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Microbial Communities Associated with Hydraulic Fracturing Fluids from Shale Gas Fields in Western Canada

by

Alexander S. Elliott

A THESIS

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Abstract

Hydraulic fracturing has revolutionized the natural gas industry and has become a prominent process in Western Canada. Since its introduction to Canada in 2005 no work regarding the associated microbial communities has been conducted. Microbes are introduced with the fracturing fluid during fracturing. Early flowback water has increased microbial biomass relative to the fracturing fluid. As flowback proceeds physicochemical conditions become increasingly saline and there is a rapid decrease in biomass. The microbial community changes reflect the changing conditions with a decrease in diversity and abundance. Community composition shifts accordingly from one resembling the source water to a halophilic community that is more adapted to the flowback water conditions. The lack of thermophiles indicates that temperature is the limiting factor that accounts for low amounts of biomass. This indicates that microbial activity will not negatively impact hydraulic fracturing operations.

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List of Symbols, Abbreviations and Nomenclature

APB	Acid Producing Bacteria
BTEX	Benzene, Toluene, Ethylene and Xylene
cP	Centipoise
EUR	Estimated Ultimate Recovery
GC-MS	Gas Chromatography-Mass Spectophotometrey
gDNA	Genomic DNA
GHB	General Heterotrophic Bacteria
hNRB	Heterotrophic Nitrate Reducing Bacteria
IRB	Iron Reducing Bacteria
mD	milliDarcy
Meq	Molar equivalent
MIC	Microbially Influenced Corrosion
MPN	Most Probable Number
N_2CO_2	90% N_2:10% CO_2
$N_2H_2CO_2$	85% N_2:5% H_2:10% CO_2
OTU	Operational Taxonomic Unit
PAM	Polyacrylamide
RPM	Rotations Per Minute
SRB	Sulfate Reducing Bacteria
Symbol	Definition
TDS	Total Dissolved Solutes
TOC	Total Organic Content
TY	Tryptone-Yeast Extract

Chapter One: Introduction

1.1 Natural Gas and Current energy Demand

Canada is the 5th largest energy producer in the world (U.S. Energy Information Administration 2014a). The majority of its resources are located in the Western Canadian Sedimentary Basin, in particular in the Alberta oil sands, which are responsible for over 60% of Canada's energy production (Conti et al. 2014; U.S. Energy Information Administration 2014a). The oil market in Canada is expected to continue growing in to the foreseeable future even with the recent drop in oil price. These resources are very energy intensive to produce and have a very large carbon footprint relative to other fuel types (Zhou et al. 2008; Murphy & Hall 2010; Gupta & Hall 2011). While it is expected that these zcome, other forms of non-renewable energy resources are gaining attention (U.S. Energy Information Administration 2014a). Factors driving this trend are reduced carbon emissions during combustion and reduced price which in part, has stimulated the extraction of natural gas from unconventional resources that were previously overlooked (Q. Wang et al. 2014).

Canada is currently the fifth largest producer of natural gas in the world, and is ranked 17th in terms of conventional natural gas reserves (Canadian Association of Petroleum Produceres 2014; U.S. Energy Information Administration 2015a). These estimates are expected to increase as technology improves leading to the discovery of more deposits and making them accessible for processing (Gadonniex et al. 2010).

World consumption of natural gas is expected to double by 2040 due to its low cost relative to oil and reports that suggested natural gas would act as a transition fuel, from fuels with higher greenhouse gas emissions, further spurring its popularity (U.S.

Energy Information Administration 2015b; Moniz et al. 2010; Canadian Association of Petroleum Produceres 2014; Holtberg et al. 2014). However, there is rising evidence that greenhouse gas emissions for unconventional natural gas may exceed that of conventional natural gas production over the life time of a well due to methane being released in to the atmosphere during production (Wood et al. 2011; Howarth et al. 2011). This however has done nothing to curtail natural gas production and it is expected that natural gas could represent as much as 20% of North America's energy portfolio in the coming years (Howarth et al. 2011; Murali Mohan et al. 2013).

Natural gas is comprised predominantly of methane (> 98 %) and other hydrocarbon components such as butane, ethane, propane and other gases (< 2%) (Arthur & Langhus 2008; Arthur, Bohm & Coughlin 2008). It can be formed in two different ways: 1) Biotically by the systematic breakdown of larger organic compounds through fermentation by microorganisms. Methanogens metabolize acetate, carbon dioxide and hydrogen to produce methane as an end product (Head et al. 2003; Zengler et al. 1999). Once the redox potential is sufficiently low these processes dominate an environment. These biotic deposits are found closer to the surface where temperatures are sufficiently cool (< 75ºC) and water in sufficient supply to support microbial life. Microbial activity is generally restricted to less than 600 m depth (Leckie & Maness 2002; Rice & Claypool 1981; Rokosh et al. 2009). 2) Thermogenic gas production occurs at much greater depths where temperatures are high enough to invoke categenic conditions that convert the organic precursors to oil and gas. Gas produced through thermogenic conditions has a higher fraction of CH_4 than gas formed biotically which has higher fraction of C_2-C_5 components (Rooney et al. 1995).

1.2 Shale Gas Formations in Western Canada

Historically natural gas was primarily extracted from high permeability, over pressured reservoirs, considered conventional gas recovery. These reserves are located in formations below impermeable cap rocks where natural gas has migrated through the permeable formation and has accumulated over geological time. These hydrocarbons are easily recovered at low cost, once the cap rock is penetrated the formation pressure forces hydrocarbon out of the well for easy recovery. These reserves however are becoming depleted.

Attention is shifting towards unconventional natural gas, this includes shale gas and tight gas, coal bed methane and gas hydrates. However only shale gas will be discussed in this work (Wang et al. 2014). Shale gas formations are found throughout the world but predominantly in North America (Smead & Pickering 2008; Gadonniex et al. 2010). Over the past decade the amount of natural gas being produced in Canada has decreased, but a larger portion of that has been produced by unconventional extraction techniques (U.S. Energy Information Administration 2015a). The most active areas in western Canada are from shales in the Horn River Basin and the Montney (Rivard et al. 2013). These formations were laid down over geological time with many fine grained sediments in marine basins and contain a high organic content (Rokosh et al. 2009). The formations were predominantly formed in fine grained sandstone, clay, siltstone, quartz and dolomite with permeabilities of less than 1 mD (Figure 1-2) (Rivard et al. 2013; Wang et al. 2014; Slatt 2011). High salt concentrations have accumulated in shale formations primarily due to evaporative concentration of solutes during formation (Slatt

2011; Blauch et al. 2009). The high organic content in these formations would also have accumulated when sediments were laid down. The organic matter is heterogeneous throughout formations and can be as high as 23 wt %, but in the Montney rarely exceed 4.7% (Riediger et al. 1990). Higher organic content is an indicator of higher production potential but this depends on many other factors (Rokosh et al. 2009). Due to the formation process of shale gas formations have a network of discontinuous pores that can be less than 0.005 μm in size, smaller than all microbes (Davis et al. 2012). This low permeability does not allow hydrocarbons even as small as methane (CH_4) to flow through the formation, making the formation both the source rock where the gas is formed and cap rock, which acts as a barrier preventing the gas from migrating in the subsurface. This is different from conventional reservoirs that can have permeabilities as high as 100 mD (1 μm) allowing oil to migrate beneath an impermeable cap rock (King 2012).

One of the largest shale gas formations is the Montney formation in northern British Columbia and parts of Alberta. It is 4000 m deep and is up to 300 m thick with a permeability between 0.001 and 0.05 mD (Figure 1-1) (Norton et al. 2010). This formation is predominantly comprised of siltstone and shale which were laid down during the early Triassic period (Riediger 1990). The Montney has a TOC that ranges from less than 1% (w/w) to 5% (w/w) in some regions (Riediger 1990). The organic matter found is predominantly of marine origin whose composition is well suited for oil and gas formation (Slatt 2011; Baveye et al. 1998; Tissot & Welte 1984). This formed over geological time as the Montney was buried and raised to a temperature exceeding 400°C, the formation has since been forced upwards causing it to cool to 70-110°C (Rokosh et al.

2009). Its peak temperature exceeded the temperatures required to thermogenically produce natural gas and far exceeded the temperature that life had been observed to exist at (80ºC) in the subsurface (Wilhelms et al. 2001). Natural gas production from the Montney formation alone is expected to reach $100\times10^6 m^3/d$ by 2016, up from $54\times10^6 m^3/d$ in 2013 (National Energy Board 2014).

1.3 Hydraulic Fracturing

The largest problem with shale gas production is the low permeability of the formation preventing hydrocarbon from easily flowing to the well bore so that it can be recovered (King 2012). In order to sufficiently increase the permeability, hydraulic fracturing is employed. While hydraulic fracturing is currently a hot media topic it is not a new technology. The first commercially fractured well was drilled in the United States in the 1950s, with the first horizontal wells being introduced by the 1970s (Curtis 2002; Hubbert & Willis 1972; King 2012). It was not until 2005 that hydraulic fracturing first came to Canada, since then more than 1000 wells have been hydraulically fractured in the Montney (Rivard et al. 2013).

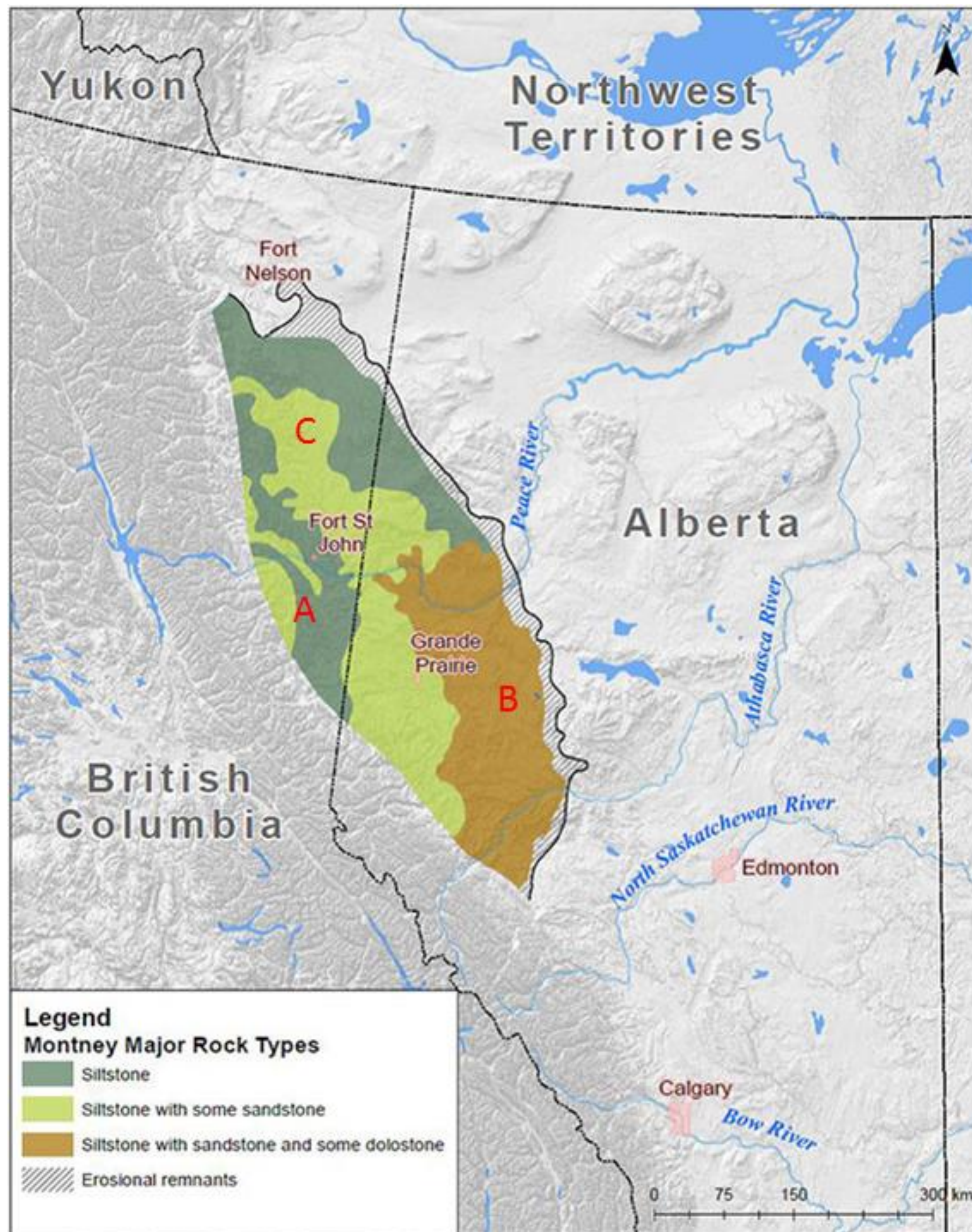


Figure 1-1: Map of western Canada's Montney formation. Sampling sites are highlighted: A) Groundbirch, B) Fox Creek and C) Kobes. Taken from National Energy Board, 2013.

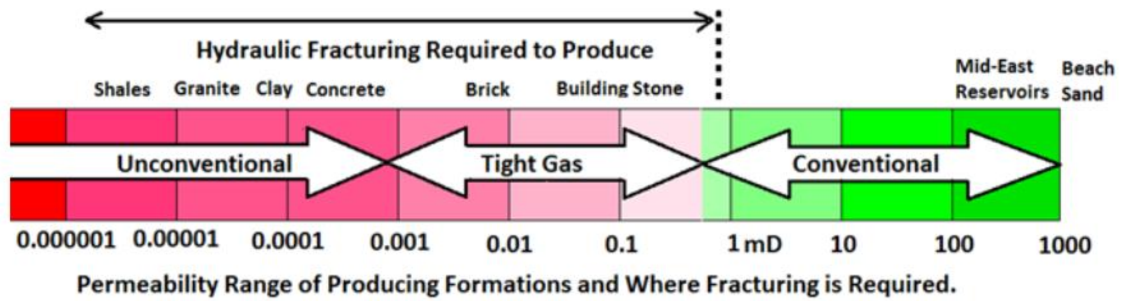


Figure 1-2: Permeability of different types of oil and gas reservoirs. Formations that are less than 1 mD require hydraulic fracturing for hydrocarbon recovery at an economical rate. Taken from King 2012.

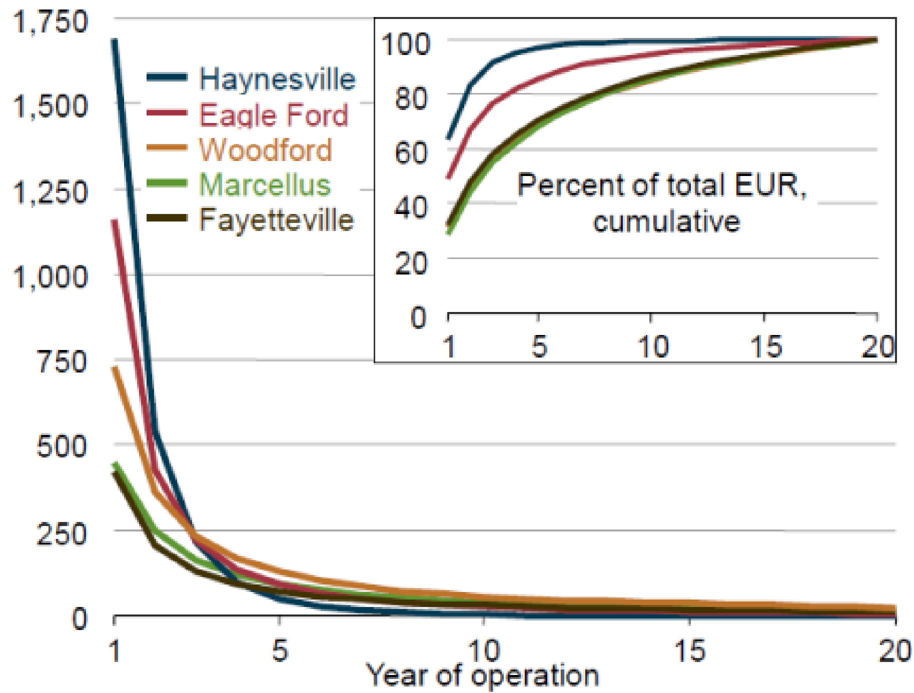


Figure 1-3: Average production profiles of a shale gas wells in the United States over 20 years. Measured in million cubic feet per year. Estimated Ultimate Recovery (EUR) shown top right. Taken from U.S. Energy Information Administration 2014.

For hydraulic fracturing large volumes of chemically augmented water are injected into subsurface formations at high pressure. The high pressure of the fluid causes the rock to "fracture" creating a dendritic network expanding from the wellbore (Hubbert & Willis 1972; Arthur, Bohm & Coughlin 2008; King 2012). The fractures spread away from the well bore and penetrate deep into the formation reaching lengths of up to 600 m (King 2012). These fractures connect the discontinuous pores of the formation, creating channels for hydrocarbons to flow through. Multiple fractures are made sequentially over the length of the horizontal section of the well beginning at the furthest end of the well and proceeding towards the wellbore (Figure 1-4). Once a fracture stage has been completed it is sealed off from the rest of the well and the next stage is fractured. This proceeds until all stages have been fractured. The fracturing process can take up to a week to complete and then the plugs separating the stages are removed. Once fracturing is completed, the well company disassembles and removes all of its surface equipment and a well service company moves in to initiate the flowback stage, this can take up to a week. For flowback, pressure in the well is relieved at the surface and fluids and gas are allowed to flow back to the surface these fluids are called "flowback" or "produced" fluids (Arthur, Bohm & Coughlin 2008). When the pressure from the formation is relieved fluid and gas flow back to the surface along the same path that the fluids were injected (King 2012). Production from hydraulically fractured wells is initially very high but rapidly diminishes over the first year of production (Figure 1-3). A lower production rate is maintained for the remainder of the well's life (Conti et al. 2012). A single well can be fractured multiple times to increase its production rate (King 2010).

1.3.1 Technological Advances

It has not been until recently that hydraulic fracturing has seen widespread use. This trend was made possible by the refinement of existing techniques as well as the development of new ones. Drilling horizontal wells over 2 km throughout a formation and fracturing multiple times along this length has decreased the number of wells that needs to be drilled and increased the amount of gas that can be recovered (Figure 1-4) (Arthur, Bohm & Layne 2008; King & Leonard 2011). Advances in the formulation of fracturing fluid have increased the efficiency of fracturing to create a larger fracture network. Real time monitoring techniques allow operators to make quick adjustments to the fracturing procedure further increasing the efficiency of the fracturing process (King & Leonard 2011).

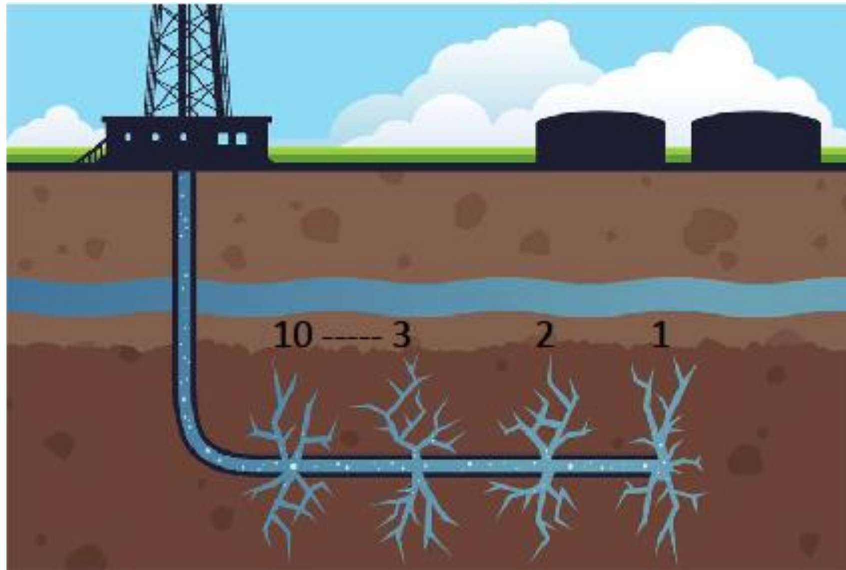


Figure 1-4: Schematic of horizontally drilled well for hydraulic fracturing. Stages are fractured sequentially in the order labelled. Taken from Barnell & Williams 2012.

1.3.2 Fracturing Fluid

Fracturing fluid is one of the key components required for hydraulic fracturing. The fluid is composed predominantly of water (~90%) and sand (~10%), with a volume of up to 20 000 m^3 being used for a single well (Gregory et al. 2011; Arthur, Bohm & Layne 2008). The water required can be obtained from a variety of sources such as deep water aquifers, ponds, lakes and treated waste water effluent. There is increased pressure to use reuse hydraulic fracturing flowback fluid. In some cases it can account for up to 20% of the water used. The problem is that it complicates the chemistry required to formulate the fracturing fluid and maintain its effectiveness (Gregory et al. 2011; Kaufman et al. 2008). The water required is transported to the well location either by pipe or truck and is stored in open ponds (Wood et al. 2011). These source waters contain many environmental microbes. Biocides can be added to diminish their numbers as many operators believe them to interfere with the fracturing processes. Many operators try to eliminate microbes completely, but even with the most rigorous biocide treatments some microbes will survive (Struchtemeyer & Elshahed 2012). Biocides can be added to waters while they are being stored in wait for fracturing as well as at the blender when fluids are being mixed. Immediately prior to fracturing the source water is mixed with sand and a chemical concoction making the fracturing fluid (Arthur, Bohm & Layne 2008; Struchtemeyer & Elshahed 2012). Microbes can enter the system during any point of the operation, including proppant, chemicals, drilling mud, contact with equipment used and the formation itself (Struchtemeyer et al. 2011). Specific chemicals used to formulate the fracturing fluid are specifically tailored to individual wells and can be modified during fracturing, using many of the same chemicals in varying quantities (Table 1-1). The fluid

formulation is determined by the formation characteristics. Tracers can also be introduced into the fracturing fluid for a variety of analyses of the well performance and to better understand its downhole characteristics. The fluids for each fractured stage have a unique tracer added that can be quantified when it returns to the surface.

There are two major types of systems used for hydraulic fracturing: 1) slick water systems, that use low viscosity fluids and high pressure and 2) gel based systems that use high viscosity cross linked polymers to suspend proppant, allowing much lower velocities to be employed. For gel based systems the viscosity of the initial fluid is higher than 100 cP. Guar gum, a galactomannan, is typically employed for this purpose. Once fractures have been created a high viscosity is no longer required and impedes flow back of water and gas to the surface. Polymer breakers are employed, enzymes for wells less than 50ºC and oxidizing agents are used for hotter wells to break polymer backbones into smaller molecules, reducing the viscosity (Reddy & Tammishetti 2004). Guar gum also represents a good carbon and energy source that can readily be metabolized by many microbes (Balascio et al. 1980; Tomlin et al. 1986). Some microbes are capable of degrading guar gum, breaking the polymer gives smaller carbon products that can be metabolized by a larger variety of microbes.

As the water used to formulate fracturing fluid can come from a variety of sources the composition of microbial communities in it can vary. Waters taken from above ground environmental locations are dominated by *Alphaproteobacteria* (Mohan et al. 2014). Microbes from flowback waters are added when these waters are mixed with fresh waters to make fracturing fluid. The use of flowback waters can thus potentially introduce a seed community that is more adapted to the conditions of the hydraulic

fracturing process and down hole conditions (Struchtemeyer & Elshahed 2012). This includes halophilic organisms including *Halobacillus* and *Halomonas* (Cluff et al. 2014).

1.3.3 Flowback Fluids

1.3.3.1 Chemical characteristics

Flowback fluids return to the surface after the pressure of a fractured well is relieved. This includes the fracturing fluid and any formation waters. The amount of fracturing fluid that can be recovered is between 20 and 70% (King 2012). The remainder of the fluid is lost due to adsorption in the formation, leak off to parts of the formation that become closed off, and capillary forces. The fluids that return to the surface more closely resemble conditions in the formation that they were subjected to (Table 1-2) (Cluff et al. 2014). The amount of dissolved salts in the flowback waters makes the waters difficult to remediate, generating an incentive to reuse the fluids for fracturing. However the chemical composition of the fluids makes this difficult, although modifications in fracturing fluid chemistry are being made to compensate for the high total dissolved solutes (TDS). After fracturing, these flowback waters are stored in open, above ground ponds where they can remain for months. Eventually flowback waters make their way to waste water treatment plants, injection into deep aquifers or reuse for fracturing (Gregory et al. 2011). If tracers were used they can be quantified in the flowback waters to determine which fracture stage the fluid belongs to.

Table 1-1: Roles of common chemicals added to hydraulic fracturing fluids

Additive	Compound	Role
Salt	KCl	Stabilizes clay to prevent it from swelling allowing fractures to initiate
Acid	HCl	Dissolves rock to help initiate fractures
Complexing agent	Na Citrate	Binds iron to prevent it from precipitating
Biocides	Gluteraldehyde, quaternary amine, chlorine dioxide, THPS	Reduces microbial populations
Friction reducer	Polyacrylamide, Hydrotreated petroleum distillate	Decreases turbulent flow reducing friction
Gelling agent	Guar Gum	Used as a viscosifying agent to suspend sand
Surfactant	Isopropanol	Reduces surface tension
Oxygen scavenger	Ammonium bisulfite	Removes oxygen, helps control corrosion and microbial activity
Corrosion inhibitor	N,N-dimethyl formamide	Reduces corrosion
Scale inhibitor	Ethylene Glycol	Prevents the formation of scale
Breakers	Ammonium persulfate, Enzymes	Form radicals to break bonds of polymers; breaks polymers enzymatically
Proppant	Silica, quartz	Maintain fractures
Foaming agents	Dinitrogen, carbon dioxide	Reduces clay swelling, reduces amount of water required
Tracer	Undisclosed	Tracks fluid returning to surface from different stages for analysis

(Arthur, Bohm & Layne 2008; King & Leonard 2011; Stringfellow et al. 2014; Gregory et al. 2011; Kaufman et al. 2008; Struchtemeyer & Elshahed 2012; Mohan et al. 2014; Arthur & Langhus 2008)

Table 1-2: General chemical characteristics of flowback fluids collected from several sources

Compound	Amount (mg/L)
Sodium (Na^{+})	11 100-26 500
Chloride (Cl^{-})	14 742- 95 100
Barium (Ba^{2+})	9.5-418
Iron Fe(tot)	0.1-64.9
Potassium (K^{+})	161-420
Magnesium (Mg^{2+})	44-1 188
Strontium (Sr^{2+})	707-1493
Bromine (Br^{-})	344-615
Iodine (I^{-})	4.4-10.3
Sulfate (SO_3^{2-})	15.1-236
Phosphate (PO_4^{3-})	ND
Nitrate (NO_3^{-})	ND
pH	6.5-7.2
H_2S	ND
TOC	968-4523

(Murali Mohan et al. 2013; Struchtemeyer & Elshahed 2012; Strong et al. 2013; Wuchter et al. 2013; Barbot et al. 2013; Cluff et al. 2014)
TOC-Total Organic Content
ND – Not Detected

1.3.3.2 Microbial characteristics

Despite the harsh conditions and best efforts of many operators, microbes are able to survive the harsh conditions they are subject to during the hydraulic fracturing process. The factors working against microbes are great; shear force, temperature, osmotic pressure and pH, are all rapidly changing. Observed microbial communities collected from shale gas operations in the United States, show microbial communities in flowback fluids are distinct from those of the initial fracturing fluid (Struchtemeyer & Elshahed 2012; Cluff et al. 2014). Diversity and abundance have been shown to decrease in flowback samples, which contain increasingly large fractions of facultative and anaerobic microbes (Struchtemeyer & Elshahed 2012; Cluff et al. 2014). Many of these belong to the *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Gammaproteobacteria* such as *Marinobacter* and *Pseudomonas* (Struchtemeyer & Elshahed 2012; Wuchter et al. 2013; Cluff et al. 2014; Davis et al. 2012). Sequencing has also indicated that mesophilic, halophilic and fermentative organisms are in higher abundance (Wuchter et al. 2013; Mohan et al. 2014). Obligate anaerobes are also observed from the genera *Dethiosulfovibrio*, *Thermovirga*, *Thermotoga,* and *Petrotoga* (Struchtemeyer & Elshahed 2012). An increase in microbes capable of spore formation is also observed in flowback from thermogenic wells (Struchtemeyer & Elshahed 2012). Despite the anaerobic conditions in shale reservoirs, sequences associated with aerobic species are continually found in flowback fluids (Cluff et al. 2014; Davis et al. 2012). The genera *Petrotoga* and *Thermovirga,* have also been detected in many wells, and could contribute to souring (Struchtemeyer & Elshahed 2012). However many of these studies did not test the viability of the organisms observed. *Archaea* are typically present in flowback fluids as

well but comprise less than 1% of the total the community (Wuchter et al. 2013; Cluff et al. 2014). After extended periods of time microbial communities in flowback waters are predominated by anaerobic halophiles (Cluff et al. 2014).

1.3.4 Impoundment Ponds

The sheer volume of flowback water produced in the initial stages of well flowback are too great to treat or dispose of on the fly. Instead they are stored in large, man-made aboveground ponds where they can undergo a variety of treatments to control unwanted biogeochemical changes such as precipitation and the dissolution of metals (Murali Mohan et al. 2013). Storage ponds are lined with plastic barriers to prevent fluids from leaking in to the environment. These ponds are exposed to the atmosphere as well as rain water adding to the existing community in the flowback waters. Microbial communities develop to reflect the conditions that are present in the storage ponds and differ from the fracturing fluid and immediate flowback water (Murali Mohan et al. 2013). While microbial communities in the impoundment ponds differ depending on the treatment that they receive they reflect communities that are commonly found in saline brines, and other oil and gas wells. This includes many halotolerant species, which can withstand but do not require high salt concentrations, belonging to the *Alpha*- and *Gammaproteobacteria* and *Firmicutes* (Murali Mohan et al. 2013).

1.4 Issues Associated with Microbes

Microbes have been observed to negatively impact hydraulic fracturing operations in many of the same ways that they interfere with conventional hydrocarbon extraction

methods (Gieg et al. 2011; Kraan et al. 2012). These includes biofouling, fracture plugging, microbially influenced corrosion (MIC) and souring (Struchtemeyer et al. 2011). Souring is the production of hydrogen sulfide (H_2S) from sulfur-containing anions such as sulfate but also thiosulfate and sulfite by microbial metabolism (Voordouw 2011; Muyzer & Stams 2008). The formation of hydrogen sulfide is undesirable because it is corrosive and toxic, creating a health hazard for workers. Removing sulfide also incurs significant cost, reducing the value of the produced gas (Prabha & Rathish 2014). Many of the organisms associated with souring belong to the class *Deltaproteobacteria* but they also exist in the phylum *Firmicutes*, and include some *Archaea.* Most of these are classified as sulfate reducing bacteria (SRB) (Barton & Fauque 2009). Many of these are well known types of strict anaerobes that use lactate as an electron donor and sulfate as an electron acceptor (Muyzer & Stams 2008).

Microbially influenced corrosion (MIC) is another concern that continually plagues oil fields and is of concern for hydraulic fracturing. This extends beyond the ability of microbes to produce corrosive sulfur compounds such as elemental sulfur (S^0) and hydrogen sulfide. MIC also includes the ability of microbes to create environments where they can use electrons from steel pipes causing Fe^0 to be converted to Fe(II) (Okoro et al. 2014; Little et al. 2000). The overall effect is corrosion product that decreases infrastructure integrity.

In conventional waterflood systems the concept of generating biomass to increase oil production is currently under investigation as a source of microbial enhanced oil recovery (Voordouw 2011). However in hydraulic fracturing plugging is undesirable and can reduce production rates. The created fractures have a small volume and a high

surface area for microbes to adhere to. Even formations that were sterilised during gas formation are at risk of plugging due to microbial communities that have been introduced into the formation (Moore et al. 2010). These fractures do not require much biomass to block gas flow (Ross et al. 2001; Heimovaara et al. 2010). The harsh conditions pressure microbes to form complex biofilm communities to better withstand the down hole environment. Formation of biofilms reduces the size of fractures or blocks them entirely, impeding gas flow (Heimovaara et al. 2010). Plugging can also arise from inorganic sources, many as salts containing sulfate are highly insoluble and their precipitates can accumulate in the pore network resulting in reduced gas flow and eventually, plugging.

1.5 Community Analysis

The ability to determine the types of microbes present helps in understanding their effect on shale gas systems that have been hydraulically fractured. There are many culture based methods that test for certain types of microbes such as SRB, acid producing bacteria (APB), iron reducing bacteria (IRB) and general heterotrophic bacteria (GHB). These tests only capture microbes that grow under the conditions being tested which is less than 1% of microbes. In order to capture the whole community and obtain a more representative analysis of microbial communities present, culture independent methods of community analysis need to be employed (Tringe & Hugenholtz 2008).

Next generation sequencing allows for the identification of thousands of microbes in parallel by sequencing a region of their 16 rDNA gene and comparing it to a database of known sequences. This allows for all microbes in a community to be identified without the use of culturing (Metzker 2010). Sequencing however gives no indication as to which

microbes are active. The information obtained still gives invaluable insight into the nature of the microbial community and indicates issues that may arise based on the presumed activity of the microbes identified (Langille et al. 2013).

Microbial communities can be identified using variable regions of the 16S rDNA. This gene is selected because it is present throughout *Archaea* and *Bacteria*, and contains conserved and variable regions within the gene. A variety of PCR primers have been created for the conserved regions that flank the variable regions which are used for phylogenetic identification (Baker et al. 2003; Wang et al. 2014). Data generated from high throughput sequencing requires bioinformatics tools for analysis to handle the large amounts of data that are generated. There are many bioinformatics tools available for processing and analysing the data such as MOTHER and QIIME (Schloss et al. 2009; Caporaso et al. 2010). The relative abundances of species within samples are then generated for further analysis.

Bioinformatics applied to the results obtained gives further insight into individual communities as well as between communities. Alpha diversity is for analyses within an individual sample such as the Chao1, Simpson index and Shannon index. The Shannon Index and Simpson Index measure sample diversity while the Chao1 index measures species richness (Pinto & Raskin 2012; Shannon 1948; Chao 2015). The Simpson Index uses the natural logarithm of species proportions while the Shannon Index uses the squared reciprocal. This results in the weight of abundant species being reduced for the Shannon index and the weight of rare species being reduced for the Simpson Index. Different samples can also be compared using a Bray Curtis analysis that groups more similar samples with one another (Bray & Curtis 1957).

Culture based methods can be complementary to next generation high throughput sequencing because they give insight into microbial community activity (Orphan et al. 2000). Culture based tests can only identify a portion of microbes as many are not currently culturable. They identify how many viable cells there are that can perform a certain metabolic process under a given set of laboratory conditions.

1.6 Microbial Metabolism

1.6.1 Acid Producing Bacteria (APB)

APB have diverse metabolic capabilities allowing them to utilize a variety of organic substrates as carbon and energy sources. Many organisms are capable of metabolizing large polysaccharide. Under aerobic conditions these polymers are capable of being entirely oxidized to H_2O and CO_2. Under anaerobic conditions other electron acceptors such as nitrate, sulfate and iron(III) are used to generate smaller amounts of energy than in the presence of oxygen (Chidthaisong & Conrad 2000). When a microbe does not possess an oxidative pathway or the required electron acceptors are absent, fermentation is employed. Carbon containing substrates are not broken down entirely to CO_2 and H_2O; instead smaller organic acids are produced such as lactate, acetate, propionate and butyrate. The latter three are classified as volatile fatty acids (VFA). Acetate can be further metabolized to CH_4 and CO_2 by methanogens. The production of volatile fatty acids, which are weak acids, can result in a decrease in pH. In biofilms the pH can be 2 units lower than that of the surrounding environment. A low pH has implications for microbially influenced corrosion (MIC) and in some circumstances can

account for greater MIC by APB than by SRB, which are historically considered the culprits for such activity (Gu 2014).

The drop in pH has an effect on microbial communities as many of them have pH optima that restrict growth to a narrow pH range; changes in pH would result in a shift in the microbial community. Microbes employ a variety of different mechanisms to tolerate a decreased pH, these include but are not limited to the use of proton pumps to increase intracellular pH, production of alkaline agents such as ammonia, which raises intracellular pH, alter gene expression to produce heat shock and chaperone proteins to refold denatured proteins and form biofilms to create a microenvironment with a less acidic pH (Cotter & Hill 2003; Russell 1997; Russell 1991).

1.6.2 Sulfate Reducing Bacteria (SRB)

Sulfur is one of the most abundant elements on the planet and is essential for life, not only as a building block for many proteins but also in the form of sulfur or sulfate as an electron acceptor for many organisms. The majority of sulfate reducing microbes are *Deltaproteobacteria* (Barton & Fauque 2009). The diversity of SRB is also reflected in the variety of electron donors they are capable of utilizing. Electron acceptors for SRB are VFAs, oil organics, carbon monoxide, aromatics and alcohols (Muyzer & Stams 2008; Barton & Fauque 2009). The majority of studies concerning SRB have been done in the presence of lactate. However it is known that SRB also use thiosulfate and sulfite (Muyzer & Stams 2008).

Hydrogen sulfide (H_2S or HS^-) produced by SRB has many negative effects on industry and human health (Gu 2014; Gieg et al. 2011). H_2S is also closely associated

with MIC, creating a corrosion cell that strips metal of electrons that can be utilized by microbes.

1.6.3 Iron-Reducing Bacteria (IRB)

The activity of iron reducing bacteria plays an important role in global iron cycling. Once thought to be an entirely chemical process, it is now widely known to involve microbes that are capable of utilizing iron as an electron acceptor. Iron is the fourth most abundant element on Earth making it believed that it was one of the first electron acceptors used by microorganisms (Weber et al. 2006). It is an important electron acceptor in reduced anoxic sediments providing greater free energy than sulfate, but less than nitrate. In reduced anoxic sediments iron(III) forms a variety of insoluble oxy- and oxyhydroxide compounds, of which the biological availability varies but in general is very low (House 1983). Due to their insoluble nature it is not easy for microbes to utilize iron oxy- and oxyhydroxides as terminal electron acceptors. A couple of methods have been proposed as to how IRB transfer electrons to iron(III) oxides. One method is through physical contact either directly or with nanowires, to transfer electrons directly to iron across the membrane using conductive pili (Reguera et al. 2005). Extracellular electron shuttles have also been proposed (Weber et al. 2006). The use of chelating agents has also been proposed which are employed by the cell to solubilize iron(III), making it more available in solution. The solubilisation of iron also releases other compounds that bind with it in its insoluble form, these include trace metals and phosphorous that are normally limiting nutrients in environmental systems that restrict growth (Lovley & Phillips 1988).

The produced Fe(II) is much more soluble than its oxidized counterpart Fe(III). When Fe(II) is exposed to oxygen it becomes oxidised resulting in the formation of insoluble iron species. The reactions consume OH^- ions from solution dropping the pH of the solution (Millero et al. 1987). Changes in pH and redox potential have adverse effects on the state of iron (Carroll 1958). At pH 7, Fe(III) is predominantly insoluble, as pH decreases its solubility increases and below a pH of 4, Fe(III) is soluble (Weber et al. 2006).

The microbial use of iron(III) is closely linked to the use of nitrate. Many of the organisms that are capable of reducing iron also have nitrate reductases. Growth of these organisms in the presence of both electron acceptors can be much higher than on either one alone. The presence of nitrate has also been shown to stimulate the use of iron after nitrate has been depleted (Sorensen 1982).

1.6.4 Halophiles

Halophiles are found in many regions arround the globe and have developed a variety of mechanisms to cope with the osmotic stress imposed by high salt concentrations. Organisms that can survive at high salt concentrations employ one of two known strategies. 1) They produce organic molecules in high abundance as osmoregulators allowing them to exclude salt but maintain a high internal osmotic pressure (Oren 1999). In general this technique is employed by organisms that do not live in energy constrained environments such as aerobes and phototrophs but to the high amount of energy required to produce these molecules. 2) They maintain a high internal salinity to match that of the environment (Oren 1999). This mechanism is favoured

among organisms with more stringent energy constraints as it does not require the production of costly osmotic solutes. It does however require adaptations at a molecular level to maintain enzymatic activity. Organisms that use this method have evolved to have proteins with a higher portion of acidic residues making them less susceptible to denaturation at high salt concentrations (Paul et al. 2008).

The physiological adaptations required to survive in saline environments are energy intensive for microbes and restricts the types of metabolism that organism can use in saline environments. Organisms that rely on phototrophic processes have been observed in salinities up to saturation (≈ 6 M NaCl) while methanogens are rarely seen in salt concentrations over 1 M NaCl (Oren 1999). Fermenting organisms are also commonly observed in anaerobic saline environments. Even though fermentation reactions produce small amounts of energy, microbes that utilize minimal energy to maintain an osmotic balance are able to survive. While halophiles have been observed in environments with pHs as low as 6.0, they generally prefer circumneutral or alkaline pHs (Oren 2011), this is due to more free energy being released at neutral or alkaline pHs for many reactions (Oren 2011).

1.6.5 Thermophiles

Thermophiles are microorganisms that are capable of growing at elevated temperatures that exceed 45°C and can be as high as 120°C. Thermophilic microbes have specially adapted proteins that prevent them from becoming denatured and maintain optimal enzymatic activity as well as a different membrane lipid composition to prevent leakage of compounds across the cellular membrane (Russell & Fukunaga 1990).

Organisms are able to withstand higher temperatures by changing their gene expression and using chaperone proteins that refold denatured proteins back to their functional conformation. It takes time for microbes to alter their gene expression to cope with their changing environment and is also energy intensive. Some thermotolerant organisms can withstand higher temperatures but have limited growth potential. Obligate thermophiles and hyperthermophiles require high temperatures for survival and growth and have specially adapted proteins that maintain their functionality at high temperatures with reduced activity at lower temperatures (Berezovsky & Shakhnovich 2005).

Chapter Two: Research Objectives

Microbial communities present throughout hydraulically fractured tight and shale gas formations are poorly characterized, especially with respect to wells drilled in the Montney formation which resides entirely in Canada. The goal of this research is to better characterize the types of microbes that are present throughout hydraulically fractured systems and determine their influence if any on the hydraulic fracturing process. To understand their impact the following will be studied:

I. Evaluate the physical and chemical properties of water used for hydraulic fracturing; source, flowback, impoundment and separator waters.

II. Determine the microbial community composition in the above mentioned waters and relate this to conditions present in the waters.

III. Evaluate the metabolic capabilities of the above mentioned communities with respect to known microbial activity associated oil fields.

Chapter Three: Methods

3.1 Sampling Sites

3.1.1 Groundbirch

The sampling sites were located at the Groundbirch field owned by Shell in Northern British Columbia as well as the town of Fox Creek in Alberta (Figure 1-1: A). Samples were collected from impoundment ponds that flowback water was being stored in as well as two separators that were receiving fluids from different wells. One of them received flowback fluids water from a well that was amended with polyacrylamide (PAM) and the other, with guar gum during the fracturing process. A sample from the fresh water storage pond that supplied water for formulating fracturing fluid was also obtained. The location of the sites sampled and their position in the water flow are depicted in Figure 3-1. The operations at Groundbirch are considered a model for hydraulic fracturing for their ability to treat and recycle much of the flowback waters that are produced from the well (Shell Canada 2015).

Shell also supplied additional samples that were collected from Fox Creek located in western Alberta (Figure 1-1: B). Samples were collected as previously described. Source water was received from two locations, combined for fracturing (no sample) and then used for hydraulic fracturing (Table 3-1: 8, 9). A sample of flowback water was also received (Table 3-1: 10).

3.1.2 Kobes

Samples collected from the Altera field near Kobes in northeast British Columbia were supplied by Suncor (Figure 1-1: C). A sample of source water from a local was collected and used to formulate the fracturing fluid. After the fracturing process was completed and fluid began to flow back, samples were collected from the well head as previously described approximately every 4 hours (Table 3-2). This offered a unique opportunity to analyse flowback water communities from the early stages of flowback and observe how they change during this time frame. This is contrary to most studies that have used samples collected at relatively few time points that range from months to years after a well has been in production.

3.2 Sample Collection

Samples were collected on site by field personnel at the well head or from water storage ponds. 1 L sterile Nalgene bottles were filled to the brim, closed and sealed with tape to prevent contact with the atmosphere. Upon arrival at the University of Calgary samples were catalogued and stored in Coy anaerobic hoods with an atmosphere of 90% N_2 – 10% CO_2 (N_2CO_2). Aliquots of the samples were removed from the hood for benchtop analysis.

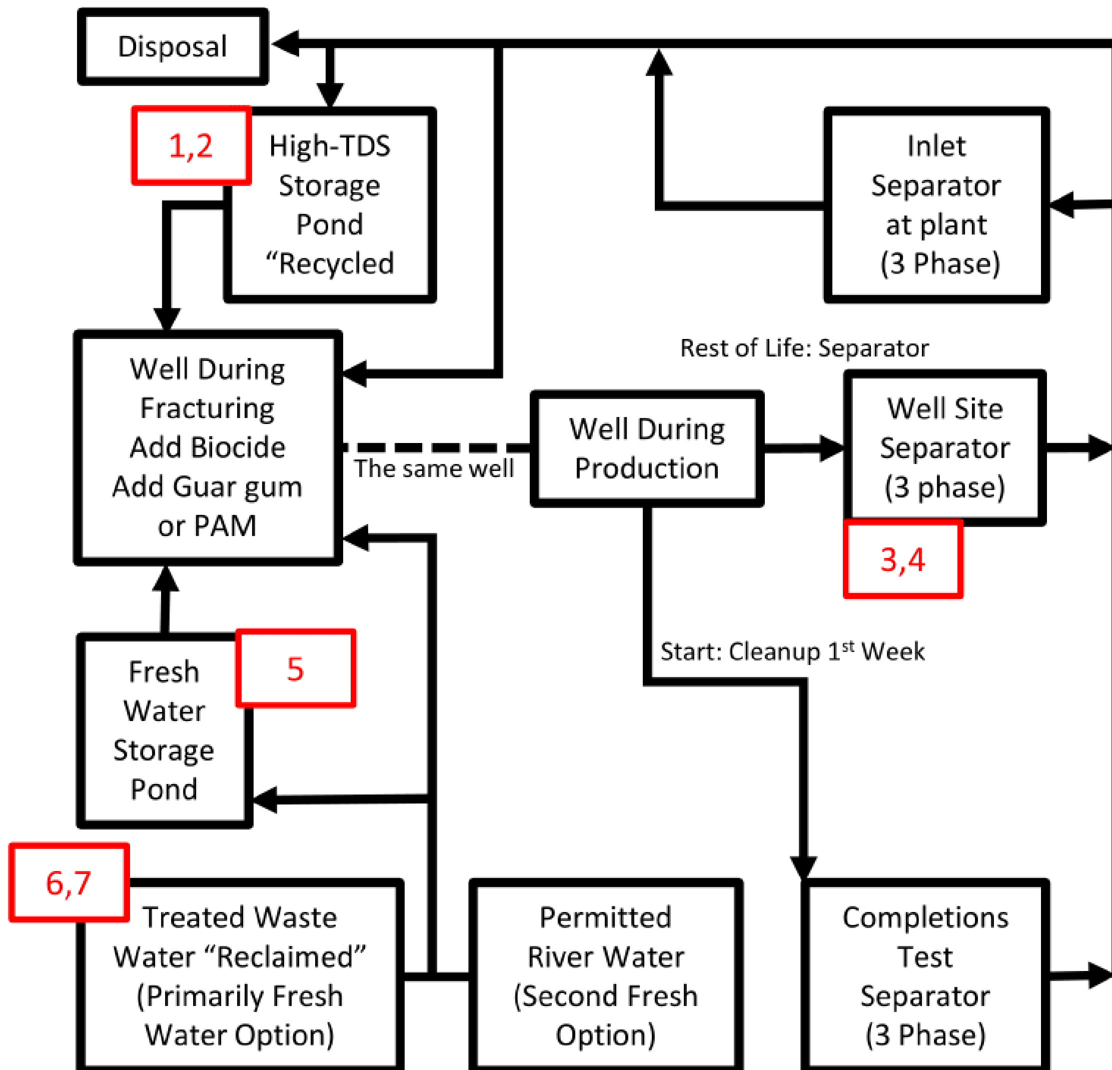


Figure 3-1: Water system flow diagram for shale gas operations at Groundbirch. Numbers in red boxes are indicative of sample points in this study (Table 3-1). Solid lines represent water flow via pipe, but there is inevitably some trucking to complement.

Table 3-1: Samples received from the Groundbirch field (1-5), two treated waste water samples (6,7) and samples from Fox Creek (8-10)

	Sample Name	Designation	#	Date sampled	Date Received
Groundbirch	Water Storage Pond 1	WSP1	1	17 June 2013	18 June 2013
	Water Storage Pond 2	WSP2	2	17 June 2013	18 June 2013
	Well Separator 1 (PAM)	WS1_PAM	3	17 June 2013	18 June 2013
	Well Separator 2 (Guar Gum)	WS2_GG	4	17 June 2013	18 June 2013
	Fresh Water Storage Pond 1	FWSP1	5	17 June 2013	18 June 2013
	AB1 Field Source (Treated Waste Water)	AB1_WS	6	25 June 2013	27 June 2013
	AB2 Field Source (Treated Waste Water)	AB2_WS	7	4 July 2013	5 July 2013
Fox	FC Source water stream	SH_SWS	8	25 March 2014	26 March 2014
	FC Source water lagoon	SH_SWL	9	25 March 2014	26 March 2014
	FC Flowback Water	SH_FBW	10	25 March 2014	26 March 2014

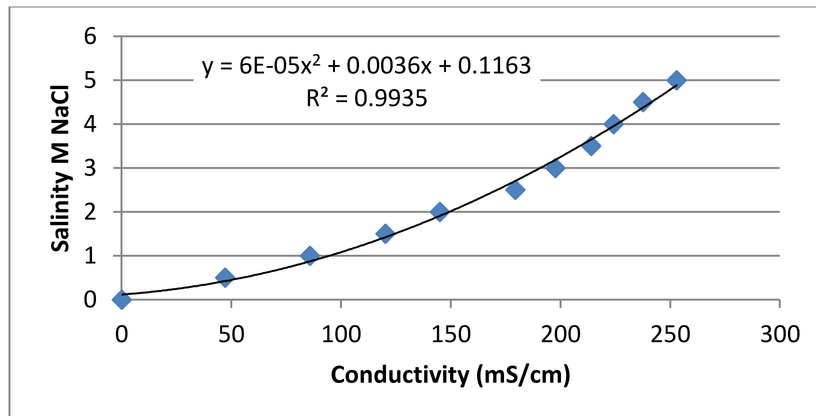
Table 3-2: Samples received from the Altera field near Kobes BC

Sample #	Date collected	Time collected	Time after flowback (h)	Cumulative flowback volume (m^3)
Source	2014-02-08	--	N/A	N/A
1	2013-11-08	16:00:00	0	13.71
2	2013-11-08	20:00:00	4	32.88
3	2013-11-09	0:00:00	8	53.66
4	2013-11-09	4:00:00	12	80.29
5	2013-11-09	8:00:00	16	107.74
6	2013-11-09	12:00:00	20	131.99
7	2013-11-09	16:00:00	24	158.49
8	2013-11-09	20:00:00	28	186.24
9	2013-11-10	0:00:00	32	210.88
10	2013-11-10	4:00:00	36	236.11
11	2013-11-10	8:00:00	40	259.99
12	2013-11-10	12:00:00	44	282.43
13	2013-11-10	16:00:00	48	318.59
14	2013-11-10	20:00:00	52	355.55
15	2013-11-11	0:00:00	56	391.27
16	2013-11-11	4:00:00	60	436.12
17	2013-11-11	8:00:00	64	482.51
18	2013-11-11	12:00:00	68	529.73
19	2013-11-16	23:30:00	199.5	591.58
20	2013-11-17	3:30:00	203.5	630.32
21	2013-11-17	7:30:00	207.5	680.14
22	2013-11-17	11:30:00	211.5	726.16
23	2013-11-17	15:30:00	215.5	776.22
24	2013-11-17	19:30:00	219.5	828.92
25	2013-11-17	23:30:00	223.5	880.24
26	2013-11-18	3:30:00	227.5	919.43
27	2013-11-18	7:30:00	231.5	971.69
28	2013-11-18	11:30:00	235.5	1028.43
29	2013-11-18	17:30:00	241.5	1096.34
30	2013-11-18	19:30:00	243.5	1160.51
31	2013-11-18	23:30:00	247.5	1231.29
32	2013-11-19	3:30:00	251.5	1278.17
33	2013-11-19	7:30:00	255.5	1341.63
34	2013-11-19	11:30:00	259.5	1403.92
35	2013-11-19	15:30:00	263.5	1466.75

Sample #	Date collected	Time collected	Time after flowback (h)	Cumulative flowback volume (m^3)
36	2013-11-19	19:30:00	267.5	1522.25
37	2013-11-19	23:30:00	271.5	1573.20
38	2013-11-20	3:30:00	275.5	1634.35
39	2013-11-20	7:30:00	279.5	1694.73
40	2013-11-20	11:30:00	283.5	1754.76
41	2013-11-20	15:30:00	287.5	1366.80
42	2013-11-20	19:30:00	291.5	1874.30
43	2013-11-20	23:30:00	295.5	1947.86
44	2013-11-21	3:30:00	299.5	1986.46
45	2013-11-21	7:30:00	303.5	2049.19
46	2013-11-21	11:30:00	307.5	2103.37
47	2013-11-21	15:30:00	311.5	2148.37
48	2013-11-21	14:30:00	310.5	2220.44
49	2013-11-21	23:30:00	319.5	2284.38
50	2013-11-22	3:30:00	323.5	2313.28
51	2013-11-22	7:30:00	327.5	2373.67
52	2013-11-22	11:30:00	331.5	2425.79
53	2013-11-22	15:30:00	335.5	2034.80
54	2013-11-22	19:30:00	339.5	2536.80
55	2013-11-22	23:30:00	343.5	2572.11
56	2013-11-23	3:30:00	347.5	2620.40
57	2013-11-23	7:30:00	351.5	2667.42
58	2013-11-23	11:30:00	355.5	2713.03
59	2013-11-23	15:30:00	359.5	2761.37
60	2013-11-23	19:30:00	363.5	2812.21
61	2013-11-23	23:30:00	367.5	2861.32
62	2013-11-24	3:30:00	371.5	2906.80
63	2013-11-24	7:30:00	375.5	2957.02
64	2013-11-24	11:30:00	379.5	3005.90
65	2013-11-24	15:30:00	383.5	3059.69
66	2013-11-24	19:30:00	387.5	3091.81
67	2013-11-24	23:30:00	391.5	3140.58
68	2013-11-25	3:30:00	395.5	3183.62
69	2013-11-25	7:30:00	399.5	3228.59
70	2013-11-25	11:30:00	403.5	3266.78
71	2013-11-25	15:30:00	407.5	3304.67
72	2013-11-25	19:30:00	411.5	3337.46
73	2013-11-25	23:30:00	415.5	3374.19

Sample #	Date collected	Time collected	Time after flowback (h)	Cumulative flowback volume (m^3)
74	2013-11-26	3:30:00	419.5	3410.46
75	2013-11-26	7:30:00	423.5	3677.40
76	2013-11-26	11:30:00	427.5	3477.60
77	2013-11-26	15:30:00	431.5	3511.80
78	2013-11-26	19:30:00	435.5	3548.54
79	2013-11-26	23:30:00	439.5	3579.37
80	2013-11-27	3:30:00	443.5	3617.34
81	2013-11-27	7:30:00	447.5	3653.35
82	2013-11-27	11:30:00	451.5	3684.57
83	2013-11-27	15:30:00	455.5	3714.65
84	2013-11-27	19:30:00	459.5	3734.50
85	2013-11-27	23:30:00	463.5	3775.84
86	2013-11-28	0:33:00	464.55	4037.26
87	2013-11-28	7:30:00	471.5	3841.01
88	2013-11-28	11:30:00	475.5	3873.47
89	2013-11-28	15:30:00	479.5	3901.11
90	2013-11-28	19:30:00	483.5	3916.19
91	2013-11-29	0:00:00	488	3945.07
92	2013-11-29	3:30:00	491.5	3970.40
93	2013-11-29	7:30:00	495.5	3997.58

Figure 3-2: NaCl Standard curve and line of best fit for converting conductivity to salinity of NaCl Meq.



3.3 Tracer Data

Tracer data was provided by Suncor for the Kobes well. The horizontal section of the well had 10 different stages that were fractured sequentially starting with stage 1 that was furthest from the well as in Figure 1-4. A unique tracer was added to the fracturing fluid for each of the fractured stages. The concentration of each tracer was measured in 18 of the flowback samples and used to calculate the amount of fluid from each stage in the flowback fluid at the well head which was provided by Suncor.

3.4 Wet Chemistry

3.4.1 Salinity

Salinity was determined using an Accumet Research AR20 pH/Conductivity probe using NaCl standards. A parabolic calibration curve can be seen in Figure 3-2 using the equation provided.

3.4.2 pH

Upon arrival at the lab the pH was measured using an Orion pH meter (model 370). Samples were removed from the hood in 50 ml Falcon tubes and were only open to the atmosphere during the measuring process. pH measurements were also provided by Suncor (Appendix 4).

3.4.3 HPLC

3.4.3.1 Organic Acids

Organic acids (lactate, acetate, propionate and butyrate) were determined using an HPLC equipped with a UV detector (Waters 2487 Detector) and an organic acids column (Alltech, 250 x 4.6 mm) eluted with 25 mM KH_2PO_4 buffer at pH 2.5. To verify the presence of butyrate, samples were amended with butyrate and peaks observed.

3.4.3.2 Anions

Sulfate was analyzed by ion chromatography using a conductivity detector (Waters 423) and an IC-PAK anion column with borate/gluconate buffer at a flow rate of 2 mL/min (4 x 150 mm, Waters). Nitrite and nitrate were also detected using ion chromatography using a UV detector (Gilson, USA).

3.4.4 Ammonium

Ammonium concentration were assayed in triplicate with the indophenol blue method (Koroleff 1969). MQ water (pH 3) (950 µl) was mixed with 30 µl of sample, 100 µl reagent A (2.9 % w/v phenol in water, 0.03 g/L nitroprusside), 100 µl reagent B (2 % w/v sodium hydroxide, 1 % v/v hypochlorite) and then thoroughly vortexed. After 1 h incubation in the dark, the absorbance was read at 635 nm using a UV-1800 Shimadzu spectrophotometer.

3.4.5 Iron

The total amount of dissolved iron for corrosion experiments was measured using the ferrozine assay (Stookey 1970). A volume of 50 µl of sample were added to 450 µl hydroxylamine solution. After 15 min of continuous stirring 100 µl of the reaction was added to 900 µl of ferrozine reagent every minute for 10 min. After another 15 min incubation the absorbance was read at 562 nm with a UV-1800 Shimadzu spectrophotometer.

3.4.6 Sulfide

The concentration of dissolved sulfide was measured using the methylene blue method in triplicate (Trueper & Schlegel 1964). A volume of 30 µl of sample was immediately added to 200 µl zinc acetate (100 mM in 4% w/v glacial acetic acid solution). A volume of 600 µl MQ water and 20 µl diamine reagent (20% w/v sulfuric acid, 14.6 mM N,N-dimethyl-p-phenylenediamine) was added and vortexed, 10 µl Fe-Alum solution (207 mM $NH_4Fe(SO_4)_2 \cdot 12\ H_2O$ in 2% sulfuric acid) was then added. After 15 min incubation, absorbance was measured at 670 nm.

This assay was modified for use in a 96 well plate to be read on an EnSpire PerkinElmer Multimode plate reader. In wells on a 96 well plate 3 µl of sample were added to 80 µl zinc acetate. 20 µl diamine reagent and 1 µl Fe-Alum solution was added. The reactions incubated for 15 min in the dark on a rotary shaker. Absorbance was read at 670 nm.

3.4.7 Guar Gum and Glucose

The concentration of guar gum in the samples was determined by a colorometric assay according to Baird and Smith (1989). In this assay the galactosyl-residues of residual guar gum are measured. Sample (500 µl) was incubated with 2 ml of a galactose oxidase reagent (SIGMA #G7400) for 1 h at 37°C. The enzyme oxidizes C6 of galactosyl-groups in guar gum to form peroxide. Horseradish peroxidase (SIGMA #77332) converts peroxide in the presence of O-toluidine (SIGMA #T8533) into a compound that can be detected at 450 nm. The reaction was terminated by adding 0.5 ml of 0.3 M EDTA and the A_{450} was then measured. The experiment was performed both with and without galactose oxidase. This corrects for components in the sample that may give a reading at 450 nm.

To determine the total amount of sugar which can be attributed to guar gum the phenol sulfuric acid method was employed (Bradley et al. 1989; Shi et al. 2012). A volume of 50 µl of 5% phenol in water was added to 50 µl of sample in a 96 well plate. A volume of 200 µl of 98 % w/v sulfuric acid was pipetted directly in to the liquid and incubated in a 90°C water bath for 15 min, then absorbance was read at 490 nm on an EnSpire PerkinElmer Multimode plate reader. This method also works for glucose determination.

3.4.8 Polyacrylamide

The polyacrylamide (PAM) concentration in the samples can be determined by measuring the amount of ammonium released from the polymer following acid hydrolysis at high temperature. Ammonium is then quantified with the indophenol blue

method, as indicated in 3.4.4. However, because very high ammonium concentrations were found in flowback waters prior to acid hydrolysis, this method could not be used. N-bromination, an alternate method for PAM determination was adapted from Lu and Wu (2001). A volume of 2 ml of sample was mixed with 1 ml of 1 M HOAC-NaOAC (pH 3.5) buffer and 1 ml of 0.04 M bromine in water solution. The mixture was incubated for 40 min at room temperature in the dark. A volume of 1 ml of 0.08 M sodium formate solution added and then incubated for 5 min. The final reagent, 1 ml of 0.25% w/v starch-0.03 M CdI_2 was added and incubated for 5 min. The resulting colorimetric change was measured at a wavelength of 570 nm.

3.4.9 Viscosity

The viscosity of the samples was determined using a Brookfield viscometer DV-II+ Pro with a CPA-42Z spindle with a 1 ml volume. The temperature was maintained constant at 25ºC using a Brookfield TC-550 circulating water bath. Data was collected and analyzed using Rheocal software v3.3.

3.5 Corrosion

The ability of the flowback waters to corrode carbon steel beads (55 mg ea.) was evaluated using anoxic (85% N_2:5% H_2:10% CO_2) $N_2H_2CO_2$ conditions while shaking. 20 carbon steel beads were incubated in a 50 ml serum bottle sealed with a butyl rubber stopper. Incubations had 25 ml of flowback water for 14 days. The amount of corrosion was determined through 2 methods; weight loss and dissolved iron. Dissolved iron was determined by solubilising iron products in 1 N HCl acid and calculating the amount of

dissolved iron over time using the ferrozine assay (Stookey 1970). To evaluate the contribution of microbes to corrosion 25 ml of unfiltered sample were compared to twice filtered sample using 0.22 µm Millipore filters. Flowback samples were incubated with 20 carbon steel beads for 16 days with an 85% N_2:5% H_2:10% CO_2 head space. Corrosion was determined using the methods mentioned above for samples K_8, 20, 28, 36, 48, 68 h.

3.6 Microbial Community Analysis

3.6.1 Microbial Counts

3.6.1.1 Aerobes

Microbes capable of aerobic respiration were evaluated using 1:10 serial dilutions of 1 ml sample in 9 ml tryptone-yeast extract (TY broth). Inoculations were incubated at 30°C for 2 weeks under aerobic conditions. Growth was indicated by turbidity in the culture tubes.

3.6.1.2 APB and SRB Counts

APB and SRB counts were evaluated using a single serial dilutions in sealed vials. A 1 ml inoculum was used with 9 ml media and serially diluted to 10^8. Vials were incubated at 30°C. SRB were tested for on Postgate B medium; the presence of SRB was indicated by the formation of a black iron sulfide precipitate. APB were tested for on

Phenol Red Dextrose Medium (ZPRA-5, DALYNN Biologicals). The presence of APB was indicated by the production of acid which turned the media from red to yellow.

3.6.2 Most Probable Number (MPN) of APB and SRB

APB and SRB numbers were evaluated using the MPN method performed in 48 well plates. A 0.1 ml inoculum was used with 0.9 ml media and serially diluted to 10^8 in triplicate. After inoculation of plates, they were sealed with a titer-top membrane and incubated at 32°C in an anaerobic hood with $N_2CO_2H_2$ atmosphere. SRB were tested for on Postgate B medium, the presence of SRB was indicated by the formation of black iron sulfide precipitate. APB were tested for on Phenol Red Dextrose Media (ZPRA-5, DALYNN Biologicals). The presence of APB was indicated by the production of acid which turned the media from red to yellow. The MPNs of each type of microbe were determined by comparing the positive pattern of the three wells with an MPN table (Oblinger & Koburger 1975).

3.7 DNA Isolation

Each sample (250ml) was centrifuged at 22000 x g for 15 min in a Beckman Coulter Avanti® J-E centrifuge at 4°C. Genomic DNA (gDNA) was extracted from the pellet by lysing intact cells by bead-beating in a FastPrep Instrument for 60 seconds at a speed setting of 6.0 in 2 mL Lysing Matrix E tubes (MP BIO, USA). DNA was isolated using a Qiagen soil fast prep kit (QiagenQIAQuick Kit, Qiagen). The amount of gDNA in each of the samples was determined by using a QubitFluorometer (Invitrogen), using a Quant-iT™ dsDNA HS Assay Kit (Invitrogen).

3.8 454 Pyrosequencing

16S rRNA genes were amplified from isolated DNA using a two-step PCR amplification (An et al. 2013). The first PCR was performed with 16S rRNA gene primers 926F (AAACTYAAAKGAATTGRCGG) and 1392R (ACGGGCGGTGTGTRC) or Epsilon specific primers, which capture a greater portion of *Epsilonproteobacteria*, 926F (AAACTYAAAKGAATWGRCGG) and 1392R (ACGGGCGGTGWGTRC) when specified. PCR was performed using *Taq* Plus Master Mix (Lambda Biotech) for 5 minutes at 95^0C, followed by 25 cycles, each consisting of 30 seconds at 95^0C, 30 seconds at 55^0C and 1 minute at 72^0C, followed by a final step at 72^0C for 5 minutes. The presence of a PCR product was confirmed by running 2 µl on a 1.5% agarose gel. The second PCR used the first PCR product as the DNA template and consisted of 10 cycles. This PCR was done using FLX titanium amplicon primers 454T_RA_X and 454T_FwB. These primers have the sequences for 16S primers 926Fw and 1392R as their 3'-ends. Primer 454T_RA_X has a 25 nucleotide A-adaptor (CGTATCGCCTCCCTCGCGCCATCAG) and a 10 nucleotide multiplex identifier barcode sequence X. Primer 454T_FwB has a 25 nucleotide B-adaptor sequence (CTATGCGCCTTGCCAGCCCGCTCAG). The resulting PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). The final 16S PCR amplicons were sent for pyrosequencing (16S profiling) to the Genome Quebec and McGill University Innovation Centre, Montreal, Quebec. Pyrosequencing was performed with a Genome Sequencer FLX Instrument, using a GS FLX Titanium Series Kit XLR70 (Roche Diagnostics Corporation). The raw data obtained from the sequencing centre was

analyzed using Phoenix 2, a 16S rRNA gene data analysis pipeline (Soh et al. 2013). Sequences with low quality scores (less than 99.8 % accuracy) as well as sequences that were less than 200 bp were removed. Sequences that represented less than 0.01 % of total reads, which include singletons and other rare OTUs, were also removed as they could have arisen from sequencing errors and could skew the computed results (Soh et al. 2013).The remaining sequences were clustered into operational taxonomic units (OTUs) at 3% distance. The OTUs were assigned to taxa by comparing their sequences to the SILVA Small Subunit rRNA Database release 108 using Phoenix 2 (Soh et al. 2013). The Bray-Curtis Index was used as a measure of dissimilarity between communities.

On select samples the eukaryotic primers for the V4 region, TAReuk454FWD1 (5'-CCAGCA(G/C)C(C/T)GCGGTAATTCC-3', *S. cerevisiae* position 565-584) and TAReukREV3(ACTTTCGTTCTTGAT(C/T)(A/G)A-3', *S. cerevisiae* position 964-981) were used for PCR to test for a eukaryotic community (Stoeck et al. 2010). Thermocycling conditions were 95°C for 3 min, 15 cycles of 95°C for 30s 53°C for 45s and 72°C for 60s. Another 20 cycles of 95°C for 30s, 48°C for 45s and 72°C for 60s. A final step of 72°C for 5 min was performed before the presence of a product was checked as described above. No PCR product could be obtained and further study was not performed.

3.9 Halophilic Microbe Isolation

3.9.1 Pure culture isolation

Sample K_336 h (100 µl) was spread on to a plate of TY agar with 2 M NaCl with a sterile glass rod and incubated at 30ºC for 2 weeks. Individual colonies were transferred on sterile toothpicks to 1 ml of 2 M NaCl TY broth. After two weeks incubation at 30ºC, they were streaked on 2 M NaCl plates with an inoculation loop to ensure that the culture was pure. DNA was extracted using the aforementioned method.

3.9.2 Sanger Sequencing of Isolates

16S rRNA gene amplicons were obtained by PCR using primers 27F (AGAGTTTGATCCTGGCTCAG) and 1525R (AAGGAGGTGATCCAGCC) from the aforementioned cultures. PCR was performed using 25 µl Qiagen HS, 0.2 µl each forward and reverse primer, 0.5 µl $MgCl_2$, 2 µl genomic DNA for a final volume of 50 µl with H_2O. PCR was for 5 minutes at 95^{0}C, followed by 25 cycles, each consisting of 30 seconds at 95^{0}C, 30 seconds at 55^{0}C and 1 minute at 72^{0}C, followed by a final step at 72^{0}C for 5 minutes. Reactions were performed in duplicate and pooled during purification with QIAquick PCR Purification Kit (Qiagen). The PCR product was verified to be the correct size of approximately 1500 bp and to be primer dimer free by running them on a 0.7 % agarose gel. The gel was stained with 2 µl SYBR Safe (Invitrogen). A 10 µl volume of the purified product (50-100 ng) along with 2 µl of either the forward or reverse primer were mixed in 0.2 ml PCR tubes and sent to the University

of Calgary's Core DNA services for Sanger sequencing using an Applied Biosystems 3730XL 96 capillary sequencer.

3.9.3 Strain Identification

Forward and reverse sequences were aligned using SeqTrace and the chromatogram was manually checked for quality (Stucky 2012). The generated consensus sequence was analyzed through NCBI blastn (Madden & Morgulis 2007). The species with the highest similarity were selected and sequences for type strains were obtained from NCBI. Sequences were aligned using Clustal X2.1's multiple alignment mode and treed with the neighbour-joining methos with 1000 bootstraps (Larkin et al. 2007). Editing of the tree was performed in TreeGraph 2 (Stover & Muller 2010).

3.10 Media

3.10.1 Anaerobic media

All anaerobic media was dispensed using a Widdel flask with an N_2CO_2 head space. A 45 ml volume was dispensed into serum bottles under flowing a N_2CO_2 gas. Bottles were sealed with butyl rubber stoppers and crimped aluminum seals.

Modified CSBK medium was used as per Table 3-3Table 3-4. Salt concentrations were increased to better simulate the physicochemical conditions that the samples were exposed to in their native environment.

3.10.2 Aerobic medium

Variations of nutrient growth media were used to culture and identify the presence of aerobes (Table 3-5). Liquid broth was used to culture microbes before plating. NaCl concentration was also increased to better simulate the conditions that were native to the sample.

3.10.3 Stock solutions

Stock solutions of electron acceptors, donors and other essential nutrients were prepared and added to incubations as the experiment required (Table 3-6, Table 3-7, Table 3-8, Table 3-9).

3.11 Metabolic Experiments

3.11.1 Anaerobic Sampling Technique

Liquid samples were periodically taken from serum bottles for analysis. Samples were removed by flushing a sterile syringe with N_2CO_2 gas 3 times and filling it with the amount of anaerobic gas equivalent to the amount of sample to be withdrawn. The anaerobic gas was injected through the butyl rubber stopper, the serum bottle was agitated and the samples were drawn into a syringe. Liquid samples were stored at -20°C until further processing.

Table 3-3: CSBK minimal salts medium

Component added	Amount per L
NaCl	1.5 g[1,2]
KH_2PO_4	0.05 g
NH_4Cl	0.32 g
$CaCl \cdot 2H_2O$	0.21 g
$MgCl \cdot 6H_2O$	0.54 g
KCl	0.1 g
After Autoclaving	
$NaHCO_3$ (1 M)	30 ml
[3]Trace elements	1 ml
[4]Selenate/tungstate	1 ml
Na_2S	1 ml
Adjust pH to 7	Drops of HCl
5Electron donor/acceptor	Added as needed

[1]High salinity CSBK had 116.88 g NaCl
[2]Low Salinity CSBK had 29.22 g NaCl
[3]Trace elements (Table 3-7)
[4] Selenate/Tungstate solution (Table 3-8)
[5]Electron acceptors and donors added from stock solutions

Table 3-4: Low salinity defined CSBK-0.5M-0.1GG medium with guar gum

Component added	Amount per L
NaCl	29.22 g
KH_2PO_4	0.05 g
NH_4Cl	0.32 g
$CaCl_2 \cdot 2H_2O$	0.21 g
$MgCl_2 \cdot 6H_2O$	0.54 g
KCl	0.1 g
Guar Gum	1 g
After Autoclaving	
$NaHCO_3$ (1 M)	30 ml
[1]Trace elements	1 ml
[2]Selenate/tungstate	1 ml
Adjust pH to 7	Drops of HCl
[3]Electron donor/acceptor	Added as needed

[1] Trace element stock solution (Table 3-7)
[2] Selenate/Tungstate solution (Table 3-8)
[3] Electron acceptors added from stock solutions

Table 3-5: TY medum

Component added	Amount per L
Tryptone	10 g
Yeast Extract	5 g
[1]NaCl	8 g
[2]Agar	15 g
pH 7	Drops NaCl or NaOH

[1]High salinity medum (2 M) had 116.88 g or low salinity (0.5 M) 29.22 g NaCl
[2]Agar was omitted if medum was used as a broth

Table 3-6: Stock solutions of electron acceptors and donors in water

Solution	**Concentration [M]**
Sodium acetate	2.5
Sodium sulfate	2.5
Sodium nitrate	5.0
[1]Glucose	1.8

Solutions were autoclaved
[1]Filter sterilized through a 0.22 μm filter as opposed to autoclaving

Table 3-7: Trace Elements

Compound added	Amount per L
Na_2EDTA	5.20 g
$FeSO_4 \cdot 7H_2O$	2.10 g
H_3BO_3	30.0 mg
$MnCl_2 \cdot 4H_2O$	100 mg
$CoCl_2 \cdot 6H_2O$	190 mg
$NiCl_2 \cdot 6H_2O$	24.0 mg
$CuSO_4 \cdot 5H_2O$	3.00 mg
$ZnCl_2$	68.0 mg
$NaMoO_4 \cdot H_2O$	36.30 mg
2M HCl	Adjust to pH 6.5

Table 3-8: Selenate/tungstate solution

Component added	Amount per L
NaOH	400 mg
$Na_2Se_2O_3 \cdot 5H_2O$ (Na Selenite)	6.00 mg
$Na_2WO_4 \cdot H_2O$ (Na Tungstate)	8.00 mg

Table 3-9: Volatile Fatty Acids (3 M stock)

Component added	Amount per L
Sodium acetate	123 g
Sodium propionate	144.1 g
Sodium butyrate	165.1 g

Bubbled with N_2CO_2 anaerobic gas and then autoclaved

3.11.2 Metabolism Incubations

The experimental setup for microbial incubations is in Table 3-10.

3.12 Hydrocarbon Toxicity

Liquid hydrocarbon was collected from all Kobes samples where an organic layer accumulated on the surface of samples when they were left standing undisturbed. The organic layers that formed in the 1L storage bottles in the anaerobic hood were decanted into a 50 ml Falcon tubes and centrifuged. The volume of the organic layer was measured and the organic layers were pooled together. The composition of the organic layer was determined using Agilent Technologies 7890A GC-MS system and analysed using Chemstation.

Some hydrocarbons have been shown to have toxic effects on some microbes (Sikkema & Jan 1995). Incubations were performed using CSBK media (Table 3-3). Kobes sample K_12 was used, due to its large biomass and species diversity. Sample (1 ml) was incubated at 30°C in the presence and absence of varying amounts of hydrocarbon and glucose as depicted in Table 3-11. A volume of 19 ml CSBK (Table 3-3) in 50 ml serum bottles sealed with butyl rubber stoppers and filled with an N_2CO_2 headspace.

Table 3-10: List of incubations established to assess the ability of microbial communities collected from source and flowback waters associated with hydraulic fracturing to use different electron acceptors and donors under different temperature and saline conditions.

#	Experiment	Samples	Electron acceptor	Electron Donor	Media	Temp (°C)
1	Acetate utilization under nitrate reducing conditions	K_S, K_0, K_8, K_16, K_64, K_68 SH_SWS, SH_SWL, SH_FBW	Nitrate (16 mM)	Acetate (10 mM)	CSBK-0.5 (Table 3-3)	30
2	Acetate utilization under sulfate reducing conditions	K_S, K_0, K_4, K_8, K_364, K_380, K_496 SH_SWS, SH_SWL, SH_FBW	Sulfate (10 mM)	Acetate (10 mM)	CSBK (Table 3-3)	30
3	Guar Gum Viscosity	WSP1, WSP2, WS1_PAM, WS2_GG, FWS1	N/A	Guar Gum 0.2 % [w/v]	CSBK (Table 3-3)	30
4	Guar Gum utilization under sulfate reducing conditions	K_8, K_32, K_56	Sulfate (10 mM)	Guar Gum 0.1 % [w/v]	CSBK-0.5 NaCl (Table 3-4)	30
5	Guar Gum utilization under Nitrate reducing conditions	K_8, K_32, K_56	Nitrate (10 mM)	Guar Gum 0.1 % [w/v]	CSBK-0.5 NaCl (Table 3-4)	30
6	Guar Gum utilization under fermentation conditions	K_8, 32, 56 h	N/A	Guar Gum 0.1 % [w/v]	CSBK-0.5 NaCl (Table 3-4)	30
7	Thermophilic activity under low salt conditions	K_40, K_204 SH_SWS, SH_SWL	Sulfate (10 mM), Nitrate (10 mM)	Glucose (5 mM), VFA (5 mM)	CSBK-0.5 (Table 3-3)	60

N/A, not applicable

Table 3-11: Design of hydrocarbon toxicity experiment.

Condition	Addition		
	Sample (1 ml)	Glucose (22 µl)	Hydrocarbon (µl)
SG	+	+	0
SG100	+	+	100
SG200	+	+	200
SG300	+	+	300
SG400	+	+	400
S	+	-	0
G	-	+	0
200	-	-	200
S200	+	-	200
G200	-	+	200

+, indicates that either 1 ml of sample or 22 µl of 1.8 M glucose was added
-, indicates that either sample or glucose was not added

Chapter Four: Results-Analysis of Groundbirch shale gas field operations

4.1 Introduction

The Groundbirch field has been in production for at least 5 years. The field is considered a model system by the company as the majority of flowback waters produced are recycled with very little water being sent for disposal (Shell Canada 2015). The field itself is comprised predominantly of siltstone, sandstone, and shale located between 2000 and 3000 m in the upper and middle regions of the montney formation which were formed during the Triassic era and is currently around 78°C at the bottom of the wellbore (Adam 2012). Production from these wells is predominantly natural gas along with some liquids.

Waters that were used for hydraulic fracturing at this location were drawn from a variety of sources, including effluent from treated waste water plants and high TDS storage ponds (Figure 3-1). Both slick water and gelled fracturing systems were employed for fracturing these wells. Produced waters and gas were sent to the separators. The water phase was sent for storage and eventualy treatment while the hydrocarbons were sent to a refinery for processing and eventually for sale.

Questions with respect to the role of microbes in shale gas operations at Groundbirch are whether these (i) contribute to degradation of polymers in fracturing fluid prior to injection, (ii) contribute to well plugging, limiting gas flow or (iii) contribute to souring and corrosion due to SRB activity.

4.2 Analysis of Groundbirch Samples

4.2.1 Samples Received

Samples 1 through 5 were collected from the Groundbirch tight gas field's water system (Table 4-1). Samples 1 and 2 (WSP1, WSP2) were from high total dissolved solids (TDS) storage ponds used for recycling flowback fluid after they are used for fracturing and produced water from the wells throughout the field. These samples contained particulate matter, but were otherwise mostly clear. Sample 3 (WS1_PAM) was a separator water sample taken from a separator for a well that was hydraulically fractured using a PAM-based friction reducer in 2010; the sample was clear. Sample 4 (WS2_GG) was taken from a different separator fed from wells that used a guar gum based gelling agent during stimulation in 2012. This sample had a red color with lots of particulate matter that settled out upon standing, the remainder of the sample maintained an opaque appearance which could be removed with centrifugation. Sample 6 (AB1_WS) and sample 7 (AB2_WS) were treated waste water effluent, captured from two different treatment plants, that are intended to be used for hydraulic fracturing. The samples were both clear in appearance.

Samples 1 to 5 represent the fracturing operation as follows: FWSP1/fresh water is used to make fracturing fluid. After this has been used for fracturing, it is sent to separators to separate gas (WS1_PAM, WS2_GG). These wells have been in production for years, and it should be noted that the separators receive fluids from multiple wells. The water is then stored in ponds (WSP1, WSP2) until it is disposed of or reused. The treated waste waters (AB1_WS, AB2_WS) may be used for making hydraulic fracturing

fluid in future fracturing operations. A water chemistry report was received for AB1_WS (Appendix 1).

Table 4-1 Physicochemical description of samples from Groundbirch

Designation/ Sample type	Code	#	pH	NaCl Meq	Viscosity (cP) at 25°C and 380/s	PAM % [w/v]	Guar Gum % [w/v]
High TDS Water Storage Pond 1	WSP1	1	5.1	1.984	1.52	0.18	0.00
High TDS Water Storage Pond 2	WSP2	2	4.1	1.569	1.27	0.04	0.00
Well Separator 1 (PAM)	WS1_PAM	3	4.7	1.646	1.40	0.21	0.02
Well Separator 2 (Guar Gum)	WS2_GG	4	5.1	1.984	1.66	0.23	0.00
Fresh Water Storage Pond 1	FWSP1	5	6.9	0.010	0.69	0.00	0.00
AB1 Field Water Source (Treated Waste Water)	AB1_WS	6	7.8	0.010	0.85	0.00	0.00
AB2 Field Water Source (Treated Waste Water)	AB2_WS	7	7.8	0.002	0.73	0.00	0.00

Table 4-2: Chemical analysis of samples. Nitrite was analysed but not found.

Designation/Sample type	Nitrate [mM]	Sulfate [mM]	Lactate [mM]	Acetate [mM]	Propionate [mM]	Butyrate [mM]	Ammonium [mM]
WSP1	0.01	0.32	0.00	0.73	0.55	2.96	70.40
WSP2	0.00	0.35	0.00	0.72	0.55	0.39	70.00
WS1_PAM	0.00	0.39	0.00	1.06	0.58	1.92	54.50
WS2_GG	0.11	0.42	4.59	0.81	0.56	0.35	62.50
FWSP1	1.22	1.62	0.00	0.41	0.55	0.35	2.20
AB1_WS	0.01	0.31	0.00	0.40	0.55	0.35	0.15
AB2_WS	0.09	0.37	0.00	0.40	0.55	0.35	0.49

4.2.2 Physicochemical properties

Samples FWSP1/fresh water, AB1_WS/treated waste water and AB2_WS /treated waste water had a low salinity (equivalent to 0.002-0.010 M NaCl) and a near-neutral pH (6.9-7.8) (Table 4-1). Following hydraulic fracturing, flowback waters from the separators and ponds had a pH of 4-5 and a salinity of 1.6-2.0 Meq NaCl. In sample WS2_GG /separator, guar gum was no longer found. There was a marginal amount of guar gum found in sample WS1_PAM/Separator despite not having been exposed to the PAM. All flowback waters appeared to have PAM (Table 4-1), even those from WS2_GG/separator which was not exposed to PAM, but these separators receive fluids from multiple wells whose histories are unknown. The viscosities of the flowback fluids were only marginally higher than those of the fresh and treated waste waters. There were only minor differences in sample viscosity. Samples 1 to 4 had viscosities of 1-2 cP while samples 5 to 7 had viscosities below 1 cP.

HPLC analysis indicated that sulfate, which can be reduced to H_2S by SRB, was at its highest concentration (Table 4-2: 1.6 mM) in FWSP1/fresh water. The concentrations of sulfate in the flowback water samples were lower (0.3-0.4 mM), as were those in the treated waste waters. In contrast, the concentrations of acetate were lowest in the fresh and treated waste waters and highest in the flowback waters. All flowback waters had high concentrations of ammonium (55-70 mM), compared to the fresh and treated waste waters (0.2-2.0 mM). The treated waste water samples were similar in composition to the FWSP1/fresh water sample with the exception of elevated nitrate, sulfate and ammonium levels.

4.2.3 Microbial counts

The flow back samples when tested at low salinity (WS1_PAM and WS2_GG) had low counts of APB (10^2/ml) and no SRB at 30°C (Table 4-3). No SRB were found at 60°C in these samples. The fresh and treated waste water samples (FWSP1, AB1_WS and AB2_WS) had 10^4/ml, 10^5/ml and 10^4/ml of APB and 10^1/ml, 0/ml and 10^2/ml of SRB at 30°C. The AB1_WS treated waste water sample also had an APB count of 10^1/ml at 60°C. This was the only sample to show activity at 60°C.

4.2.4 Microbial community Analysis

16S rRNA genes were amplified for pyrosequencing using both sets of primers. These are referred to as AB1_WS and AB1_WS_E and as AB2_WS and AB2_WS_E where E denotes the use of Epsilon primers that were designed to capture a larger portion of *Epsilonproteobacteria*. A total of 884 to 3285 pyrosequecing reads were obtained for each of the samples following quality control. The Phoenix 2 bioinformatics pipeline placed these reads into groups with 97-100% identity. These groups are referred to as operational taxonomic units (OTUs), which can be regarded as representing distinct microbes at the genus level. All of the samples received from the Groundbirch field were sequenced successfully, and the raw data was uploaded to the Phoenix 2 bioinformatics pipeline (Soh et al. 2013). The number of operational taxonomic units (OTUs) that Phoenix 2 was able to classify sequences from 101 (AB1_WS_E) to 243 (FWSP1) OTUs per sample based on the 0.03 cut off (Table 4-5). The estimated number of OTUs based on the Chao1 index ranged from 203 (AB1_WS_E) to 510 (WS1_PAM). The two high

TDS storage pond samples had similar Chao1 indexes while estimates from the two separator samples WS1_PAM and WS2_GG had very different estimates, 510 and 261 respectively despite both being flowback samples.

All of the samples had a high Shannon index of 2.78-3.98 despite having varying amounts of OTUs (Table 4-5). Diversity as indicated by the Shannon index, was highest in sample FWSP1 (3.98), but was not drastically reduced in the high TDS storage pond samples WSP1, WSP2 (3.81, 3.82). Diversity was more reduced in the separator samples WS1_PAM and WS2_GG (3.53, 3.58). Diversity was lowest in the treated waste water samples AB1_WS and AB2_WS (3.38, 2.78), this was also true when the Epsilon specific probes were used (2.89, 3.75).

An informative way to compare community compositions for the different samples is through the dendrogram shown in Figure 4-1. The shorter or longer distance between branches representing different communities indicates whether these are more or less similar, respectively. Figure 4-1 indicates that the compositions obtained for the same sample with different primers were similar for AB2_WS (Figure 4-1: AB2_WS and AB2_WS_E) but less for (Figure 4-1: AB1_WS and AB1_WS_E). Analysis of duplicate samples also gave very similar community compositions (Figure 4-1: WSP1 and WSP2).

Table 4-3: Microbial counts (#/ml) of APB and SRB measured at both 30 and 60ºC

#	Designation/Sample type	Code	APB (30°C)	APB (60°C)	SRB (30°C)	SRB (60°C)
1	Water Storage Pond 1	WSP1	10^2	0	0	0
2	Water Storage Pond 2	WSP2	10^2	0	0	0
3	Well Separator 1 (PAM)	WS1_PAM	10^2	0	0	0
4	Well Separator 2 (Guar Gum)	WS2_GG	0	0	0	0
5	Fresh Water Storage Pond 1	FWSP1	10^4	0	10^1	0
6	AB1Treated Waste Water Source	AB1_WS	10^5	10^1	0	0
7	AB2Treated Waste Water Source	AB2_WS	10^4	0	10^2	0

Table 4-4: Sample description and processing dates for DNA extraction from Groundbirch samples

Designation	Sample Type	Date collected	Date received	Date DNA extracted
WSP1	Water Storage Pond	June 17, 2013	June 18, 2013	June 20, 2013
WSP2	Water Storage Pond	June 17, 2013	June 18, 2013	June 20, 2013
WS1_PAM	Separator	June 17, 2013	June 18, 2013	June 20, 2013
WS2_GG	Separator	June 17, 2013	June 18, 2013	June 20, 2013
FWSP1	Fresh Water	June 17, 2013	June 18, 2013	June 20, 2013
AB1_WS	Treated waste water	June 25, 2013	June 27, 2013	June 27, 2013
AB2_WS	Treated waste water	July 4, 2013	July 27, 2013	Aug 22, 2013

Table 4-5: Summary of sequencing data for Groundbirch samples.

#	Sample	Sample Type	Sequence Identifier	# reads	OTUs	Chao	Shannon Index	Simpson Index	% Coverage
1	WSP1	Water Storage Pond	12.798.V32_1461	1872	172	295	3.81	0.04	95.78
2	WSP2	Water Storage Pond	12.798.V32_1463	1875	186	328	3.92	0.04	95.31
3	WS1_PAM	Separator	12.798.V32_1462	2705	262	510	3.53	0.09	94.71
4	WS2_GG	Separator	12.798.V32_1460	2701	179	261	3.58	0.05	97.26
5	FWSP1	Fresh Water	12.798.V32_1464	2007	243	529	3.98	0.04	93.12
6	AB1_WS	Treated waste water	1.795.V30_1366	2903	215	395	3.38	0.09	96.18
6	AB1_WS_E	Treated waste water	2.802.V33_1516	884	101	203	2.89	0.12	91.69
7	AB2_WS	Treated waste water	2.802.V33_1483	3285	118	260	2.78	0.15	92.07
7	AB2_WS_E	Treated waste water	1.795.V30_1372	2779	147	350	3.75	0.05	90.27

Despite the large differences in physicochemical properties between fresh and treated flowback waters samples a clear difference in community compositions was not observed between these sets of samples. The community composition of the WS1_PAM/separator, which has a low pH and high salinity, grouped with those of the fresh and treated waste water samples (Figure 4-1). At the phylum level (Figure 4-1: middle) the communities consisted mostly of *Proteobacteria* (28-83%) and *Bacteriodetes* (6.5-59%). The phylum *Proteobacteria* consisted of classes *Betaproteobacteria* (15-59%), *Alphaproteobacteria* (3-41%), *Epsilonproteobacteria* (0-16%) and *Gammaproteobacteria* (1.3-9%). *Deltaproteobacteria*, the class to which most SRB belong, along with *Firmicutes* and *Archea* were in low abundance, which is in agreement with the low SRB counts observed for the samples. Other phyla were the *Bacteroidetes* (4.9-62%), *Euryarchaeota* (0-13%) and *Actinobacteria* (0.5-4.6%).

The *Betaproteobacteria* consisted of the orders *Burkholderiales* (11-27%), *Rhodocyclales* (0.6-22%) and *Methylophilales* (0.3-16%). The *Burkholderiales* include mostly aerobic bacteria, which are capable of using nitrate as an electron acceptor (Garrity et al. 2005a). Most *Rhodocyclales* are mesophiles whereas most *Methylophilales* consist of families/genera growing by aerobic oxidation of single carbon compounds such as methanol, but not methane (Garrity et al. 2005b). The *Bacteroidetes* consist of the orders *Sphingobacteriales* and *Flavobacteria*. The *Sphingobacteriales* are facultative aerobes (Krieg et al. 2010b), whereas the *Flavobacteria* are facultative with some aerobic species, requiring NaCl in concentrations similar to those found in seawater (0.5 M) (Krieg et al. 2010a). The *Euryarchaeota* consist of the classes *Methanobacteria* and *Methanomicrobia* (Figure 4-1). These are methanogens, that can produce methane from

H_2 and CO_2 and acetate in anoxic environments (Anderson et al. 2009; Nakamura et al. 2011). They were found in the AB1_WS/fresh water and flowback water samples, but not in the two treated waste water samples.

4.3 Discussion

The analysis of physical and chemical parameters indicated that processed or stored flowback waters (WSP1, WSP2, WS1_PAM, WS2_GG) were distinct from the fresh water (FWSP1) used at Groundbirch for making fracturing fluid. Flowback waters had high salinity, low pH and high ammonium concentrations compared to the source water. The microbial counts for these flowback waters were lower than for the fresh water (Table 4-3). The microbial community composition of the flowback samples did not indicate the presence of thermophiles or halophiles. The microbial community composition for WS1_PAM/separator was more similar to the compositions of fresh and treated waste water samples than to those of other flowback samples (Figure 4-1). The most reasonable interpretation of these results is that the Montney formation is sterile due to its geothermal history and low porosity, the microbes that are being introduced with the fracturing fluid are not suited for down hole conditions (Fichter et al. 2012). If there are microbes native to the formation they are in such low numbers that they have evaded sequencing they did not survive the fracturing process. Even though microbes can not penetrate the low porosity of shale formations they may be able to access the interior of the formation through fractures in the shale that connect to adjoining formations (Zolfaghari Sharak et al. 2014).

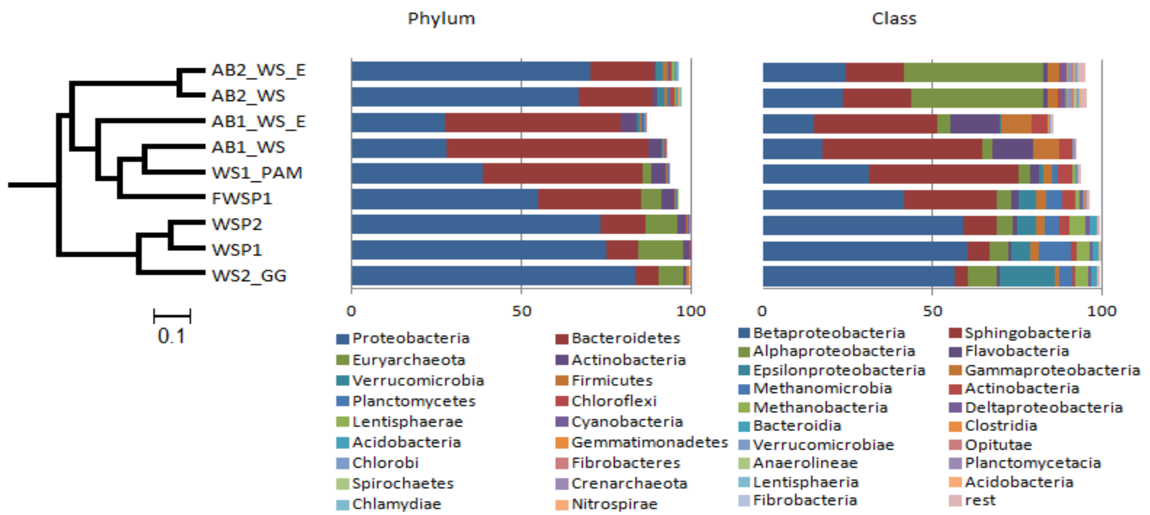


Figure 4-1: (Left) Dendrogram of community composition for the samples (Bray-Curtis), together with a representation of these compositions at the phylum (middle) and class (right) levels. The dendrogram indicates the relatedness of the microbial communities. Compositions, which tree closely together (eg. AB2_WS and AB2_WS_E, or WSP1 and WSP2), are similar

Table 4-6: Relative abundance of microbes identified by 454 pyrosequencing up to the genus level using a 0.03 % cutoff. Taxon above 4% relative abundance are shown along with the total fraction of known SRB (*Deltaproteobacteria*, *Firmicutes* and *Archaea*) are shown.

Kingdom;phylum;class;order; family;genus	AB1_WS	AB1_WS_E	WS2_GG	WSP1	WS1_PAM	WSP2	FWSP1	AB2_WS	AB2_WS_E
Bacteria;Bacteroidetes; Sphingobacteria; Sphingobacteriales; Chitinophagaceae; Sediminibacterium	35.89	25.23	1.85	3.21	37.19	6.35	22.82	12.94	10.08
Bacteria;Proteobacteria; Betaproteobacteria; Rhodocyclales;Rhodocyclaceae	0.28	0.23	16.44	12.50	2.74	12.48	5.58	4.08	4.17
Bacteria;Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae	0.17	0.23	7.18	4.01	11.42	4.80	9.72	0.34	0.22
Bacteria;Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae;Acidovorax	4.38	3.73	2.85	2.24	1.59	3.20	4.48	6.48	7.23
Bacteria;Proteobacteria; Epsilonproteobacteria; Campylobacterales	0.00	0.00	15.77	5.45	1.22	5.28	5.43	0.00	0.00
Bacteria;Proteobacteria; Betaproteobacteria; Methylophilales; Methylophilaceae;LD28	2.96	2.94	4.67	8.71	1.07	10.61	2.74	0.09	0.25
Bacteria;Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Polaromonas	0.03	0.00	7.96	6.09	1.11	4.96	2.89	0.00	0.04
Bacteria;Bacteroidetes; Flavobacteria;Flavobacteriales; Flavobacteriaceae; Flavobacterium	10.78	12.67	1.00	0.86	2.55	1.07	1.55	1.31	1.40
Bacteria;Proteobacteria; Betaproteobacteria; Methylophilales; Methylophilaceae	0.00	0.00	2.30	6.94	0.41	5.01	0.85	0.12	0.04
Bacteria;Proteobacteria; Alphaproteobacteria; Rhodospirillales;Oleomonas	0.00	0.00	0.00	0.00	0.00	0.00	0.00	31.63	33.00

Kingdom;phylum;class;order; family;genus	AB1_WS	AB1_WS_E	WS2_GG	WSP1	WS1_PAM	WSP2	FWSP1	AB2_WS	AB2_WS_E
Bacteria;Proteobacteria; Betaproteobacteria; Rhodocyclales; Rhodocyclaceae;uncultured	0.00	0.00	0.04	0.05	0.00	0.16	0.25	5.75	5.58
Bacteria;Proteobacteria; Alphaproteobacteria; Rhodospirillales; Rhodospirillaceae; Magnetospirillum	0.00	0.00	0.00	0.00	0.04	0.00	0.00	4.72	5.79
Bacteria;Proteobacteria; Betaproteobacteria; Burkholderiales; Alcaligenaceae	4.27	2.83	0.63	0.43	4.51	1.44	2.99	0.91	0.97
Bacteria;Actinobacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae;Candidatus_Limnoluna	3.14	4.07	0.07	0.21	3.96	0.48	2.94	0.52	0.32
Archaea;Euryarchaeota; Methanobacteria; Methanobacteriales; Methanobacteriaceae; uncultured	0.00	0.00	3.41	3.31	0.44	4.37	1.20	0.00	0.00
Bacteria;Proteobacteria; Betaproteobacteria; Rhodocyclales;Rhodocyclaceae;Ferribacterium	0.03	0.11	4.33	3.74	0.74	2.67	2.59	0.00	0.00
Bacteria;Proteobacteria; Alphaproteobacteria; Sphingomonadales	0.69	1.47	4.52	3.58	1.22	2.35	0.95	0.03	0.07
Bacteria;Bacteroidetes; Sphingobacteria; Sphingobacteriales; Sphingobacteriaceae; Sphingobacteriaceae; Pedobacter	3.55	4.19	0.00	0.00	0.96	0.21	0.85	0.82	0.90
Archaea;Euryarchaeota; Methanomicrobia; Methanomicrobiales; Candidatus_Methanoregula	0.10	0.00	1.78	5.02	1.18	1.33	2.99	0.00	0.00
Deltaproteobacteria	0.00	0.00	1.11	0.91	0.19	1.07	0.85	1.21	1.44
Firmicutes	0.27	0.45	0.48	0.32	0.30	0.32	0.20	0.79	1.12
Archaea	0.31	0.23	7.48	13.30	2.55	9.49	6.18	0.03	0.04
Fraction of taxa not included in table	33.22	41.62	21.32	27.45	26.26	28.05	26.17	28.20	27.36

Although microbes may contribute to polymer degradation in fracturing fluid, especially when guar gum is used, it is not a large concern. The amount of time that microbes are in contact before the polymer is intentionally broken is short and not expected to be a concern unless left in storage (Rahim & Holditch 2003). A larger concern is, (ii) well plugging by growth downhole. At the field being studied this is also not much of a concern due to the harsh physicochemical conditions that are observed in waters emerging from the well and the lack of organisms with suitable adaptations (Heimovaara et al. 2010). The formation waters are likely to be (iii) corrosive towards metals because of their low pH and high salt concentration, not because of high numbers of microbes (Nešić 2007).

Polymers such as guar gum and PAM in flowback waters have not been able to be definitively quantified in this study, possibly because these waters have aged in the formation allowing for the polymers to degrade below detectable levels. Guar gum is broken with a chemical oxidising agent, it would however be expected to be present in a fresh flowback sample (Pope et al. 1995; Hong et al. 2010).

When guar gum, is broken by an oxidizing agent the smaller organic components that are released are easier for organisms to metabolize as they do not require the enzymes for breaking the 1-6 α-D-galactopyranosyl and 1-4 β-D-mannopyranosyl bonds that hold the sugar residues together (Kawamura 2008). PAM is a more difficult compound to degrade, there are not many organism that are known to be able to use PAM. Those identified, are mostly able to utilize the amide group as a nitrogen source (Bao et al. 2010).

It is difficult to draw conclusions from the relative abundance of taxa in microbial communities as it does not give an absolute number of organisms. While a particular member of the community may increase it does not indicate that it has grown in number. An increase in relative abundance could indicate that it is more resistant to the conditions that it is subjected to relative to the other organisms in the community assuming that there was no native microbial community present in the formation or the taxon was picked up from another source of contamination.

Counts of APB were higher in the low salinity waters than in high salinity waters. SRB counts were only observed in the fresh water storage pond (FWSP). Low pH in the flowback samples could also contribute to the low counts of APB. Community analysis did not revealed a lot of facultative microbes that possess photoautotrophic capabilities. These were not expected due to the lack of light that these organisms require in the subsurface.

Many of the same organisms are present in all of the samples in different proportions. Minor components of the microbial communities that were not observed in all samples. One of the dominant organisms in many of the samples was *Sediminibacterium*. *Sediminibacterium* is a member of the *Bacteroidetes* and comprises up to 37% of OTUs in the low salinity samples. *Sediminibacterium* was least prominent in WS_GG and is commonly identified in oil fields (Li et al. 2012; Callbeck et al. 2013; Wei et al. 2014). *Sediminibacterium* species are typically aerobes with some facultative activity, and can withstand 2% NaCl [w/v] (Kim et al. 2013; Kang et al. 2014). Due to its low salt tolerance and decreased abundance in flowback waters, it is unlikely that it is proliferating down hole, but rather does well in the less saline storage ponds. The

Rhodocyclaceae from the *Betaproteobacteria,* were found in all samples but were present in higher abundance in samples that had higher salt concentrations. Organisms belonging to this family are commonly associated with oil fields and are capable of degrading hydrocarbons (Strong et al. 2013; Folarin et al. 2013).

Counts showed that fresh water storage pond (FWSP1) and treated waste water source 2 (AB2_WS) had SRB activity, sequencing showed the presence of SRB in the majority of samples. Sequencing showed organisms belonging to the class *Deltaproteobacteria*, which are commonly found in many oil fields and contribute to souring (Kryachko et al. 2012; Grabowski et al. 2005; Callbeck et al. 2011). A wider variety of growth tests on different media would likely have revealed more SRB activity (Muyzer & Stams 2008). Many of the same sequences were found in the separators, source water and storage ponds. There was no change in the relative abundances of *Deltaproteobaceria* that were identified. Despite the presence of sequences belonging to organisms associated with SRB activity in the majority of samples, the risk of souring is expected to be low or non-existent due to the low SRB counts and the lack of sulfate in the waters tested. Some sulfate or sulfide in the formation would likely precipitate out along with barium or iron respectively, which are commonly found in high abundance in shale gas formations (Table 1-2) (Kraan et al. 2012; Barbot et al. 2013).

4.4 Conclusion

The analysis of the samples from the Groundbirch field offers valuable insight into the subsurface environment of the shale formations especially which microbes that are present at different points of a hydraulic fracturing and flowback operation. The

physicochemical conditions of the flowback waters are distinct from the source waters, contributing to differences in community composition. Sequencing revealed that it is unlikely that there are any microbes native to the formation. There were no organisms observed with known metabolisms that could be supported at the temperatures and salinities in the formation. This lack of extremophiles reduces the likelihood of biofouling and subsequent fracture plugging in the formation. Souring is unlikely to be a concern due to the lack of sulfate in the source and flowback waters. There is some SRB activity in the source waters used when sulfate is introduced but the microbes responsible do not appear to be able to survive the fracturing process.

Chapter Five: Results-Analysis of Kobes Flowback Waters

5.1 Introduction

West of Fort St. John (BC) in the Altares field, hydraulic fracturing is becoming more common. This area lies over top of the north eastern region of the Montney formation. The well being studied has a UWI of 200/B-049-D/094-A-15/00 with a surface location of 15-12-84-26W6 being drilled to a final depth of 4112 m and in the Kobes field (Figure 1-1). The formation is composed predominantly of shale, siltstone and sandstone giving it a permeability of less than 0.05 mD and porosities ranging from 1-4.7% (Norton et al. 2010). During the Triassic period the formation was laid down from marine sediments. It has since undergone thermal maturation, reaching a maximum temperature that exceeded 450°C (Riediger 1990). This well exceeds the temperatures needed to put the formation in the gas window and sterilize the formation (Peters & Cassa 1994). The formation has since cooled to 70-110°C. This temperature is higher than many microbes can withstand, but could conceivably support thermophilic life in cooler regions of the formation. Microbial activity in the subsurface has not been observed above 80°C in hydrocarbon reservoirs (Head et al. 2003).

The well was fractured using source water from a local river; no recycled flowback water was used. A hybrid fracturing system was used that employed both guar gum and PAM as thickening agent and friction reducer respectively. Waters were heated to 45°C and mixed with fracturing chemicals prior to injection. The entire fracturing process took 5 days. There was a three day turnover period where the well was shut in and surface crews prepared for flowback which commenced once surface crews were ready. Flowback then proceeded for the next 500 h with natural gas that was produced, being

flared as the appropriate infrastructure for processing natural gas had not yet been put in place. This infrastructure will be installed once the feasibility of production from this field has been determined. After 500 h of flowback the well was capped and shut in until further action was required. The scope of this work does not extend beyond the well being shut in, instead this work takes a detailed look at early flowback communities and the parameters surrounding them as well as potential concerns regarding flowback waters.

5.2 Methods

Flowback samples were collected every 4 hours for the duration of the flowback period with the exception of when the well was shut in. All samples were collected at the well head by field employees in 1 L sterile Nalgene bottles. All samples were stored on site (7-27 days) and received at the University of Calgary in a single shipment. Samples listed in Table 3-2 were immediately catalogued and stored in Coy anaerobic hoods. Samples 250 ml) were removed from the anaerobic hood for DNA isolation. Samples were centrifuged at 35000 x g for 15 min to obtain a pellet. Pellets were resuspended in 1 ml of supernatant and transferred to 2 ml microfuge tubes and centrifuged at 17 000 x g on a table top centrifuge for 5 min. Pellets of gDNA were frozen at -20°C until they could be extracted using the Qiagen soil fast DNA kit (QiagenQIAQuick Kit, Qiagen) kit. gDNA was quantified using a Qubit Fluorometer (Invitrogen), using a Quant-iT™ dsDNA HS Assay Kit (Invitrogen), to estimate the amount of biomass in each sample. Due to the number of samples, it took 2 days to obtain pellets for all of the samples. DNA was prepared for community analysis by PCR.

Aliquots of samples were removed from the anaerobic hood in 50 ml Falcon tubes and tested for electron donors (acetate, lactate, propionate and butyrate), electron acceptors (sulfate, nitrate, nitrite, iron) as well as sulfide as previously described (3.4). Field observations were received from Suncor that outlined some of the parameters that were measured at the well head. This included on site pH values and tracer data. pH was also measured at the University of Calgary to determine if there was any change during transport and storage. Hydrocarbon analysis was performed on a sample of combined hydrocarbon that was collected from all of the samples. Data was collected on the GC-MS and analysed using Chemstation.

5.3 Results

5.3.1 Tracer data analysis

Tracer data was provided by Suncor (Appendix 2). During the fracturing process of a multistage well, stage 1 is fractured at the end of the horizontal section, furthest from the wellbore. The remaining stages 2-10 were completed sequentially proceeding towards the wellbore. There were 10 fracturing stages and 18 of the flowback samples collected were tested for the tracers used. Fluid from different fracture stages were seen to flow back at different times (Figure 5-1). When a particular stage began to flow back it comprised a larger portion of the flowback volume, its contribution to the total flowback volume then decreased. As the later flowback stages began flowing they provided less of a contribution to the total amount flowback volume.

Initial flowback waters comprised mostly of fluids that were used to fracture stage 8 which made up 85% of the flowback volume (Figure 5-1). At 20 h after flowback the contribution of Stage 8 had decreased to 38% while the contribution of stage 7 had increased from 2% to 38%, this was the highest value that was seen for stage 7 and it decreased and then remained constant over the remaining flowback period. Fluid from stage 6 had also increased from 9% to 20%. At 32 h fluid from stage 6 make up the largest portion of the flowback fluid at 29%. This was the highest value seen for stage 6 which decreased steadily for the remainder of the flowback period. The contribution of stages to the total volume of flowback waters was less for the remainder of the flowback period. Fluid from stage 9 and 5 peaked at 6.5% and 11.5% at 52 h respectively. The appearance of fluid from these stages was quite sudden, after their initial appearance their contribution to the total flowback fluid decreasesed. Stages 1 and 3 were the only stages that did not begin flowing back until after the shut in stage. After the shut in stage the contribution of each of the stages remained relatively constant for the remainder of the flowback period.

5.3.2 Samples

Flowback water samples (93) were received as well as a source water sample. The source water sample was a fresh water sample taken from a local river (Table 3-2). It was clear in appearance and had no notable appearance or odour. No particulate matter settled out of the source water sample when left standing. The appearance of flowback fluids gradually changed as flowback proceeded. Earlier flowback samples were mostly clear with no strong odor or particulate matter. Later flowback samples, became increasingly

turbid with an increasingly strong odour of organic solvent and had red-brown precipitate that settled out when left to stand in the hood, some red-brown matter still remained suspended. Later samples had an increasingly large organic layer accumulate on the surface of later flowback samples. The flowback rate remained constant for the first 68 h at approximately 7 m^3/h. From 68 – 200 h the well was shut in. After flowback resumed the rate was approximately 13 m^3/h from 200-500 h until the well was shut in.

5.3.3 Physiochemical Parameters

The physicochemical parameters of the flowback waters changed as flowback progressed. The salinity of the source water was 0.0055 Meq NaCl, which is characteristic of fresh water (Table 5-1). The first flowback water sample had a salinity of 0.4 Meq NaCl (Figure 5-2: A). Salinity increased to 1.9 Meq NaCl by the end of the flowback period. The increase did not reflect the contributions of different flowback stages.

There was no ammonium detected in the source water (Table 5-1). In the first flowback samples the ammonium concentration was 10 mM and this followed a similar trend as salinity, reaching a concentration of 50 mM by the end of the sampling period (Figure 5-2: B).

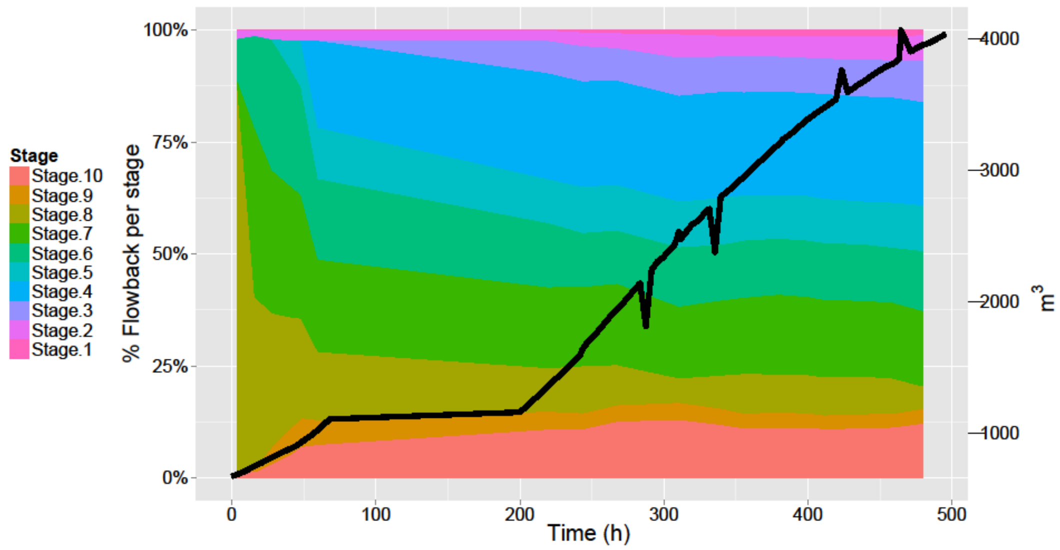


Figure 5-1: Kobes cumulative flowback volume for well 200/B-049-D/094-A-15/00. Flowback water was produced at a constant rate of approximately 7 m^3/h from 0-68 h of flowback and of 13 m^3/h when flowback resumed at 200 h. The relative contribution of individual fractured stages are shown based on the relative abundance of tracers found in the flowback fluid.

The amount of dissolved iron that was present in the flowback waters was greater than that in the source water (0.6 mM). The amount of ferric iron in initial flowback waters was 0.8 mM and remained constant for the first 68 h before the well was shut in (Figure 5-3: A). Fe(III) concentrations decreased after the shut in period but remained around 0.6 mM. There was no sulfide detected in the source water sample received (Table 5-1), but there were small amounts of sulfide detected in some of the flowback samples, with a slight increase in sulfide concentration to 0.3 mM, towards to the end of the sampling period (Figure 5-3). The largest change in sulfide concentration was observed after the shut in period at 200 h with a reading of 0.2 mM, which quickly decreased to below detectable levels. It was not until after 400 h after flowback that sulfide was observed as high as 0.4 mM in some of the samples. A water chemistry report received from the field did not show any sulfide at 500 h (Appendix 3).

The change in pH follows a different trend than the dissolution of ions. In the source water sample the pH measured in the lab was just below neutral (6.6) (Table 5-1). The first flowback water sample had a slightly reduced pH (6.4) and this decreased to a pH of 5.6 by the end of the sampling period (Figure 5-2: C). The pH measured at the well head by the company personnel was constantly 7 throughout the flowback period. The discrepancy with pH measurements in the lab are likely the result of oxidation of Fe(II) iron or sulfide that occurred during the time in storage.

Of the electron acceptors that were tested, no sulfate, nitrate or nitrite were detected, except iron(III) (Figure 5-3:A). Of the organic acids that were tested (acetate, lactate, propionate, butyrate) only acetate and lactate were detected. Lactate was absent from early flowback samples but increased gradually throughout the sampling period.

Acetate was not observed in the source water sample. In the initial flowback samples there was also no acetate detected, but 0.4 mM of acetate was detected at 60 h to the well being shut in. After flowback resumed the concentration of acetate remained constant at 0.4 mM for the remainder of the sampling period, which coincided with flowback fluid from stage 4 being in its greatest proportion at the well head.

5.3.4 Hydrocarbon analyses

When samples were left standing in the Coy anaerobic hood, an organic layer formed on the top of the later flowback samples. The layer was colorless and had a strong odour. There was no hydrocarbon observed with any of the samples until 350 h after flowback where the amount of hydrocarbon observed increased rapidly (Figure 5-4). This analysis does not take in to account gaseous hydrocarbons that would include methane which makes up more than 98 % of hydrocarbons in thermogenic natural gas well like the one being studied (Peters & Cassa 1994).

Table 5-1: Kobes source water characteristics

NaCl Meq	Fe (tot) [mM]	pH[1]	pH Co[2]	gDNA [ng/ml]	Acetate [mM]	Sulfide [mM]	NH_4^+ [mM]	APB /ml	SRB/ ml
0.0055	0.60	6.63	7	8	0	0	0	10^4	<33

[1]pH of the samples upon arrival at the University of Calgary
[2]pH values provided by Suncor that were collected at the well head

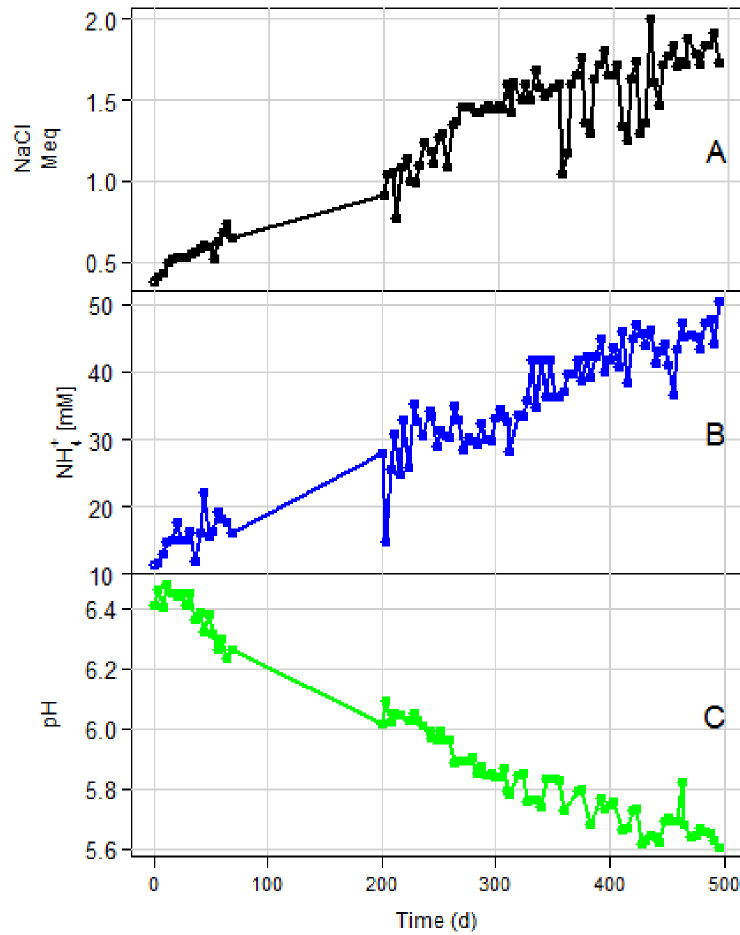


Figure 5-2: Physicochemical parameters of flowback water for the first 500 h of flowback. A) NaCl Meq, B) NH_4^+ and C) pH.

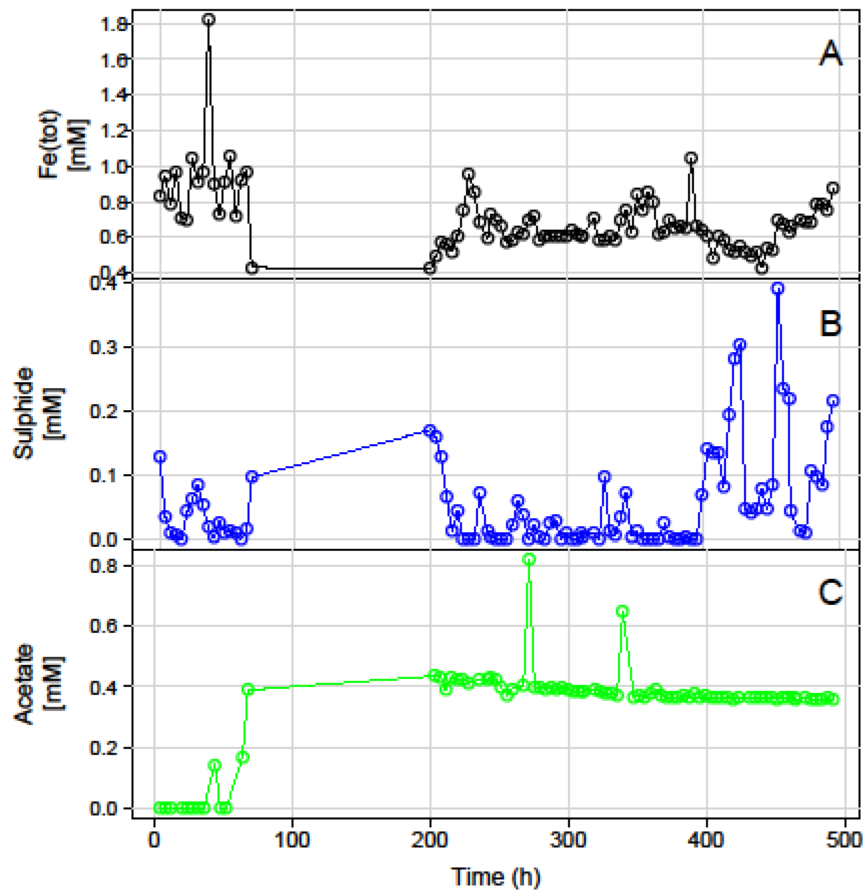


Figure 5-3: Physicochemical parameters of flowback water from the first 500 h from the Kobes field. A) Fe(tot), B) Sulfide and C) Acetate.

Analysis of the combined hydrocarbon fractions that were collected revealed that they were very light in nature (Table 5-2). The heaviest compound detected was hexadecane, 2,6,10,14-tetramethyl- with a molecular weight of 282 $g \cdot mol^{-1}$. The lightest compound identified was cyclohexane with a molecular weight of 84 $g \cdot mol^{-1}$. The majority of the hydrocarbons identified are branched alkanes. Approximately 3.3 % of the compounds identified had cyclic structures.

The amount of light hydrocarbons is likely underestimated due to volatilization in to the head space which is justified by the odour that was detected when samples were removed from the hood and opened for analyses. These observations agree with the hydrocarbon analyses received (Appendix 5 and 6)

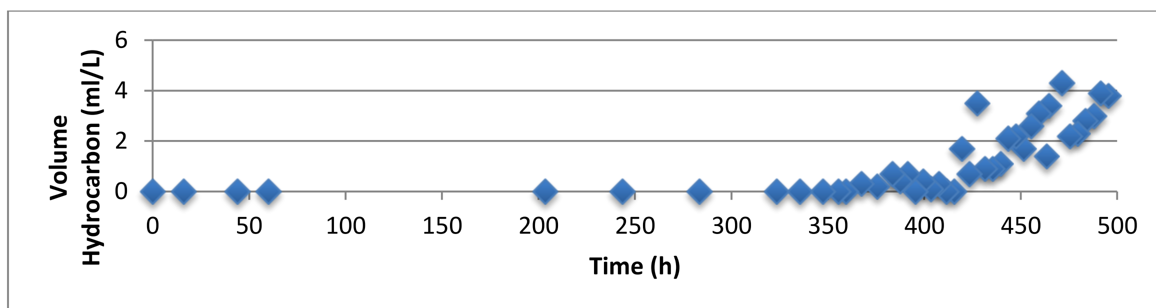


Figure 5-4: Volume of hydrocarbon recovered from Kobes samples for the first 500 h of flowback.

Table 5-2: Relative abundance (%) of hydrocarbon components in the combined hydrocarbons recovered from Kobes samples for components greater than 1% abundance.

Hydrocarbon Name	Mol. weight	#C	#H	relative abundance
Dodecane	170	12	26	3.08
Tridecane	184	13	28	2.85
Undecane	156	11	24	2.71
Undecane, 2,6-dimethyl-	184	13	28	2.45
Tetradecane	198	14	30	2.23
Unidentified				2.04
Tridecane, 7-methyl-	198	14	30	2.02
Pentadecane	212	15	32	1.84
Nonane, 2,2,4,4,6,8,8-heptamethyl-	226	16	34	1.77
Hexadecane, 2,6,10,14-tetramethyl-	282	20	42	1.60
Nonane, 2,6-dimethyl-	156	11	24	1.58
Dodecane, 2,6,10-trimethyl-	212	15	32	1.54
Unidentified				1.38
Hexadecane	226	16	34	1.25
Nonane	128	9	20	1.22
Unidentified				1.15
Octane, 2-methyl-	128	9	20	1.10
Pentadecane, 2,6,10,14-tetramethyl-	268	19	40	1.07
Pentadecane, 2,6,10-trimethyl-	254	18	38	1.07
Total cyclic				3.31
Total branched				20.28
Total linear				6.69
Total Identified				30.28
Not Identified				36.15

5.3.5 Microbial community analysis

The amount of recoverable gDNA from the source water and flowback samples was quantified to estimate the relative amount of biomass that was present in the sample. This is not a perfect indicator of biomass as different organisms have different sized genomes that could skew the results based on microbial community composition. The increased amount of solutes and organic content such as humic acids can interfere with DNA extraction and quantification, resulting in an underestimation of gDNA (Kreader 1996; Wintzingerode et al. 1997). The amount of gDNA in early flowback samples was up to 5 fold higher than that of the source water (Table 5-1: 8 ng/μl). The amount of gDNA in the flowback waters remained higher than that seen in the source water for much of the period before flowback was halted at 68 h. After 68 h there was almost no detectable gDNA in any of the flowback samples (Figure 5-5). There were 4 increases in gDNA during the first 68 h, the intensity of the spikes of gDNA decreased as flowback progressed until they were no longer observed. These spikes of gDNA corresponded to the times that a newer flowback stage was initiated. The larger the contribution of that particular stage to the flowback volume the larger the increase in gDNA that was observed. Larger spikes in gDNA were also associated with less formation exposure than samples that were exposed to the formation for longer periods of time. High amounts of gDNA were extracted from samples that had lower salinity.

The numbers of aerobes detected in the samples was highest in the first flowback samples and decreased throughout the first 68 h (Figure 5-5). There was an increase at 32 h where the highest number of aerobes 10^8/ml were detected, this is the same time that flowback from stage 6 was at its highest (Figure 5-6). The MPN of APB, like the amount

of gDNA and aerobes, were high before 68 h of flowback similar to the total amount of extracted gDNA (Figure 5-5). After 68 h the MPN of APB had decreased to below detectable limits where they remained for the remainder of the sampling period. There were never any SRB detected in either the source water sample or any of the flowback samples.

Sequencing returned 835-11108 reads for each sample (Table 5-3). Species diversity was determined by both the Shannon and the inverse Simpson Index. Both indices gauge species diversity, however the Simpson Index puts less weight on rare species while the inverse Shannon index puts less weight on abundant species (Shannon 1948; Simpson 1949). The indices for both of these measurements are highest in the source water (Figure 5-7, Table 5-3). The diversity according to the Shannon Index is next highest in the initial flowback samples but decreased rapidly and remained relatively low for the remainder of the sampling period. This shows that microbial communities after the first 10 h maintain low diversity. The inverse Simpson Index shows a similar trend with diversity and eveness being highest in the initial flowback waters and decreasing to almost 0 by 68 h after flowback. This demonstrates a drastic unevenness in the microbial community that is either dominated by *Shewanella*, *Pseudomonas* or *Halomonas*.

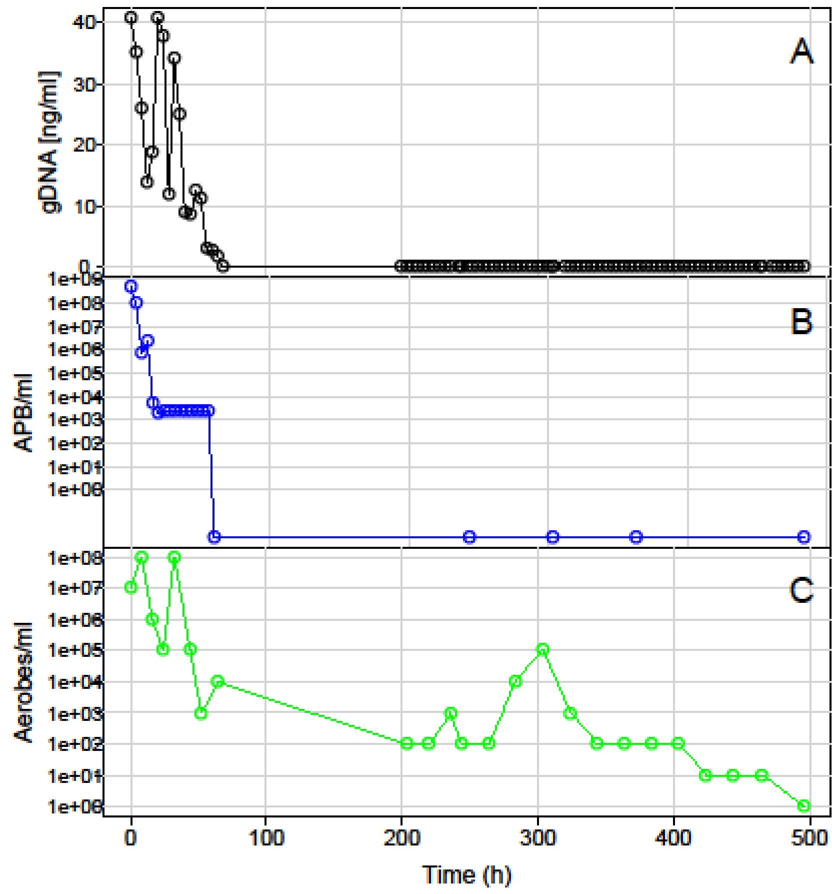


Figure 5-5: Detectable biomass in the first 500 h of flowback From the Kobes well. A) Extracted gDNA, B) Log MPN of APB/ml and C) Log counts of aerobes/ml.

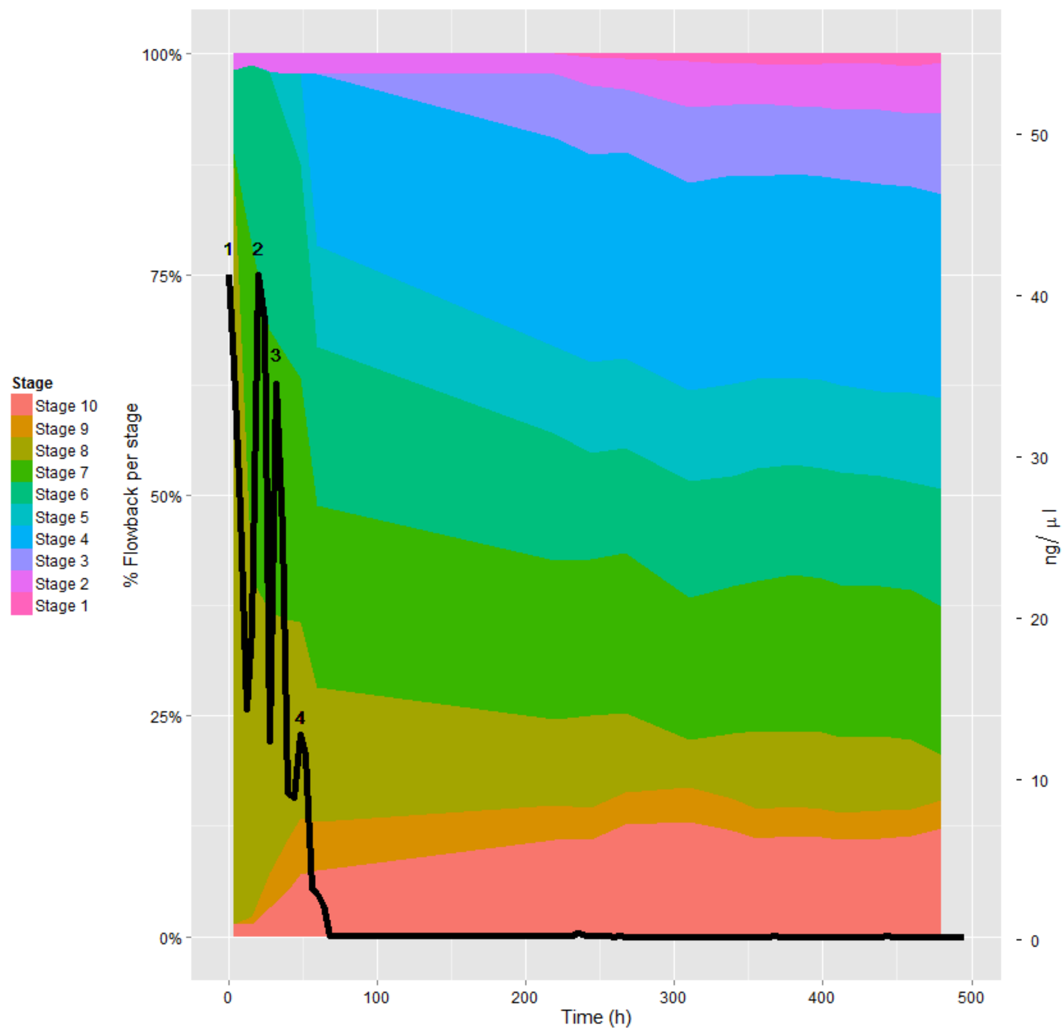


Figure 5-6: The amount of recoverable gDNA from Kobes flowback samples with respect to the relative contribution of each flowback stage. Four increases of gDNA were observed (1-4).

Table 5-3: Sequencing data for all Kobes samples that were sent for sequencing.

Sample (h)	Sequence Identifier	# reads	OTUs	chao	shannon	invsimpson	coverage
source	2.892.V43_2097	6975	727	1383.26	4.300	19.321	0.946
0	2.892.V43_2097	5996	111	233.77	2.353	6.616	0.990
4	2.892.V43_2097	5453	58	87.55	1.718	3.315	0.995
8	2.892.V43_2097	2007	41	68.14	1.746	4.287	0.990
12	2.892.V43_2097	3207	27	72.33	0.849	2.100	0.995
16	2.892.V43_2097	8313	72	158.67	0.780	1.427	0.995
20	2.892.V43_2097	11108	38	155.00	0.384	1.222	0.998
24	2.892.V43_2097	6358	31	46.11	0.137	1.041	0.997
28	2.892.V43_2097	7605	34	224.00	0.939	2.060	0.997
32	2.892.V43_2097	6468	68	234.50	1.844	4.803	0.994
36	2.892.V43_2097	6441	18	109.00	0.085	1.024	0.998
40	2.892.V43_2097	7053	26	179.00	0.752	2.013	0.997
44	2.892.V43_2097	835	15	22.50	1.047	2.061	0.993
48	2.892.V43_2097	4951	29	64.00	0.806	1.575	0.997
52	2.892.V43_2097	8540	24	46.75	0.370	1.228	0.998
56	2.892.V43_2097	4709	39	85.20	1.115	2.391	0.995
60	2.892.V43_2097	10257	53	107.38	0.863	2.100	0.997
64	2.892.V43_2097	6287	33	60.20	0.424	1.205	0.997
68*	2.892.V43_2097	2738	58	100.27	1.745	3.128	0.974
200	2.892.V43_2097	2738	90	163.50	1.209	1.610	0.982
204	2.892.V43_2097	1213	49	88.43	1.218	1.674	0.980
208	2.892.V43_2097	2022	63	154.11	0.922	1.444	0.980
272*	2.892.V43_2097	4043	81	141.00	1.160	1.733	0.990
276*	2.892.V43_2097	7574	129	214.80	1.375	2.085	0.991
280*	2.892.V43_2097	5034	87	172.00	0.857	1.516	0.990
292*	2.892.V43_2097	1307	28	63.00	0.718	1.454	0.989
368*	2.892.V43_2097	3375	275	497.13	2.853	4.817	0.957
424*	2.892.V43_2097	2206	96	214.46	1.470	2.014	0.975
436*	2.892.V43_2097	1206	73	216.50	1.640	2.296	0.965
444	2.892.V43_2097	5177	41	173.00	0.097	1.022	0.994

*Sequencing results showed >30% *Escherichia* which was attributed to *Escherichia coli* used to manufacture *Taq*. On average samples that had *Escherichia* removed averaged 75%.

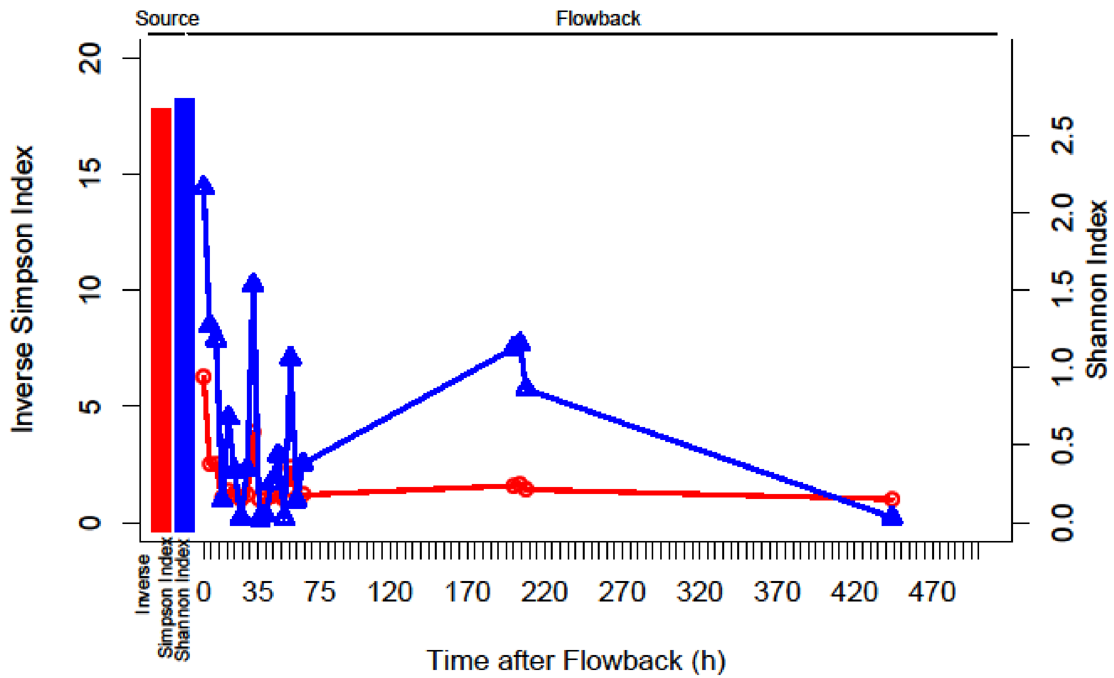


Figure 5-7: Changes in diversity of Kobes flowback and source water microbial communities as determined by the inverse Simpson index (O) and Shannon index (Δ) as calculated by the Phoenix 2 bioinformatics pipeline (Soh et al. 2013).

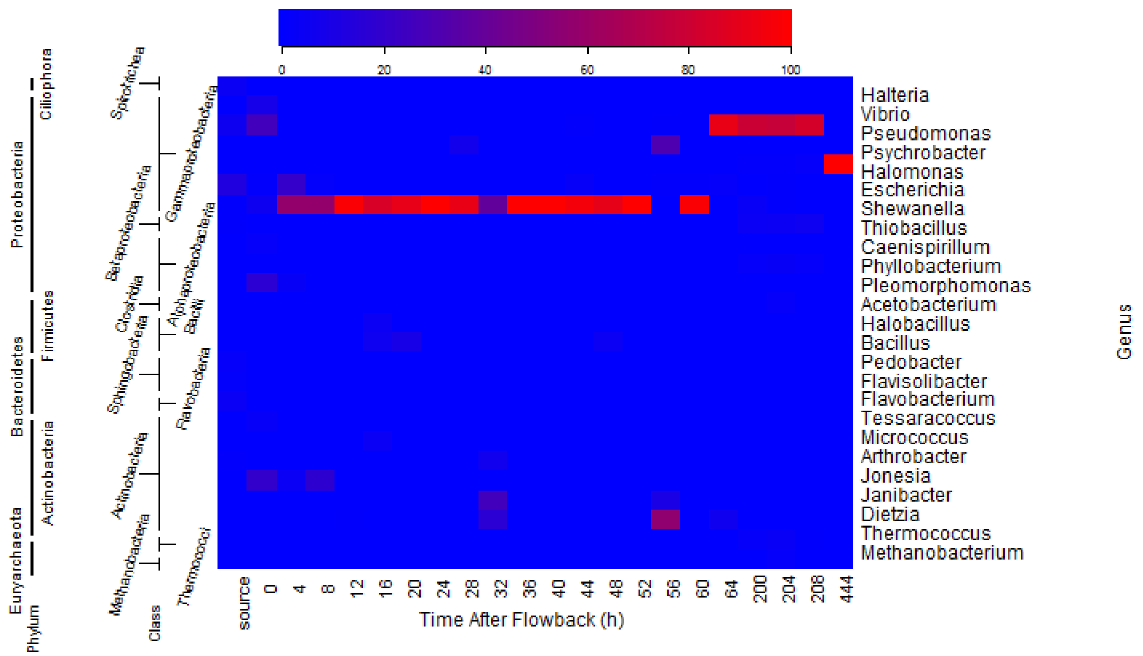


Figure 5-8: Heatmap of taxa in sequenced Kobes samples identified to the genus level by 454 pyrosequencing of the V6-V8 16S rDNA variable region. The left sidebars depict the phylum and class levels that each genus belongs to.

Table 5-4: Relative abundance of 16S rDNA pyrosequencing results. Taxa with fractions above 5 % in at least 1 sample are shown along with the total amount of *Deltaproteobacteria*, *Firmicutes* and *Archaea.*

superkingdom;phylum;class;order;family;genus	source	0	4	8	12	16	20	24	28	32	36	40	44	48	52	56	60	64	200	204	208	444
Bacteria;Proteobacteria;Gammaproteobacteria;Alteromonadales;Shewanellaceae;Shewanella;	0.5	5.2	55.6	53.5	97.7	84.2	90.3	99.4	91.6	38.3	99.6	99.3	89.7	89.7	99.5	0.3	97.6	0.1	3.3	0.5	0.0	0.0
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas;	5.1	25.9	0.0	0.1	0.1	0.0	0.0	0.1	0.1	0.1	0.0	0.1	0.8	0.1	0.0	0.5	0.4	91.3	79.1	77.5	83.3	0.0
Bacteria;Actinobacteria;Actinobacteria;subActinobacteridae;Actinomycetales;subCorynebacterineae;Dietziaceae;Dietzia;	0.0	0.0	0.0	0.2	0.7	0.0	0.0	0.1	0.0	16.6	0.0	0.0	0.0	0.0	0.1	56.6	0.0	6.3	0.1	0.0	0.0	0.0
Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacteriales;Enterobacteriaceae;Enteric_Bacteria_cluster;Escherichia;	12.9	1.1	13.3	1.3	0.2	0.1	0.0	0.1	0.3	0.2	0.2	0.3	2.8	0.2	0.2	0.9	1.1	1.8	0.2	0.4	0.2	0.1
Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacteriales;Enterobacteriaceae;Enteric_Bacteria_cluster;	0.1	0.9	14.1	21.7	0.3	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.5	5.0	0.0	0.1	0.3	0.1	1.6	0.4	0.4	0.0
Bacteria;Actinobacteria;Actinobacteria;subActinobacteridae;Actinomycetales;subMicrococcineae;Jonesiaceae;Jonesia;	0.0	20.0	3.6	18.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.1	0.0	0.0
Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;	0.0	0.0	0.0	0.0	0.0	5.7	9.1	0.0	0.0	0.0	0.0	0.0	0.0	4.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Bacteria;Actinobacteria;Actinobacteria;subActinobacteridae;Actinomycetales;subMicrococcineae;Intrasporangiaceae;Janibacter;	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25.8	0.0	0.0	0.0	0.0	0.0	10.2	0.0	0.0	0.1	0.0	0.0	0.0
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Psychrobacter;	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.2	0.0	0.0	0.0	0.0	0.0	0.0	30.7	0.0	0.0	0.1	0.0	0.0	0.0
Bacteria;Proteobacteria;Betaproteobacteria;Hydrogenophilales;Hydrogenophilaceae;Thiobacillus;	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.1	3.8	4.5	5.5	0.0
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Methylocystaceae;Pleomorphomonas;	0.0	17.5	3.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0

superkingdom;phylum;class;order;family;genus	source	0	4	8	12	16	20	24	28	32	36	40	44	48	52	56	60	64	200	204	208	444
Bacteria;Proteobacteria; Gammaproteobacteria;Oceanospirillales;Halomonadaceae;Halomonas;	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.8	1.9	99.1
Bacteria;Actinobacteria;Actinobacteria; subActinobacteridae;Actinomycetales;subMicrococcineae;	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	11.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
Bacteria;Actinobacteria;Actinobacteria;subActinobacteridae;Actinomycetales; subMicrococcineae;Micrococcaceae;Arthrobacter;	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Incertae_Sedis;	0.0	10.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Alcaligenaceae;	5.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae; Polaromonas;	5.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
Total Deltaproteobacteria	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total Firmicutes	0.3	14.1	0.0	0.1	0.0	10.9	9.4	0.0	0.1	0.0	0.0	0.0	0.2	4.6	0.0	0.0	0.0	0.0	1.4	2.2	0.2	0.0
Total Archaea	0.1	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.5	7.0	0.8	0.0
% of sequences not shown in table	51.6	7.0	2.9	4.7	1.1	4.2	0.2	0.3	0.7	1.3	0.1	0.2	5.9	0.5	0.1	0.6	0.4	0.3	6.2	6.2	7.5	0.8

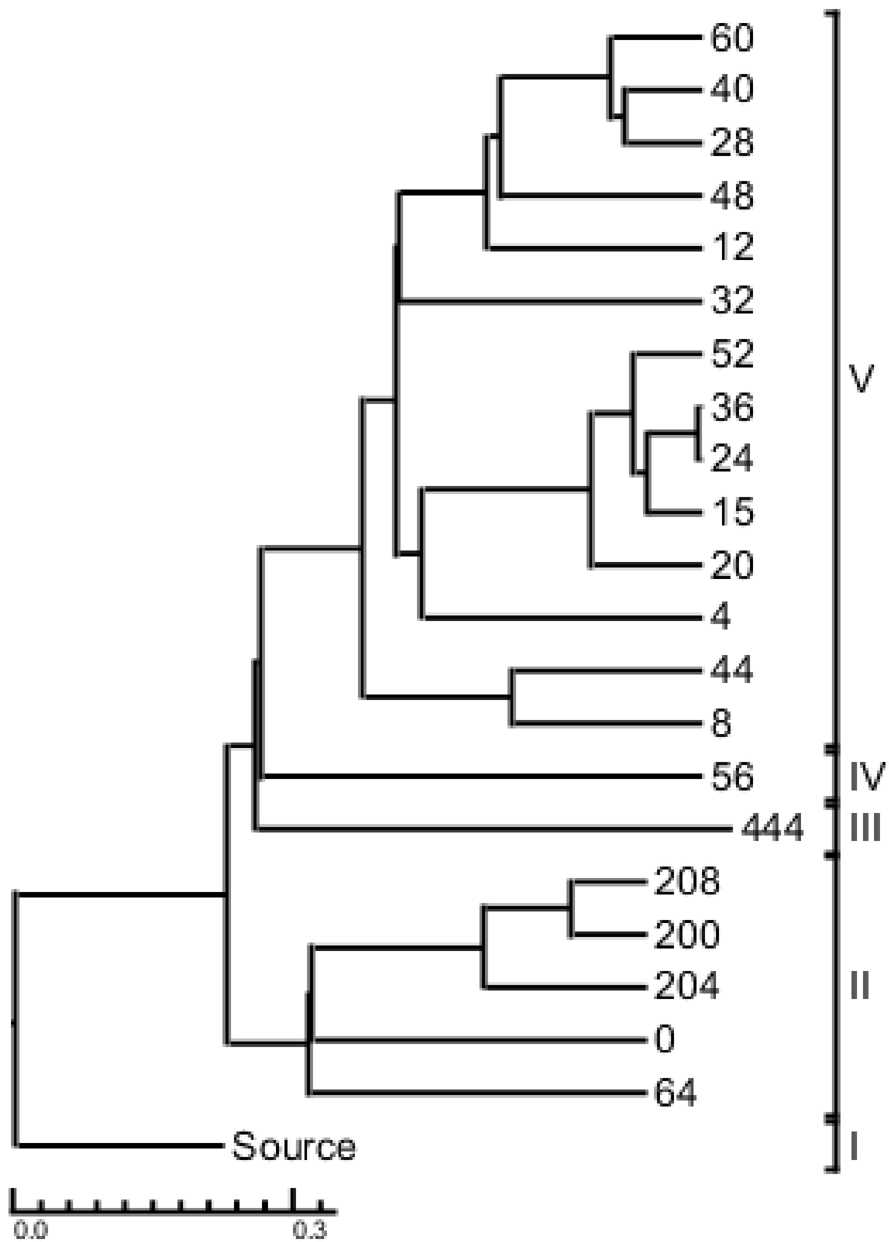


Figure 5-9: Relationship tree of successfully sequenced Kobes samples using the Bray Curtis clustering algorithm of Phoenix 2. Clusters were determined by a 0.3 cut off.

The source water sample was dominated by members of the phylum *Proteobacteria* (67.7%) (Table 5-4). This comprised the classes *Gammaproteobacteria* (21.3%), *Deltaproteobacteria* (1.3%), *Betaproteobacteria* (33.2%), and *Alphaproteobacteria* (11.6%). The phylum *Bacteroidetes* (11.0%) consisted of *Sphingobacteria* (7.1%) and *Flavobacteria* (3.9%). Members of the phylum A*ctinobacteria* represented 7.8% of the total reads. *Verrucomicrobia* represented a very small portion of the sequenced reads (0.76%) and were only found in the source water sample. *Firmicutes* consisted of less than 0.4%. The phylum *Planctomycetes* represented 4.3% of total reads. The fraction of *Archaea* present in the source water sample was less than 0.1%.

Almost 5% of the reads belonged to *Eukaryota*. The primers used for PCR were not designed to amplify regions of 18S eukaryotic rDNA but it is still possible for the primers to amplify eukaryotic sequences. This fraction of eukaryotic reads was an order of magnitude larger than any of the flowback fluids. *Thermococcus* was one of the only thermophilic taxa identified (0.14%).

The microbial community in the source water was very different from that of the flowback samples received and clustered by itself (I) (Table 5-4, Figure 5-9). Of the flowback samples sequenced, the most similar were from 0, 64, 200, 204 and 208 h forming cluster II (Figure 5-9). These are, the first flowback sample, one just before the well was shut in and the first three as soon as flowback was resumed. The community of the sample collected at 0 h was not dominated by any organism in particular. This community had a large fraction of the phylum *Actinobacteria* (22.4%) higher than in the source water (7.8%). This consisted of *Jonesia* (19.9%) and *Tessaracoccus* (2.4%),

neither of which were detected in the source water sample (Table 5-4). *Firmicutes* (14.1%) comprised another large fraction which is up from the 0.3% in the source water. The population in the 0 h sample consisted predominantly of members of the family *Lachnospiraceae* (10.5 %) which were not detected in the source water. *Proteobacteria* (63.4 %) dominated the sample and had a similar abundance as in the source water sample (67.7 %), the majority of which was comprised of *Gammaproteobacteria* (43.1 %) which was up from 21.3 % in the source water with *Pseudomonas* being the dominant member (25.9 %). There was also a large fraction of *Vibrio* (8.5 %) and a large fraction of the genus *Pleomorphomonas* (17.5 %) belonging to the phylum *Alphaproteobacteria* 20.0 % up from 11.6 %. There were no *Betaproteobacteria* (0.0 %) sequences detected in this sample, this was a decrease from the fraction of *Betaproteobacteria* (33.2 %) that was seen in the source water sample. These samples likely clustered together due to the amount of *Pseudomonas* that they contained ranging from 26 % to 79 %.

The next set of samples that were collected from the well from 4 to 60 h clustered together (Figure 5-9: V) and was almost entirely dominated by *Proteobacteria* (84.7-99.9%) (Figure 5-9, Table 5-4). The *Gammaproteobacteria* were almost entirely dominated *Shewanella*. In samples collected from 4 and 8 h after flowback community analysis comprised of only 57 % *Shewanella*. These samples had a large proportion of *Enterobacteriaceae* (35.5 and 23.3 % respectively). These samples had a decreasing amount of extractable gDNA. While the proportion of *Sheawnella* remained high through this period of flowback the biomass concentration decreased. The sample collected at 56 h clustered by itself (IV) due to the large amount of *Dietzia* (57 %) that it containted.

The majority of the samples sequenced clustered together whowever the sample collected at 444 h did not (Figure 5-9). This was the sample collected latest in the flowback period that was successfully sequenced. This sample was the only sample received that was dominated by *Halomonas* (>99 %) and clustered by itself (Figure 5-9: III). This was the only organisms observed that is known to be a halophile. Species of *Halomonas* can endure a wide range of salinities up to 25 % [w/v] and temperatures up to 45ºC (Martínez-Checa et al. 2005). Many species are also able to grow on mannose, galactose and acetate, all compounds that were present during the fracturing process at one point or another.

The fraction of *Deltaproteobacteria* decreased in flowback waters to almost undetectable levels (<0.5 %) (Table 5-4). The only halophilic organisms observed were *Halobacillus* (<5 %), *Halothiobacillus* (<3 %), *Halomonas* (99.6 %). These organisms are typically present in the source water in much smaller fractions. It is not until the later flowback samples that larger fraction of these halophiles are observed, in particularly for *Halomonas*. The only organisms observed to have thermophilic capabilities belonged to the genus *Thermococcus* and *Archaea*. There was no increase in their relative abundance.

5.4 Discussion

5.4.1.1 Effect of Temperature

Similar to sea water injections into hot reservoirs, injection of fracturing fluids into a hot reservoir creates a temperature gradient (Liebensteiner et al. 2014; Okoro et al. 2014; Callbeck et al. 2011; Voordouw et al. 2009). Water is warmed on the surface, up to

40ºC and then injected up to 4 km in to the subsurface. On the path down the wellbore the fluid is initially exposed to cooler temperatures which transition from surface ground temperature to reservoir temperature i.e. 0-70ºC (Majorowicz et al. 2005). The narrow diameter of the well bore and the long distance means that once the fluid reaches down hole it is cooler than the reservoir (Hagoort 2004; Ramey 1962). As the fluid exits the horizontal section and makes its way into the formation the temperature of the water increases (Hoang et al. 2013). As more fluid is injected into the first stage a cooler zone is created that propagates into the formation (Roodhart et al. 1993). When the next stage is fractured, fluid stops flowing into the previous stage and begins flowing into the newer stage. The fluid in the previous stage beings to warm up through heat diffusion, slowly decreasing the size of the cooled zone. This proceeds throughout all of the stages of fracturing. The cooled zone continues to decrease in size as time proceeds during the shut in stage. Once flowback is initiated fluid begins to flow back along the same path that was initially used to fracture the system. This draws warmer water from deeper in the formation causing what is left of the cooled region to decrease at an accelerated rate (Hoang et al. 2013). Flowback will cause the formation to return to its native temperature of 70-110ºC at a quicker rate, which is too hot for most subsurface microbial life. During the injection period, temperatures in the formation may be cool enough for mesophilic life to exist. Sequencing analysis did not reveal a readily apparent thermophilic community (Figure 5-8, Table 5-4). In the initial stages of flowback there was much more biomass than in the source water that was used to formulate the fracturing fluid, likely due to guar gum fermentation (Figure 6-11). Unless the subsurface is acting as a filter to accumulate biomass near the well head, the increase in biomass would have to be

attributed to cell growth downhole (Ross et al. 2001), possibly during the extended period of stopped flow. During the fracturing process there would be very little opportunity for biomass to accumulate because of the rapid injection rate of fluid and short time period of 5 days during which fracturing occurs. Biofilms would have a more difficult time forming during this time due to the high shear rate (Donlan 2002). If any biofilms were able to form or if microbes were to get caught on the surface of a fracture they would be subjected to a continual supply of nutrients fresh, water, and removal of end product metabolites until fracture stimulation was complete. This would present a brief opportunity to increase biomass with favourable temperatures and surrounding conditions. Diffusion would be a limiting factor due to the small volume of fractures (Jardine et al. 1999).

The reduced temperatures are a temporary condition that would exist in hot wells. A different model would be needed for a cooler well that could potentially support mesophilic or thermophilic life indefinitely. When the well is opened for flowback, the biomass is carried out of the well. The amount of biomass observed exiting the well while initially very high, rapidly decreased (Figure 5-5). Additional increases in gDNA were observed as new fracture stages flew back as observed by the tracer data (Figure 5-6). Microbes at the near wellbore region for each of the fractured stages would be in cooled regions.

Once the fluid reached the formation’s temperature, it would only allow for the survival or proliferation of thermophilic microbes, but sequencing did not reveal a substantial thermophilic community (Table 5-4). The increasing temperatures expected

for each of the flowback stages and the lack of thermophiles suggest that temperature plays a role in the amount of organisms recovered in the flowback samples.

Fluids that have reached the formation temperature would cool on their way to the surface of the well bore and in the storage ponds. Surface temperatures would fluctuate based on daily and seasonal factors. Flowback occurred in the winter exposing the flowback waters to close to freezing temperatures upon reaching the surface.

The microbes may have generated a large amount of biomass during the shut in stage before the increasing temperature decreased the growth of mesophilic organisms. Individual cooled zones existed near each of the fractured stages that rapidly decreased during the flowback stage. Here mesophiles identified by sequencing had an opportunity to grow in a reservoir that prior to injection had a temperature only suited for thermophiles.

5.4.1.2 Effect of changing chemical conditions

The hot temperature and transient nature of temperature zones in the fractured formation is only one of the conditions that can affect microbial life and explain some of the observations. Unlike the temperature of the fluid which increases in the formation and then cools on the return to the surface, chemical conditions of the water do not revert to the pre-fractured state when they flowback to the surface nor when they are stored in ponds on the surface (Zolfaghari et al. 2014). Pond conditions only change due to environmental factors such as rain or more treatment.

The salinity in the flowback samples increased over time. While the data set did not show it, the salinity of the injected fracture fluid would have increased from the

moment it was injected into the formation through diffusion. The first flowback sample that returned from the well had a salinity of 0.4 Meq NaCl. This concentration is similar to that of sea water, which was high enough to limit growth of freshwater organisms due to the increased osmotic stress. The increased salinity means that organisms have to divert more energy to maintaining ion concentration gradients (Oren 1999). The concentration of salt increased gradually to 2 Meq NaCl. Only obligate halophiles are capable of growing under these conditions.

The ammonium concentrations in the flowback fluids were very high. High levels of nutrients can contribute to rapid increases in biomass (Howarth & Marino 2006). The higher ammonium concentration in formation waters could have contributed to the large concentration of biomass that were observed in the initial flowback samples. (Figure 5-5).

Fe(III) can be used as an electron acceptor by some organisms (Widdel & Rabus 2001; Lovley & Phillips 1986). Fe(III) had precipitated in later flowback samples. Iron may also have been in the form Fe(II) when it exited the well and may have precipitated out due to oxidation during the storage and transportation of the samples. Fe(III) solubility is highly pH dependent, the lower the pH the greater its solubility (Carroll 1958). The pH at the well head was determined to be 7 throughout the entire flowback period from field sampling (Appendix 4). During sample storage the pH of the samples decreased to the values observed (Figure 5-2: C), likely due to the reaction of dissolved Fe(II) with oxygen forming ferric (oxy)hydroxide species (Millero et al. 1987). These reactions result in a reduced pH which is a common observation in open pit storage ponds. Decreases in pH vary, but are often lower by a couple of units, which would be attributed to the oxidation of Fe(II), especially since the fracturing fluids are buffered to a

pH of 7 before they are used (Rahim & Holditch 2003). The amount of iron in solution can also be affected by the presence of other molecules such as barium and sulfate. These molecules form highly insoluble compounds which can precipitate within the formation (Barbot et al. 2013).

Of the organic acids measured acetate had the largest change. Unlike sulfide and iron, changes in acetate concentration are dependent on biological activity. Acetate is formed from the metabolism of larger compounds. Shale formations inherently have large amounts of organic matter which could be metabolized (Riediger et al. 1990). Large amounts of carbon are also introduced through the fracturing process due to carbohydrates that are used to formulate the fracturing fluid, in particular, guar gum. The amount of guar gum used to formulate the fracturing fluid was not disclosed, but its use can range from 4-12 L/m^3 of fracture fluid. The lack of guar gum in flowback waters is partially a result of the breaker that were used to reduce the viscosity of the fluid, easing the flowback process. Acetate was not present in the source water used, nor was it observed in the first 64 h of flowback water. The constant concentration of 0.4 mM acetate was surprising, if it had originated from the formation it would have been expected that it would have followed the same trend as ammonium and salinity (Figure 5-2, Figire 5-3).

Salinity and ammonium concentration of flowback changed similarly throughout flowback. Even when flowback was halted between 68 and 200 h the concentration of these solutes continued to increase at a constant rate suggesting that formation exposure time is the factors contributing greatest to solute dissolution into the fracture fluid. Different fracture stages flowing back do not affect the rate at which salinity increased. If

they did, the rate at which the concentration of these dissolved solutes would be different at the same time that changes in gDNA were observed and new stages were observed to flowback.

5.4.1.3 Microbial composition

The process of hydraulic fracturing occurs at a rapid rate, subjecting microbes to greatly different conditions than they were subjected to in the source water. This was demonstrated by the decrease in both number of OTUs and the eveness of the community (Table 5-3). The source water, having the most even microbial community had a higher fraction of *Proteobacteria*, including *Gammaproteobacteria*, *Deltaproteobacteria*, *Betaproteobacteria*, and *Alphaproteobacteria*. There was also a small amount of *Planctomycetes* (Table 5-4). The source water sample showed the highest number of eukaryotic reads by over an order of magnitude. The primers used for sequencing were not designed for the 18S rDNA gene of eukaryotes but were able to amplify some *Eukaryotic* reads (Table 5-4). The proportion of eukaryotic reads would be expected to be underestimated based on the sequencing performed. Universal eukaryotic primers were used to determine if there was a large fraction of eukaryotic sequences that were being missed. Not enough PCR product was obtained to send for sequencing suggesting that eukaryotic sequences represent a small portion of the source water community.

The elevated biomass in the initial flowback waters was attributed to microbial growth. In the 0 h sample where gDNA was highest (Figure 5-5). The fraction of *Alphaprototeobacteria* was higher than in the source water (Table 5-4). *Alphaproteobacteria* are common in marine environments with low amounts of nutrients,

as many of these organisms are oligotrophs (Bouvier & Giorgio 2002; Fuchs et al. 1999). These conditions are short lived due to the increasing salinity and increased concentrations of nutrients.

Despite having similar nutritional requirements as *Alphaproteobacteria* (Stackebrandt & Murray 1991), *Betaproteobacteria* were almost entirely absent from early flowback samples (Table 5-4). The fact that *Betaproteobacteria* are not typically found in environments with elevated salinity accounts for their absence in flowback waters, which have salinities ranging from 0.5-2.0 Meq NaCl.

Gammaproteobacteria represented 21.3 % of the source water community (Table 5-4). By 0 and 4 h after flowback they represent more than 40 and 90 % of the microbial community respectively, *Gammaproteobacteria* are predominantly facultative anaerobic bacteria allowing them to be metabolically suited for both the surface and the subsurface environment (Stackebrandt & Murray 1991). At 0 h after flowback where gDNA was highest, the community was dominated by *Pseudomonas* and *Vibrio* species. *Pseudomonas* is a facultative aerobe capable of hydrocarbon degradion that is commonly isolated from oil fields while *Vibrio* is a facultative marine fermenter (Sánchez et al. 2014; Park et al. 2011; Shieh et al. 2000).

Flowback waters collected at 12 h after flowback began were almost entirely dominated by *Shewanella* (Table 5-4, Figure 5-8). Their fractions remained high until the well was shut in at 68 h. The relative abundance of *Shewanella* remained high but the amount of gDNA decreased indicating that their numbers were diminishing. This is likely due to the increasing temperature as only one species of *Shewanella* has been observed to have thermophilic activity (Ghosh et al. 2003). *Shewanella* species have been isolated

from a variety of freshwater marine environments, from as deep as 9000 m, shale rocks as well as oil pipelines (Fredrickson et al. 2008; Ghosh et al. 2003). *Shewanella* is suited to tolerate much of the ionic stress and pressure that it would be subjected to while down hole. The metabolic capabilities *Shewanella* are very diverse allowing it to utilize a large variety of substrates, this includes acetate and lactate (Hansel et al. 2003). Of the electron acceptors tested, acetate was absent when *Shewanella* was present and observed after they disappeared. Acetate was not introduced with the fracturing fluid (Table 5-1) meaning that it must have arisen either from the metabolism of carbohydrates that were introduced as part of the fracturing fluid, or must have originated from within the formation.

Shewanella can utilize a large variety of electron acceptors this includes but is not limited to nitrate, thiosulfate , iron(III) and a variety of heavy metals making it an ideal candidate for bioremediation (Fredrickson et al. 2008; Ghosh et al. 2003). Sulfate is not used as an electron acceptor but *Shewanella* is able to reduce sulfur containing compounds such as thiosulfate. The reduction of thiosulfate by *Shewanella* is sometimes used as a diagnostic test (Fredrickson et al. 2008). The ability of *Shewanella* to use thiosulfate and not sulfate means that it could have been missed in the SRB MPN test that was performed which would explain the low SRB counts and the high amount of gDNA and relative abundance of *Shewanella* which could result in souring. Thiosulfate is not overly abundant in the subsurface; any sulfide formed would readily precipitate out with iron which is in abundance in flowback water.

The ability of *Shewanella* to perform dissimilatory Fe(III) reduction is typical for *Shewanella* and *Geobacter* (Lovley 2006). Iron cycling is a biotic and abiotic process that

is heavily driven by microbial activity. *Shewanella* is able to use forms of poorly crystalline Fe(III) as a terminal electron acceptor. The insoluble nature of iron requires special mechanisms to transfer electrons to the solid phase iron such as direct contact mechanisms or electron shuttles (Lovley 2006). Insoluble ferric (oxy)hydroxides may have formed by oxygen exposure during storage that may have led to the growth of *Shewanella* during the storage period and increased both the relative abundance of *Shewanella* in the sample as well as the amount of gDNA recovered. This could have also resulted in the metabolism of acetate in the samples where it was not detected. These findings exemplify the importance of receiving and analysing samples as quickly as possible once they are collected.

Shewanella disappeared almost entirely at 64 h after flowback (Table 5-4, Figure 5-8). The community became dominated by *Pseudomonas,* which was the predominant community member until 208 h after flowback (41-91 %). The amount of recovered gDNA was almost undetectable for these samples (Figure 5-5). *Pseudomonas* species are facultative aerobes with some strains being able to use nitrate as a terminal electron acceptor (Fewson & Nicholas 1961). It should be noted that species of *Pseudomonas* have been identified that are capable of using Fe(III) as an electron acceptor which could also explain its presence in the flowback samples (Obuekwe et al. 1981; Arnold et al. 1988).

The microbial community sampled at 444 h after flowback was almost entirely dominated by *Halomonas* (99.6 %). This is the only substantial halophilic component observed. The genera *Halomonas* and *Halanaerobium* are commonly identified in later flowback samples (Cluff et al. 2014; Murali Mohan et al. 2013). *Halomonas* has been

identified in high temperature wells and is able to grow up to 45°C and at salinities above 4 M (Martínez-Checa et al. 2005; Orphan et al. 2000). Some strains are also capable of producing H_2S (Martínez-Checa et al. 2005). Many strains are capable of growing on mannose and galactose, which are the monomers that make up guar gum. *Halomonas* is a facultative aerobe that can use acetate, nitrate and nitrite and grow by fermentation (Garrity et al. 2005b).

Deltaproteobacteria were found in their highest relative abundance in the source water at 1.3 %. This is a small portion of the community, no *Deltaproteobacteria* were found during flowback (Table 5-4). The lack of sulfate detected would not support the growth of sulfate reducing bacteria.

5.5 Conclusion

The sample set received allowed an in depth analyses of microbes in a hydraulically fractured operation in Canada for the first 500 h after fluids began to flowback. Concentrations of dissolved solutes were high in flowback waters increased regardless of the contribution of different fractured stages. There was a short period of time after fracturing when the formation was cool enough to allow microbial growth. These occurred during the initial flowback of each of the fractured stages and had increased amounts of recoverable gDNA. There was an increase in the abundance of halophiles towards the end of the sampling period, which has also been observed in other wells. The resident temperature of the Montney is too hot to support mesophilic microbes. The lack of thermophilic growth as indicated above by the lack of thermophilic

sequences and by the low amounts of gDNA, supports the idea that downhole growth is not supported after initial flowback.

Chapter Six: Metabolic activity of microbial communities associated with hydraulic fracturing

6.1 Introduction

The analysis of microbial communities using sequencing techniques offers invaluable insight to the members of the community and allows for subsequent interpretation of the communities' activity. However sequencing is no substitute for activity testing to determine how a microbial community will behave under a specific set of conditions. Testing microbial metabolism under different conditions, processes that may occur under simulated field conditions can be observed. By testing microbial activity under different nutrient conditions, temperatures, and salinities, help to better understand what might occur *in situ*.

Microbial communities associated with hydraulic fracturing (source water, flowback water and separator water) were tested for by their ability to metabolize different electron acceptors and donors to determine how they may impact a hydraulic fracturing operation. Whether or not the microbial communities associated with hydraulic fracturing can utilize guar gum, acetate, nitrate and sulfate were evaluated. Whether or not the microbial communities associated with hydraulic fracturing contribute to corrosion or are inhibited by the presence of hydrocarbons were also evaluated.

6.2 Nitrate utilization in the presence of acetate under mesophilic conditions

Nitrate is a common additive in oil fields being subjected to water flooding as a method of controlling SRB populations (Hubert & Voordouw 2007; Myhr et al. 2002;

Gieg et al. 2011; Voordouw et al. 2009). Nitrate is an electron acceptor that offers a greater amount of free energy for NRB than sulfate does for SRB (Weber et al. 2006). This allows NRB to outcompete SRB for organic compounds and other essential nutrients (Hubert & Voordouw 2007). The major benefit of exploiting NRB is their ability to convert nitrate to nitrite which is a potent inhibitor of SRBs DsrAB enzyme responsible for the final catalytic step in converting sulfate to sulfide in the dissimilatory sulfate reduction pathway (Gieg et al. 2011; Kaster et al. 2007). In the presence of nitrite SRB levels are greatly reduced. If the microbial communities present in flowback waters are able to utilise nitrate and produce nitrite, it is possible that if ever SRB and souring became a problem nitrate could be applied to control them as opposed to adding more biocide.

6.2.1 Methods

As per Table 3-10 #1 samples from Kobes source water, Kobes flowback water from 0, 8, 16, 64 and 68 h after flowback and SH_SWL, SWS and FBW from Fox Creek were used as inoculum. In 100 ml serum bottles, 5 ml of sample was used to inoculate 45 ml of CSBK-0.5 which had a salinity of 0.5 M NaCl. The head space was made anaerobic with N_2CO_2 and the bottles were closed with a butyl rubber stopper and aluminum crimped seal. Acetate (200 µl of 2.5 M) to a final concentration of 10 mM, and nitrate (160 µl 5.0 M) to a final concentration of 16 mM were added through the butyl rubber stoppers using syringes that were flushed with N_2CO_2 gas (Table 3-6). These were incubated in the dark at 30ºC, while being shaken at 125 RPM.

6.2.2 Results

Source and flowback waters were tested that were received from Kobes and Fox Creek locations. Source water samples that have not been used for hydraulic fracturing are K_Source, SH_SWS and SH_SWL (Table 3-1, Table 3-2). The only one of these samples to show any nitrate reduction was SH_SWL which was a freshwater sample collected from an open pit pond. Nitrate reduction was not observed in sample SH_FBW, even though that the source water used to make the fracturing fluid (SH_SWL) showed the ability to reduce nitrate (Figure 6-1). The ability of the microbial community to reduce nitrate was not retained during the fracturing process as no nitrate reducing activity was observed in SH_FBW.

Sample K_0 h which was the first flowback water sample collected from the Kobes well coupled acetate oxidation to nitrate reduction at a rapid rate without nitrite being observed (Figure 6-2). In sample K_68 h nitrate and acetate were consumed after 40 days. This took much longer than K_0 h that was collected. Nitrite was also produced in this sample and persisted in the experiment until its end at 80 days (Figure 6-2). Sample SH_SWL like K_0 h showed rapid consumption of both acetate and nitrate consumed very rapidly with nitrite accumulating in the system by 7 days. After 7 days the concentration of nitrite in K_68 began to drop. At 40 days only half of the nitrite had been consumed, and remained constant for the remainder of the incubation.

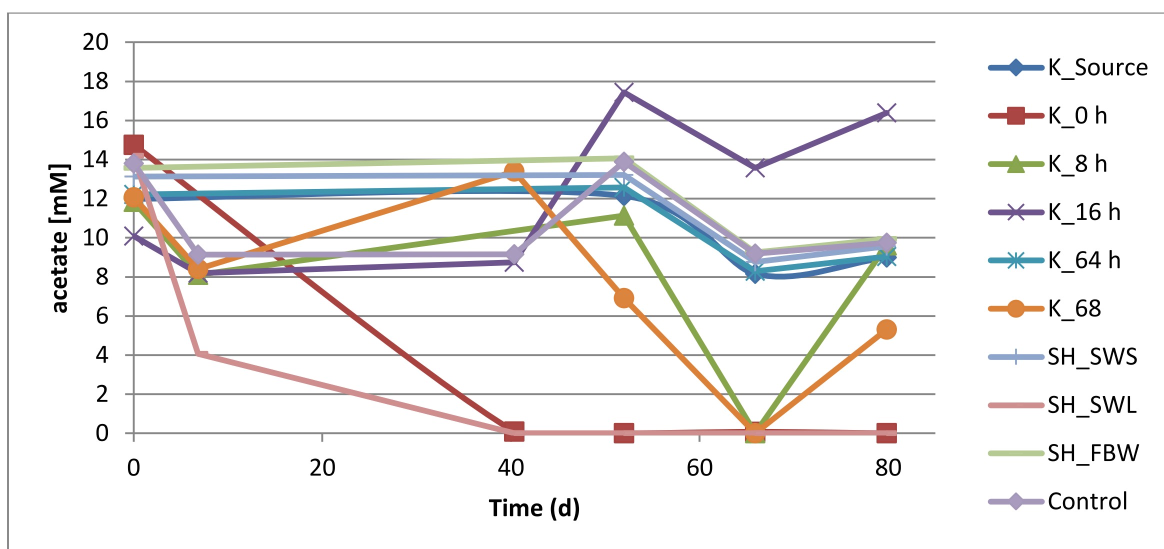


Figure 6-1: Change in acetate concentration in the presence of nitrate incubated with either source or flowback waters received from Kobes and Fox Creek at 30ºC and 0.5 M NaCl.

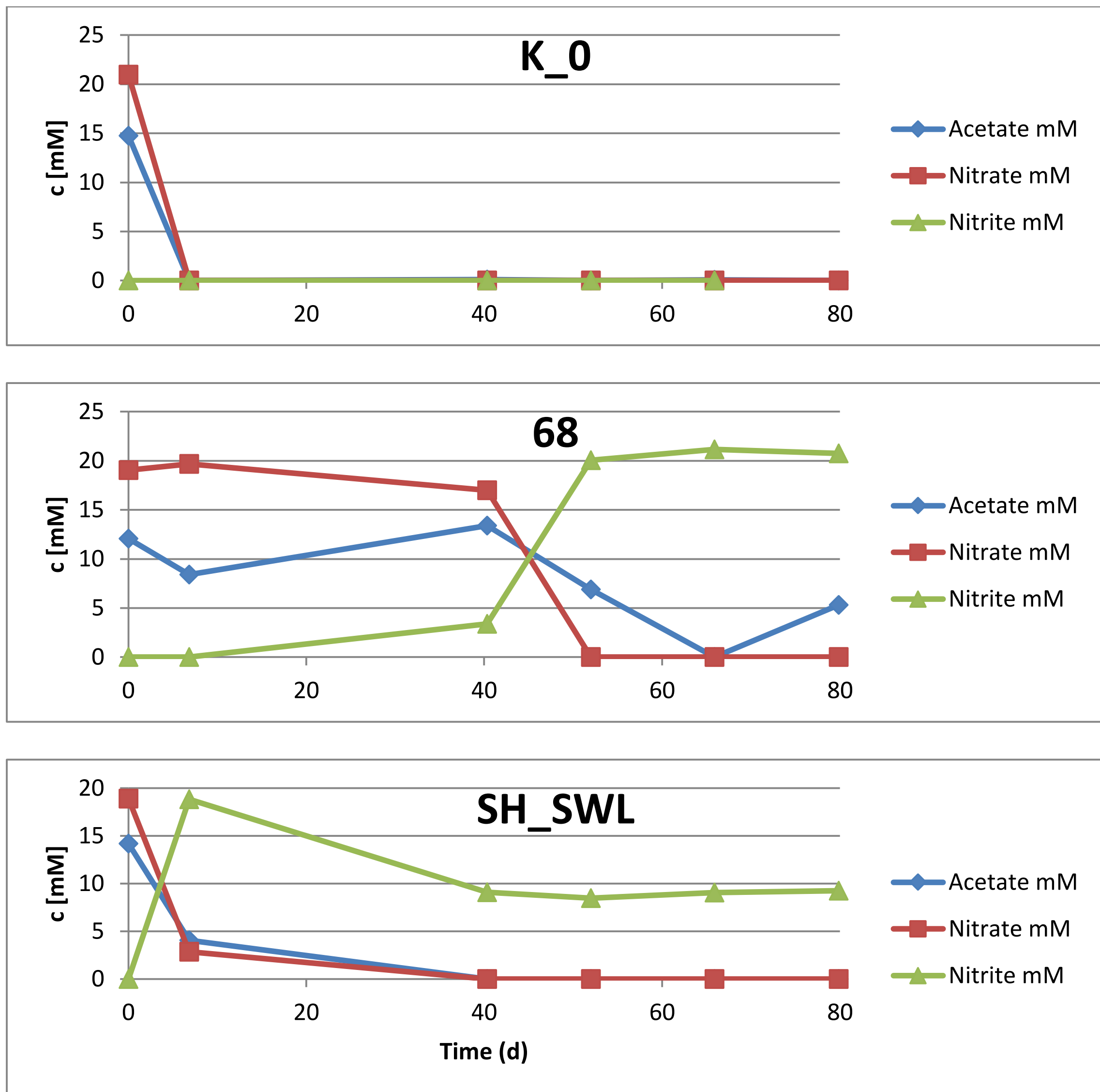


Figure 6-2: Change in acetate and nitrate concentrations in incubations that showed acetate utilization in the presence of nitrate.

6.2.3 Summary

Heterotrophic nitrate-reducing bacteria (hNRB), using acetate as an electron donor were found in waters obtained following hydraulic fracturing. While organisms that are capable of reducing nitrate to nitrite can survive the fracturing process they are more active when they have been exposed to the formation for a reduced amount of time. The higher activities that were observed for K_0 h and SH_SWL were likely the result of larger biomass concentrations in the inoculum (Figure 5-5). K_0 h had the largest biomass concentration and reduced nitrate without nitrite accumulation.

6.3 Sulfide production

The production of sulfide is not expected due to the lack of sulfate and SRB as shown by both 454 sequencing and MPN tests as previously described (Figure 5-3: B, Figure 5-5). These tests do not take in to account growth on substrates other than lactate or rare organisms that could have been missed or overlooked in the 454 analysis. The only organic acid that was detected in any substantial quantity was acetate and this may be used by a variety of organisms, including autotrophic methanogens. Samples were tested from Kobes flowback and Fox creek.

6.3.1 Methods

As per Table 3-10:2 samples from Kobes source water, Kobes flowback water from K_0, 4, 8, 364 and 496 h (Table 3-2) after flowback and SH_SWS, SWL and FBW from Fox Creek were used (Table 3-1). In 100 ml serum bottles, 5 ml of sample was used to inoculate 45 ml of CSBK which had a salinity of 0.026 M NaCl. The head space was

made anaerobic with N_2CO_2 and the bottle was closed with a butyl rubber stopper and aluminum crimped seal. Acetate (200 µl of 2.5 M) to a final concentration of 10 mM and sulfate (200 µl 2.5 M) to a final concentration of 10 mM were added through the butyl rubber stoppers using syringes that were flushed with N_2CO_2 gas (Table 3-10: 2). These were incubated at in the dark at 30ºC, while being shaken at 125 RPM.

6.3.2 Results

The ability of organisms to utilize acetate as a sole electron donor in the presence of sulfate was rare in the samples tested. Only one of the source water samples (SH_SWL), had such activity which took 30 days before any sulfidogenic activity was observed (Figure 6-3). This activity was not seen in the flowback water sample SH_FBW. The SRB that produced this activity did not survive the fracturing process and were eliminated from the community. The acetate and sulfate were consumed and sulfide was produced in 1:1 stoichiometric ratio as would be expected (Figure 6-4).

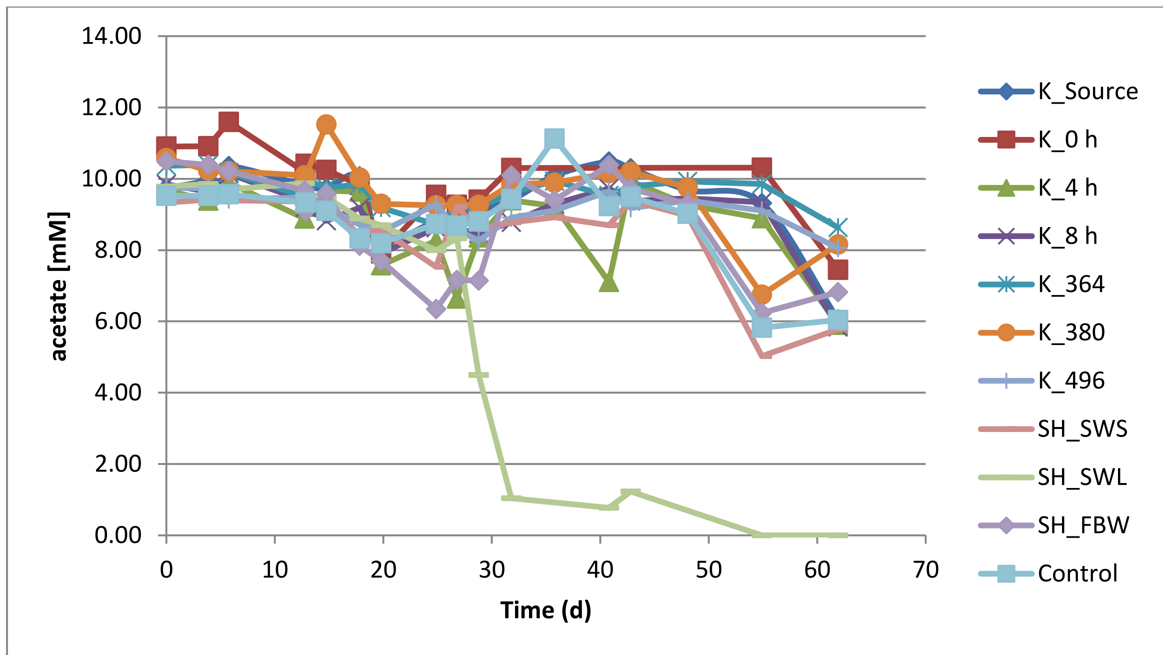


Figure 6-3: Reduction of acetate concentration in the presence of sulfate.

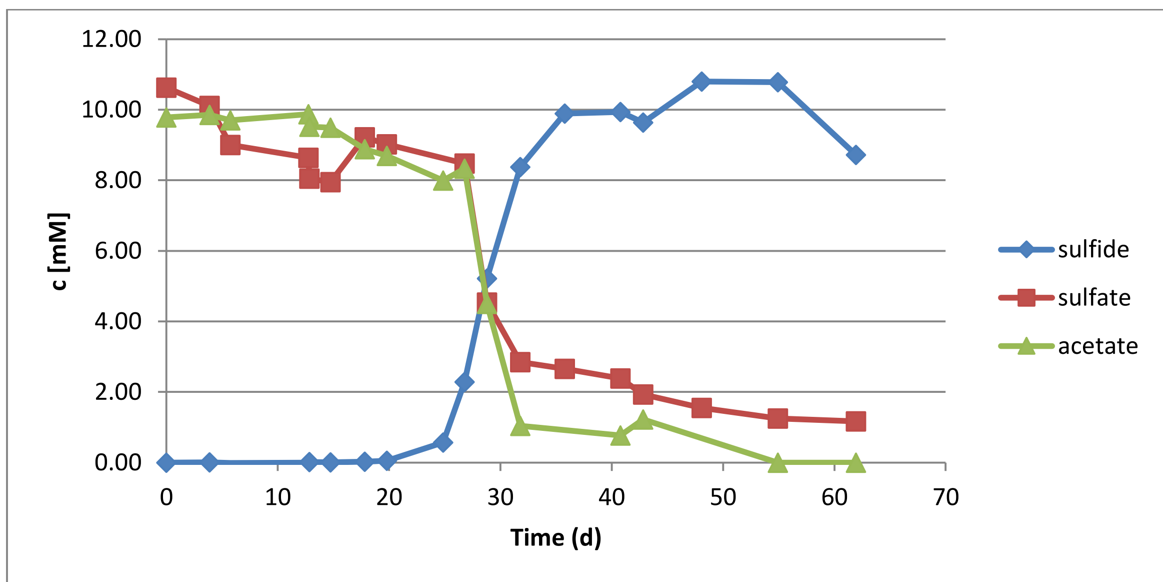


Figure 6-4: Acetate and sulfate consumption to produce sulfide in source water sample SH_SWL.

6.4 Guar Gum utilization

6.4.1 Introduction

Guar gum is commonly used throughout hydraulic fracturing operations for increasing the viscosity of the fracturing fluid. The increased viscosity helps proppant (sand) to remain suspended in the fluid and helps deliver and distribute it throughout the formation. Guar gum is a large molecular weight galactomannan with mannose:galactose ratios ranging from 1.55 to 2 (Weaver et al. 2003; Hong et al. 2010; Wientjes et al. 2000; Kawamura 2008). The larger the molecular weight of guar gum molecules the greater the viscosity of the fluid (Wientjes et al. 2000). Other factors that affect viscosity include temperature, the presence of other chemical species, polymer-polymer interactions and shear force (Robinson et al. 1982; Morris et al. 1981). Viscosity decreases with temperature, the greatest demonstration of this is bitumen which is almost solid at room temperature but begins to flow once it is heated through Steam Assisted Gravity Drainage (Cyr et al. 2001). Chemical interactions can change the folding of polymers, either increasing or decreasing the overall volume that the polymer occupies, a larger volume results in a higher viscosity. If there are no charged groups on the polymer like guar gum, it is not drastically affected by ionic interactions (Ma & Pawlik 2007). Enough guar gum is added to hydraulic fracturing fluids to have a viscosity of 100-1000 of cP, in comparison to the viscosity of water which is 1 cP.

6.4.2 Changes in Viscosity and Guar Gum Concentrations in Groundbirch Samples

Only one of the samples collected from Groundbirch was from a separator handling fluids from a well exposed to guar gum when fractured (WSP2_GG) (Table 3-1). Since fracturing the well has not been exposed to guar gum in many years the microbial community composition does not reflect this as there are many similarities to the sample that was not fractured with guar gum (Figure 4-1).

6.4.2.1 Methods

Samples collected from Groundbirch were incubated with CSBK medium amended with 0.2% [w/v] of guar gum (Table 3-3, Table 3-1:1-7). Sulfide was typically added to these incubations but its addition resulted in the rapid reduction in the viscosity of the media and the degradation of guar gum. Hence it was omitted from the incubation (Brown & Norris 1994). In 100 ml serum bottles, 5 ml of sample was used to inoculate 45 ml of CSBK which had a salinity of 0.026 M NaCl. The head space was made anoxic with N_2CO_2 and the bottle was closed with a butyl rubber stopper and aluminum crimped seal. These were incubated at in the dark at 30ºC, being shaken at 125 RPM. Sample (1 ml) was removed from the serum bottle by inserting a syringe through the butyl rubber stopper to get a sample for the viscosity measurements which were performed under aerobic conditions. The concentration of guar gum was determined using the enzymatic method described in section 3.4.7.

6.4.2.2 Results

Concentrations of guar gum remained high for the first few days of incubation and then decreased to 0 % [w/v] with the exception of the incubation with WS1_PAM (Figure 6-5: Top). The community present in this sample was incapable of degrading guar gum in the 26 day time frame. The decrease in guar gum concentration did not correlate identically to the decrease in viscosity. The viscosity in sample WSP2_GG, which had been previously exposed to a well that was fractured with guar gum, and AB2_WS, a treated waste water sample, decreased to that of water (1 cP) after 2 days of incubation. The viscosity of WSP2 and AB1_WS decreased to almost that of water by day 3 of incubation. The viscosity of WSP1 and FWSP1 took longer to decrease. The viscosity of the control and WS1_PAM remained high for most of the 26 day incubation time with final values of 4 and 7 cP respectively (Figure 6-5).

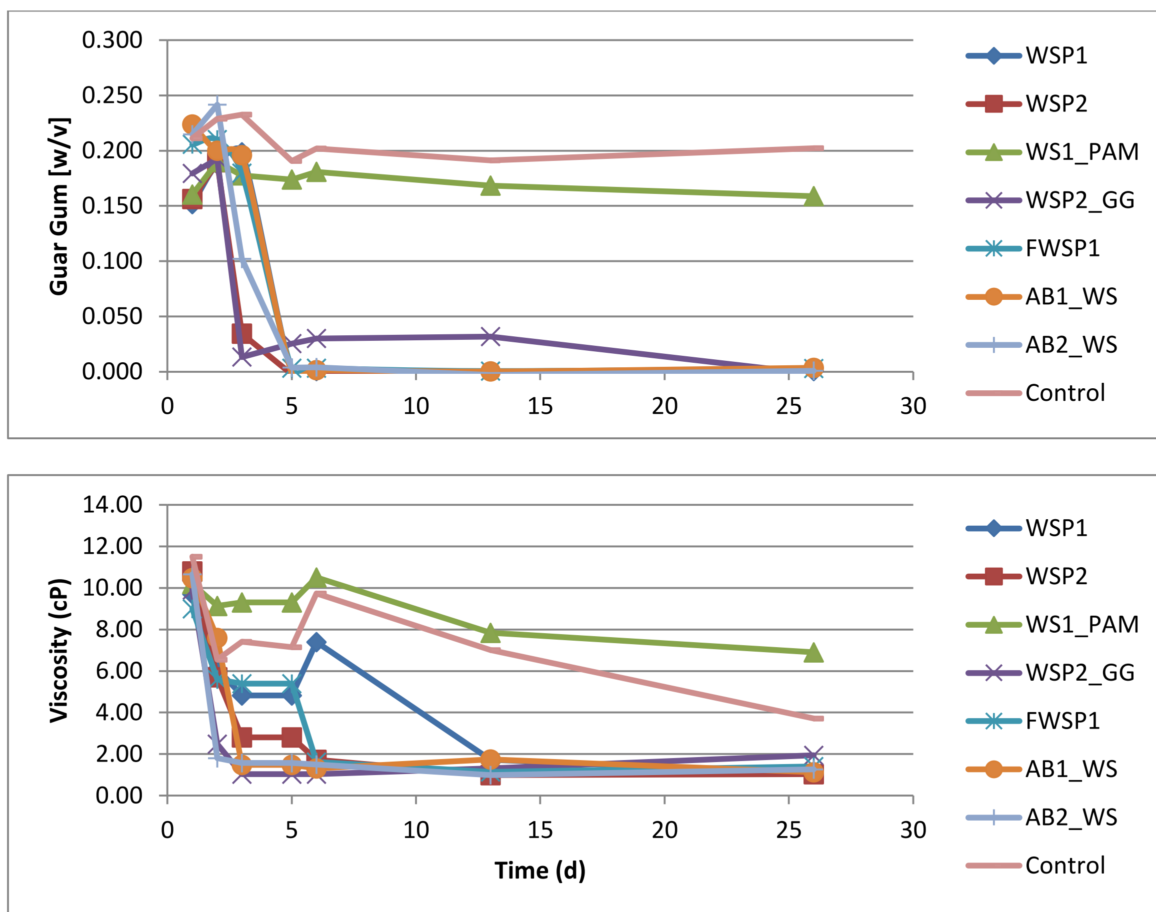


Figure 6-5: Changes in (top) guar gum concentrations and (bottom) viscosity with time for incubations with Groundbirch samples.

6.4.2.3 Discussion

It was originally thought that changes in viscosity could be used as a proxy for microbial metabolism in a well that was fractured using guar gum. This method is not able to be applied for this purpose due to breakers that are employed to degrade polymers in wells after they have been fractured. A breaker, typically an oxidizing agent for high temperature wells, is employed to degrade the polymer's backbone and reduce the viscosity making it easier for the fluids in the well to flow back to the surface (Sullivan et al. 2004). This also makes the polymer more available for microbial metabolism as smaller molecules are easier to metabolise than larger ones (Reddy & Tammishetti 2004). There are many other factors in the formation that can affect the viscosity of polymers such as salinity (Morris et al. 1981; Ryles 1988).

There are two methods of degradation that microbes can employ on polymers, endo- and exo- enzyme degradation (Wheatley & Moo-Young 1977). Exo- degradation attacks terminal residues of the polymer chain while endo- degradation targets the backbone at random which results in a more rapid decrease in molecular weight. A combination of both mechanisms results in a more rapid synergistic breakdown in molecular weight. The rapid decrease in viscosity for samples WSP2_GG and AB2_WS relative to the decrease in guar gum concentration is likely due to members of the microbial population attacking the backbone of the polymer (Figure 6-5). Other samples such as FWSP1 which had the viscosity and guar gum concentration decrease at a similar rate were likely degraded systematically from the end of the polymer. The degradation is likely a combination of both processes.

Even though WSP1 and WSP2 were collected from the same pond the results suggest different types of microbial degradation with exodegradation dominating WSP1 and endodegradation dominating WSP2. Guar gum was not being degraded in the incubation with WSP1_PAM. WSP2_GG had both guar gum concentration and viscosity decrease at a rapid rate suggesting that the backbone is being degraded along with the ends. This is not surprising as the sample originated from a well that was fractured with guar gum, this is likely what initiated the seed community (Murali Mohan et al. 2013). Incubation with FWSP1, the freshwater source water sample had its viscosity and guar gum concentration decrease at a similar rate suggesting that the polymer was degraded almost entirely by microbes attacking the ends. Changes in viscosity and relative guar gum concentration can be used to infer the type of microbial activity being used to degrade polymers but has limited use for making field observations.

6.4.3 Degradation of guar gum in Kobes samples

Guar gum was used to fracture the Kobes well from which samples were obtained. While there was no nitrate or sulfate detected, the samples were tested using those electron acceptors to determine how much of an issue sulfate would present if the flowback waters from this well were to be reused for another fracturing job that containe sulfate. Testing guar gum degradation in the presence of nitrate was also done to determine how the community would respond to souring control through the addition of nitrate. There is also the concern that if used, nitrate would allow microbes to produce more biomass than they would under fermenting conditions, resulting in decreased porosity. Changes in APB were measured in each of the three samples under fermenting

conditions. After 21 days an APB count was performed using the microtiter plate as described in section 3.6.2: Most probable number (MPN) of APB and SRB. Incubations were performed using low salinity medium at 30ºC.

6.4.3.1 Methods

Kobes samples collected at 8, 32, and 56 h were used to evaluate the ability of microbial communities to utilize guar gum under fermentative, nitrate and sulfate reducing conditions. Sample (5 ml) and CSBK-0.5M-0.1GG (45 ml, Table 3-4) were incubated in 100 ml serum bottles sealed with butyl rubber stoppers and a N_2CO_2 head space at 30ºC in the dark. Either sulfate (200 µl of 2.5 M) or nitrate (100 µl of 5.0 M) (Table 3-6) were added to their respective incubations for a final concentration of 10 mM (Table 3-10: 4,5,6). Guar gum concentration was measured using the phenol sulfuric acid method (Bradley et al. 1989).

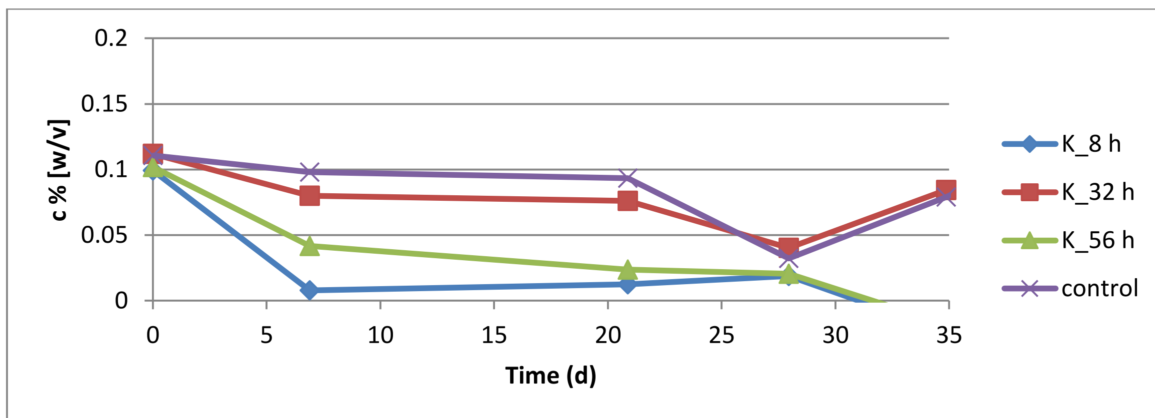
6.4.3.2 Results

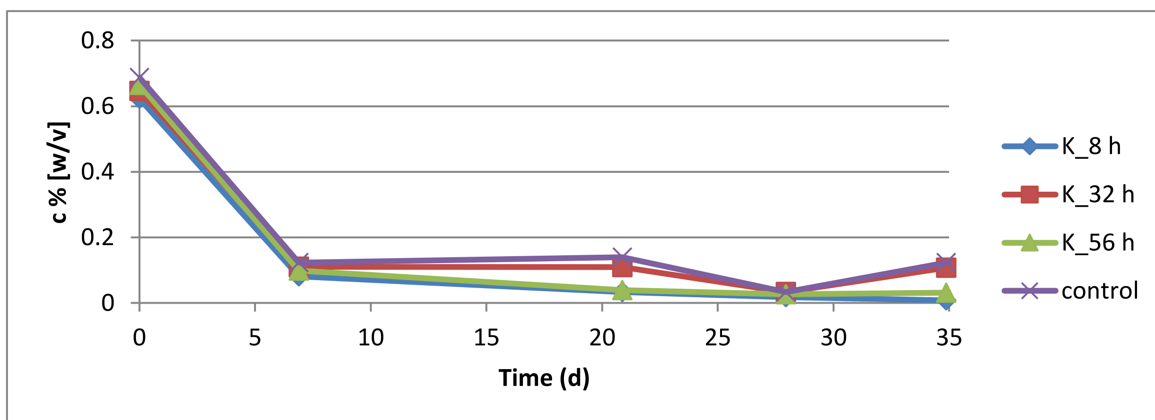
Guar gum appeared to be degraded under fermentation conditions during incubations with K_8 h and K_56 h, but not during incubation with K_32 h and the control (Figure 6-6: Top). In the presence of nitrate or sulfate, guar gum also appeared to have been used more at 35 days in incubations with K_8 h and K_56 h than the incubation with K_32 h and control (Figure 6-6). No nitrate or sulfate reduction was observed under these conditions (not shown). In the presence of sulfate and nitrate (Figure 6-6) similar trends for guar gum degradation as under fermenting conditions were

observed. Hence the changes in guar gum concentration were due to fermentation in all conditions indicated in Figure 6-6.

Under fermenting conditions (Figure 6-6: A) the production of lactate was shown in incubations with K_56 h (Figure 6-7). The production of acetate was found mostly with K_8 h and K_56 h (Figure 6-8) whereas the production of propionate and butyrate were shown for the incubation with K_8 h. Hence, (with the exception of K_32 h) the formation of fermentation products are in agreement with the consumption of guar gum due to fermentation (Figure 6-6).

APB in samples K_8 h, K_32 h and K_56 h had increased MPNs after growth on guar gum as a sole carbon source and energy source. APB increased 2 fold in K_ 8 h, 3.2 fold in K_ 32 h and a 20 fold in K_ 56 h (Figure 6-11).





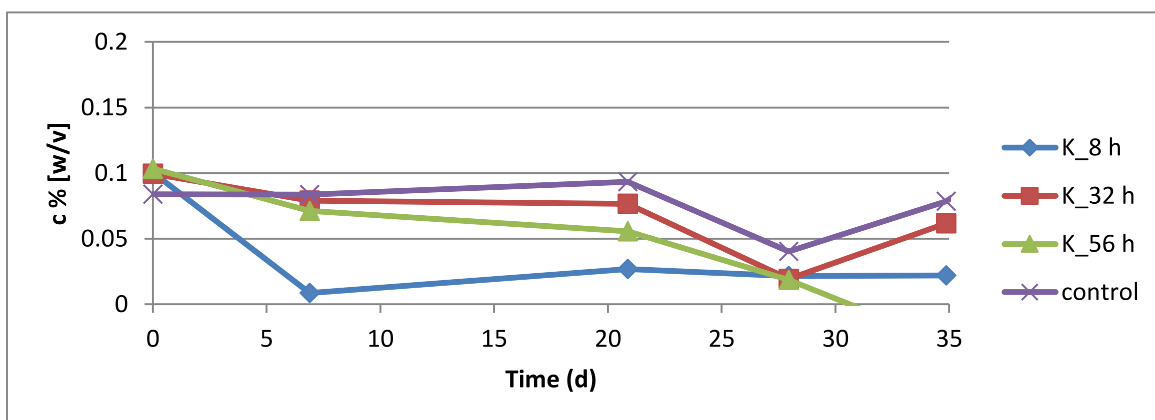


Figure 6-6: Guar gum degradation under (top) fermenting, (middle) nitrate-reducing conditions and (bottom) sulfate-reducing conditions.

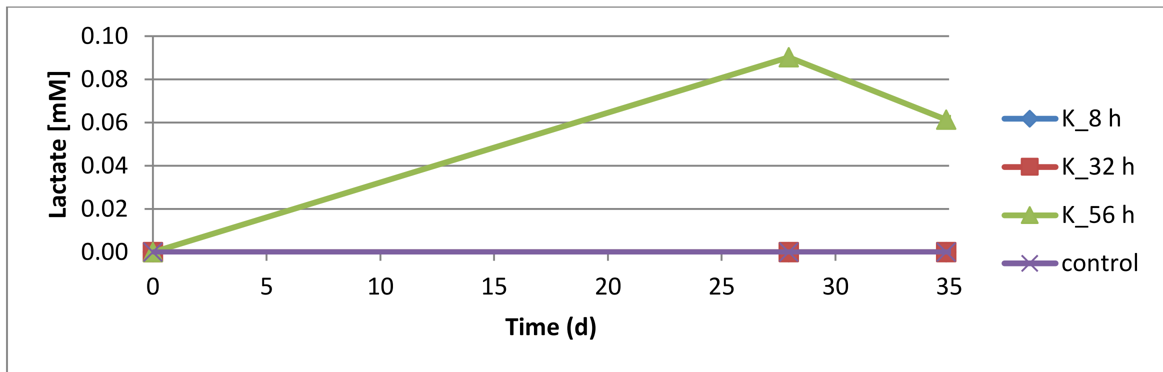


Figure 6-7: Lactate production in the presence of guar gum under fermenting conditions.

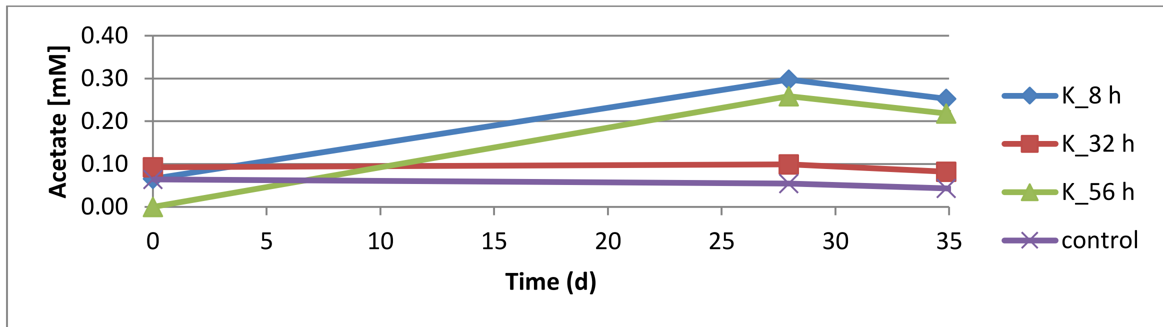


Figure 6-8: Acetate production in the presence of guar gum under fermenting conditions.

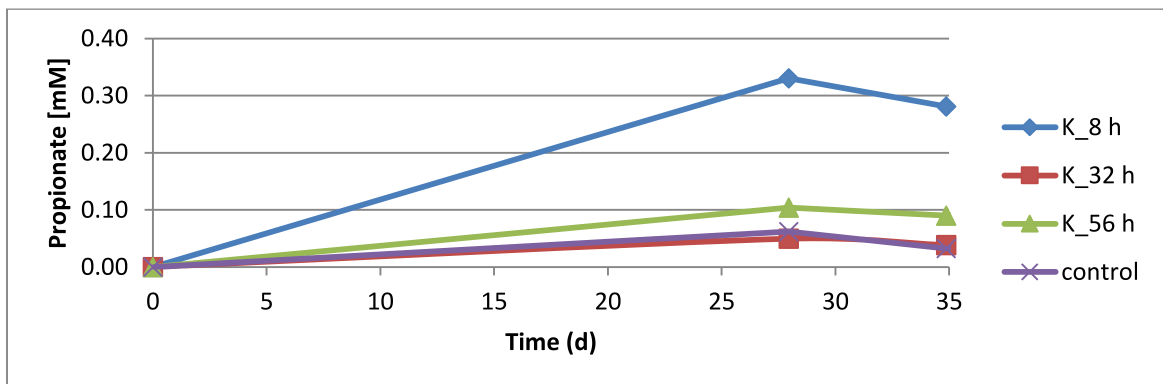


Figure 6-9: Propionate production in the presence of guar gum under fermenting conditions.

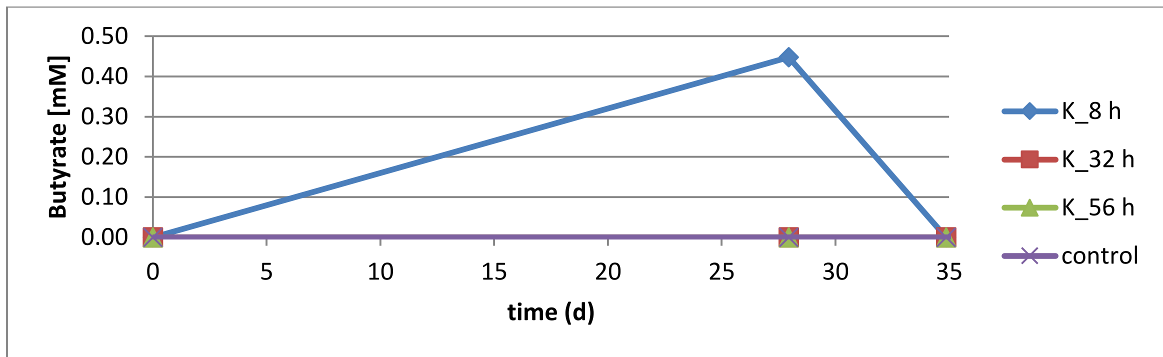


Figure 6-10: Butyrate production in the presence of acetate under fermenting conditions.

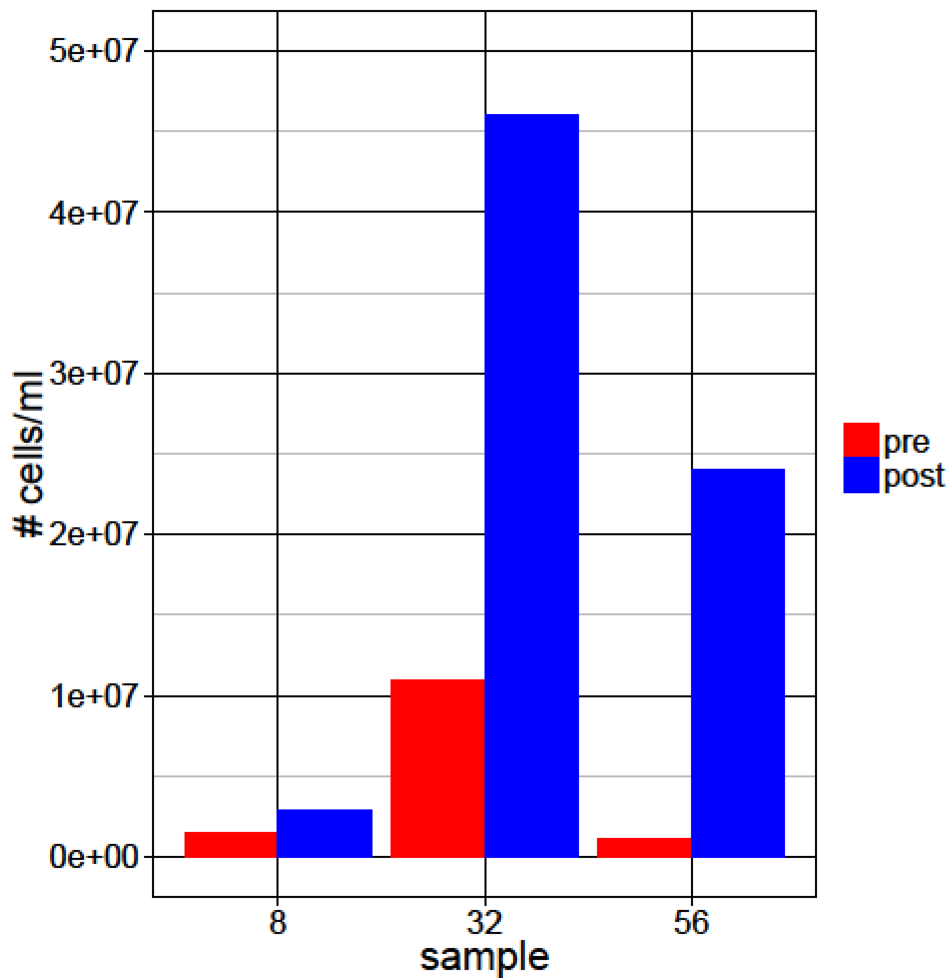


Figure 6-11: MPN counts of APB before and after growth on guar gum when incubated in CSB-K for 21 days at 30ºC.

6.4.3.3 Discussion

Under both nitrate and sulfate reducing conditions sulfate and nitrate were not reduced and the degradation of guar gum was similar to that observed under fermenting conditions. This agreed with both the SRB MPN test and the community sequencing data, which indicated a lack of SRB. The lack of organisms capable of using guar gum or its fermentation products as electron donors for nitrate reduction is surprising. Fermentative guar gum degradation occurred in samples K_8 h and K_56 h but not in sample K_32 h. The lack of guar gum degradation in the sample collected at 32 h after flowback is not in agreement with the increase seen using MPN counts (Figure 6-11). Replication of this experiment could have clarified the findings. Acetate production from guar gum was observed in both samples K_8 h and K_56 h. This was the only organic acid that was observed in the flowback water samples from 64 h onwards (Ficure 5-3: C). The microbial community in the flowback samples has the ability to produce acetate from guar gum at 0.5 M Meq NaCl. The absence of acetate in the earlier samples received suggest that the microbial community is capable of consuming acetate. As the conditions become less favourable due to increasing temperature and salinity the microbial community is no longer capable of using acetate, hence its accumulation in later flowback waters.

The production of propionate was observed in the samples K_8 h and K_56 h especially in the incubation with the sample from K_8 h after flowback (Figure 6-9). Butyrate was only detected in the incubation with sample K_8 h (Figure 6-10). These results suggest that the organic acids do not persist in the well for any length of time as they are consumed more readily than they are produced. The metabolism of guar gum

would explain the large amounts of biomass that were observed in initial flowback water samples received as shown by the increase in MPN numbers (Figure 6-11). The increased biomass concentrations that were able to be generated in the experiment using later flowback water samples support the theory that prolonged exposure to the conditions of hydraulic fracturing stimulates the formation of a seed community that is suited for guar gum fermentation at low salinities. This could be disadvantageous when reusing flowback waters for subsequent hydraulic fracturing operations. This same observation was not made in the samples received because the downhole conditions restricted microbial growth due to the harsh conditions.

6.5 Halophilic Organisms

6.5.1 Introduction

Due to the high salinity of the flowback samples received the presence of halophiles would be expected. However, sequencing revealed very few microbes with halophilic capabilities. Also, low amounts of gDNA were present in high salinity flowback waters. The low biomass resulted in difficulties obtaining sequencing data.

6.5.2 Methods

To test for the presence of halophilic organisms saline enrichments were performed aerobically using high nutrient medium TY agar. Colonies were isolated growing on TY agar with 2 M NaCl after 2 weeks at 30ºC. DNA was isolated from one of

the colonies and identified using Sanger sequencing. An attempt was made to isolate organisms using this method under anaerobic conditions.

Individual colonies were picked with a toothpick and transferred to 2 ml of 2 M NaCl TY broth and incubated at 30ºC for 14 days. A single colony from K_336 h was selected for sequencing. DNA was extracted as previously described using the DNA fast spin kit. The 16S rDNA gene was amplified using PCR with primer 27F(AGAGTTTGATCCTGGCTCAG) and 1525R(AAGGAGGTGATCCAGCC) using 25 cycles under conditions that were previously described. PCR product (10 µl) was sent with 2 µl of one of the primers to the University of Calgary's University Core DNA Services for Sanger sequencing using BigDye Terminators version 3.1 kit using an Applied Biosystems 3730xl 96 caplillary DNA Analyzer.

Results were downloaded from their website (http://www.sequencing.ucalgary.ca/). A consensus sequence was constructed using SeqTrace and visually checked for quality control (Stucky 2012). The consensus sequence was entered into blastn. The consensus sequences was aligned against the top 22 results using Clustal W (Larkin et al. 2007).

6.5.3 Results

The consensus sequence generated was entered in to the NCBI nucleotide BLAST tool. The top results returned had 99% similarity with the isolated consensus sequence. The top 22 unique species were selected to evaluate phylogeny with the obtained isolate. The sequence obtained most closely resembled that of *Oceanobacillus iheyensis* (Figure 6-12).

6.5.4 Summary

Oceanobacillus iheyensis is a marine biofilm producer (Kavita et al. 2014). This organism was first isolated from deep marine sediments (1050 m). It is a strict aerobe that can withstand salinities from 0 to 21 % w/v or about 4 M, and prefers alkaline conditions (Lu et al. 2001). The genus *Oceanobacillus* was not detected in the 454 results (Table 5-4). The subsurface environment is anaerobic which would not support a strict aerobe like *O. iheyensis*. In order to survive the unfavourable conditions, *O. iheyensis* would have to form an endospore until conditions became more favourable, such as the culture conditions used. It is more difficult to extract DNA from spores and they could have been missed by sequencing (Kuske et al. 1998; Bürgmann et al. 2001).

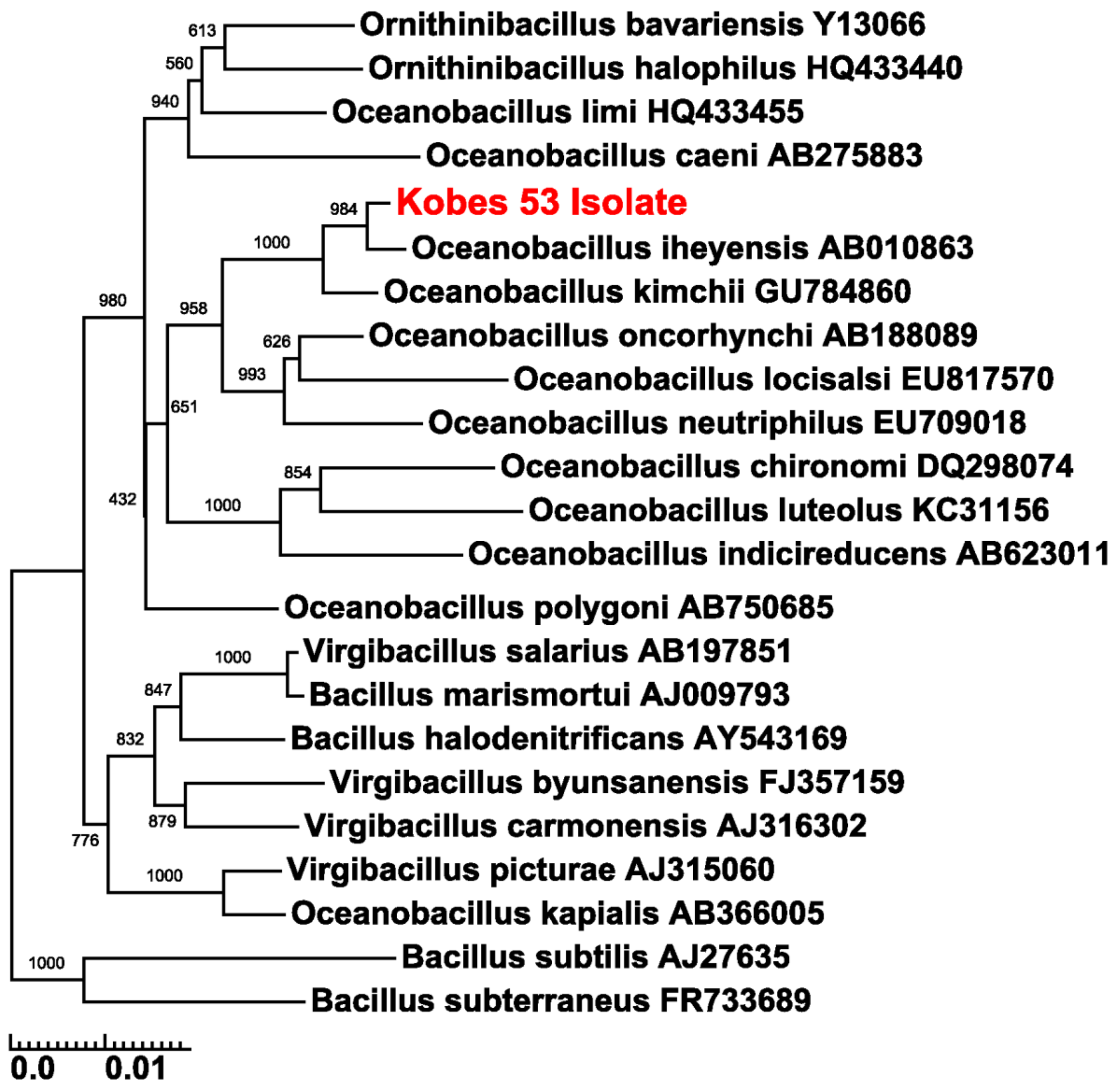


Figure 6-12: Phylogenetic tree of isolated 16S rDNA sequence obtained with most similar BLAST searches. Clustering was performed using nearest neighbour joining method with 1000 bootstraps.

6.6 Heat Tolerance

The thermal gradient that microbes are exposed to during hydraulic fracturing is steep and can severely limit the number of microbes that can survive. Organisms that are capable of surviving these high temperatures are the most likely the organisms that could become established in a formation after hydraulic fracturing. Determining the metabolic ability of the organisms present in the flowback community could be indicative of problems associated with biofouling, fracturing plugging and more rapid decline in production rates.

6.6.1 Methods

Samples (250 ml) of K_40 h, K_204 h, SH_SWS and SH_SWL were vacuum filtered on a 0.45 µm filter on top of a 0.22 µm filter (Table 3-10: 7). The filters were used as inoculum and placed in 100 ml serum bottles with 50 ml CSBK-0.5 (Table 3-3). A head space of N_2CO_2 was used and bottles were sealed with butyl rubber stoppers. Glucose (140 µl 1.8 M) and VFA (84 µl of 3 M) were added to final concentrations of 5 mM (Table 3-6). Sulfate (200 µl of 2.5 M) and nitrate (100 µl of 5 M) were added to a final concentration of 10 mM. The incubations were performed in the dark at 60ºC being shaken at 125 RPM.

6.6.2 Results

Metabolic activity in the incubations varied between the different samples. Glucose was metabolized at 60ºC in SH_SWL and K_204 h but not in K_40 h and SH_SWS (Figure 6-13). Despite not seeing glucose being metabolized in all of the

samples, there is some production of either lactate, acetate, propionate and/or butyrate in each of the samples. Lactate was only observed in sample K_204 h and accounts for all of the glucose that was consumed. Acetate increased in K_204 h, SH_SWS and SH_SWL. The concentration of acetate in K_40 h initially decreased over the first 45 h but increased over the remainder of the experiment. Propionate was only produced in K_204 h. Butyrate began to accumulate in SH_SWL in small amounts towards the end of the experiment. Nitrate was consumed in its entirety for all K_204 h, SH_SWS, SH_SWL but not K_40 h. Nitrite was produced for K_204 h and SH_SWS but there is about a 25 % discrepancy between the amount of nitrite that is expected based on the amount of nitrate that was consumed. Nitrite was not observed in SH_SWL. There was no change in sulfate or sulfide concentrations (Not shown).

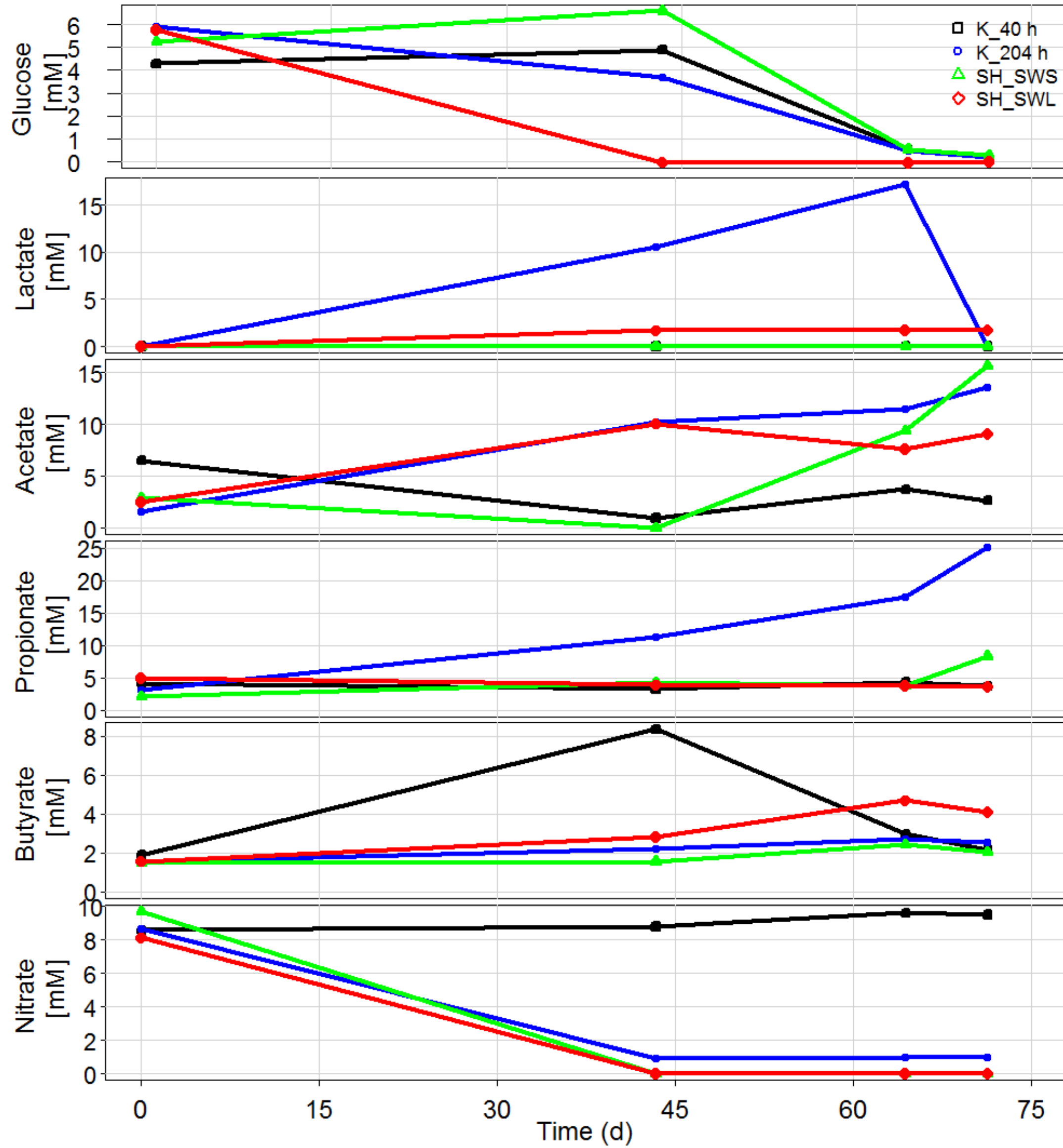


Figure 6-13: Metabolic activity of K_40 h, K_204 h, SH_SWS and SH_SWL at 60ºC. All concentrations are in mM. A) Glucose, B) Lactate, C) Acetate, Propionate, D) Butyrate, E) Nitrate, F) Nitrite. Controls not shown as there was no change. Sulfide and sulfate concentrations are not shown as there was no change.

6.6.3 Summary

The consumption of glucose and changes in concentrations of organic acids demonstrates that the microbial communities are capable of thermophilic activity at 60ºC at 0.5 M NaCl (Figure 6-13). While this does not mimic field conditions it does show that thermophilic activity is possible from these communities. Furthermore it should be noted that nitrate was reduced in the majority of the samples that were tested, a phenomenon not seen at lower temperatures and salinities. Sulfate was not observed to be reduced in any of the incubations. In samples K_204 h, SH_SWS and SH_SWL sulfate would not be expected to be consumed while organisms are consuming nitrate because nitrate has a higher redox potential and is preferentially used, allowing NRB to outcompete the SRB (Myhr et al. 2002). Once all of the nitrate was consumed in SH_SWL SRB would have been able to utilize the sulfate, which they did not. Sulfate reduction would not be expected to occur in K_204 h and SH_SWS because nitrite persists in the system once it has been produced. These are however batch incubations and the persistence of metabolites is different under flow conditions which would be found *in situ*. Nitrite is a powerful inhibitor of the dsrAB enzyme that is used to perform the final reaction producing sulfide by SRB.

6.7 Hydrocarbon Toxicity

6.7.1 Introduction

The types of hydrocarbons present in aquatic systems can have adverse effects on microbial populations. Lighter unbranched hydrocarbons such as alkanes and

cycloalkanes can be used as a carbon and energy sources by many microbes (Atlas 1981; Rueter et al. 1994; Pérez-Jiménez et al. 2001; Widdel & Rabus 2001). Conversely, many hydrocarbons such as BTEX compounds are toxic to many microbes due to interactions between hydrophobic regions with cell membranes and amphipathic regions (Sikkema & Jan 1995). Later Kobes flowback samples had an organic layer containing light branching alkanes and some cyclic compounds (Table 5-2).

To determine whether or not these light hydrocarbons were having a toxic effect and contributing to the lower amounts of biomass that were seen in later flowback samples K_12 h, an earlier flowback samples with a diverse microbial community was incubated with different amount of the extracted hydrocarbon and glucose, a compound that would be preferentially used over the hydrocarbons. Changes in the ability of the microbial community to utilize glucose would be indicative of an effect from the hydrocarbons.

6.7.2 Methods

Sample K_12 (1 ml) and CSBK (19 ml) were used for hydrocarbon toxicity incubations (Table 3-3). Different amounts of hydrocarbon that were collected from later Kobes samples were added to some of the samples as per Table 3-11, ranging from 0 to 400 µl. Glucose (22 µl of 1.8 M) was added to a final concentration of 2 mM to select incubations as per Table 3-11. Incubations were performed in 50 ml serum bottles with a N_2CO_2 head space and sealed with butyl rubber stoppers. Samples were kept at 30ºC in the dark, with shaken at 125 RPM.

6.7.3 Results

Of the five experimental conditions that were employed no differences between conditions were observed. There was no change in glucose concentration until after 4 days. Over the next 4 days all of the glucose in the incubation was consumed regardless of the amount of hydrocarbon that was present (Figure 6-14).

6.7.4 Summary

There was no substantial difference in glucose utilization between any of the experimental conditions that were tested. The composition of the hydrocarbons that were present in the Kobes formation would not be expected to have a toxic effect on the microbes due to the linear branched nature of many of the hydrocarbons (Table 5-2). BTEX compounds were not identified in the hydrocarbons analysis and cyclic alkanes accounted for less than 3% of the total amount of hydrocarbons that were recovered (Table 5-2, Appendix 6). The hydrocarbons recovered would more likely serve as a carbon source as they are predominantly linear alkanes which are among the easiest hydrocarbons to degrade. The organic layer indicates that the recovered flowback fluids are saturated with these compounds, but it is unlikely that they would be the target of microbial degradation as there are easier compounds present in the fractured system to degrade such as fragmented polymer and acetate (Figure 5-3: C).

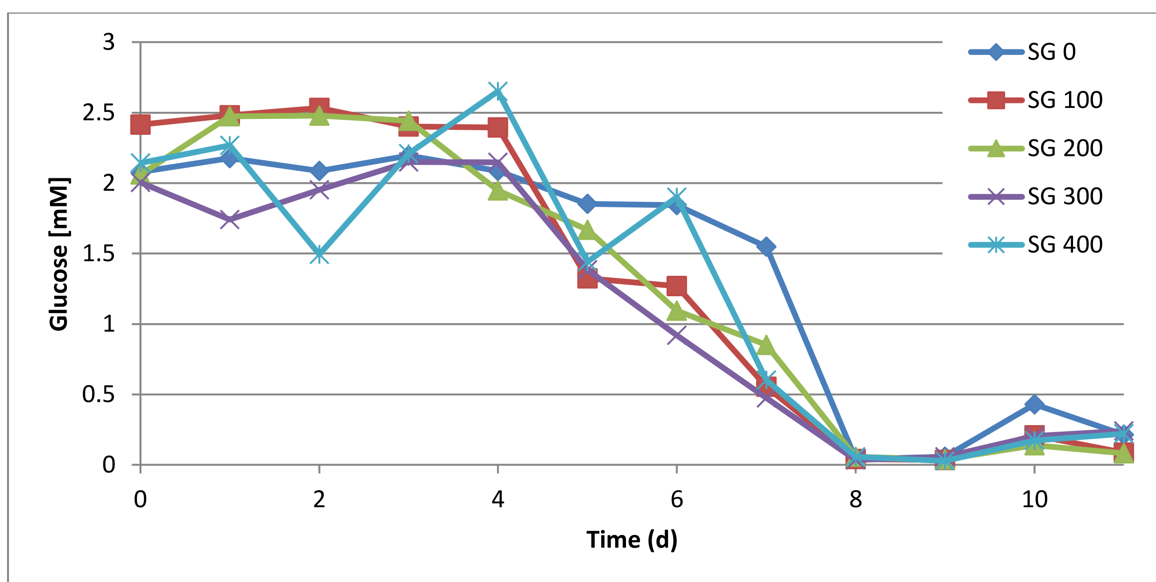


Figure 6-14: Degradation of glucose in the presence of different amounts of hydrocarbons extracted from Kobes samples. Microbial activity was measured under low salinity and low temperature conditions (30ºC).

6.8 Corrosion

Corrosion is a concern for hydraulic fracturing that could potentially arise through microbially influenced corrosion (MIC). High salinity and low pH conditions found in the flowback waters could contribute to corrosion. The ability of changing physicochemical conditions and microbial communities could also contribute to different corrosion rates during the flowback period.

6.8.1 Methods

Incubations using 20 ml samples of flowback waters K_8, 20, 28, 36, 48, and 68 h were in 50 ml serum bottles with a $N_2H_2CO_2$ head space and sealed with butyl rubber stoppers. Twenty carbon steel beads (55 mg ea.) were included in each incubation. Serum bottles were laid on their sides at 30ºC while shaking at 125 RPM for the incubation. Incubations were performed in duplicate, once with the untreated sample and once with the sample filtered through two 0.22 µm Millipore filters to remove any microbes. Corrosion rates were determined by both weight loss and the amount of dissolved iron as determined by the Ferrozine assay.

6.8.2 Results

Corrosion rates did not differ substantially between the filtered and unfiltered samples. Filtered samples for K_28 and 36 h had elevated corrosion rates for both weight loss and dissolved iron methods used to measure corrosion (Figure 6-15). The weight loss method for determining corrosion gave slightly higher values of corrosion than the dissolved iron method. No substantial change were observed for the corrosion rates of

samples collected at different times after flowback, nor was there a substantial difference between the filtered and unfiltered condition.

6.8.3 Summary

There was no substantial difference in corrosion rates in the samples measured even though the samples before 68 h had notable levels of biomass (Figure 5-5). The microbial communities in these flowback waters do not appear to be contributing to corrosion in the native well samples. MIC is usually associated with SRB belonging to the *Deltaproteobacteria,* which were almost entirely absent from the samples (Table 5-4, Figure 5-4) (Gu 2014; Gieg et al. 2011). The inactivity of SRB is likely due to a lack of sulfur compounds like sulfate (Appendix 3). Measured corrosion rates did not change substantially with the time that they were collected after flowback. The latest sample measured was collected before the well was shut in and had a salinity of less than 0.7 Meq NaCl. Overall the microbial communities associated with the hydraulic fracturing fluids do not appear to be contributing to MIC in tests under laboratory conditions.

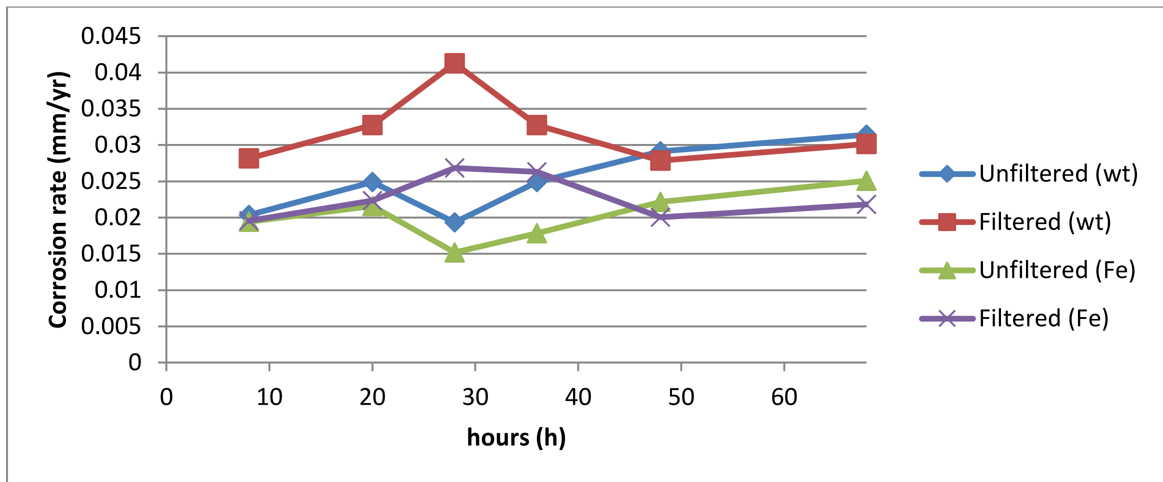


Figure 6-15: Corrosion rates measured by weight loss and dissolved iron methods for Kobes flowback samples that were untreated or filtered to remove biomass. (wt) weight loss, (Fe) dissolved iron. Corrosion rates measured in samples collected at different flowback times. The corrosion rates were measured by weight loss (wt) or by measuring dissolved iron (Fe) in samples that were either filtered or unfiltered to remove biomass.

6.9 Discussion

The microbial communities associated with the water samples received were mostly fermentative in nature. They were able to utilise glucose, and the more complex compound, guar gum, which is a high molecular weight polymer consisting of mannose and galactose subunits. These compounds were broken down in to a variety of organic acids including lactate, acetate, propionate and butyrate. Under constant lab conditions with defined media in most cases it took multiple weeks to see changes in the concentrations of glucose or guar gum. This is in part due to the low amounts of biomass in the inoculum used for each of the incubations. Anaerobic degradation is a slow process with most of the metabolic energy going towards cell maintenance instead of biomass (Mccarty 1964). The presence environmental stresses would have required larger amounts of energy to be used for maintenance instead of growth (Tijhuis et al. 1993).

The use of electron acceptors such as sulfate and nitrate was fairly limited in both the flowback and source water samples that were tested. The lack of organisms that are capable of utilizing these electron acceptors is likely due to the lack of nitrate and sulfate in the environment. The only evidence of sulfate reduction occurred in the presence of acetate for the source water (SH_SWL) that was used to formulate fracturing fluid. Under controlled lab conditions sulfate reduction took almost 30 days (Figure 6-3, Figure 6-4). The ability for the culture to reduce sulfate to sulfide occurred very rapidly once there was sufficient biomass and occurred in a stoichiometric ratio as would be expected between sulfate and acetate. The flowback waters obtained from the well fractured with this source water did not share this characteristic, presumably because the organisms that

were able to reduce sulfate in the presence of acetate did not survive the fracturing process.

The ability of microbial communities associated with hydraulic fracturing to reduce nitrate appears to be more common than that to reduce sulfate. This was observed to occur under both low and high temperature conditions, but was not observed for all samples that were analysed. Of the Kobes samples analysed at low temperature in the presence of acetate K_0 h and K_68 h were able to reduce nitrate but K_Source, K_8 h, K_16 h and K_64 h were not (Figure 6-2). This shows that at least some of the microbial communities are capable of reducing nitrate. Only the source water sample SH_SWL, was able to reduce nitrate in the presence of acetate under mesophilic conditions. This was the same sample that was able to reduce sulfate. The lag time of the aforementioned samples to reduce nitrate ranged anywhere from a couple of days (SH_SWL) to a couple of weeks (K_0 h and K_68 h). Nitrate and acetate were consumed in stoichiometric ratios as was expected. Nitrite was only observed in K_68 h and SH_SWL at 30ºC. In K_68 h all of the nitrate was converted to nitrite and persisted in the system; there did not appear to be any organisms present that were capable of reducing nitrite further under the incubation conditions and time. For SH_SWL all nitrate was converted to nitrite after a couple of days, with only 50 % of the nitrite reduced further. This demonstrates the ability of microbial communities associated with hydraulic fracturing to utilize nitrate as an electron acceptor. The ability of the microbes present in some of the samples to utilize nitrate at high temperatures was also demonstrated. SH_SWL is able to reduce nitrate within a couple of days at 60ºC, as it did in the mesophilic experiment.

Based on the metabolic activities observed under controlled laboratory conditions, the microbial communities present in hydraulic fracturing flowback fluids are capable of fermenting guar gum at high temperature which can be attributed to the high amounts of biomass that were observed in the first 74 h of flowback fluids. Nitrate is capable of being reduced under both thermophilic and mesophilic conditions but microbes that are capable of reducing sulfate do not appear to be able to survive the fracturing process. Many of these observations took a long time to manifest, *in situ* activity would be expected to be much slower or non-existent due to the higher temperatures. The chemical conditions microbes would be subjected to would also be different. While subsurface microbiology is performed with minimal growth medium, it does not accurately represent the chemical conditions that are encountered in hydraulically fractured shale formations. The amount of dissolved solutes is much higher in flowback waters than source waters. Nutrients in flowback fluid are also not limiting, there are large amount of organic carbon as well as many other compounds such as nitrogen which are limiting nutrients in many environments. Trace elements that microbes typically hoard such as iron are also in abundance, which presumably creates a nutrient rich environment that microbes could exploit if they are capable of surviving the harsh conditions associated with hydraulic fracturing. The work presented here is a good first step in determining the potential impact that microbes can have on hydraulic fracturing operations but merits years of study.

Chapter Seven: Conclusion

Hydraulic fracturing has only been performed in Canada for the last decade. While many advances have been made from an engineering perspective very little has been done regarding the associated microbiology. By looking at both microbial community characteristics and associated flowback fluid characteristics, as was done in this work, a greater understanding of microbes and their activities associated with hydraulic fracturing has been obtained.

Microbial communities and physicochemical conditions in flowback waters were distinct from the initial source water used. Earlier flowback samples more closely resemble the source water used and became increasingly different as flowback proceeded. Initial flowback water samples had the highest amount of biomass as well as the highest amount of diversity. This was attributed to having the least amount of formation exposure and high nutrient levels which were primarily attributed to the addition of guar gum. As flowback proceeded the conditions became more saline and selected for a microbial community that was more suited for a saline environment (0.5 Meq NaCl). The microbial community was initially dominated by a single organism (*Shewanella*). Concurrently, the amount of biomass rapidly decreased due to the increasingly harsh conditions associated with increased formation exposure in the form of both increasing temperatures and salinity. Community diversity rapidly decreased and became dominated by organism that were more suited for a saline environment (2 Meq NaCl). More biomass was able to be generated closer to the horizontal wellbore where temperatures would have been coolest.

As flowback proceeded the amount of biomass decreased and only organisms that are able to survive in the hot saline conditions were able to be detected. Low amounts of

biomass were also detected in flowback samples from a shale gas field that had been producing for many years. The current study does not indicate that microbes pose much of a concern for hydraulic fracturing operations. The conditions are too harsh to support a substantial microbial community that could result in fracture plugging.

While the current study is one of the first to offer detailed insights into the changing microbial community with respect to physicochemical conditions in the early flowback period of a hydraulically fractured well in Canada, the sample set received had been left standing on site for some time before it was received in the lab. Chemical changes could be observed in the samples based differences in pH measurements made on site and in the lab. Receiving samples in a more timely fashion would make for a more accurate analysis of microbial community composition.

It is the hope that these findings will help dictate biocide regimes employed for hydraulic fracturing. Low amounts of biomass detected by the end of early flowback periods and in older wells, along with low fractions of thermophiles and halophiles identified by sequencing does not indicate that issues due to microbes could arrise. This suggests that the amount of biocide needed to control microbial populations could be reduced or eliminated. Having the microbial populations dominated by a single organism could also help in the selection of the most effective biocide. More testing would needed to confirm these because the systems studied were subjected to biocides before a microbial concern was established. Subjecting source water samples to conditions encountered in hydraulic fracturing would give further insight for optimizing biocide regimes.

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Appendix

Appendix 1: Fox Creek Water chemistry that corresponds to samples AB1_WS.

Shell Global Solutions Canada A Division Of Shell Chemicals Americas Inc.
Calgary Research Centre

Submission # 7563
Sample ID # 19721

Water Analysis Reported: 2013-June-28

U.W.I.	Well or Sample Name: Fox Creek	KB Elevation/m	GL/m
Field or Area	Pool or Zone	Sampler	
Test Type	No	Recovery	
Point of Sample: FCC Treated Waste Water	Remarks: filtrate -- clear and yellow		
Test Interval/m			
Perforations/m			
Date Sampled (Year/Month/Day): 2013-05-27	Received (Year/Month/Day): 2013-June-18	Analyst: MH/NJR	

Cations	(mg/L)	Mass Fraction	(meq/L)
Na	160	0.1858	6.96
K	10	0.0116	0.26
Ca	57	0.0662	1.42
Mg	18	0.0209	0.74
Fe	<0.1		

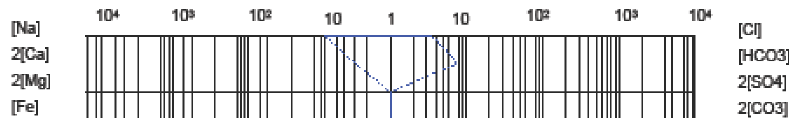
Additional Cations	(mg/L)
Mn	0.4
Ba	0.1
Sr	0.6

Anions	(mg/L)	Mass Fraction	(meq/L)
Cl	126	0.1463	3.55
HCO3	468	0.5437	7.68
SO4	22	0.0255	0.23
CO3	0	0.0000	0.00
OH	0	0.0000	0.00

Additional Anions	(mg/L)

Relative Density @15°C,g/cc	1.00071	Observed pH @ 22.2 °C	7.26
Refractive Index @ 21.9 °C	1.3330	Resistivity @ 25°C, Ω * m	8.44
Total Alkalinity (as CaCO3) (mg/L)	384	Spot test for H2S	No
Total Hardness (as CaCO3)(mg/L)	216	Spot test for Iron	

TD Solids (g/m³) calc. 862
Evaporation @105°C



Comments:

Appendix 2: Kobes well tracer data

Suncor Energy, Inc. 15-12-84-26W6 Normalized Data Table

Key
>200
150 to 200
100 to 150
70 to 100
50 to 70
35 to 50
25 to 35
17 to 25
12 to 17
8 to 12
5 to 8
3 to 5
2 to 3
1 to 2
0.05 to 1

				Normalized Chemical Frac Tracer Concentration, ppb											
			Traced Segment	10	9	8	7	6	5	4	3	2	1		
			Stim Date	11-5-13	11-5-13	11-5-13	11-3-13	11-3-13	11-2-13	11-2-13	11-1-13	10-30-13	10-29-13	Totals	Avgs
			Traced Fluid vol (m3D)	1,900	1,695	1,755	1,602	1,765	1,818	1,903	1,914	1,459	596	16,407	1,641
	FPE	6.8	CFT Injected (g)	1,455	1,364	1,346	1,273	1,309	1,364	1,309	1,455	1,182	364	12,418	1,242
			% Injected	11.7%	11.0%	10.8%	10.2%	10.5%	11.0%	10.5%	11.7%	9.5%	2.9%		
	Cum Vol*	Sample Date	Sample Type	CFT 2100	CFT 2000	CFT 1900	CFT 1700	CFT 1500	CFT 1400	CFT 1300	CFT 1200	CFT 1100	CFT 1000	CFT Total ppb	Calc Chlorides Total
1	33	11-8-13 20:00	Water (Produced)	3.1	0.0	205.2	4.3	22.2	0.0	0.0	0.0	5.4	0.0	240.2	15,792
2	108	11-9-13 8:00	Water (Produced)	3.2	2.4	95.4	103.0	50.8	0.3	0.0	0.0	3.6	0.0	258.6	18,364
3	186	11-9-13 20:00	Water (Produced)	7.1	9.4	69.5	81.8	67.7	0.3	0.0	0.0	5.6	0.0	241.3	20,164
4	260	11-10-13 8:00	Water (Produced)	10.7	13.3	55.8	72.0	58.2	13.0	0.0	0.0	5.9	0.0	228.9	21,502
5	319	11-10-13 16:00	Water (Produced)	13.6	13.9	46.7	63.5	50.4	20.8	0.0	0.0	5.6	0.0	214.5	22,879
6	436	11-11-13 4:00	Water (Produced)	15.4	12.7	33.8	50.1	39.8	24.5	39.8	0.0	6.1	0.0	222.2	26,819
7	829	11-17-13 19:30	Water (Produced)	18.0	7.4	17.3	35.2	25.5	16.8	38.8	11.8	4.9	0.0	175.7	46,431
8	1161	11-18-13 19:30	Water (Produced)	16.8	6.1	17.5	32.2	20.0	16.5	36.1	11.9	6.2	2.0	165.3	49,415
9	1522	11-19-13 19:30	Water (Produced)	19.1	6.1	14.7	32.4	19.2	16.1	35.0	10.7	6.6	2.9	162.6	55,122
10	2220	11-21-13 19:30	Water (Produced)	16.9	5.5	7.6	24.5	18.4	13.8	30.4	10.9	8.8	3.5	140.3	61,352
11	2542	11-22-13 20:00	Water (Produced)	15.0	5.1	10.1	25.0	16.8	13.7	29.7	9.9	7.6	4.6	137.6	64,781
12	2718	11-23-13 12:00	Water (Produced)	13.6	4.6	11.7	24.6	16.8	12.9	28.0	9.9	7.1	4.6	133.6	63,805
13	2820	11-23-13 20:00	Water (Produced)	13.8	4.6	11.3	25.7	16.5	12.5	28.1	9.4	7.3	4.7	134.0	64,986
14	3013	11-24-13 12:00	Water (Produced)	13.3	4.3	11.0	24.4	15.9	12.3	27.0	9.1	7.6	4.4	129.2	67,052
15	3343	11-25-13 20:00	Water (Produced)	12.5	3.9	10.4	23.3	15.6	11.7	26.6	8.9	7.7	4.2	124.7	68,844
16	3736	11-27-13 0:01	Water (Produced)	12.3	3.9	10.0	22.1	14.9	11.0	25.7	9.1	7.5	4.1	120.6	70,830
17	3858	11-27-13 20:00	Water (Produced)	12.4	3.8	9.2	21.9	14.4	11.5	25.5	9.0	7.6	4.8	120.0	71,610
18	3893	11-28-13 16:00	Water (Produced)	12.5	3.7	5.6	20.4	14.7	10.9	23.5	9.3	7.7	3.3	111.5	72,601
	193		Avg ppb	12.7	6.1	35.7	38.1	27.7	12.1	21.9	6.7	6.6	2.4	170.0	49,019
	Avg m3D		% total ppb from Stage	7.5%	3.6%	21.0%	22.4%	16.3%	7.1%	12.9%	3.9%	3.9%	1.4%	100.0%	
			% total ppb @ last sample	11.2%	3.3%	5.0%	18.3%	13.2%	9.7%	21.1%	8.3%	6.9%	3.0%	100.0%	
			Mass Balance Recov'd (g)	67.6	24.5	76.5	125.0	89.2	58.1	117.2	41.8	26.1	3.4	629.3	
			% of Total Recovery	10.7%	3.9%	12.2%	19.9%	14.2%	9.2%	18.6%	6.6%	4.1%	0.5%	100.0%	
			SLR	9.2	3.5	10.0	10.0	10.0	8.4	10.0	5.7	4.4	1.8	7.3	
			No Flow Zones			0	Deduct								
			Heel/Toe Ratio			0.5	Deduct								
			14 Day flow decline	14%		0	Deduct								
			Traced Fluid Recovered	5.1%											

Segment Load Recovery (SLR) - A grade of 0-10 given to each traced segment based on the % of Total Recovery divided by the % CFT injected.

Flow Profile Effectiveness (FPE) - A total well score (0-10) based on the weighted avg SLR with deductions for no flow, poor heel/toe ratio, high flow declin

* Red Values denote assumed volumes based on Avg m3D.

Appendix 3: Trace sulfur analysis from received from Suncor's Kobes field. Sample was collected at 450 h after flowback began**.**

TRACE SULPHUR ANALYSIS

CONTAINER IDENTITY	METER ID	WELL LICENSE NUMBER	LABORATORY FILE NUMBER
V0009541 - 2		28394	52136-2013-6752

OPERATOR	PAGE
Suncor Energy Oil and Gas Partnership	3

LOCATION (UWI)	WELL NAME	KB ELEV (m)	GR ELEV (m)
200 b-49-D/94-A-5/02	Suncor HZ Altares 15-12-84-26	780.29	770.90

FIELD OR AREA	POOL OR ZONE	SAMPLER
Altares	Upper Montney	Opsco Energy

TEST TYPE AND NO.

TEST RECOVERY

POINT OF SAMPLE: HP Pressure Tank

SAMPLE POINT ID

TEST INTERVAL or PERFS (meters): 2523.0 - 3985.0

PUMPING FLOWING GAS LIFT SWAB

WATER 148.608 m³/d OIL 6.192 m³/d GAS 146647 m³/d

Pressures, kPa (gauge): SEPARATOR 220 RESERVOIR OTHER CONTAINER WHEN SAMPLED @ °C CONTAINER WHEN RECEIVED @ °C

Temperatures, °C: SEPARATOR 7 OTHER

at 09:45 hrs

DATE SAMPLED (Y/M/D)	DATE RECEIVED (Y/M/D)	DATE ANALYZED (Y/M/D)	ANALYST	AMT. AND TYPE CUSHION	MUD RESISTIVITY
2013 11 29	2013 12 04	2013 12 11	RS		@ °C

Trace Sulphur Analysis by Chemiluminescence (SCD)

Component	Result	Detection Limit
Hydrogen Sulphide:	N.D.	0.1
Carbonyl Sulphide:	N.D.	0.1
Methyl Mercaptan:	N.D.	0.1
Ethyl Mercaptan:	N.D.	0.1
Dimethyl Sulphide:	N.D.	0.1
Iso-Propyl Mercaptan:	0.3 mg/kg	0.1
Tert-Butyl Mercaptan:	N.D.	0.1
N-Propyl Mercaptan:	0.2 mg/kg	0.1
Methyl Ethyl Sulphide:	N.D.	0.1
Sec-Butyl Mercaptan/Thiophene:	0.5 mg/kg	0.1
Iso-Butyl Mercaptan:	N.D.	0.1
Diethyl Sulphide:	0.3 mg/kg	0.1
N-Butyl Mercaptan:	N.D.	0.1
Dimethyl Disulphide:	N.D.	0.1
Diethyl Disulphide:	N.D.	0.1

Remarks: N.D. - Not Detected.

Appendix 4: Water analysis of Kobes samples flowback water provided by Suncor. The samples was collected at 450 h after flowback began.

WATER ANALYSIS

Field	Value
CONTAINER IDENTITY	174 - 4
METER ID	
WELL LICENSE NUMBER	28394
LABORATORY FILE NUMBER	52136-2013-6752
OPERATOR	Suncor Energy Oil and Gas Partnership
PAGE	5
LOCATION (UWI)	200 b-49-D/94-A-5/02
WELL NAME	Suncor HZ Altares 15-12-84-26
KB ELEV (m)	780.29
GR ELEV (m)	770.90
FIELD OR AREA	Altares
POOL OR ZONE	Upper Montney
SAMPLER	Opsco Energy
TEST TYPE AND NO.	
TEST RECOVERY	
POINT OF SAMPLE	Stock Condensate
SAMPLE POINT ID	
PUMPING / FLOWING / GAS LIFT / SWAB	
TEST INTERVAL or PERFS (meters)	2523.0 - 3985.0
WATER	148.608 m³/d
OIL	6.192 m³/d
GAS	146647 m³/d
Pressures, kPa (gauge): SEPARATOR / RESERVOIR / OTHER	
CONTAINER WHEN SAMPLED @ °C / CONTAINER WHEN RECEIVED @ °C	
Temperatures, °C: SEPARATOR	40
Temperatures, °C: OTHER	
DATE SAMPLED (Y/M/D)	2013 11 29 at 09:45 hrs
DATE RECEIVED (Y/M/D)	2013 12 04
DATE ANALYZED (Y/M/D)	2013 12 11
ANALYST	CD
AMT. AND TYPE CUSHION	
MUD RESISTIVITY	@ °C

CATIONS

ION	mg/L	mg Fraction	meq/L
Na	40,075	0.3182	1,743.2
K	1,304	0.0104	33.3
Ca	5,027	0.0399	250.8
Mg	586	0.0047	48.2
Ba	251	0.0020	3.6
Sr	768	0.0061	17.5
Fe	0.79	0.0000	0.0
Mn	0.86	0.0000	0.0

ANIONS

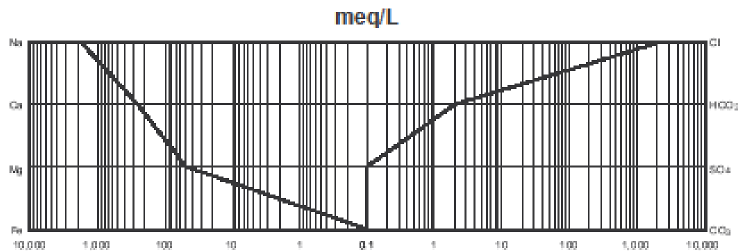
ION	mg/L	mg Fraction	meq/L
Cl	77,822	0.6178	2,195.1
Br			
I			
HCO_3	119	0.0009	2.0
SO_4	4.0	0.0000	0.1
CO_3	0.00	0.0000	0.0
OH	0.00	0.0000	0.0
H_2S	N.D.		

Total Dissolved Solids (mg/L)

Property	Value
By Evaporation @ 110 °C	Not Requested
By Evaporation @ 180 °C	Not Requested
Calculated	125958
Specific Gravity	1.0867 @ 15.6 °C
Refractive Index (n_D)	1.3543 @ 22 °C
pH	7.0 @ 25.0 °C
Resistivity (Ohm-Meters)	0.066 @ 25 °C

LOGARITHMIC PATTERNS OF DISSOLVED IONS

meq/L



REMARKS: N.D. - Not Detected.

Appendix 5: Extended gas analysis of Kobes samples provided by Suncor

EXTENDED GAS ANALYSIS

CONTAINER IDENTITY	METER ID	WELL LICENSE NUMBER	LABORATORY FILE NUMBER
V0000308 - 1		28394	52136-2013-6752

OPERATOR	PAGE
Suncor Energy Oil and Gas Partnership	1

LOCATION (UWI)	WELL NAME	KB ELEV (m)	GR ELEV (m)
200 b-49-D/94-A-5/02	Suncor HZ Altares 15-12-84-26	780.29	770.90

FIELD OR AREA	POOL OR ZONE	SAMPLER
Altares	Upper Montney	Opsco Energy

TEST TYPE AND NO. | TEST RECOVERY

POINT OF SAMPLE: HP Meter Run | SAMPLE POINT ID

TEST INTERVAL or PERFS (metres): 2523.0 - 3985.0

PUMPING | FLOWING | GAS LIFT | SWAB

WATER 148.608 m³/d | OIL 6.192 m³/d | GAS 146647 m³/d

Pressures, kPa (gauge): SEPARATOR 2000 at 09:45 hrs | RESERVOIR | OTHER | CONTAINER WHEN SAMPLED @ °C | CONTAINER WHEN RECEIVED 2040 @ 22 °C

Temperatures, °C: SEPARATOR 28 | OTHER

DATE SAMPLED (Y/M/D)	DATE RECEIVED (Y/M/D)	DATE ANALYZED (Y/M/D)	ANALYST	AMT. AND TYPE CUSHION	MUD RESISTIVITY
2013 11 29	2013 12 04	2013 12 05	MF		@ °C

COMPONENT	MOLE FRACTION AIR FREE AS RECEIVED	MOLE FRACTION AIR FREE ACID GAS FREE	mL/m³ AIR FREE AS RECEIVED
H_2	Trace	Trace	
He	0.0001	0.0001	
N_2	0.0027	0.0027	
CO_2	0.0013	0.0000	
H_2S	0.0000	0.0000	
C_1	0.8570	0.8581	
C_2	0.0872	0.0873	309.9
C_3	0.0285	0.0286	104.7
iC_4	0.0062	0.0062	27.1
C_4	0.0076	0.0076	32.0
iC_5	0.0024	0.0024	11.7
C_5	0.0022	0.0022	10.6
C_6	0.0020	0.0020	10.8
C_7	0.0012	0.0012	6.7
C_8	0.0010	0.0010	5.9
C_9	0.0004	0.0004	2.8
C_{10}	0.0001	0.0001	0.8
C_{11}	0.0001	0.0001	0.8
C_{12+}	Trace	Trace	Trace
Total	1.0000	1.0000	523.8

CALCULATED GROSS HEATING VALUE, MJ/m³ @ 15°C & 101.325 kPa (abs.): MOISTURE FREE 44.20 | MOISTURE & ACID GAS FREE 44.25

CALCULATED VAPOR PRESSURE, kPa (abs.) @ 40 °C: PENTANES PLUS 77.2

CALCULATED TOTAL SAMPLE PROPERTIES (AIR=1) @ 15°C & 101.325 kPa, MOISTURE FREE AS SAMPLED: DENSITY 0.819 kg/m³ | RELATIVE DENSITY 0.669 | RELATIVE MOLECULAR MASS 19.4

CALCULATED PSEUDOCRITICAL PROPERTIES:

- AS SAMPLED: pPc 4587.6 kPa (abs) | pTc 211.7 K
- ACID GAS FREE: pPc 4584.0 kPa (abs) | pTc 211.6 K

C_{7+} PROPERTIES @ 15°C & 101.325 kPa: DENSITY 745.1 kg/m³ | MOLECULAR WEIGHT 105.8

HYDROGEN SULPHIDE: MOLE FRACTION 0.0000000 | LOCATION Field | METHOD Gastec

AROMATICS	MOLE FRACTION	NAPHTHENES	MOLE FRACTION
Benzene (C7)	0.0001	Cyclopentane (C6)	0.0001
Toluene (C8)	0.0002	Methylcyclopentane (C7)	0.0001
Ethylbenzene (C9)	0.0001	Cyclohexane (C7)	0.0002
Xylenes (C9)	Trace	Methylcyclohexane (C8)	0.0003
1,2,4 Trimethylbenzene (C10)	Trace		

REMARKS:

H2S was not detected in the field by Gastec.

Benzene Content (mole fraction) = 0.00009

NOTE: THE GROSS HEATING VALUE HAS BEEN CALCULATED IN ACCORDANCE TO AGA REPORT #5 AND ALL PROPERTIES HAVE BEEN CALCULATED UTILIZING PHYSICAL CONSTANTS AND BOILING POINT GROUPING.

Appendix 6: Hydrocarbon liquid analysis of Kobes samples provided by Suncor. The sample was collected at 450 h after flowback.

HYDROCARBON LIQUID ANALYSIS

Container Identity	Meter ID	Well License Number	Laboratory File Number
V0009541 - 2		28394	52136-2013-6752

Operator	Page
Suncor Energy Oil and Gas Partnership	2

Location (UWI)	Well Name	KB Elev (m)	GR Elev (m)
200 b-49-D/94-A-5/02	Suncor HZ Altares 15-12-84-26	780.29	770.90

Field or Area	Pool or Zone	Sampler
Altares	Upper Montney	Opsco Energy

Test Type and No.	Test Recovery

Point of Sample	Sample Point ID
HP Pressure Tank	

Test Interval or Perfs (meters): 2523.0 - 3985.0

Pumping: | Flowing: | Gas Lift: | Swab:

Water: 148.608 m³/d | Oil: 6.192 m³/d | Gas: 146647 m³/d

Pressures, kPa (gauge): Separator: 220 | Reservoir: | Other: | Container when sampled: @ °C | Container when received: 241 @ 22 °C

Temperatures, °C: Separator: 7 | Other:

Date Sampled (Y/M/D)	Date Received (Y/M/D)	Date Analyzed (Y/M/D)	Analyst	Amt. and Type Cushion	Mud Resistivity
at 09:45 hrs 2013 11 29	2013 12 04	2013 12 11	AJ		@ °C

Component	Mole Fraction	Mass Fraction	Liquid Volume Fraction	mL/m³
N_2	Trace	Trace	Trace	Trace
CO_2	0.0001	Trace	Trace	0.2
H_2S	0.0000	0.0000	0.0000	0.0
C_1	0.0127	0.0018	0.0043	28.7
C_2	0.0212	0.0056	0.0114	75.3
C_3	0.0360	0.0140	0.0200	132.3
iC_4	0.0194	0.0099	0.0128	84.7
C_4	0.0361	0.0185	0.0230	151.9
iC_5	0.0295	0.0187	0.0218	144.0
C_5	0.0365	0.0232	0.0267	176.6
C_6	0.0886	0.0670	0.0734	486.4
C_{7+}	0.7199	0.8413	0.8066	5,336.5
Total	1.0000	1.0000	1.0000	6,616.6

Observed properties of C_{7+} residue (15/15°C)

Density	Relative Density	API @ 15.5 °C	Relative Molecular Mass
758.8 kg/m³	0.7595	55.0	133

Calculated properties of total sample (15/15°C)

Density	Relative Density	API @ 15.5 °C	Relative Molecular Mass
727.5 kg/m³	0.7281	63.0	113.60

Gas equivalent: 0.1514 10^3m^3 Gas/m³ Liquid (E³m³/m³)

REMARKS: Saturation Pressure could not be determined. Refer to page 2a for extended analysis.

NOTE: All Properties have been calculated utilizing physical constants.