdot 2H_2O$ (0.05 g), NH_4Cl (0.25 g), KH_2PO_4 (0.1 g), and Na_2SO_4 (0.71 g). The media was then sparged with 70:30 N_2:CO_2 gas for 1 hour, dispensed in 100 mL aliquots to 158 mL serum bottles that were flushed with N_2:CO_2 during transfer, which were sealed with butyl stoppers and autoclaved. After autoclaving, the media bottle headspace was flushed with N_2:CO_2 gas to maintain anaerobic conditions, and each bottle received 0.1 mL of the vitamin solution, 0.5 mL of the trace element solution, 3 mL of the bicarbonate solution, and 3 mL of the

sodium sulfide solution before storage at 4°C. These were added after autoclaving because they were previously sterilized and the autoclaving conditions may denature them.

Methanogenic medium preparation involved mixing two mineral stock solutions that were then added to the media. The first mineral solution contained NaCl (5 g), $CaCl_2 \cdot 2H_2O$ (1 g), NH_4Cl (5 g), and $MgCl_2 \cdot 6H_2O$ (1 g) all dissolved in 100 mL distilled water. The second mineral solution contained $(NH_4)_6Mo_7O_{24} \cdot 2H_2O$ (1 g), $ZnSO_4 \cdot 7H_2O$ (0.01 g), H_3BO_3 (0.03 g), $FeCl_2 \cdot 4H_2O$ (0.15 g), $CoCl_2 \cdot 6H_2O$ (1 g), $MnCl_2 \cdot 4H_2O$ (0.003 g), $NiCl_2 \cdot 6H_2O$ (0.003 g), and $AlK(SO_4)_2 \cdot 12H_2O$ (0.01 g) dissolved in 100 mL distilled water. The next step in the procedure involved mixing 7 mL of mineral solution 1, 0.7 mL of mineral solution 2, 7 mL of resazurin, 7 mL of a 50 g/L KH_2PO_4 solution, and 5.7 g of $NaHCO_3$. Resazurin was a redox indicator used to indicate the presence or absence of oxygen in the media. 500 mL of boiled distilled water is added to the mixture, and after the ingredients are fully dissolved, the media was sparged with N_2:CO_2 for 1 hour and then dispensed in 100 mL aliquots into serum bottles which were then sealed, autoclaved, and then flushed with N_2:CO_2 in the media bottle headspace. Bottles were then sealed with butyl stoppers. After sterilization, each bottle received 1 mL of anaerobic 25 g/L sodium sulfide and 0.5 mL of a sterilized, anaerobic vitamin B solution before storage. This solution contained pyridoxine (0.025 g), thiamine (0.005 g), nicotinic acid (0.01 g), pantotheinic acid (0.0025 g), Vitamin B12 (0.01 g), and p-aminobenzoic acid (0.005 g) in 100 mL of distilled water. After the addition of sodium sulfide, the media undergoes a colour change from bright pink to colourless to indicate that the oxygen in the bottle had reacted with the sodium sulfide, and eventually a black precipitate forms in the bottle. Bottles were then stored in the fridge.

Aerobic soil cultures were incubated with sterile aerobic Bushnell-Haas media, which contained K_2HPO_4 (1 g) , KH_2PO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $CaCl_2 \cdot 2H_2O$ (0.02 g), $FeCl_3$

(0.05 g), and NH_4NO_3 (1 g). These compounds were added to 1 L of deionized water, which was then autoclaved in a sealed 1 L glass container and stored in the fridge before use.

3.2.5 Experimental Setup

A total of 48 serum bottles (158 mL) were cleaned and autoclaved, then 8 bottles were set aside to later receive FFT as a microbial inoculate. The remaining 40 bottles each received 50 g of soil, and then bottles were then labelled and divided into the 4 redox conditions (10 bottles per redox condition). Then, 7.5 mL of each microcosm's associated media was dispensed into the bottles (ex. Nitrate-reducing media in nitrate-reducing bottles, Bushnell-Haas media in aerobic bottles) such that 60% water holding capacity was achieved, and then sealed with butyl stoppers cut to 1/3" thickness. The 8 remaining microcosms received ~70 mL FFT rather than 50 g of soil; this volume was chosen because FFT was added on a weight basis and this volume was equivalent to 50 g of FFT. Similarly, FFT microcosms received 7.5 mL of methanogenic media, and then the 38 anaerobic microcosms were flushed with N_2:CO_2 for 6 minutes to ensure that there was no residual oxygen in the microcosm headspace after sealing with cut butyl stoppers.

The soil microcosms were then divided into different experimental treatments. Within each redox condition, 2 bottles were set aside and autoclaved once a day for four consecutive days and thereby served as sterilized controls to determine whether any microcosmal changes were due to non-biological processes/reactions. Two other microcosms were also separated from the others and received no hydrocarbon amendment; these were referred to as unamended controls, and were monitored to also examine whether any other carbon sources apart from cycloalkane amendments were present and degradable by indigenous microbial communities. The remaining 6 bottles were amended with hydrocarbons, with half amended with 300 mg/L of cyclohexane and the other half amended with 300 mg/L of cyclopentane. To ensure that the

hydrocarbon was adequately mixed with the soils and therefore accessible for soil microbiota, each cycloalkane was dissolved in a 100 mL methanol stock solution such that the concentration was 17550 mg/L. Each microcosm would receive 1 mL of the stock solution to have a microcosm hydrocarbon concentration of 300 mg/L. However, due to an initial miscalculation, stock solutions of only 1755 mg/L were made, and microcosms were therefore amended with 30 mg/L of cyclohexane and cyclopentane. However, these solutions were immediately remade at the proper concentration and the remaining 270 mg/L (0.9 mL) was added to all microcosms, with the exception of the unamended controls. Each bottle therefore received a total 1.9 mL of methanol containing the hydrocarbon amendment. Additional media was also added to the anaerobic soil microcosms on day 60 after determining that residual oxygen may have inhabited the soil pores and could be inhibiting anaerobic hydrocarbon degradation. Originally, 6 mL were intended to be added to reach 100% water holding capacity, but an additional 10 mL of media was added to ensure saturated conditions for a total of 23.5 mL media per microcosm. Additional media was identical to the initial media recipe with the exception of resazurin; in this case, resazurin was added to all anaerobic medias (including nitrate and sulfate reducing) to determine whether the residual oxygen had compromised the anaerobic conditions within the microcosms. If so, the resazurin would be slightly oxidized and turn into resorufin (pink); however, no signs of discoloration were found in the bottles after supplementing with anaerobic media, suggesting that anaerobic conditions were maintained. For aerobic media, no additional media was added since soil pore oxygen would assist in aerobic degradation rather than inhibit. For methanogenic FFT microcosms, it was determined that no additional methanogenic media was required because FFT is already saturated with water and is likely anaerobic. After this initial setup, microcosms received no further media over the course of the long-term incubation.

The FFT microcosm experiment also differed in how their cycloalkane amendments were set up; rather than include sterile controls, different mixes of cyclohexane, cyclopentane, and *n*-heptane (300 mg/L each) were used to test co-metabolic processes in FFT microbial cultures. In addition to cyclohexane and cyclopentane amendments, 1,1,3-trimethycyclohexane was also added to the soil microcosms to serve as an internal standard to verify hydrocarbon degradation. 1,1,3-trimethylcyclohexane has been determined in previous research to be highly recalcitrant to most biodegrading species across different redox conditions, and therefore helps to account for any measurement error when identifying any reduction in hydrocarbon concentration (Siddique et al., 2007). For microcosms containing FFT as a microbial inoculate, it was determined that FFT contains a diverse collection of endogenous hydrocarbons, including 1,1,3-trimethylcyclohexane at a high enough concentration to be used as an internal standard. Therefore, the 8 FFT microcosms did not require and received no internal standard amendment. In the other microcosms, 50 mg/L (3.01 µL per microcosm, calculated based on the liquid phase volume) of internal standard was added on Day 258 and then measured on the GC to verify that the concentration was detectable and could be monitored for any changes. After internal standard addition, the microcosms were then monitored for over 700 days for any hydrocarbon changes. All microcosms were incubated in the dark at ambient room temperature (20°C). Anaerobic microcosms were incubated on benchtop without any agitation, while aerobic incubations were placed on a covered gyrotory shaker shaking at 180 rpm for at least 12 hours per day. Bottles remained sealed for the entire incubation duration with intermittent headspace gas analysis. FFT microcosms wer also monitored via hydrocarbon liquid extraction after methane generation to determine whether any of the added linear chain hydrocarbons or cycloalkanes were used as a carbon source and thereby biodegraded.

3.2.6 Carbon Dioxide (CO_2 Analysis)

Carbon dioxide analysis was performed by injecting 100 µL of headspace gas from the microcosm (after shaking the bottle to release any trapped gas) into a gas chromatograph (GC) equipped with a thermal conductivity detector (TCD) using a 28G 500 µL disposable insulin syringe. Carbon dioxide standards (0.16%, 1%, 2%, 4%, 8%, 15%, and 30%) were prepared in sterile 158 mL serum bottles and used to prepare a standard curve to determine the percentage of carbon dioxide in the microcosm headspace. Additionally, pressure was measured in each microcosm using a 25G needle and pressure meter. The measured pressure and % CO_2 was then used to calculate the amount of carbon dioxide (µmol) using the Ideal Gas Law (PV=nRT).

3.2.7 Methane (CH_4) Analysis

Similar to carbon dioxide analysis, anaerobic microcosms were shaken before measurement to release gas trapped in the liquid phase, and 100 µL was extracted from the headspace using a disposable 28G 500 µL syringe. This volume was injected into a GC equipped with a flame ionization detector (FID). Methane standards (0.16%, 1%, 4%, 8%, 15%, 30%) were prepared in sterile 158 mL serum bottles and then used to generate a standard curve to calculate headspace methane. Pressure was measured, and the methane percentage and pressure were then used to calculate µmol of methane produced using the Ideal Gas Law Formula.

3.2.8 Headspace Hydrocarbon Analysis

Both aerobic and anaerobic microcosms were measured for cyclohexane, cyclopentane, and 1,1,3-trimethylcyclohexane using a gas chromatograph equipped with a mass spectrometer (GC-MS). After shaking the microcosm, 100 µL of headspace was extracted using a disposable 28G 500 µL disposable insulin syringe, with the resulting peak output covering 12 minutes post-injection. The retention time (in minutes) for cyclopentane was ~2, ~3.2 for cyclohexane, and

7.24 for 1,1,3-trimethylcyclohexane. Peak areas were monitored over the course of the incubations to determine whether biodegradation of cyclohexane or cyclopentane had occurred.

3.2.9 Liquid Extraction Hydrocarbon Analysis

For the 8 FFT bottles, the liquid extraction protocol was used to determine whether methanogenesis paired with cycloalkane degradation had taken place. 1 mL of the FFT/media mixture was extracted using a 1 mL syringe with a 22G needle, and then added to 5 mL of methanol and shaken for 30 minutes. The samples were then centrifuged at 1500 rpm for 20 minutes to separate the solids associated with FFT. The samples were then stored in the refrigerator at 4°C for 30 minutes to 2 hours to ensure adequate settling. 4.5 mL of an NaCl solution (1 g NaCl/4.5 mL) was added into GC headspace analysis vials, and 0.5 mL of the top methanol layer was extracted from the previous vial and added to the headspace vial (1 per sample). The mixture was then sealed, and analyzed via GC-MS analysis. Additionally, 8 hydrocarbon standards were prepared (0 ppm, 50 ppm, 100 ppm, 150 ppm, 200 ppm, 300 ppm, 400 ppm), wherein FFT was amended with known concentrations of cyclohexane or cyclopentane and was processed as above to generate a standard curve, which was then used to calculate concentration. Concentration values were then compared to headspace hydrocarbon values to verify that any changes were due to biological activity rather than measurement error.

3.3 Results

3.3.1 Carbon Dioxide (CO_2) Production

In the aerobic soil microcosms, the headspace was initially composed of atmospheric air and after ~170 days of inactivity, it was inferred that lack of activity was attributable to headspace oxygen depletion. Bottles were injected with 2497 μmol of pure oxygen (60 mL volume at 1 atm pressure) and then monitored to determine if there was any changes in pressure

or carbon dioxide production (Figure 3-1). Unlike the active FFT bottles, only small pressure drops were observed in the aerobic soil bottles that were likely due to headspace gas sampling and monitoring after oxygen addition rather than biological activity. Carbon dioxide in the cyclohexane and cyclopentane amended bottles did not increase significantly, experiencing trends very similar to the sterile controls. The only significant carbon dioxide increase occurred in the unamended control microcosms. Therefore, oxygen (2497 μmol) was once again added to all 10 aerobic soil bottles on day 249, and similar trends were observed. No significant carbon dioxide increase was observed in either the sterile and amended microcosms, while in the unamended controls, carbon dioxide increased significantly and subsequently plateaued after oxygen within the microcosms had been replenished. Therefore, the only carbon dioxide increase in the aerobic microcosms was observed in the treatment where no hydrocarbons were added, while all other aerobic microcosms (sterile and amended) did not exhibit significant generation.

3.3.2 Methanogenesis

Microcosm methane generation was monitored in 10 anaerobic soil microcosms and 8 FFT microcosms, both of which receiving methanogenic media initially. In the soil microcosms, no significant methane generation was observed over the course of the incubation for any replicates. However, significant methane generation was observed in select FFT microcosms, with higher methane concentrations being measured after ~day 75 of the experiment (Figure 3-5). At different points in the incubation, bottles amended with cyclohexane, cyclopentane, and both cycloalkanes at once exhibited substantial methane production that was higher than the theoretical maximum for the hydrocarbon amendments added. However, no methane production was observed in the two microcosms amended with cyclohexane, cyclopentane, and n-heptane wherein methane levels remained consistently lower during the experiment.

3.3.3 Hydrocarbon Biodegradation

Cyclopentane and cyclohexane concentrations were monitored during the incubation in sterile controls and active bottles over a period of over 700 days. In this time, the soil microcosms did not exhibit any significant hydrocarbon reduction over the course of the incubation with any observed concentration reduction also being observed in the sterile controls as well, and wass therefore unlikely to be attributable to biodegradation (Figures 3-2 and 3-3). In fact, some redox conditions (nitrate-reducing and methanogenic) had more significant hydrocarbon decreases in the sterile control bottles when compared to active amended bottles. Changes in the hydrocarbon peak areas were consistent across different treatments and therefore attributable to multiple explanations such as GC measurement variation, analytical variation in the GC-MS software, or abiotic processes such as hydrocarbon absorption.

In the FFT preliminary experiment, all bottles had a brief two month phase of inactivity where no methane production was detected; after methane production drastically increased in multiple bottles (with the exception of the two bottles that received cyclohexane, cyclopentane, and n-heptane), hydrocarbon measurements were performed and determined that all 8 bottles had considerably less of all amended hydrocarbons when compared to initial peak areas (Figure 3-4). Since the two microcosms amended with all 3 hydrocarbons did not produce methane, they were used as pseudo controls to establish whether the lower hydrocarbon concentrations were due to methanogenic microbial communities using either cycloalkane as a carbon source. These two microcosms did not produce any methane and had hydrocarbon reductions comparable to the other six bottles; therefore, it is unlikely that methane generation in the other bottles is due to hydrocarbon biodegradation and more likely due to biodegradation of methanol, which was used to dissolve cycloalkanes and ensure their uniform distribution within the soils.

3.4 Discussion

Unlike the previous chapters, the soil experiment outlined here had no indications of successful hydrocarbon biodegradation under any redox condition tested. No significant hydrocarbon biodegradation was observed in any of the 48 established microcosms, including the 8 that had received FFT as a microbial inoculate rather than the pristine soils. The aerobic soil microcosms were the first to indicate that there was a potential problem with the microcosm setup since degradation was expected in aerobic conditions first. In these conditions, oxygen is the primary electron acceptor and is the highest energy redox state wherein quick and kinetically favorable reactions are typically most common. After a relatively long period with complete inactivity in the hydrocarbon amended aerobic microcosms, it was determined via GC headspace analysis that the microcosm was sealed with insufficient oxygen and required more to survive and potentially degrade the desired carbon source. However, once the necessary oxygen was added, carbon dioxide increased in the unamended controls, while in the other aerobic microcosms, the carbon dioxide concentration was reduced briefly before returning to its previous concentration. This decline only lasted for a single timepoint, and is most likely due to the GC measurements underestimating the percentage of carbon dioxide in the headspace after diluting with oxygen. Even after repeated oxygen additions, the amended bottles displayed no substantial carbon dioxide increase and had cyclohexane and cyclopentane peak area reduction comparable to the sterile control bottles, suggesting that no cycloalkane biodegradation had occurred. Given that carbon dioxide had increased in the unamended controls only, this suggests that amendment with cyclohexane and cyclopentane was the determining factor in carbon dioxide production. Hydrocarbon addition is likely to be responsible for inhibiting native microbial community respiration in pristine soil microcosms. Furthermore, the soil microbial

communities demonstrated a capability of recovery after an extended period in anaerobic conditions due to the activity of the unamended microcosms after oxygen addition. Although the aerobic microcosms spent over two months in anaerobic conditions with no oxygen replenishment, subsequent carbon dioxide production in the unamended microcosms may establish that aerobic communities can survive in anaerobic conditions over this long-term period. After considering these factors, it was determined that the most probable explanation is that the cycloalkane amendment was toxic to soil microbial communities, preventing recovery after oxygen addition. In this experiment, cyclopentane and cyclohexane were dissolved in methanol to ensure that the cycloalkanes would be uniformly mixed in with the soils rather than being lost to the headspace where microbes would not be able of physically accessing the hydrocarbons. In the soil bottles, 1.9 mL of methanol was added along with the cycloalkane amendment, and was likely the cause of toxicity as it has been established to be harmful to microbial communities and is sometimes used as a lab disinfectant. Additionally, only 300 mg/L of cyclopentane and cyclohexane were added to the bottles, which is a relatively insignificant volume compared to the large volume of methanol it was dissolved in. It was concluded that the aerobic soils were incapable of degrading both the solvent and methanol.

After determining why activity was not observed in the aerobic microcosms, similar results exhibiting no significant degradation in nitrate reducing, sulfate reducing, and methanogenic soil microcosms are also explainable by methanol toxicity. No hydrocarbon reduction was measured, and no changes in headspace gas was observed (ie. generation of methane and depletion of electron acceptors). In the methanogenic FFT microcosms, however, large quantities of methane generation was detected in multiple bottles, with the exception of the bottles amended with all three hydrocarbons: cyclohexane, cyclopentane, and n-heptane.

Comparing hydrocarbon concentrations in these microcosms with those that are not generating methane shows similar amounts of reduction, suggesting that the hydrocarbon reduction may be due to physical factors such as absorption rather than biological activity. Therefore, the methane produced in these bottles was attributable to microbial consumption of methanol, which was then used as a carbon source to eventually produce methane. The toxicity of methanol was further demonstrated in the bottles that received all three hydrocarbons, since each was initially dissolved separately such that 1 mL of methanol was added per hydrocarbon. No activity was seen in these microcosms, and was likely due to the higher methanol concentration.

3.5 Conclusions

In this initial microcosm experiment, microbial communities found in soils were found to be incapable of degrading cyclohexane and cyclopentane when exposed to a high concentration of methanol in which the cycloalkanes were dissolved. No biodegradation was observed over multiple redox scenarios, including aerobic, nitrate-reducing, sulfate-reducing, and methanogenic conditions. This is attributable to excessive toxicity affecting microbial survival and growth.

Table 3-1. Experimental design for microcosm experiment containing soils. Each microcosm amended with cyclopentane or cyclohexane initially contained a concentration of 300 mg/L. Aerobic microcosms were combined and sealed under atmospheric conditions, while the anaerobic microcosms were flushed with 70:30 N_2:CO_2 balanced gas after sealing. Additionally, aerobic microcosms received 7.5 mL of media while anaerobic microcosms received 23.5 mL.

Redox conditions	**Treatment**	**Replicates**	**Monitoring parameters**	**Substrate**
Aerobic	Unamended	2	CO_2 production	A_e horizon Luvisol
	Cyclopentane	3		
	Cyclohexane	3		
	Heat-killed Cyclohexane	2		
Nitrate-reducing	Unamended	2	HC degradation followed by confirmation of nitrate depletion	
	Cyclopentane	3		
	Cyclohexane	3		
	Heat-killed Cyclohexane	2		
Sulfate-reducing	Unamended	2	HC degradation followed by confirmation of sulfate depletion	
	Cyclopentane	3		
	Cyclohexane	3		
	Heat-killed Cyclohexane	2		
Methanogenic	Unamended	2	CH_4 production	
	Cyclopentane	3		
	Cyclohexane	3		
	Heat-killed Cyclohexane	2		
Methanogenic	Cyclopentane	2	CH_4 production	FFT
	Cyclohexane	2		
	Cyclopentane + Cyclohexane	2		
	Cyclopentane + Cyclohexane + n-heptane	2		

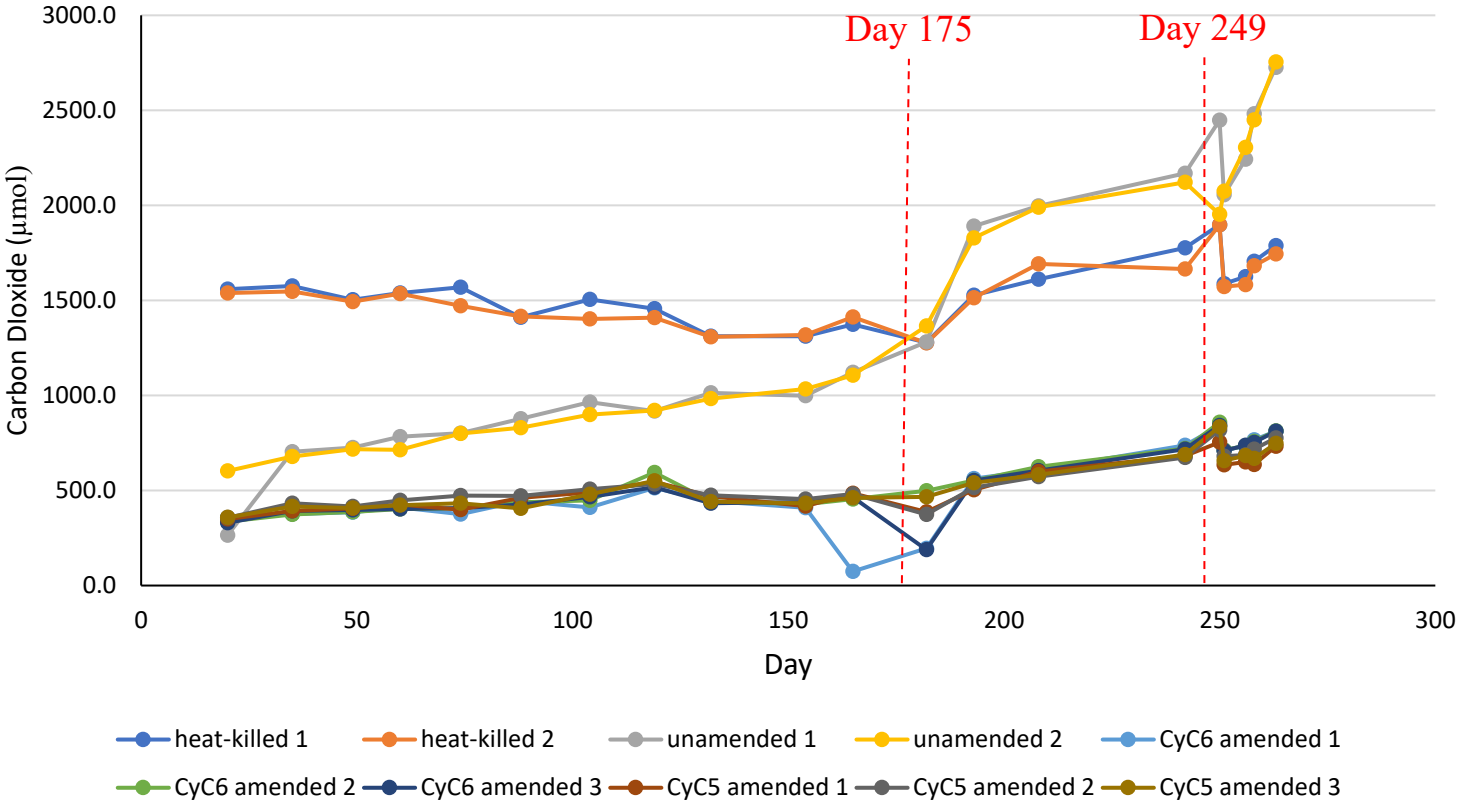


Figure 3-1. Carbon dioxide generation in aerobic soil microcosms amended with either cyclohexane or cyclopentane. After 175 days of inactivity, all 10 microcosms received 2497 µmol of pure oxygen on Day 175 and again on Day 249 and then monitored via GC analysis for any changes in headspace carbon dioxide generation. Amended microcosms received 300 ppm of either hydrocarbon dissolved in methanol.

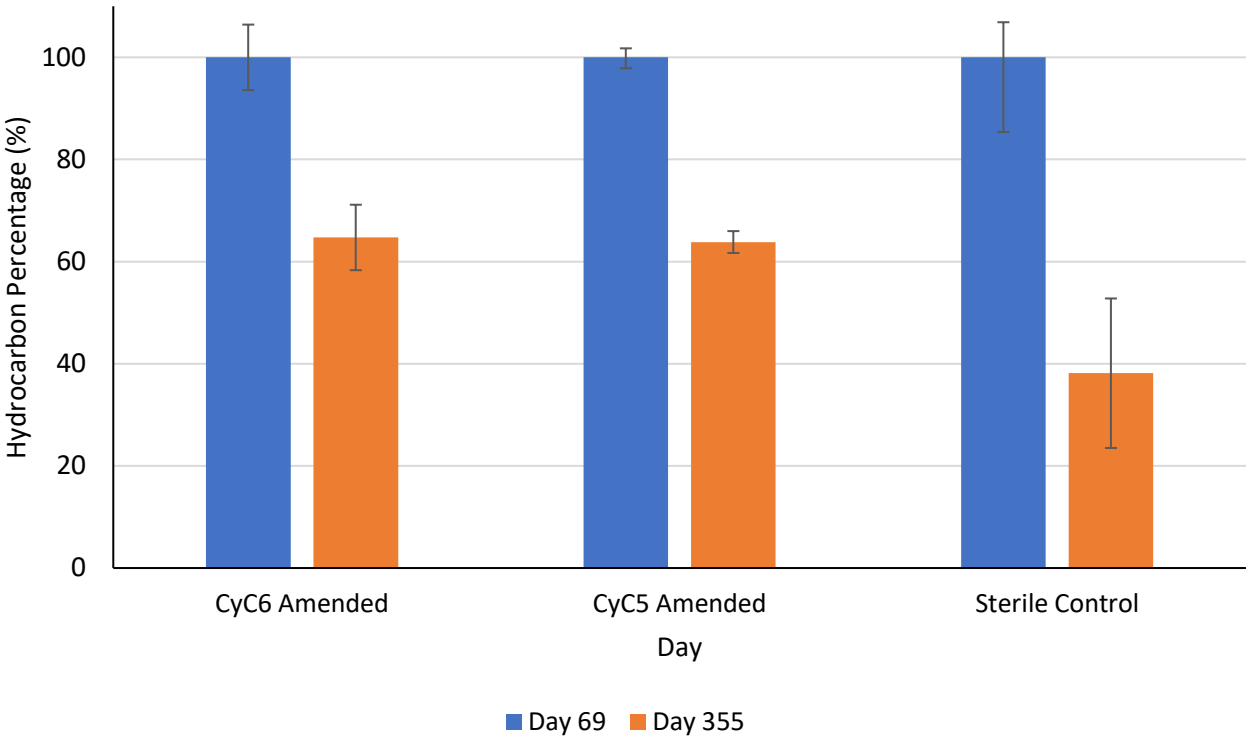
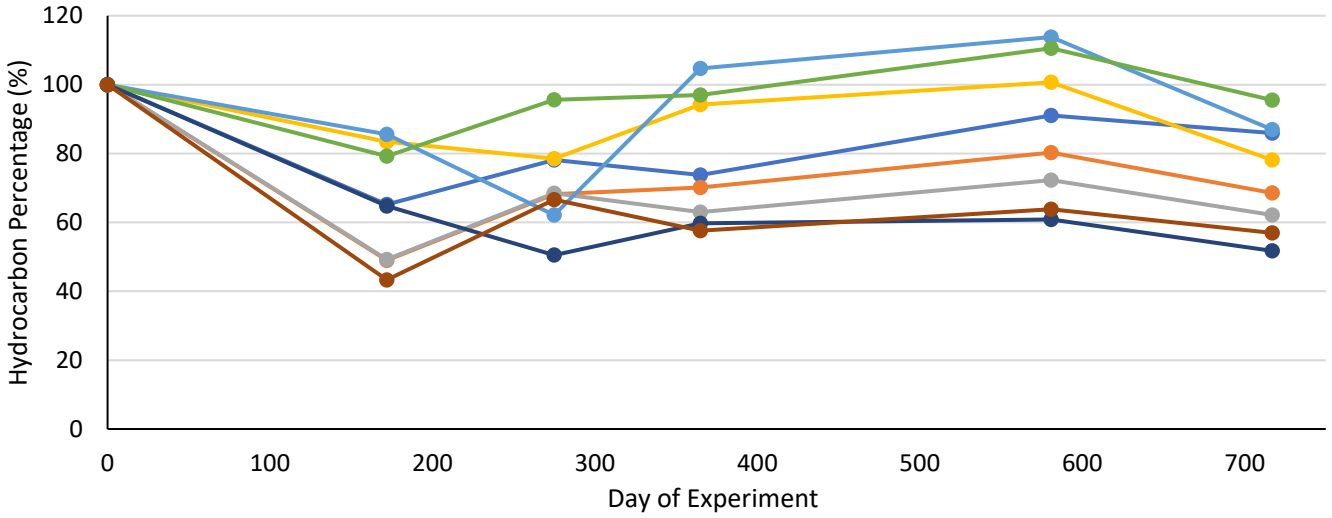
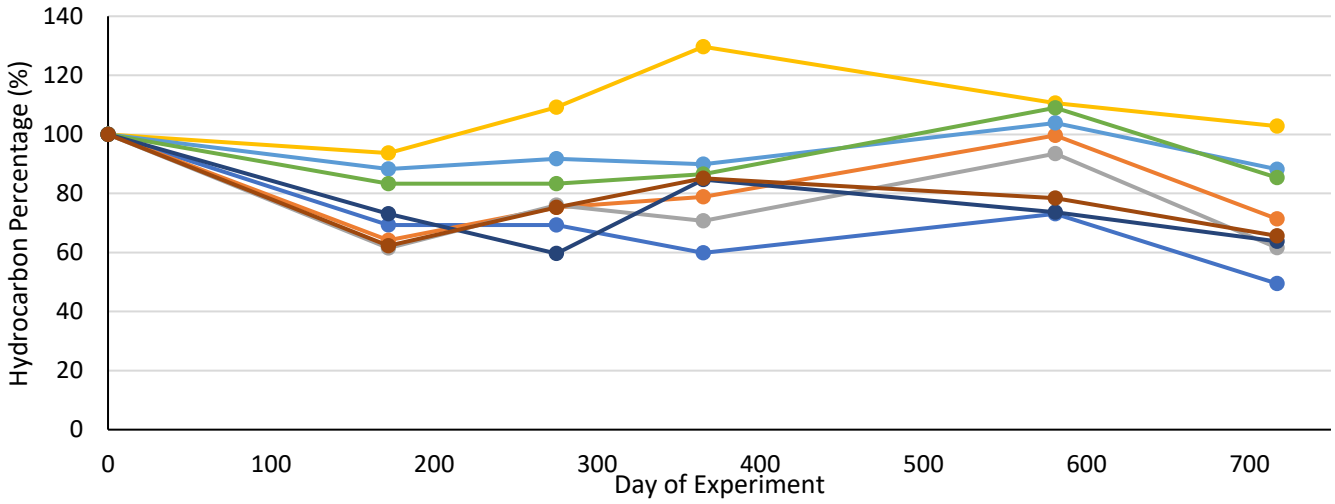


Figure 3-2. Comparison of GC-MS peak areas in aerobic soil microcosms for cyclohexane and cyclopentane on Day 69 of the incubation vs. Day 355. The sterile control was also amended with cyclohexane at the beginning of the experiment. Standard error bars are included for each experimental subset.

(A)


Hydrocarbon Percentage (%)
120
100
80
60
40
20
0
0
100
200
300
400
500
600
700
Day of Experiment

(B)


Hydrocarbon Percentage (%)
140
120
100
80
60
40
20
0
0
100
200
300
400
500
600
700
Day of Experiment

(C)

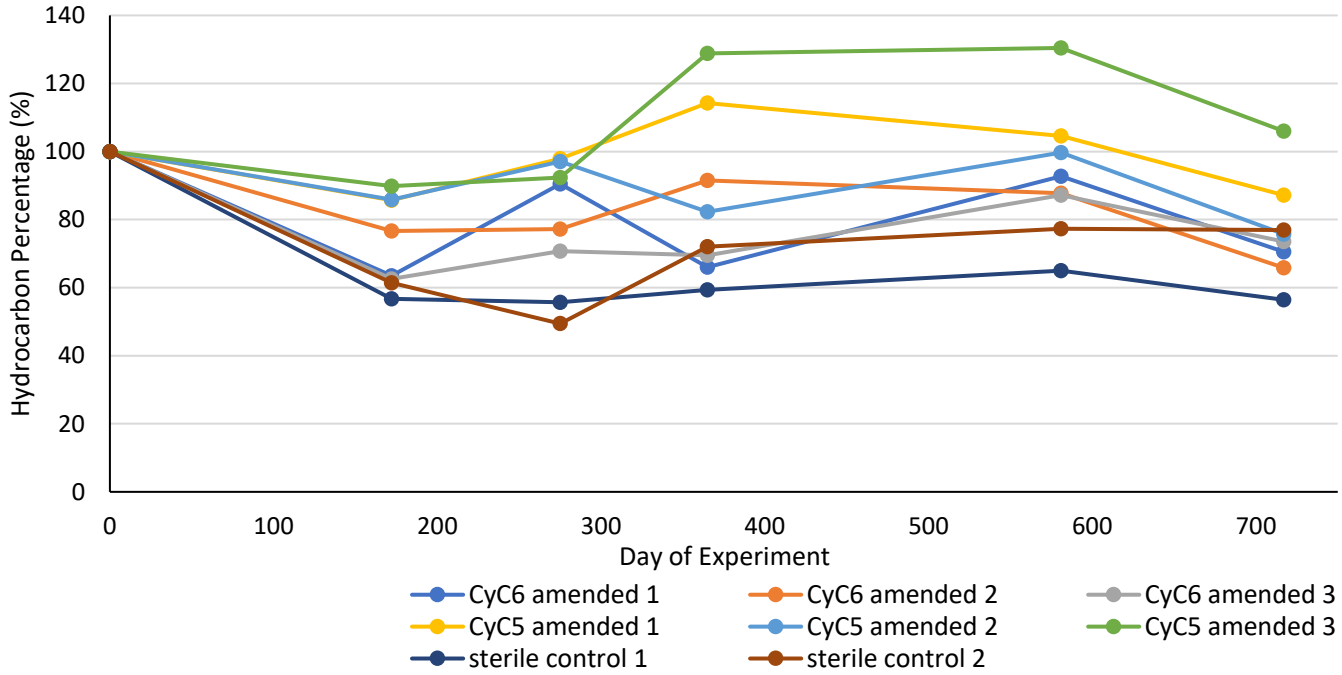

Hydrocarbon Percentage (%)
140
120
100
80
60
40
20
0
0
100
200
300
400
500
600
700
Day of Experiment
CyC6 amended 1
CyC6 amended 2
CyC6 amended 3
CyC5 amended 1
CyC5 amended 2
CyC5 amended 3
sterile control 1
sterile control 2

Figure 3-3. GC-MS analysis of headspace hydrocarbon peaks over the course of the soil incubation experiment. (A) Changes in the peak area of cyclohexane (CyC6) and cyclopentane (CyC5) after ~700 days of incubation in amended bottles under nitrate-reducing redox conditions. (B) Changes in the peak area of cyclohexane and cyclopentane after incubation in amended bottles under sulfate-reducing conditions. (C) Changes in the peak area of cyclohexane and cyclopentane after incubation in amended bottles under methanogenic conditions. CyC5 refers to cyclopentane while CyC6 refers to cyclohexane. Sterile controls were amended with cyclohexane while unamended controls received no additional hydrocarbons. All amended bottles received 300 ppm of each hydrocarbon dissolved in methanol.

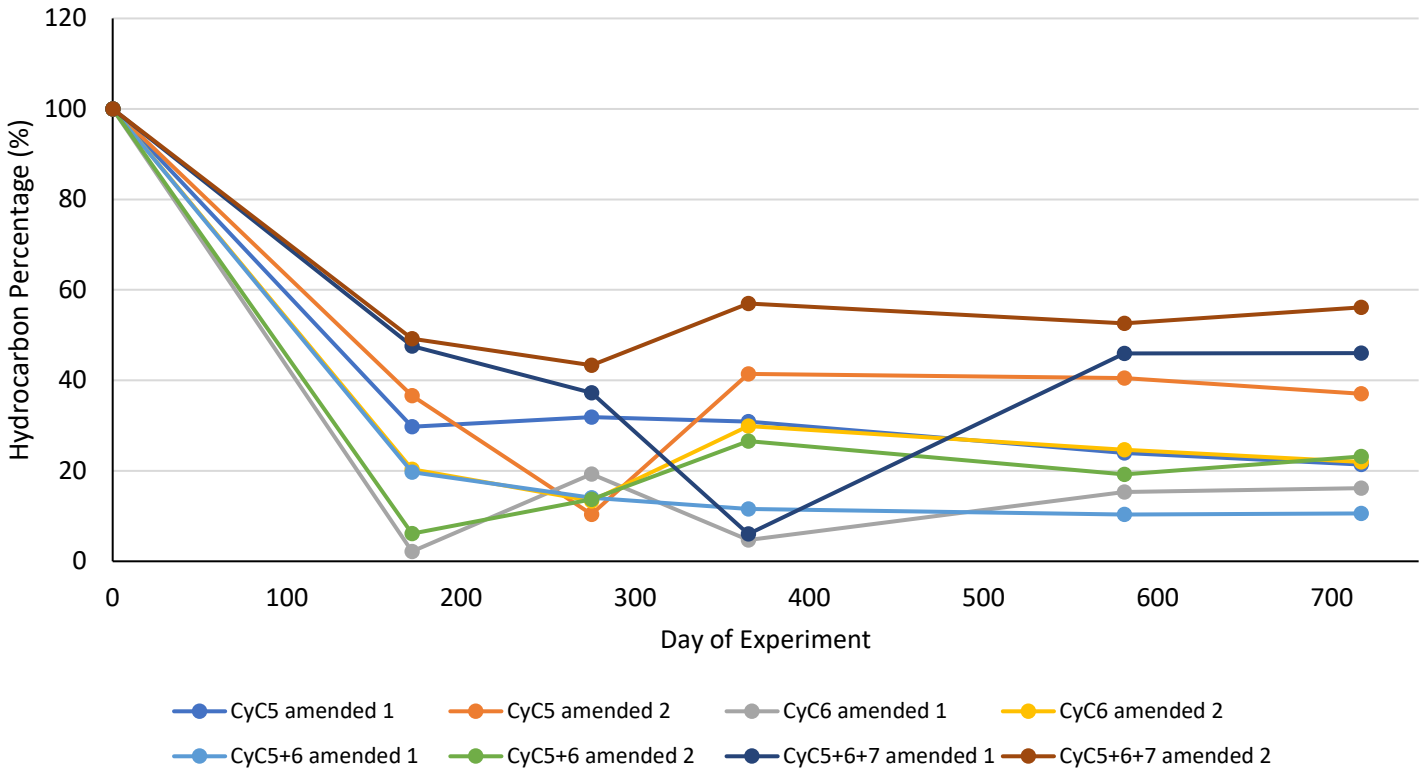


Figure 3-4. Cyclohexane and cyclopentane peak area change over the course of the methanogenic FFT incubation experiment spanning over 700 days measured via GC-MS. CyC5 refers to cyclopentane amended microcosms, CyC6/C6 refers to cyclohexane amended microcosms, and nC7 refers to microcosms which also received an n-heptane amendment. For all microcosms, 300 ppm of each hydrocarbon (dissolved in methanol) was added to each bottle.

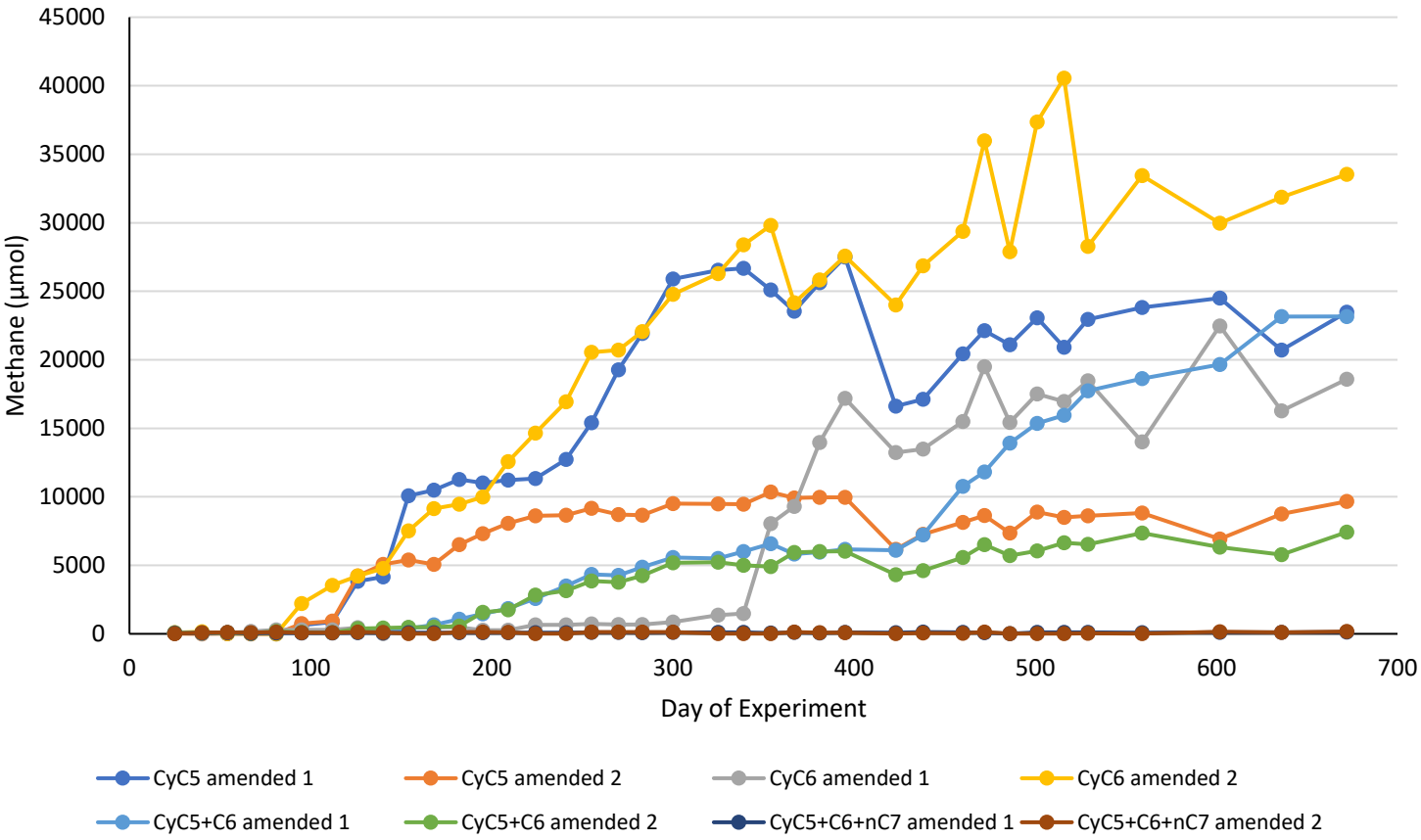


Figure 3-5. Methane generation in methanogenic microcosms using FFT as a microbial inoculate. CyC5 refers to cyclopentane amended microcosms, CyC6/C6 refers to cyclohexane amended microcosms, and nC7 refers to microcosms which also received an n-heptane amendment. For all microcosms, 300 ppm of each hydrocarbon (dissolved in methanol) was added to each bottle.

Chapter 4: Conclusions and Recommendations for Future Work

In all experiments performed, three factors were common across all microcosms that were found to be capable of cycloalkane biodegradation: aerobic redox conditions, sufficient concentrations of nitrogen and phosphorus, and the use of fluid fine tailings (FFT) as a source of hydrocarbon-degrading microbial communities. In all other treatments, significant cycloalkane biodegradation has yet to be observed due to multiple potential explanations. Pristine soils, oil sands process affected waters (OSPW), and non-aqueous extraction dry solids likely do not contain sufficient concentrations of microbial species that are capable of biodegradation and therefore cannot incorporate cycloalkanes into their metabolic pathways. Furthermore, multiple anaerobic redox conditions were established that have not exhibited biodegradation during the allotted 2 year incubation, including nitrate-reducing, sulfate-reducing, iron-reducing, and methanogenic conditions. To induce these conditions, media was added that provided large quantities of the preferred electron acceptor as well as any necessary micro and macronutrients for growth. Although ideal anaerobic growth conditions were provided, no cycloalkane degradation was observed over the course of an incubation spanning almost two years, suggesting that either anaerobic conditions require longer periods to degrade cycloalkanes or that microbial activity utilizes other substrates and utilizing available electron acceptors. Most biodegradation research has found that anaerobic communities require long incubation periods in order to detect significant substrate reductions. Another possibility is a paucity of anaerobic hydrocarbon-tolerant communities, which is much more unlikely due to anaerobic conditions being observed in situ in microbially active tailings ponds.

The aerobic microbial communities were determined to require nitrogen and phosphorus after comparing the incubation time required to degrade similar concentrations of cycloalkanes: in the initial FFT experiment, neat cyclohexane amendments (300 mg/L) were degraded after

almost half a year with regular oxygen inputs while in the second experiment which included NAE dry solids, a combined total concentration of 400 mg/L of neat cyclohexane/cyclopentane was completely degraded in a matter of days, despite receiving inoculum from the same initial FFT source. Similar degradation rates were then also observed in the following stable isotope probing experiment involving isotopic and non-isotopic cyclohexane amendments at an identical hydrocarbon concentration of 300 mg/L (Appendix B). After taking nutrient samples at the end of the initial aerobic FFT experiment, it was found that microbial activity had almost completely consumed the abundant nutrients initially provided by the aerobic media, likely slowing biodegradation considerably after the second cyclohexane amendment. In the second experiment, adequate nutrients were provided by a minimal nutrient media and additional oxygen was provided to all microcosms, allowing cycloalkane biodegradation to occur uninhibited. This experiment also demonstrated the ability of tailings communities to bioaugment NAE solids.

These findings will be valuable in future biotechnologies wherein scaled up bioreactors may be used to manage the dry tailings derived from non-aqueous extraction processes and ensure that the system has minimal environmental impact upon deposition of extracted ores. Based on the experimental results, these bioreactors will require adequate supplies of nitrogen, phosphorus, and oxygen, as well as microbial cultures from FFT to provide hydrocarbon-degrading microbial communities that are not present in the dry solids or surrounding soils. Future research could address toxicity and determine the maximum cycloalkane concentration that communities can tolerate and degrade by creating similar microcosm experiments at lower range of concentrations in multiple redox conditions. Alternatively, future research could also elucidate which specific genes are responsible for cycloalkane degradation via functional gene analysis, or test the biodegradation of metabolites suggested in the cycloalkane metabolic path.

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Appendix A: List of Reagents

Chemicals/Reagents

Aerobic media

- Potassium phosphate (monobasic): NF/FCC, Fisher Scientific
- Calcium chloride: USP/FCC, Fisher Scientific
- Potassium phosphate (dibasic): ACS certified, Fisher Scientific
- Magnesium sulfate: ACS certified, Fisher Scientific
- Ammonium nitrate: ACS certified, Fisher Scientific
- Iron (III) chloride: reagent grade, Sigma Aldrich

Methanogenic media

- Sodium chloride: certified for biological work, Fisher Scientific
- Calcium chloride: USP/FCC, Fisher Scientific
- Ammonium chloride: GR ACS, EMD
- Magnesium chloride: ACS certified, Fisher Scientific
- Ammonium molybdate: ACS certified, Fisher Scientific
- Zinc sulfate heptahydrate: ACS certified, Sigma Aldrich
- Boric acid: analytical grade, BDH
- Iron (II) chloride tetrahydrite: ACS certified, Fisher Scientific
- Cobaltous chloride hexahydrate: ACS certified, Fisher Scientific
- Manganese (II) chloride tetrahydrite: ACS certified, Fisher Scientific
- Nickel (II) chloride hexahydrate: ACS certified, Fisher Scientific
- Potassium alum: reagent grade, BDH
- Potassium phosphate (monobasic): NF/FCC, Fisher Scientific
- Sodium bicarbonate: ACS certified, Sigma Aldrich

Nitrate-reducing media

- Potassium phosphate (monobasic): NF/FCC, Fisher Scientific
- Ammonium chloride: GR ACS, EMD
- Magnesium sulfate: ACS certified, Fisher Scientific
- Calcium chloride: USP/FCC, Fisher Scientific
- Sodium nitrate: ACS Certified, Caledon

Sulfate-reducing media

- Sodium chloride: certified for biological work, Fisher Scientific
- Potassium chloride: ACS certified, Fisher Scientific
- Magnesium chloride: ACS certified, Fisher Scientific
- Calcium chloride: USP/FCC, Fisher Scientific
- Ammonium chloride: GR ACS, EMD
- Potassium phosphate (monobasic): NF/FCC, Fisher Scientific
- Sodium sulfate: Sigma Grade, Sigma Aldrich

Iron-reducing media

- Ammonium chloride: GR ACS, EMD
- Sodium phosphate (monobasic): ACS Certified, Fisher Scientific
- Sodium bicarbonate: ACS certified, Sigma Aldrich
- Potassium chloride: ACS certified, Fisher Scientific
- Nitrilotriacetic acid: ACS certified, Sigma Aldrich

DNA-related Reagents

- Tris: ultrapure, ICN Biomedicals
- Monobasic sodium phosphate: laboratory/manufacturing grade, Fisher Scientific
- Dibasic sodium phosphate: enzyme grade, Fisher Scientific
- Sodium dodecyl sulfate (SDS): >98.5% for molecular biology, Sigma Aldrich
- Ammonium acetate: for laboratory use, MP Biomedical
- Chloroform: HPLC grade, ACS certified, Fisher Scientific
- Isoamyl alcohol: ACS certified, Anachemia
- Isopropanol: anhydrous 99.5%, Sigma Aldrich
- Agarose: genetic analysis grade, Fisher Scientific

Other

- Sodium hydroxide: ACS certified, Fisher Scientific
- Ferrozine: 97%, Sigma Aldrich
- HEPES: for molecular biology, Fisher Scientific
- Ammonium iron (II) sulfate: >98%, Sigma Aldrich
- Methanol: for high resolution gas chromatography suitable for organic residue analysis, Fisher Scientific
- Cyclohexane: 99.5% extra dry, Acros Organics
- Cyclopentane: HPLC Grade, Alpha Aesar
- Carbon-13 Isotopic Cyclohexane: 99% isotopic purity, Sigma Aldrich
- N-heptane: anhydrous 99%, Sigma Aldrich
- 1,1,3-trimethylcyclohexane: 99%, ChemSampCo

Appendix B: Stable Isotope Probing of Microbial Communities Using ^{13}C Cyclohexane

B.1 Introduction

Fluid fine tailings (FFT) have been established in our previous chapter to be capable of cyclohexane degradation under aerobic conditions. Although a significant shift in microbial communities was observed over the course of the incubation as cyclohexane biodegradation was occurring, this does not necessarily confirm that the microbial species that became more prominent in cyclohexane-rich conditions are directly involved in the metabolism of cyclohexane. Other explanations for microbial community changes includes certain species being selected for in aerobic conditions that may be degrading other carbon sources or occupying a particular niche that was previously taken by another species before introducing oxygen and cyclohexane. Therefore, determining which members of the microbial consortium that are directly involved in cyclohexane biodegradation is difficult when amending microcosms with conventional cyclohexane. Stable isotope probing (SIP) is a method used in microbial ecology that is capable of identifying microbes which consume specific substrates without requiring cultivation of microbial isolates (Neufeld et al., 2007). In this specific case, microcosms were amended with stable-isotope-labeled cyclohexane, wherein each carbon on the six-membered ring is replaced with isotopic carbon-13. When biodegraded, labeled cyclohexane is broken down and a percentage of the constituent carbon-13 isotopic atoms are incorporated into cellular structures, including DNA. Carbon-13 labeled DNA is slightly heavier than unlabeled carbon-12 DNA and can therefore be separated via ultracentrifugation. After separation, quantitative PCR and sequencing are capable of determining which specific members of the cyclohexane-degrading community contain isotopic DNA and therefore actively contributed to the biodegradation of cyclohexane within the microcosm. This provides information that will further elucidate the metabolic pathway of aerobic cyclohexane degradation.

B.2 Materials and Methods

B.2.1 Chemicals and Reagents

All chemicals were purchased from Fisher Scientific, located in Ontario, Canada. For a complete list of reagents and chemicals used in the lab, as well as manufacturers and grade, refer to the List of Reagents in Appendix A.

B.2.2 Gases

Pure O_2 canisters were purchased from Praxair, located in Edmonton, Alberta, Canada.

B.2.3 Microbial Inoculate Collection

Fluid fine tailings (FFT) were collected in bulk in 2016 from a tailings pond located near Fort McMurray at a depth of 10.5 metres below the surface and stored in pails in the dark at a temperature of 4°C. After removal from the refrigerator, samples were immediately used in the microcosm culture setup in 158 mL sealed serum bottles.

B.2.4 Media Preparation

Aerobic Bushnell-Haas media was prepared for all microcosms, which contained K_2HPO_4 (1 g), KH_2PO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $CaCl_2 \cdot 2H_2O$ (0.02 g), $FeCl_3$ (0.05 g), and NH_4NO_3 (1 g) dissolved in 1 L of distilled water and then autoclaved before use.

B.2.5 Experimental Setup

Serum bottles (158 mL) were autoclaved, and 50 mL of FFT was added to each bottle in ambient atmospheric conditions. 50 mL of Bushnell-Haas media was then added to each bottle, and then bottles were sealed using a butyl stopper cut to 1/3”, then crimped with an aluminum cap. From the set, two bottles were set aside and autoclaved once per day for 4 consecutive days,

serving as a sterile control to determine whether any physical or chemical changes are responsible for any changes in headspace carbon dioxide/cyclohexane concentrations. Before amending microcosms with 300 ppm of cyclohexane, the theoretical oxygen demand for cyclohexane was stoichiometrically calculated and determined to be 1214.3 µmol (29 mL pure O_2 at 1 atm of pressure). All 8 microcosms received 60 mL of oxygen (2497 µmol) and left to incubate for 4 days on a gyratory shaker at 180 rpm, and then flushed with sterile atmospheric air for 8 minutes. Since the heat-killed controls had already been amended with cyclohexane from a previous iteration of the experiment, these two bottles were flushed for an additional 24 minutes to remove any residual cyclohexane from the previous incubation. Bottles then received additional oxygen once again (2497 mol), and two were then separated to receive no hydrocarbon amendment (unamended control). The sterile microcosms, as well as two more microcosms, were amended with rudimentary cyclohexane composed of carbon-12, the most common carbon isotope that is found in nature. The non-sterile microcosms serve as a ^{12}C control. However, the final two microcosms were amended with cyclohexane composed of carbon-13, a stable carbon isotope that is naturally rare and can therefore be traced within the microcosm via stable isotope probing techniques. Microcosms were then incubated on a 180 rpm gyratory shaker and monitored via headspace gas chromatography over the next two weeks.

B.2.6 Carbon Dioxide (CO_2) Analysis

Carbon dioxide analysis was performed by injecting 100 µL of headspace gas from the microcosm (after shaking the bottle to release any trapped gas) into a gas chromatograph (GC) equipped with a thermal conductivity detector (TCD) using a 28G 500 µL disposable insulin syringe. Carbon dioxide standards (0.16%, 1%, 2%, 4%, 8%, 15%, and 30%) were prepared in sterile 158 mL serum bottles and used to prepare a standard curve to determine the percentage of

carbon dioxide in the microcosm headspace. Additionally, pressure was measured in each microcosm using a 25G needle attached to a pressure meter. The measured pressure and % CO_2 was then used to calculate the amount of carbon dioxide (μmol) using the Ideal Gas Law.

B.2.7 Headspace Hydrocarbon Analysis

A gas chromatograph equipped with a mass spectrometer was used to detect the amount of cyclohexane in the headspace of each microcosm. After shaking the bottle, 100 μL of headspace is extracted using a 28G 500 μL disposable insulin syringe. The analysis and resulting peak output covers 12 minutes post-injection, with the retention time for both ^{12}C and ^{13}C cyclohexane being ~3.2 minutes. Peak areas were monitored and recorded over the course of the incubations to determine whether biodegradation of either isotope of cyclohexane had occurred.

B.2.8 Liquid Extraction Hydrocarbon Analysis

While headspace analysis measures the presence of hydrocarbons in the gaseous microcosm headspace, liquid extraction was used at the beginning and end of the incubation to determine the total change in cyclohexane concentration and confirm the validity of the headspace measurements. 1 mL of the FFT/mixture is extracted using a 1 mL syringe with a 22G needle, and then added to 5 mL of methanol in clean 30 mL EPA vials and shaken for 30 minutes. The samples are then centrifuged at 1500 rpm for 20 minutes to separate the solids associated with FFT. The samples are then stored in the refrigerator at 4°C for 30 minutes to 2 hours to ensure adequate settling. 4.5 mL of an NaCl solution (1 g NaCl/4.5 mL) is added into GC headspace analysis vials, and 0.5 mL of the top methanol layer is extracted from the previous vial and added to the headspace vial (1 per sample). The mixture is then sealed, and analyzed via GC-MS analysis. Additionally, 8 hydrocarbon standards are prepared (0 ppm, 50 ppm, 100 ppm, 150 ppm, 200 ppm, 300 ppm, 400 ppm), wherein FFT is amended with known concentrations of

cyclohexane and is processed as above to generate a standard curve, which is then used to calculate concentration. Values are then compared at the beginning and end of the incubation to confirm the complete degradation of cyclohexane within the active and amended bottles.

B.2.9 Oxygen Analysis

Oxygen analysis was performed using a separate GC-TCD equipped with a molecular sieve that allows it to distinguish between the dinitrogen and oxygen peaks on the chromatograph output. Aerobic microcosms were measured at the beginning of the experiment immediately after hydrocarbon amendment to ensure that relatively equal amounts of oxygen was present in each bottle. Similar to other headspace analyses, 100 µL of headspace was extracted from each microcosm and injected into the GC-TCD, with resulting peak areas then recorded. Different volumes of atmospheric air were then injected to provide an oxygen standard curve (250, 200, 150, 100, 50, and 25 µL). The standard curve was then used to convert peak areas to molar amounts of oxygen present in each microcosm.

B.2.10 Microbial Community Extraction and Analysis

Samples of the media/FFT mixture (10 mL) were extracted from the microcosms for the purposes of microbial community analysis when a measurable reduction in cyclohexane had occurred or was currently taking place. Samples were extracted by shaking the microcosm and injecting a 22G needle with an attached 10 mL syringe (both sterile). The volume was extracted from each bottle and stored at -20°C in sterile 15 mL centrifuge tubes before DNA extraction.

The night before the sample underwent DNA extraction, the sample was moved into the 4°C refrigerator to thaw. The next day, 4 mL of the sample was then transferred to two 2 mL eppendorf tubes, and the remaining sample was then refrozen. The tubes were centrifuged at 15000 rpm for 8 minutes, and the supernatant was discarded. 250 mg of each pellet was extracted

with a sterilized scupula and weighed into sterile 2 mL screw cap tubes containing 0.5 g of 2.3 mm and 0.5 g of 0.1 mm zirconium-silica beads. 300 µL of phosphate buffer (pH=8) is then added to the tubes, which was prepared previously and contains both monobasic ($NaH_2PO_4 \cdot H_2O$) and dibasic (Na_2HPO_4) phosphate (sterilized via autoclave). Next, 300 µL 10% SDS lysis buffer was added, which was also previously prepared by mixing 48 mL H_2O (millipore), 2 mL of NaCl (5M), 50 mL of 1M Tris (pH=8), and 10 g of sodium dodecyl sulfate (SDS). The mixture is then sterilized via autoclave before use. Finally, 300 µL of 24:1 chloroform:isoamyl alcohol solution is added to the tubes before sealing. Tubes were then shaken via beadbeater using the Powerlyzer 24 for 45 seconds at 3400 reciprocations per minute in order to lyse the cells. The tubes were then centrifuged at 15000 rpm for 5 minutes, and the supernatant was transferred into a sterile eppendorf tube. Fresh buffers were then added into the same screw cap tube and beadbeaten and centrifuged again to ensure as much DNA was extracted from the sample as possible. This process generates a total of 1 mL of supernatant, which then receives 560 µL of ammonium acetate to obtain a fianl concentration of 2.5M, causing a precipitate to form in the tube. The tubes were then hand mixed gently and centrifuged at 15000 rpm for 7 minutes. The supernatant (~1.25 mL) was then transferred into another sterile 2 mL eppendorf tube and received 675 µL of isopropanol. Samples were then incubated overnight at 4°C. The next day, the precipitated DNA was recovered by centrifuging the eppendorf tubes at 15000 rpm for 30 minutes. The isopropanol was then discarded and the samples were then centrifuged again for 1 minute to collect any remaining supernatant. A 20-200 µL micropipette was then used to extract any remaining supernatant. The tubes were then left on a sterilized countertop to dry for 1 hour, and then suspended in 50 µL of sterile nuclease-free water. The sterile water was pipetted up and down until the DNA precipitate is properly suspended, and then the volume is transferred to the other

fraction-containing eppendorf that contained DNA originating from the same original sample. The water was pipetted up and down once again to suspend the new DNA, and the sample is placed on ice. A Qubit was used to measure the concentration of DNA and ensure that the total volume was higher than the minimum 500 ng required for SIP analysis.

B.2.11 SIP Sample Processing and qPCR

The project is currently in progress Dr. Siddique's lab for further processing, including separation and identification of active microorganisms involved in cyclohexane degradation.

B.3 Results

B.3.1 Carbon Dioxide Production

Carbon dioxide generation was monitored over 14 days using the GC-TCD, and sterile controls remained consistently low at approximately 100-150 µmol of carbon dioxide (Figure B-1). The unamended controls, however, exhibited a typical microbial growth curve, with carbon dioxide increasing from ~350 µmol to almost 1500 µmol in an eight day span. Similarly, the ^{12}C controls and ^{13}C amended microcosms exhibit a similar trend, starting with a concentration of ~340 µmol and increasing to an average of 1600 µmol over eight days.

B.3.2 Hydrocarbon Biodegradation

Hydrocarbon biodegradation was monitored via GC-MS headspace analysis as well as liquid extraction performed at the beginning and end of the experiment (Figure B-2). Although the first sterile replicate exhibited some variation in headspace cyclohexane measurements, the amount measured at the beginning and end of the experiment were relatively consistent, similar to the second replicate. Conversely, the headspace cyclohexane measurements for the ^{12}C and ^{13}C controls displayed clear degradation almost immediately after amendment, with the majority occurring between Day 3 and 4 of the experiment. At Day 6, the cyclohexane has become

degraded almost completely and is below the detection limit by Day 7. This is confirmed by liquid extraction; the sterile controls start with an average of 361.5 ppm of cyclohexane (slightly higher due to being used in previous incubations) and were measured to have an average of 326.5 ppm by the end of the experiment. The ^{12}C controls began with 222 ppm and was undetectable by liquid extraction performed on Day 14 of the experiment. Similarly, the two ^{13}C amended replicates began with an average of 201.5 ppm of cyclohexane which was undetectable in the microcosm by Day 14, suggesting that degradation had taken place.

B.4 Discussion

When comparing the cyclohexane biodegradation rates to those observed in Chapter 2, both the isotopic and non-isotopic cyclohexane exhibit a much higher rate of degradation. These microcosms completely break down cyclohexane in a matter of days as opposed to previous aerobic microcosms requring months to degrade despite being amended with an identical concentration of cyclohexane (300 mg/L). This is likely attributable to microcosms receiving adequate amounts of both nutrients and oxygen. The microcosms set up and used for stable isotope probing received both Bushnell-Haas media and 2497 μmol of oxygen at the beginning of the experiment, while the previous aerobic FFT experiment had a paucity of either oxygen after the first cyclohexane amendment or nutrients after reamending. In the larger NAE microcosms also described in Chapter 2, FFT and NAE were mixed with a minimal nutrient media rather than Bushnell-Haas aerobic media which may have slowed cyclohexane breakdown. Since the SIP microcosms received both nutrients and oxygen, there were no limiting factors for biodegradation, which allowed it to occur at a much faster rate.

Active and amended microcosms received either ^{12}C cyclohexane as a non-isotopic control or isotopic ^{13}C cyclohexane, and these treatments exhibited extremely similar results in

both carbon dioxide production and hydrocarbon degradation. This suggests that tailings microbial communities have no preference when choosing between ^{12}C and ^{13}C cyclohexane and that isotopic cyclohexane is a suitable isotope candidate for elucidating non-isotopic cyclohexane breakdown in tailings. However, this finding is dependent on both the compound and microbial community; other research into ^{12}C and ^{13}C isotopes in aromatic hydrocarbons found that in toluene degradation, isotopic fractionation occurs, which suggests that those particular microbial communities have an isotope which is consumed preferentially (Meckenstock et al., 1999).

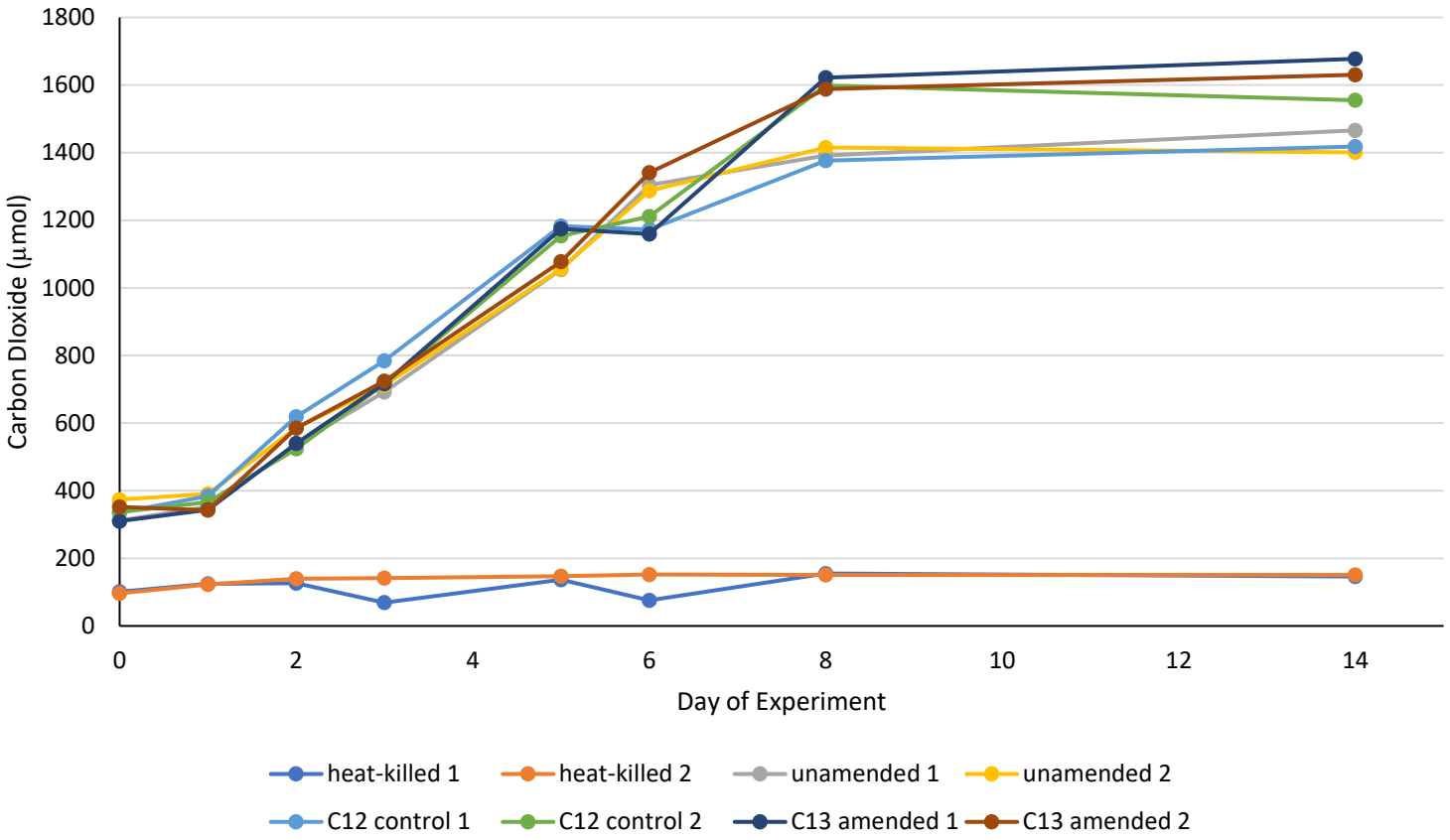

Figure B-1. Carbon dioxide generation in aerobic microcosms after 14 days of incubation. Heat-killed refers to microcosm controls that were autoclaved for 4 consecutive days, while unamended microcosms received no hydrocarbon amendment. ^{12}C controls received conventional cyclohexane (non-isotopic) while ^{13}C amended microcosms received cyclohexane marked with isotopic carbon-13 which was biodegraded over the course of the incubation.

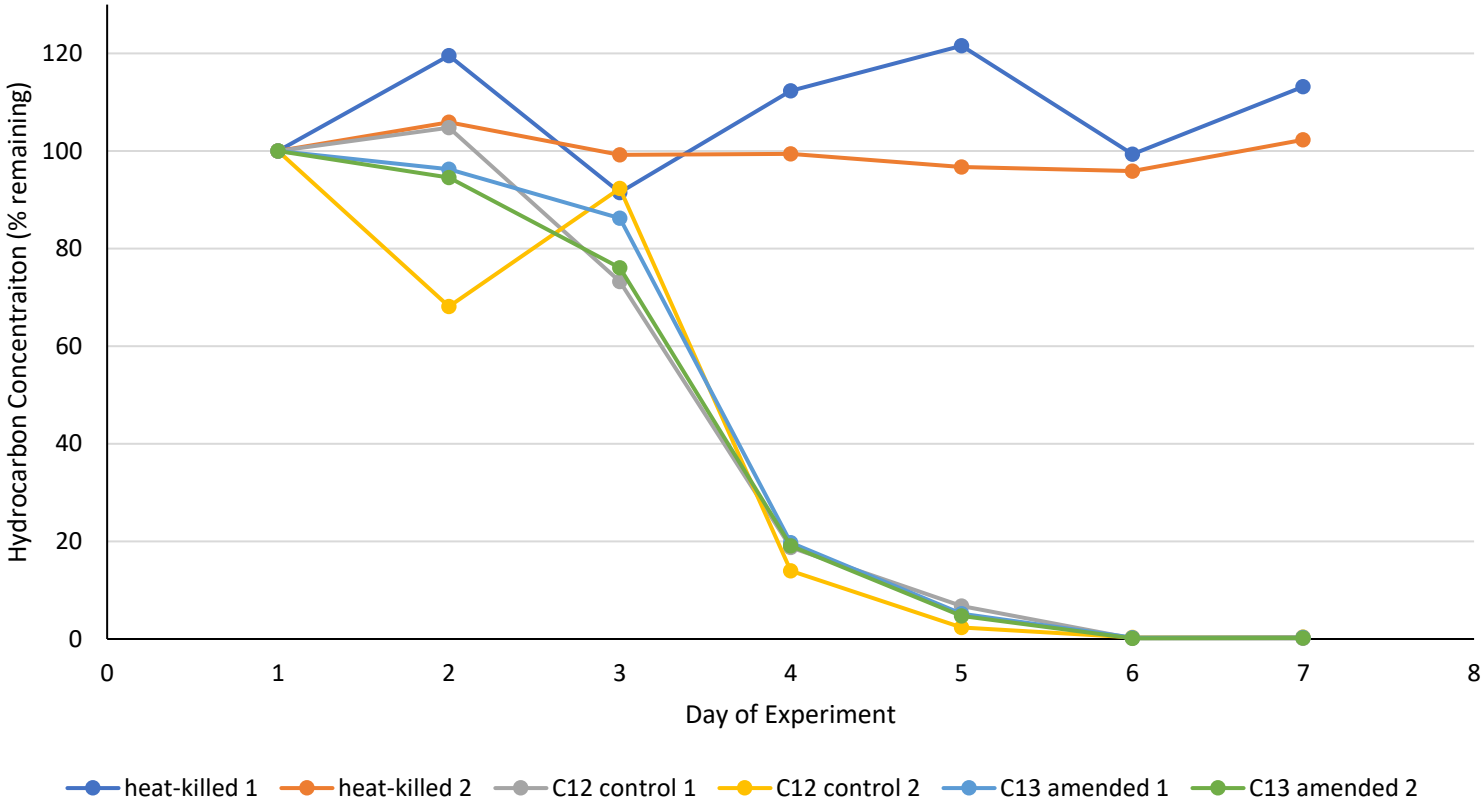

Figure B-2. Isotopic and non-isotopic cyclohexane degradation during the first seven days of the microcosm incubation determined via GC-MS headspace analysis. Heat-killed refers to microcosm controls that were autoclaved for 4 consecutive days, while unamended microcosms received no hydrocarbon amendment. ^{12}C controls received conventional cyclohexane (non-isotopic) while ^{13}C amended microcosms received cyclohexane marked with isotopic carbon-13 which was biodegraded over the course of the incubation.

Appendix C: Example Stoichiometric Calculations

Cyclohexane: $C_6H_{12} + 9O_2 \leftrightarrow 6CO_2 + 6H_2O$

Cyclopentane: $C_5H_{10} + 7.5O_2 \leftrightarrow 5CO_2 + 5H_2O$

Cyclohexane Molar Mass: 84.16 g/mol
Cyclopentane Molar Mass: 70.1 g/mol

$$300\frac{mg}{L}\ Cyclohexane\ \times 0.064L\ (microcosm\ volume) = 19.2\ mg = 0.0192\ g\ C_6H_{12}$$

$$\frac{0.0192\ g\ cyclohexane}{84.16\ g/mol} = 228.1\ \mu mol\ cyclohexane$$

Oxygen Demand: $228.1\ \mu mol\ \times \frac{9O_2}{1C_6H_{12}} = 2053\ \mu mol\ O_2$

Oxygen Already in Headspace: $n = \frac{PV}{RT} = \frac{(1\ atm)(0.096\ L)}{(0.082\frac{L\cdot atm}{mol\cdot K})(293\ K)} = 839.1\ \mu mol$

Pure Oxygen Input Volume Required: $V = \frac{nRT}{P} = \frac{(2053 - 839.1\ \mu mol)(0.082\frac{L\cdot atm}{mol\cdot K})(293\ K)}{(1\ atm)} = 29.1\ mL$

Carbon Dioxide Production: $249.5\ \mu mol\ \times \frac{6CO_2}{1C_6H_{12}} = 1497\ \mu mol\ CO_2$