BIOCIDE INHIBITION OF MICROBIAL SULFUR REDUCTION IN FRACING FLUIDS

by

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Abstract

Fracing technology has revolutionized the natural gas industry, and currently, it is the most widely used method to extract gas from shale in Western Canada. Microbial activity in fracing fluids can lead to biofouling, corrosion, and gas souring. Biocides are commonly applied to inhibit microbial activity, but in many cases biocide application is partly or even wholly ineffective. This is, in part, because biocides are rarely tested using real environmental communities relevant to fracing systems. To address this problem, I investigated the efficacy of glutaraldehyde, which is one of most commonly used biocides to control microbial activity, on microbial sulfur reduction in fracing fluids. To do this, I collected fracing fluids from the shale gas play in the Fort St. John area of northern British Columbia, Canada. In the lab, I conducted incubation experiments by amending fracing fluids with glutaraldehyde and yeast extract and incubating these fluids for 30 days at room temperature. During the incubation, I measured sulfide and sulfate concentrations to track rates of microbial sulfur metabolisms with and without glutaraldehyde and yeast extract amendments. To link these results to the relevant microbial taxa, I determined the microbial community present in the incubated fluids using 16S rRNA gene amplicon sequencing. Overall, I found that glutaraldehyde is only moderately effective in controlling microbial sulfide production in fracing fluids and that even in the presence of glutaraldehyde, amendment with reactive organic matter stimulates sulfide production.

Lay Summary

Natural gas is increasingly recovered from low permeability, low porosity rocks including shales. These unconventional gas resources can now be produced as the combined result of horizontal well drilling and hydraulic fracturing (fracing). Fracing increases the permeability of shales and other tight rocks through the high-pressure injection of fluids into reservoir formations to create micro-fractures. Microbial activity in fracing fluids causes a number of deleterious effects including well souring, corrosion, and biofouling. Well souring, in particular, represents a major liability to the production of unconventional gas resources. Souring, or the production and accumulation of hydrogen sulfide, is the result of anaerobic sulfur respiration in fracing systems. Biocides, like glutaraldehyde, are commonly applied to mitigate well souring, but this application is at best partly effective. I experimentally evaluated the efficacy of glutaraldehyde in reducing well souring, and found that its application only partly inhibited sulfide production by frac fluid microbial communities.

Preface

This dissertation is original, unpublished, independent work by the author, Amani T. Alsufyani,

Table of Contents

List of Tables

List of Figures

List of Abbreviations

APB	Acid-Producing Bacteria
CDS	Chromatography Data System
DBNPA	dibromo3-nitrilopropionamide
DDAC	didecyldimethylammonium chloride
GA	Glutaraldehyde
HAB	Heterotrophic Aerobic Bacteria
H_2S	Hydrogen Sulfide
IC	Ion Chromatography
IW	Injection Water samples
Mya	Million years ago
MIC	Minimum Inhibitory Concentration
MPN	Most Probable Number
NRB	Nitrate-Reducing Bacteria
NACE	The National Association of Corrosion Engineers
OTU	Operational Taxonomic Unit
PCR	Polymerase chain reaction
PW	Produced Water samples
SRB	Sulfate-Reducing Bacteria
Tcf	Trillion cubic Feet
TDS	Total Dissolved Solids
TOC	Total Organic Carbon
THPS	tetrakis (hydroxymethyl) phosphonium sulfate

WCSB	Western Canadian Sedimentary Basin
YE	Yeast extract

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Dedication

This thesis is dedicated to:

The sake of Allah, my creator and my master, my home country Saudi Arabia, my dearest parents, my beloved sisters, and brothers; particularly my dearest brother, Abdullah.

Chapter 1: Introduction

Canada was one of the world's largest natural gas producers in 2014, following United States, Russia, Iran, and Qatar[1]. Abundant natural gas reserves exist across Canada, but more than 70% of the current production is located in British Columbia (BC) and Alberta[2] . Fracing technology revolutionized the natural gas industry, and currently, it is the most commonly used technique to extract shale gas in Canada. Microbial activity in fracing fluids can lead to deleterious issues such as biofouling, corrosion, and gas souring. Operators add biocides to control microbial activity, however, in many cases biocide application is partly or even completely ineffective. This is, in part, because biocides are rarely tested using real environmental communities relevant to fracing systems. The following introduction provides background on shale gas resources and fracing technologies. In addition, it highlights features of fracing fluids and additives relevant to microbial community activities and their deleterious effects on shale gas well completions and sets the stage for the thesis work that follows.

1.1 Energy Demand and Natural Gas

Natural gas is currently one of the most widely used sources of energy[3]. There are many reasons that natural gas is the fastest growing primary petroleum-based energy source in the world. Natural gas is the cleanest burning alternative fuel and it emits roughly half the carbon dioxide (CO_2) of coal along with low levels of other air pollutants, practically no sulfur dioxide, and only small amounts of nitrous oxides[4]. The amount of CO_2 produced

when a fuel (e.g., coal) is burned is a direct function of the carbon content of the fuel. Natural gas is primarily methane (CH_4), and it thus has higher energy content relative to other fuels for equivalent CO_2 release. In fact, natural gas emits less carbon per unit of energy than oil and coal [5]. Natural gas thus carries a smaller greenhouse footprint than other petroleum resources[4].

Natural gas is currently used for industrial, residential, electricity generation, commercial, and transportation purposes[4], and this usage is expected to double by 2040, with the most robust growth in demand predicted internationally (U.S. Energy Information Administration, 2014)[6-8]. The production of natural gas is estimated to increase as extraction technologies improve, making new reservoirs more accessible for processing and extraction[9]. The natural gas industry is progressively extracting gas from more challenging resource types such as tight gas, shale gas, coal-bed methane, and methane gas hydrates[10]. Shale gas is produced from very low permeability shale reservoirs[10]. These reservoirs formed by the migration of gas through surrounding permeable rocks over geological time followed by gas trapping in low permeability shales[11]. The challenge with producing shale gas is thus its effective extraction from these low permeability formations which inhibit hydrocarbon flow through subsurface production infrastucture[12]. Operators therefore rely on technologies that enhance reservoir permeability to recover natural gas. The most widely used technology is hydraulic fracturing (fracing)[13,14 10].

1.2 Shale Gas Formations in Canada

Canada is currently the fifth largest producer of natural gas in the world[15]. It has many resources located in the Western Canadian Sedimentary Basin (WCSB)[16,17]. These account for 536 Tcf of the total of industrially recoverable shale gas resources in Canada[9,18]. The WCSB holds some of the world's largest reserves of petroleum and natural gas representing much of the North American gas production potential[9]. One of the most active locations, and largest shale gas formations of the WCSB, is the Montney formation[9]. The Montney formation is predominantly composed of siltstone and shale, which were laid down during the early Triassic period, over 227 million years ago (Mya)[19,20]. The Montney formation extends from the west of British Columbia into Alberta[21]. The depth to the top of the Montney increases from around 300 m in the east to more than 3000 m in the west[21]. The Montney formation comprises an average total organic carbon (TOC) content of 0.8 wt%, with a range of 0.1 to 3.6% and types II/III of organic matter[19]. The organic matter in the formation is marine in origin, with a composition appropriate for oil and natural gas generation[19]. This high organic content can lead to effective production of hydrocarbons, but the Montney formation has a permeability of less than 1 mD and pore spaces less than 0.005 μm in size, which prevents hydrocarbons from flowing[22]. Due to its low permeability, most of the Montney shale reservoir, such as that in the Fort St. John area of northern British Columbia, must be fractured to enhance permeability and recover commercial amounts of natural gas. To increase the permeability of the shale, fluids are injected under high pressure to create fractures (hydraulic fracturing). The combination of hydraulic fracturing and horizontal well completion is essential for gas extraction from tight shales like the Montney[23].

1.3 Hydraulic Fracturing

Shale gas production begins with drilling wells vertically through shale formations up to 4000 m deep and horizontally over more than 2000 m to reach the target zone for gas production, which is generally on the order of 300 m thick with permeability between 0.001 and 0.05 mD[24,25]. Fracing is used to enhance formation permeability and is conducted using large volumes of pressurized fluids that are injected into subsurface formations[26]. The pressure of the fluid causes the shale to break creating fissures and microfractures[12,27,28]. Microfractures spread away from the well bore and penetrate deep into the formation reaching up to 600 m in length[12]. These microfractures connect the formation pores, creating channels that facilitate subsurface hydrocarbon (natural gas) flow[12]. Fracing operations are made sequentially over the length of the horizontal well beginning at the well terminus and moving progressively towards the wellbore[29]. Proppants, small grains of sand, are added to fracing fluids and these grains settle in the microfractures created [30]. Once pressure is released at the end of the fracing operation, these proppants prop microfractures open and thus promote gas flow from the shale formations to the well bore[30]. Following a fracing operation, pressure in the well is relieved and fluids along with gas are allowed to return to the surface. The fluids that return to the surface following fracing are referred to as flowback fluids, and these generally comprise mostly the waters that were pumped into the formation during fracing[12]. Flowback progressively gives way to production fluids, and these are thought to generally comprise formation waters along with residual injection water. Flowback and produced fluids are saline to hypersaline as defined by total dissolved solids (TDS) concentrations that can be as high as 350,000 mg/l[14,31]. TDS is generally derived from

interaction of the flowback and formation waters with shale, with the latter generally possessing higher TDS. TDS comprises mostly inorganic ions including sodium, calcium, barium, strontium, and chloride[32].

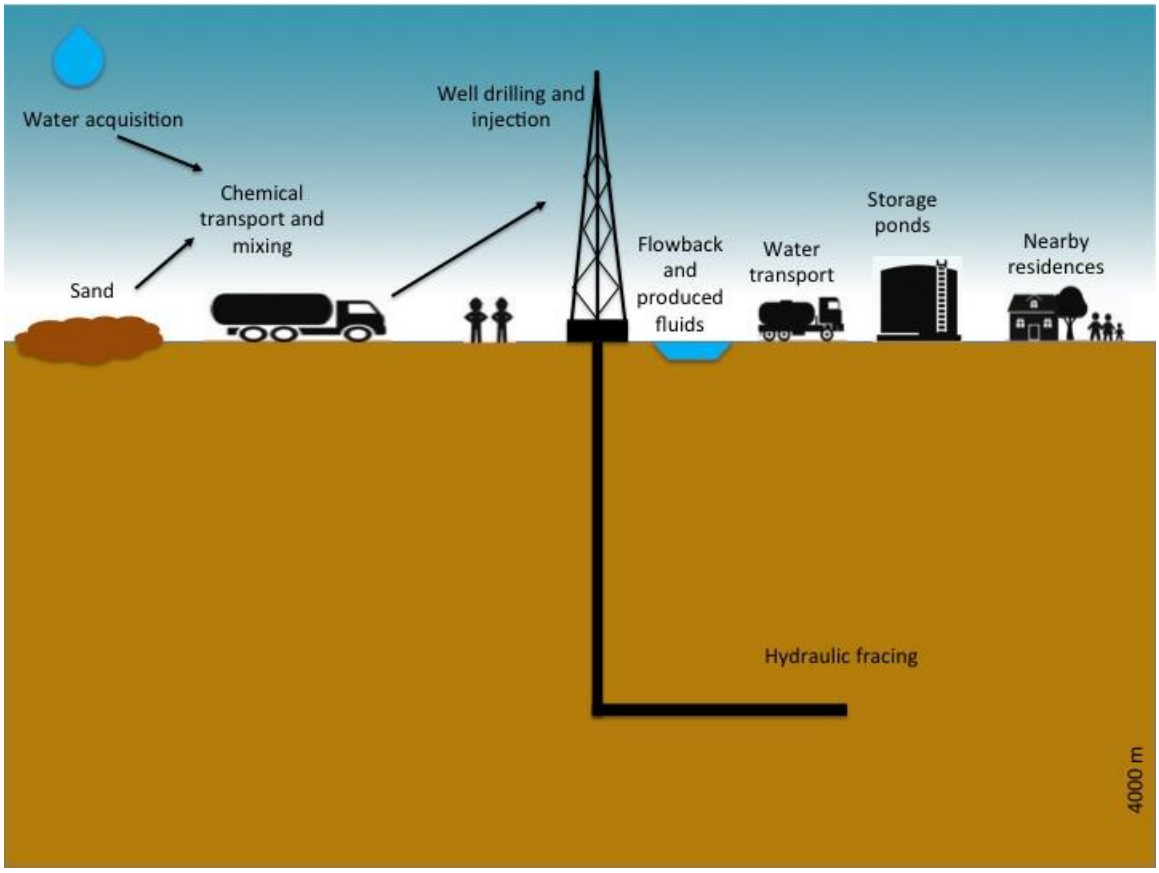

Figure 1: Schematic illustration of a hydraulic fracring operation and horizontal well.

1.3.1 Fracing fluid

Large volumes of fluid are needed for hydraulic fracturing, and these fluids are generally comprised of 15-20 million litres of water (~90% of the total fluid volume) and ~10% sand as the proppant[26]. Operators generally use water recovered from a variety of

sources including aquifers, rivers, lakes and treated wastewater effluent[14]. The water is transported to the well location by pipes or trucks and is then stored in open storage ponds[33]. Before injecting fluid into the subsurface, most fracing operations apply a cocktail of chemical additives that depend on the characteristics of the water and the shale formations to be fractured[30]. In particular, biocides comprise about 0.001 % of the total fracing fluid and these biocides are added to waters while they are stored in storage ponds. Immediately prior to pumping down hole, stored water is mixed with sand and other chemicals at the 'mixer' making up the fracing fluid [26,34].

1.3.2 Chemical characteristics of fracing fluids

Water injected into the subsurface can be retained within the shale formation, and thus the volume of the recovered (flowback and produced) water is often lower than the volume of the injection water[12,35]. Retained fracing water may also include formation waters. The volume of fluid that is recovered is generally between 20 and 70% of that injected[12]. Flowback and produced waters take on many of the chemical characteristics of the shale they pass through[12] and the fluids dissolve many of the substances (inorganic elements) naturally present in shale formations[36]. This water-rock interaction generally leads to decreases in the quality of fracing fluids, in particular increasing TDS[36]. Fracing fluids thus differ from the source fresh waters (<5,000 ppm (TDS) Total Dissolved Solids) principally due to different concentrations of TDS (5,000 ppm to 100,000 ppm TDS or higher)[31].

Three classes of inorganic components control flowback and produced water quality. The first are the components that contribute to salinity, principally Na and Cl. The rapid increase in water salinity during the first few days of a fracing operation renders the waters challenging either to reuse in fracing or to dispose of and eventually remediate[37]. There are also components generally considered toxic[38,39] and flowback and produced waters often have high concentrations of Strontium (Sr), Barium (Ba), Copper (Cu), Manganese (Mn) and other heavy metals[38,39], as well as naturally occurring shale derived radioactive elements, such as potassium (K), uranium (U) and radium (Ra)[40]. All of these components need to be considered when evaluating water management strategies.

1.3.3 Chemical additives

Chemical additives are used for diverse functions in fracing fluids. A list of common chemical additives is compiled in table 1. The use of these additives in fracing is determined by many factors, including water properties like hardness, the geology of the target shale formation to be fraced, temperature, pressure, anticipated biofouling, and souring potential[41,42]. Waters containing these additives are generally referred to as injection waters or fluids[12,26,30,34]. The types of chemical additives include acids, friction reducers, gelling agents, cross-linkers, scale inhibitors, pH buffers, iron control agents, corrosion inhibitors, and biocides. These chemical additives are used in injection fluids to optimize the performance of the fracing process and support gas extraction[43]. The addition of friction reducers lowers the surface pressures required to pump fracing fluids into the target shale and to maintain the high pressure required at depth[43]. Hydrochloric

acid (HCl) is the largest liquid component added to fracing fluid with concentrations up to 15% by volume [44]. The volume of the acid is diluted 85% with water in a solution prior to addition to the injection fluids[44]. HCl helps to dissolve carbonate minerals further opening pores and fractures in the shale. The viscosity of injection water is low, which limits the ability of water to carry the proppant, which is necessary to keep the microfractures open and release gas[45].

Table 1: Roles of common chemicals added to hydraulic fracturing fluids[3,12,30]

Additive	Example	Function
Acid	Hydrochloric acid HCl	Dissolves minerals and initiate cracks in the rock
Gelling agent	Guar Gum	Thickens the water in order to suspend the sand
Friction Reducer	Polyacrylamide	Slicks the water to minimize friction
Corrosion Inhibitor	Isopropanol, Acetaldehyde	Prevents the corrosion of the pipe
Scale Inhibitor	Sodium Polycarboxylate	Prevents scale deposits in the pipe
Surfactant	Lauryl Sulfate	Increases the viscosity of the fracture fluid
PH Adjusting	Agent Sodium Hydroxide	Adjusts the pH of fluid to maintains the effectiveness of other components
Tracer	Undisclosed	Tracks fluid returning to surface from different stages for analysis
Iron Control	Citric Acid	Prevents precipitation of metal oxide
Biocide	See table 2	Eliminates bacteria in the water that produces corrosive by-products

Thus, to increase the viscosity of injection fluids, a gelling agent is often added[45]. Guar gum is the most common compound used as a gelling agent to suspend proppant (sand)[44]. In addition to the gelling agent, cross-linkers also add viscosity to the fracing fluid by increasing polymer molecular weight[45]. Corrosion inhibitors are added to form a protective layer on metal well components, preventing corrosion by acids, salts, or corrosive gasses[46,47]. Biocides are additives that also decrease corrosion that results from microbial activity. In addition, biocides are applied to prevent biofouling and gas souring[48]. Biocides, such as Glutaraldehyde are liquid additives that are diluted in the fracing fluid before injection[49]. Generally speaking, the cost of a well completion increases as the volume and number of additives increase. Minimizing additive, in particular biocide, use is thus key for optimizing well completion costs and improving the value of natural gas resources.

1.3.4 Microbial activity in fracing fluids

One of the major challenges associated with fracing and natural gas production is microbial activity. In addition to biofouling, growth and activity of microorganisms in fracing fluids can produce acids as well as hydrogen sulfide, which can induce corrosion, and gas souring[50,51]. The National Association of Corrosion Engineers (NACE) states that ~20% of all corrosion in the oil and gas industry is caused by microbial activity[52]. Corrosion in gas and fracing systems can occur thermochemically, however, it is more frequently associated with the metabolic activities of fermentative and sulphate or sulphur reducing bacteria[53]. Microbes generate corrosive hydrogen sulfide during anaerobic cellular respiration by using energy from coupling organic matter (or

hydrogen) oxidation to the reduction of various oxidized sulfur compounds such as sulfate, thiosulfate, and sulfite to sulfide [53]. Microbial sulphide production is a key problem in shale gas completion and if left unchecked, it can lead to gas souring and an appreciable loss in the value of shale gas resources.

Hydrogen sulfide can accumulate to relatively high concentrations of more than 100s of ppm in natural gas produced from shales[54] . It can be generated in reservoirs through the activity of sulphate and sulphur reducing bacteria, as described above, or through thermochemical sulfate reduction[55,56]. Hydrogen sulfide is highly corrosive; it reacts with metal surfaces dissolving the metal, depositing ferrous sulfide (FeS) and degrading the overall strength and integrity of the equipment[53,57,58]. In addition to this equipment corrosion, sulphide production leads to souring of natural gas[53,57-59]. There are additional concerns that sulfate reducing bacteria can also contribute to biofouling[60]. Biofouling and plugging are problematic during fracing because they can reduce the rate of gas production[60,61]. Shale microfractures can be plugged both by microbial communities that have been introduced into the formation while fracing and by microbial growth during well completion. Microbial communities can adhere to the surface of the fractured shale and plug the gas stream[60,62]. Such adherent biofilms clog microfractures decrease, permeability, and prevent gas flow[63].

Corrosion and gas souring represent major liabilities for natural gas producers. For instance, in 2016 the American oil and gas industry invested around 7 billion dollars annually in direct corrosion repair and replacement in addition to the 1.4 billion dollars spent on the preservation and repair of production equipment[64]. Given these costs,

operators commonly apply expensive liquid scavengers to remove hydrogen sulfide from sour natural gas production streams at a cost of between 8 to 10 USD per pound of sulphide removed [65-67]. Operators add biocides in attempts to eliminate microbial activity entirely[34], yet despite biocide application, microorganisms are able to survive and even grow during well completion[34,68].

Microorganisms enter fracing fluids through several possible sources. The various water sources (rivers, lakes, municipal waste waters) all contain populous microbial communities as does the sand proppant and drilling mud[69]. Microorganisms may also populate the shale formations themselves, though the low inherent permeability of shales themselves makes this unlikely[34]. Well bores, however, crosscut many natural subsurface fracture and fault systems. Microorganisms populating these fracture and fault systems could thus be entrained in fracing fluids and introduced to the shale following fracturing. Fracing equipment also harbours microbial communities providing a vector for the transmission of organisms between well bores, formations, and strorage ponds[34,70]. Regardless of the provenance of particular microbial community members, the ultimate composition and activity of fracing fluid microbial communities is likely a strong function of fluid chemistry and subsurface conditions (e.g. TDS, electron donor and acceptor concentrations, P, and T)[34].

To date, there is little combined information on the ecology and activity of microorganisms in fracing fluids. Existing literature is summarized in table 2. These studies reveal that the microbial communities in flowback, and produced fluids are different from those in the injection fluids[34,68]. Notably, both microbial diversity and

abundance tend to decrease in flowback and produced waters relative to injection fluids, the former of which tend to be comprised largely of anaerobic microorganisms[34,68]. Most of the anaerobic bacteria recovered from these fluids belong to the phyla Actinobacteria, Bacteroidetes, and Firmicutes[22,34,68,71]. Gammaproteobacteria have also been detected along with members of the Pseudomonas and Marinobacter clades[22,34]. In particular, halotolerant bacteria, specifically bacteria of the genus Halanaerobium, are progressively enriched in relative abundance from injection through to flowback waters[22,34]. Microorganisms with specific metabolic capacities such as sulfate reducing, fermenting, and methanogenic guilds have also been identified or cultured[72,73]. For example, Mohan et al. recently found that members of the order Halanaerobiales were particularly abundant in produced waters[61]. Halanaerobium have been found to dominate fracing systems from Texas to Pennsylvania[61]. For instance, community surveys of frac fluids from in the Marcellus Formation in Pennsylvania have shown a shift to a dominance of halophilic microbial communities as the well proceeds from injection through to flowback and produced flow phases[61,68]. In situ, the high temperatures up to 80 of many subsurface shale formations likely restrict microbial growth, which leads to low biomass and challenges in the recovery of DNA from flowback and production fluids[74,75]. For example, a study on flowback and produced fracing fluids recovered from Montney wells have shown that there is an increase in the relative abundance of the halophilic genus Halomonas, however, there was insufficient DNA recovered from produced fluids for sequencing[74]. Akob et al. studied the microbiology of flowback and produced fracing fluids from 13 different shale gas wells in north-central Pennsylvania[73].

Table 2: Summary of previous studies having analyzed the microbial ecology of produced fracing fluids [22,55,56,68,71,73,76-79]

Year	Source	Method applied
2011	Struchtemeyer et al.	16S rRNA, MPN
2012	Davis et al.	16S rRNA
2013	Wuchter et al.	16S rRNA
2014	Cluff et al.	Metagenome
2014	Mohan et al.	Enrichment culture, 16S rRNA
2015	Akob et al.	Metagenomic binning
2016	Daly et al.	16S rRNA
2016	Vikram et al.	16S rRNA, metatranscriptome
2016	Liang et al.	16S rRNA, MPN, isolation
2017	Booker et al.	Metagenomic binning, isolation

They found evidence for acid production, sulfide, and methanogenic activity in the sampled wells. They did microbiological analyses including culture-based experiments for anaerobic fermenters, methanogens, and hydrogen based sulfate-reducing bacteria[73]. They detected anaerobic fermenters in flowback and produced fluids in all wells studied. Moreover, they detected hydrogen sulfide producing bacteria from seven wells, and methanogens from five wells. Most of the classified OTUs were affiliated with the genus Halanaerobium[73]. The high abundance of the genus Halanaerobium in these

produced fluids confirms a shift in the microbial composition from injection to production fluids and also implies a role for the genus Halanaerobium in producing sulfide in fracing operations[73].

Struchtemeyer et al. (2011) used most probable number experiments to investigate fracing fluids from mixing trucks, storage tanks or storage ponds (injection fluid) and flowback fluids from the Barnett Shale gas play[34]. Fluids were also analysed by sequencing 16S rRNA genes. These analyses revealed that storage ponds, which were comprised of Actinobacteria, Firmicutes, Bacteroides, Betaproteobacteria, and Cyanobacteria, have more diverse communities than flowback fluids[34]. The diversity of flowback communities was reduced to comprise only the taxa Bacillaceae, Clostridiaceae, Planococcaceae, and Halanaerobiaceae. Taxa affiliated with known sulfate-reducing bacteria were identified in these fluids, including members of the Desulfotomaculum and Desulfosporosinus, implying the presence of sulfate reducers in flowback fluids from the Barnett Shale.

Davis et al. (2012) examined the microbial ecology of equipment used in shale gas production[22]. They also used 16S rRNA gene sequencing and analysed materials collected from storage tanks (injection fluids) and separators (flowback/produced) at different time points from Barnett shale gas wells[22]. These results revealed more diversity in storage tanks than in the separator with taxa including members of the Marinobacter, Arcobacter, and Pseudomonas genera[22]. The separator community was characterized by high relative abundances of Firmicutes, particularly of the order Halanaerobiales. In this study, the authors confirm that the composition of microbial communities shifted from

storage tanks to separator[22]. The relatively high abundances of anaerobic and fermentative bacteria in the separator implied susceptibility to microbial corrosion[22].

While many previous studies have detected the presence of specific microbial taxa in fracing systems, these studies are largely based on recovery of DNA, which yields little information on activity. The central dogma of molecular biology describes the two-step process, transcription and translation, by which the information in genes flows to proteins: DNA → RNA → protein[80]. Genomic DNA contains all the information for the structure and function of an organism[80,81], but to activate a metabolic function, such as sulfur-metabolism, the responsible genes must be expressed, that is, transcribed into RNA. Transcription is the synthesis of an RNA copy of a segment of DNA[81]. Sulfidogenesis (hydrogen sulfide production from sulphate or other sulphur compounds) is one of the major metabolic processes that take places under anaerobic conditions[82]. In shale gas systems, our knowledge of the diversity and activity of the microbial communities responsible for sulfidogenesis processes remains limited. This is partly because of our inability to isolate the most relevant microorganisms into pure cultures that are tractable to laboratory experimentation. New molecular microbiological techniques, however, have provided alternative approaches to partly overcome problems associated with the culture-dependent analysis of microbial communities[83]. Combinations of these molecular methods with classical microbiology, such as 16S rRNA gene sequencing, enrichment culturing, metagenomics, and metatranscriptomic can, in principle, be used together to study the diversity and metabolic potential of sulfate-reducing or sulphide producing bacteria in fracing fluids. To date, there exist few studies in which the metabolically active populations were interrogated though

metatranscriptomic approaches[78,84]. Transcriptomics is the study of the transcriptome, the complete set of RNA transcripts that are produced from a genome, under a specific set of conditions or in a particular cell, using high-throughput sequencing methods[85]. Comparison of transcriptomes allows the identification of genes that are differentially expressed in different cell populations, or in response to different treatments[85]. Transcriptomics, or metatranscriptomics when applied to microbial communities, thus provides an opportunity to evaluate the active organisms in an environment and identify expressed metabolic pathways[85]. Vikram et al. (2016) evaluated the active community in produced fluids and identified the active metabolic pathways by applying metatranscriptomic tools[78]. The produced fluids were collected from the Marcellus Shale gas play. Some fluids were obtained from storage tanks (produced fracing samples), and others from mixing trucks (injection fracing fluids)[78]. Their taxonomic analyses revealed differences in community compositions estimated via 16S rRNA amplicon gene sequencing versus metatranscriptomics, which underscores the importance of considering the active community in microbial control strategies[78]. The most abundant populations in injection fluids were taxonomically affiliated with the Vibrionaceae, Enterobacteriaceae, and Bacillaceae lineages. Storage tank communities comprised Pseudomonadaceae and Burkholderiaceae. About 15% of the community in the produced fluids was Archaeal, suggesting they may also contribute to microbial community activity in fracing systems[78].

Halophilic microorganisms, including Halanaerobium, appear to be ubiquitous and abundant components of microbial communities in fracing fluids because of generally high salinities. Booker et al. (2017) isolated the first Halanaerobium strain from produced fluids[79]. Halanaerobium bacteria were isolated from produced fluids from a

Utica Shale well and were cultured onto anaerobic yeast extract-peptone-dextrose (YPD) medium[79]. This study reveals that Halanaerobium possess a sulfite reductase with capacity for reducing thiosulfate to sulfide. Metabolic reconstructions imply a fermentative metabolism in Halanaerobium in which thiosulfate is used to eliminate excess reductant[79]. In this way, Halanaerobium may contribute to well souring and sulphide production through thiosulfate-reduction in saline fracing fluids[79]. Indeed, a more recent study by Liang et al. revealed that Halanaerobium is responsible for sulfide production in produced fluids and that glutaraldehyde is not effective at controlling growth of Halanaerobium[56]. This is consistent with previous work that found more generally that glutaraldehyde was ineffective at controlling microbial communities in fracing fluids, especially produced fluids[56]. These observations underscore the need to evaluate the efficacy of biocides against uncultivated organisms to effectively mitigate deleterious biocorrosion and sulphidogenic processes in shale gas production operations.

1.3.5 Biocides

The water used in fracing contains many environmental microbes including sulfate-reducing bacteria (SRB), acid-producing bacteria (APB), nitrate-reducing bacteria (NRB), and heterotrophic aerobic bacteria (HAB)[53,86]. Bacterial activities in shale gas production systems can cause issues such as corrosion, souring, biofouling, and safety hazards[22,53]. Biocides are compounds used to arrest microbial activity and growth thereby mitigating deleterious processes[87]. Chemical compounds, with biocidal properties and used as antibacterial preparations have diverse chemical properties[48]. Biocides are added to frac fluid source waters to reduce the concentration and activity of resident bacterial

populations[48]. There are currently more than 15 different biocides in use in fracing operations[48,88]. Table 3 shows the frequently used biocides, grouped by name and mode of action. Glutaraldehyde has been in use in several industries for many years, and is widely used in fracing as an inhibitor of microbial sulphide production. Knowing the efficacy of industrial biocides in controlling the growth and activity of relevant microorganisms is important for optimizing their use[48]. Biocides are grouped based on their mode of action and not their specific chemistry (biocidal mechanism) and are generally classified into two groups[89]; 1) oxidizing compounds; and 2) non-oxidizing compounds.

Table 3: Mode of action of most used biocides in fracing operations, the table shows the frequently used biocides, grouped by name and mode of action. Glutaraldehyde has been in use in several industries for many years, widely used in fracing as an inhibitor of SRB metabolism [48,89].

Biocide	Mode of Action	Frequency of use
Glutaraldehyde	Electrophilic	27%
dibromo3-nitrilopropionamide (DBNPA)	Electrophilic	24%
tetrakis(hydroxymethyl)-phosphonium sulfate (THPS)	Electrophilic	9%
chlorine dioxide	Oxidizing	8%
didecyldimethylammonium chloride (DDAC)	Lytic	8%
tributyltetradecylphosphonium chloride (TTPC)	Lytic	4%
Soduim hypoclorite	Oxidizing	3%
Bronopol	Electrophilic	1%

Oxidizing biocides are chemical agents that oxidize cellular material, enzymes, or other proteins resulting in the death of microorganisms[48]. This mode is not commonly used in fracing because the oxidizing substances are too short-lived in the presence of abundant reductants to control microbial growth for extended periods[90]. An example of an oxidizing compound is sodium hypochlorite (bleach)[91,92]. The use of oxidizing biocides is associated with negative effects such as the interaction with other chemicals (corrosion inhibitors), potential interaction with non-metallic substances, and corrosion of metallic materials[48].Non-oxidising compounds can be sub-divided again based on mode of action into; 1) lytic biocides; and 2) electrophilic biocides[48]. Lytic biocides bind to anionic functional groups on cell membrane surfaces and cause destruction of the lipid bilayer with a subsequent loss of osmotic control and eventual lysis of the cells[93]. An example lytic biocide is didecyldimethylammonium chloride (DDAC)[94]. Electrophilic biocides react with electron-rich chemical groups in membrane proteins on bacterial cell walls causing death[94]. The common electrophilic biocides used in fracing fluids are glutaraldehyde (GA), dibromo3-nitrilopropionamide (DBNPA), and pronopol[95]. Based on data from Fracfocus, the electrophilic biocides are used in more than a quarter of fracing operations with glutaraldehyde used in 27% of operations and 2,2-Dibromo-2-cyanoacetamide (DBNPA) used in 24%[96]. Glutaraldehyde (GA) is the most commonly used electrophilic biocide in hydraulic fracturing operations; it has long been used to protect wells and pipelines from microbial fouling and corrosion[48]. In particular, aldehyde-based biocides react with cellular nucleophiles, they are aliphatic di-aldehydes, which function by cross-linking proteins[48,94,97]. Few studies have investigated the efficiency or the mode of action of biocides on environmental communities relevant to

hydraulic fracing. Kahrilas et al. reviewed current usage of biocides in fracing operations[48] and found that the use of biocides might pose environmental risk from wastewater treatment and the danger of spills. Furthermore, frequent use of biocides could lead to microbial resistance in surviving microorganisms[98].

As previously mentioned, fracing fluids from the various shale gas production systems have been shown to comprise diverse microbial communities, which can contribute to corrosion and souring. To control microbial activities, fracing well operators regularly use different types of biocides[26,34] but biocide application is not sufficient as it generally only reduced cellular abundances by 60%[50]. Some studies have examined the efficiency of biocide application on microbes. Critical reviews of the application of biocides in shale gas extraction[48] summarized the types and the concentrations of biocides used and addressed the concern that repeated use causes adaptation and resistance in surviving microbes. They further found that biocides are not generally transferred from fracing fluids to groundwater. Moreover, the efficacy of seven biocides, including glutaraldehyde, sodium hypochlorite (bleach), tetrakis (hydroxymethyl) phosphonium sulfate (THPS), and didecyldimethylammonium chloride (DDAC) was evaluated using Desulfovibrio, Desulfuricans, and a sulfate reducing enrichment culture from the Barnett Shale obtained from produced fluids [99]. The Planktonic culture and the biofilm were treated with biocide concentrations of 12.5 ppm, 400 ppm, and the Minimum Inhibitory Concentration (MIC). The results revealed that MIC values for all biocides were higher in biofilms compared to planktonic cells. Furthermore, biofilm MICs values varied among biocides. In this experiment, lower concentrations of biocides DDAC and THPS between 20 ppm to 40 ppm were sufficient to inactivate *Desulfovibrio*

desulfuricans and the sulfate-reducing enrichment culture. In contrast, higher concentrations of glutaraldehyde, up to 200 ppm, and sodium hypochlorite up to 100 ppm were needed to inactivate both *Desulfovibrio desulfuricans* and the sulfate-reducing culture[99]. These data suggest that biocides (DDAC and THPS) are more effective in controlling sulfate-reducing bacteria than glutaraldehyde and sodium hypochlorite. Furthermore, MIC data support that the biofilms have resistance to biocides. The study also examined the impacts of organic matter loading on the efficacy of the seven biocides[99]. Humic acid was used as the source of organic matter in these experiments. The MICs of all biocides increased with the addition of humic acid with the sole exception of DDAC. These findings illustrate that organic matter generally decreases biocide efficacy.

Biocides also degrade in fracing operations. Previous studies have shown degradation of the most commonly used biocides, including glutaraldehyde[48]. Degradation of glutaraldehyde becomes possible due to auto-polymerization, particularly under high pH conditions, which makes glutaraldehyde lose its efficacy over time[48]. Phosphonium sulfate (THPS), dibromo3-nitrilopropionamide (DBNPA), and tetrakis (hydroxymethyl) are also likely to degrade microbially. These data imply that it is crucial to gain more information on the effects of subsurface conditions such as temperature and pressure on biocide efficacy[48] given that both of these two factors may promote degradation. While the degradation of several biocides has been thoroughly investigated, more studies are needed to fully explore the range of conditions relevant to biocide degradation as well biocide efficacy in controlling diverse microbial taxa and processes.

Given that biocide use appears, at best, partly effective at controlling microbial processes in fracing fluids, more research is needed to explore specific biocide efficacy on relevant taxa and processes in diverse fluid compositions. I thus set up a study to test the efficacy of glutaraldehyde, which is one of most commonly used biocides, to control microbial sulfur reduction in fracing fluids from the Fort St. John area of northern British Columbia, Canada. To do this, I conducted incubation experiments in which I amended the fluids with glutaraldehyde and yeast extract and followed sulphide and sulphate concentrations for 30 days. To link these results to the relevant microbial taxa, I identified and characterized the microbial community present in the incubated fluids using 16S rRNA gene amplicon sequencing. All in all, I found that glutaraldehyde is only moderately effective in controlling microbial sulfide production in fracing fluids from the Fort St. John area, and that, even in the presence of glutaraldehyde, amendment with organic matter enhances sulfide production. These experiments are described in Chapter 2 of this thesis.

Chapter 2

Introduction

Natural gas extraction from shale formations is one of the fastest growing energy production methods, globally[100]. About 120 trillion cubic feet (Tcf) per year of natural gas is used internationally and this is expected to increase to 203 Tcf by 2040[1]. Canada has the most abundant natural gas reservoirs in the world[2]. Most of Canada's shale gas is in the Western Canada Sedimentary Basin (WCSB), which spans northern British Columbia, Alberta, Saskatchewan, Manitoba and the southern Northwest Territories[9,17]. Production data to date suggests that as much as 536 Tcf of the total recoverable shale gas in the sedimentary basins of western Canada is from shales in the Horn River Basin and the Montney formation, which are the most active plays in WCSB[9,17]. Technological combinations of horizontal drilling and hydraulic fracturing have made extraction of this enormous shale gas resource possible, thereby revolutionizing the energy production industry in western Canada[101].

Hydraulic fracturing, commonly referred to as fracing, is an unconventional method used to extract natural gas from shale[12]. Fracing operations follow vertical and horizontal drilling into the targeted shale formations, which, in western Canada, are located more than 1 km deep[3,36]. Large volumes of fluids, commonly composed of water (90%) mixed with sand (9%) and chemical additives (1%), are injected into the well at high pressures to create microfractures in the shale formation[26]. Regular fracing procedures use specific concentrations of chemical additives depending on the

characteristics of the water and the shale formation being fractured[12,26,30,34]. Additives mixed with fracing fluids include: 1) biocides to control microbial growth and activity; guar gum as a gelling agent to suspend sand[12,26,30,34]; hydrochloric acid as a corrosive agent to help dissolve minerals; and a friction reducer to reduce fluid friction that develops at the pipe interface while pumping water under high proessure[61]. Following creation of fissures and microfractures within shale formations, the pressure is released and the fracing fluids return to the surface as so called 'flowback' fluid[102,103]. Following the flowback period, formation waters and residual fracing fluids are recovered during production, and these are referred to as 'production' fluids[102,103]. Flowback and produced fluids are saline to hypersaline and are generally characterized by total dissolved solids (TDS) concentrations as high as 350,000 mg/l. These high TDS concentrations arise due to high concentrations of inorganic ions such as sodium, calcium, barium, strontium, and chloride that are derived from the shale formation itself[14,31]. In many cases, operators elect to mix produced waters with regional sources of surface water to minimize disposal costs and to avoid fresh water consumption. These water mixtures can remain in storage ponds for up to several months before their ultimate recycling as injection waters in further fracing operations[104].

Microbial community activity in fracing fluids leads to a number of deleterious effects including the production of hydrogen sulfide (H_2S)[34]. Hydrogen sulfide induces corrosion and causes the accumulation of ferrous sulfide (FeS) on metal surfaces[105]. Corrosion leads to a deterioration of equipment[53]. Sulfide production also creates safety and health hazards since exposure to H_2S gas can be toxic and even fatal[106]. The source of most of this sulphide is the metabolic activity of microorganisms, in particular sulphate

(and sulphur)-reducing bacteria (SRB)[107]. Microorganisms generate sulfide anaerobically as the result of cellular respiration with energy gained by coupling organic matter (or H_2) oxidation to the reduction of sulphur species, like sulfate, to sulfide[107]. Sulfate (or sulphur) reducing bacteria in fracing systems derive from several possible sources; drilling mud used during the vertical and horizontal drilling of the well; the source water, or the formation itself[108]. Many microorganisms derived from surface sources likely survive the fracing operation despite being subjected to large and rapid changes in temperature, pressure, salinity and pH as well as exposure to biocides during the fracing operation[48]. The combination of these effects is likely reflected in changes in microbial community composition between injection, flowback, and produced fluids[34,68].

Biocides are commonly used to control microbial growth and activity in fracing fluid. The biocides glutaraldehyde, sodium hypochlorite (bleach), and amine compounds are usually added, individually or in combination, to fracing fluids prior to injection[48]. Despite application of biocides, many microorganisms survive and even grow during fracing and well production[78]. Microbial activity is known to negatively impact hydraulic fracing in a number of ways. These include souring, corrosion, and biofouling[34]. Souring is the production of hydrogen sulfide (H_2S) from sulfur compounds such as sulfate, thiosulfate, and sulfite by microbial metabolism (through anaerobic respiration)[109,110]. Hydrogen sulfide is corrosive and toxic, and it decreases the value of extracted natural gas and poses health risks to workers[111]. The interaction between steel and the H_2S results in the formation of a FeS—a corrosion product[112]. Microorganisms in fracing fluids can form biofilms within the shale itself or on metal surfaces including well production equipment[60]. This so-called 'biofouling' clogs gas flow channels and pipelines[63,113].

Microbial growth and activity thus represent financial liabilities as a result of decreased production, decreased gas values, and additional operating costs [65,67].

Several studies have evaluated the microbial communities in fracing fluids[114,115]. A diverse suite of bacteria including those belonging to the Proteobacteria, Clostridia, Synergistetes, Thermotogae, and Bacteroidetes lineages, in addition to a number of Archaea have been detected in injection fluids[116,117]. The diversity of organisms in flowback and produced fluids is much lower and these are comprised mostly of anaerobic Firmicutes, Bacteroidetes, Actinobacteria and Gammaproteobacteria such as Marinobacter and Pseudomonas[22,34,68,71]. Studies have linked microbial communities in fracing fluids to the degradation of hydrocarbons. Specifically, halotolerant bacteria including Halanaerobium, Halomonas, diverse Vibrio, Halolactibacillus, and Marinobacter have metabolic potential for hydrocarbon oxidation, fermentation, and sulfur-cycling and these taxa can comprise >90% of the fracing fluid microbial community[118]. Thus microbial populations in fracing fluids have the potential for corrosion, biofouling, and souring effects.

Given their role in well souring, the growth and activity of sulfate-reducing bacteria are major concerns in the production of shale gas[107,119]. Application of biocides is only partly effective in controlling the activity of sulphate reducers in shale gas systems as some biocide treated wells continue to produce hydrogen sulphide. Biocides in general, however, have not been tested under controlled laboratory conditions with relevant microbial taxa. This is largely due to the fact that microbial communities in fracing fluids remain poorly characterized and that most environmentally relevant

microorganisms remain unrepresented in culture collections. In order to test the efficacy glutaraldehyde at controlling microbial sulphide production in fracing fluids, I therefore incubated fracing fluids from the Fort St. John, unconventional shale gas play, with their intact microbial communities, in the presence and absence of glutaraldehyde, as well as with supplemented electron donor. I found that glutaraldehyde is moderately effective at controlling sulphide production, but less so when organic matter is augmented.

Materials and Methods

2.2.1 Sampling

In this study, we targeted fluids from unconventional shale gas wells, from Fort St. John in northern British Columbia. The sampling site, D-26-A/94-G-8, was owned by Progress Energy and located at 57°13'08.5"N 122°11'52.7"W. Injection and produced waters were taken directly from the fracing infrastructure on site. Flowback fluids were collected after 100-250qm of water flowed back to the surface, which occurred after one day of reopening the wells. The samples from the wells were taken from the valve manifold before the sand filter or separator. The valves were fit with 1" tubing and bled directly into the bottle.

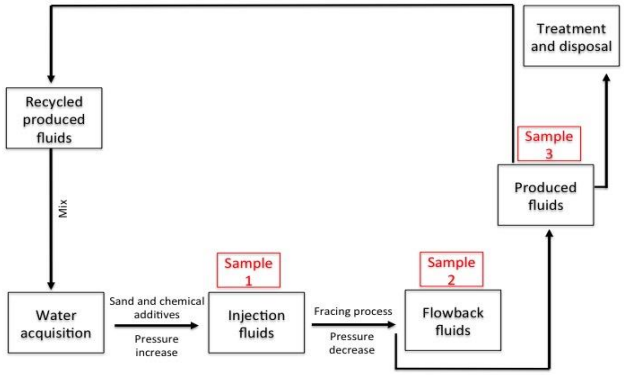

Figure 2: Schematic of fracing operations. Numbers in red boxes are indicate locations for sample collection in this study. Black squares and lines represent water flow via the pipes. However, there is some trucking to complement.

2.2.3 Experimental design

To test the effect of gluteraldehyde and organic matter on sulphate reduction and sulphide production in fracing fluids, I incubated a suite of fluids and monitored the production of sulphide and the consumption of sulphate. 60 ml of fluid was allocated to sterilized serum bottles.

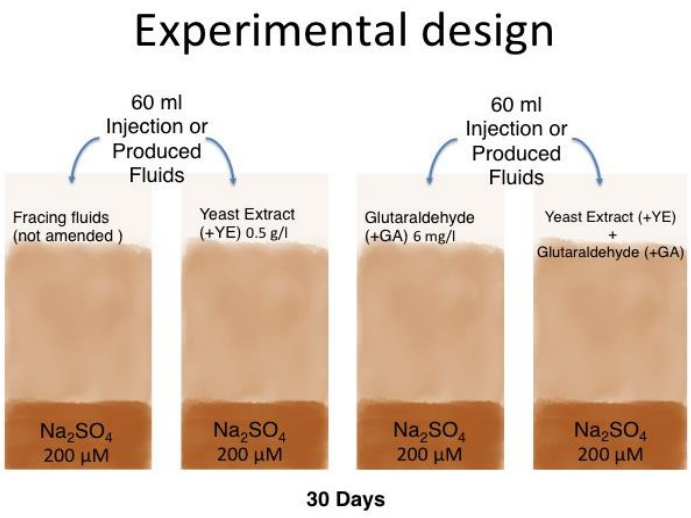

Figure 3: Experimental design—60 ml of fracing fluid was allocated to sterilized serum bottles (orange color). These were variably amended with and.5g/l yeast extract as a carbon and energy source, and 6 mg/l glutaraldehyde as a biocide. All treatments were prepared in triplicate and received 200 µM sodium sulfate as a source of sulfate. Samples incubated for 30 days at room temperature in the dark.

Some of these fluids were amended with 0.5 g/l yeast extract (YE) as carbon and energy source and 6 mg/l glutaraldehyde (GA) as a biocide (Table 3). All treatments were prepared in triplicate and amended with 200 µM sodium sulfate (Na_2SO_4) as a source of sulphate for anaerobic respiration. The fluids were incubated for 30 days at room temperature 23 ^{o}C in the dark. Over the course of the incubation, sulfide and sulfate concentrations were measured by removing a 1 ml sample from each treatment. In addition to determining sulphide production and consumption, I used amplicon sequencing to profile the resident microbial community. At the beginning and end of amending the fluids with GA and YE, and incubating anaerobically for 30 days I

recovered biomass from 50 ml of fluid passed through a sterivex filter with pores size of 0.22 μM.

2.2.4 Analytical procedures

Sulfate concentrations were measured using ion chromatography (Thermo Scientific Dionex, ICS-2100) with a Dionex Ion Pac™ AS19 RFICTM analytical column (2X250 mm), which is for routine analysis of inorganic anions and oxyhalides in addition to Dionex Ion Pac™ MFC-1, trap (3x27 mm) which used to strip transition and lanthanide impurities of fracing fluids, and Dionex Ion Pac™ NG1, guard (4x35 mm) that traps organic materials. Stock standard solutions were prepared by dissolving sulfate in Milli-Q water at a 10 mM concentration, and working standards prepared from these stocks to between 100 and 1000 μM. Sample concentrations were quantified by comparing the peak area of standard solutions to samples. Every sample was measured on a 300 μl sub-sample following centrifugation to remove particles. Sulfide concentrations were determined using the methylene blue assay[120], which was calibrated using the standard iodometric titration to determine the specific absorbance of the methylene blue reagent. To measure sulfide, 700 μl of sample was made up to 1ml with 300 μl Milli-Q and 80 μl of the methylene blue reagent was added (different concentrations). Spectrophotometric measurements were performed at a wavelength of 670 nm after 1 hour from the reagent addition.

2.2.5 Process rates

I calculated the rates of sulfate reduction and sulfide production from the decrease and increase in concentrations of sulfate and sulfide as a function of time, respectively. I first calculated the average of the triplicate concentrations of sulfide and sulfate for each treatment over 30 days. Rates were calculated through a least squares regression through the most linear portion of the incubation period. Rates were derived from the slope of this regression.

2.2.6 DNA extractions

DNA was extracted from biomass collected on sterivex by enzymatic lysis and phenol:chloroform purification as in Walsh et al [121]. Equal volumes (about 3ml) of Phenol:Chloroform:Isoamyl Alcohol (IAA) were added to the lysate contained in the sterivex filter cartridges[122]. At the end of the extraction, the DNA was washed twice or more with TE buffer to extract pure DNA[122]. The DNA quality was checked by running all samples on a gel 0.8% agarose gel stained with 1ml ethidium bromide (EtBr)[122]. The purity of and quantity of DNA were assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific) and the Picogreen® (Invitrogen) assay according to manufacturers instructions[123]. TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) was used for diluting the Quant-iT™ PicoGreen® reagent, and for diluting DNA samples[123]..

2.2.7 SSU rRNA gene amplification and iTag sequencing

Bacterial and Archaeal 16S rRNA gene fragments from the extracted genomic DNA were amplified by Polymerase chain reaction (PCR) using primers 515f and

806r[124,125]. Sample preparation for amplicon sequencing was performed as described previously[126]. In brief, the aforementioned 16S rRNA gene-targeting primers, complete with Illumina adapter, an 8-nt index sequence, a 10-nt pad sequence, a 2-nt linker and the gene specific primer were used in equimolar concentrations together with dNTPs, PCR buffer, $MgCl_2$, 2U/µl (one unit Taq DNA Polymerase is defined as the amount of enzyme that incorporates 10 nmol of total deoxyribonucleoside triphosphates into acid perceptible DNA within 60 min at +65 °C under the assay conditions stated above) ThermoFisher Platinum™ Taq DNA polymerase and PCR-certified water to a final volume of 25 µL. PCR amplification was performed with an initial denaturing step of 95°C for 2 min, followed by 30 cycles of denaturation (95°C for 20 s), annealing (55°C for 15 s), and elongation (72°C for 5 min), with a final elongation step at 72°C for 10 min. A nested-PCR approach, using primers 27F and 1492R for the initial amplification, was adopted for samples in which the original PCR did not yield any products. Equimolar concentrations of amplicons were pooled into a single library using the Invitrogen SequalPrep kit. The amplicon library was analyzed on an Agilent Bioanalyzer using the High Sensitivity DS DNA assay to determine approximate library fragment size, and to verify library integrity. Pooled library concentration was determined using the KAPA Library Quantification Kit for Illumina. Library pools were diluted to 4 nM and denatured into single strands using fresh 0.2 N NaOH as recommended by Illumina. The final library was loaded at a concentration of 8 pM, with an additional PhiX spike-in of 5 – 20%. Sequencing was conducted at the Sequencing + Bioinformatics Consortium, UBC (https://sequencing.ubc.ca)

2.2.8 Informatics

Sequences were processed using Mothur[127] and the Miseq protocol, as accessed on 071717[126]. Briefly, sequences were removed from the analysis if they contained ambiguous characters, had homopolymers longer than 8 bp or did not align to a reference alignment of the correct sequencing region. Unique sequences and their frequency in each sample were identified and then a pre-clustering algorithm was used to further de-noise sequences within each sample[128]. Unique sequences were identified and aligned against the SILVA alignment (available at http://www.mothur.org/wiki/Silva_reference_alignment). Sequences were chimera checked using VSEARCH[129] and reads were then clustered into 97% OTUs based on uncorrected pairwise distance matrices. OTUs were classified using SILVA reference taxonomy database (release 123, available at http://www.mothur.org/wiki/Silva_reference_files)

Results

2.3.1 Sulfide and sulfate concentrations

In the incubated injection fluids (IF) that had no amendments of either glutaraldehyde or yeast extract, the sulfate concentration remained constant at between 600 and 700 μM over the 30 days of incubation (Figure 4), at the same time, sulfide concentrations increased slightly from 3 to 12 μM (Figure 5).

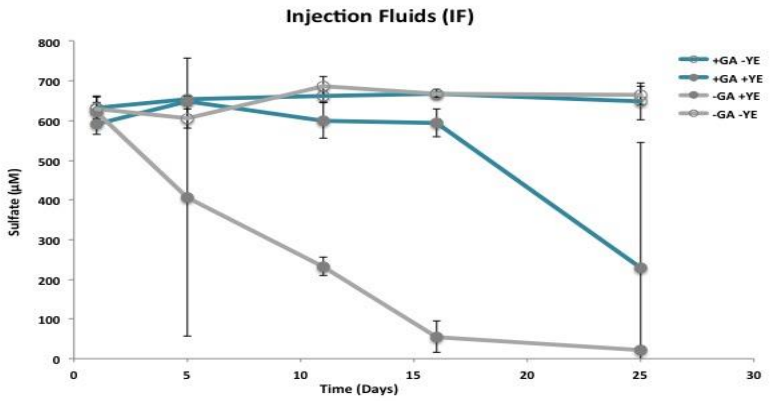

Figure 4: The concentrations of sulfate in injection fluids (IF), with and without glutaraldehyde and yeast extract amendments. Amendments are indicated by annotation GA for glutaraldehyde and YE for yeast extract. "+" Refers to the addition of GA or YE, "-" to no addition. All data are the average of three samples of each treatment with standard deviation (error bar)

Injection fluids that were only amended with glutaraldehyde also had constant sulfate concentrations, while sulfide concentrations remained below 5 µM throughout the incubation (Figure 5). In the injection fluids that were amended with yeast extract only, the sulfate concentrations decreased from 633 µM to 338 µM (Figure 4) and sulfide increased from 3 µM to 451 µM (Figure 5). In the fluids amended with both glutaraldehyde and yeast extract, sulfate concentrations decreased from 590 µM to 229 µM, but this decrease was less than the fluids amended with only yeast extract (Figure 4), while the sulfide concentrations increased 4.6 to 148 µM (Figure 5).

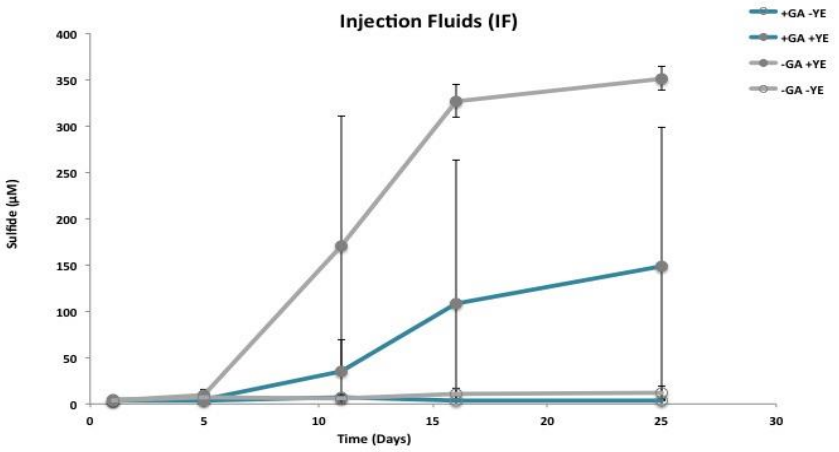

Figure 5: The concentrations of sulfide in injection fluids (IF), with and without glutaraldehyde and yeast extract amendments. Amendments are indicated by annotation GA for glutaraldehyde and YE for yeast extract. "+" Refers to the addition of GA or YE, "-" to no addition. All data are the average of three samples of each treatment with standard deviation (error bar)

The hydrogen sulfide production rate was up to 25 µM/day when only YE added and up to 15 µM/day when both yeast extract and glutaraldehyde added (Figure 6).

In the incubated produced fluids (PF) that had no amendments of either glutaraldehyde or yeast extract, the sulfate concentration remained constant at between 318 µM and 343 µM over the 30 days of incubation (Figure 7), at the same time, sulfide concentrations increased from 17 to 83 µM (Figure 8). Production fluids that were only amended with glutaraldehyde also had constant sulfate concentrations between 368 and 416 µM (Figure 7), while sulfide concentrations increased slightly from 17 to 64 µM

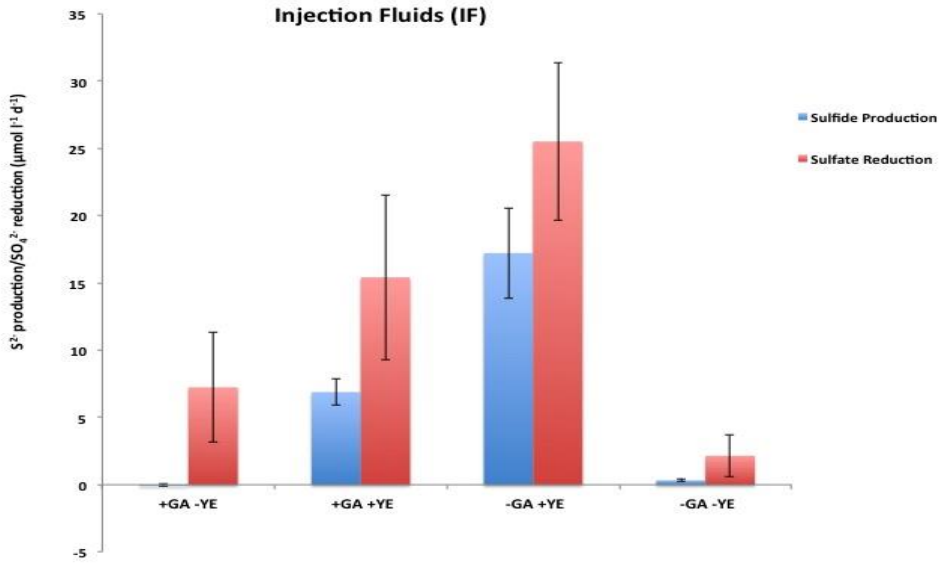

Figure 6: Rates of sulfate reduction (SO_4) and sulfide production (H_2S) in injection fluids (IF) with and without glutaraldehyde and yeast extract. Additions are indicated by annotation GA for glutaraldehyde and YE for yeast extract. "+" refers to the addition of GA or YE, "-" to no addition. All data are the average of three samples of each treatment with standard deviation (error bar).

throughout the incubation (Figure 8). In the production fluids that were amended with yeast extract only, the sulfate concentrations remained between 364 µM to 431 µM (Figure 7) and sulfide increased slightly from 17 µM to 69 µM (Figure 8). In the fluids amended with both glutaraldehyde and yeast extract, sulfate concentrations also stayed between 398 µM to 405 µM (Figure 7), while the sulfide concentrations increased from 17 µM to 68 µM (Figure 8). The hydrogen sulfide production rate was up to 3 µM/day when waters were not amended and was 2 µM/day with the amendments (Figure 6). Sulfate reduction rates in produced waters were greater than sulfide production rates

(Figure 9). In flowback fluids there was no detectable change in sulfide and sulfate concentrations in any of the amendments (Figure 10).

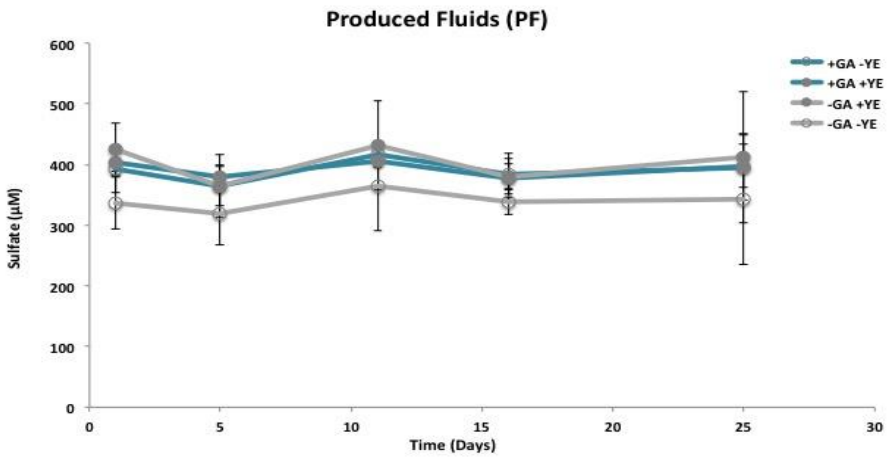

Figure 7: The concentrations of sulfate in produced fluids (PF), with and without glutaraldehyde and yeast extract amendments. Amendments are indicated by annotation GA for glutaraldehyde and YE for yeast extract. "+" Refers to the addition of GA or YE, "-" to no addition. All data are the average of three samples of each treatment with standard deviation (error bar)

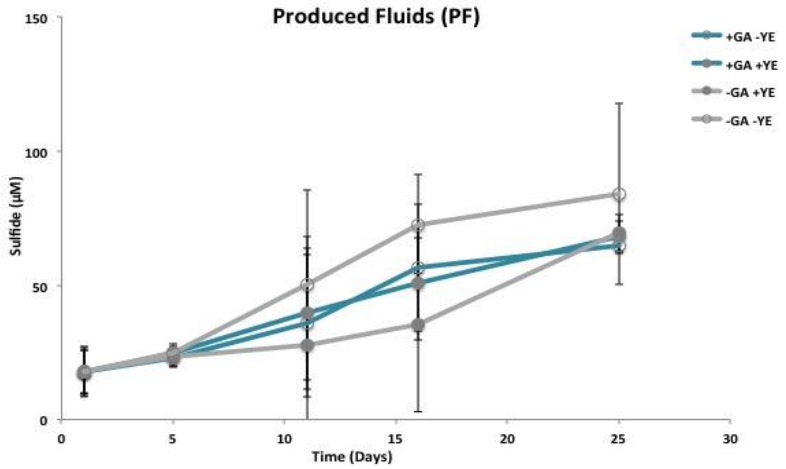

Figure 8: The concentrations of sulfide in produced fluids (PF), with and without glutaraldehyde and yeast extract amendments. Additions are indicated by annotation GA for glutaraldehyde and YE for yeast extract. "+" Refers to the addition of GA or YE, "-" to no addition. All data are average of three samples of each treatment with standard deviation (error bar)

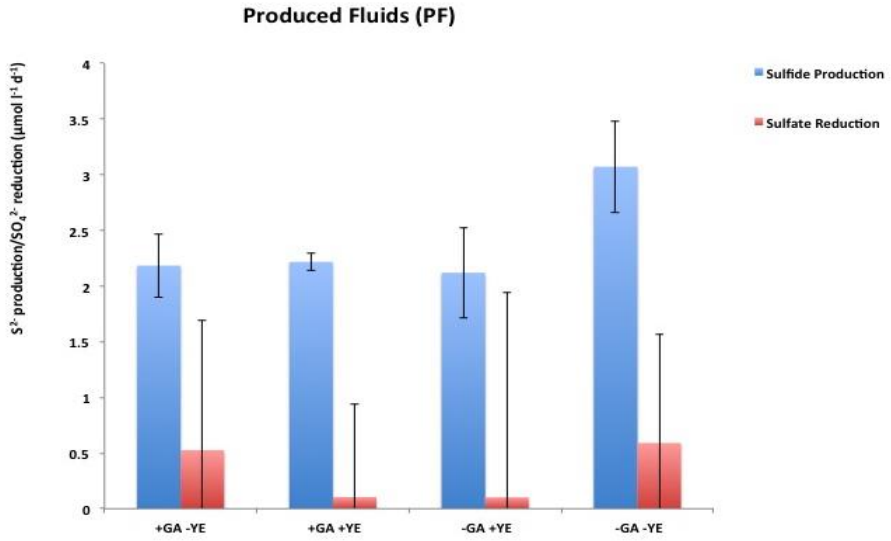

Figure 9: Rates of sulfate reduction (SO_4) and sulfide production (H_2S) in produced fluids (PW) with and without glutaraldehyde and yeast extract amendments. Amendments are indicated by annotation GA for glutaraldehyde and YE for yeast extract. "+" refers to the addition of GA or YE, "-" to no addition. All data are average of three samples of each treatment with standard deviation (error bar)

Table 4: Sulphate reduction and sulphide production rates for the injection fluid incubations.

Replicate	Sample	Sulfide (μM d^{-1})	SD	Sulfate (μM d^{-1})	SD
1	+GA -YE	1.0	0.0	1.5	0.9
	+GA +YE	14.8	4.6	-18.4	10.2
	-GA +YE	17.4	4.9	-30.2	7.3
	-GA -YE	0.2	0.2	2.7	1.2
2	+GA -YE	0.0	0.0	-0.3	2.3
	+GA +YE	0.0	0.5	-0.9	1.7
	-GA +YE	16.3	4.4	-17.4	12.1
	-GA -YE	0.7	0.1	3.4	1.2
3	+GA -YE	0.0	0.1	0.3	0.7
	+GA +YE	5.1	1.6	-24.0	8.7
	-GA +YE	16.4	4.2	-27.4	6.3
	-GA -YE	0.0	0.1	0.1	2.6
Mean	+GA -YE	0.0	0.1	7.2	0.8
	+GA +YE	6.9	1.0	15.4	6.2
	-GA +YE	17.2	3.3	25.5	5.3

	-GA -YE	0.3	0.1	2.2	1.5

Table 5: Sulphate reduction and sulphide production rates for the produced fluid incubations.

Replicate	Sample	Sulfide (μM d^{-1})	SD	Sulfate (μM d^{-1})	SD
1	+GA -YE	1.9	1.2	-3.0	1.5
	+GA +YE	1.9	1.1	-1.2	1.0
	-GA +YE	1.9	1.1	-2.0	1.3
	-GA -YE	1.9	1.0	-1.2	2.0
2	+GA -YE	2.0	1.0	2.1	2.6
	+GA +YE	2.2	1.1	-0.4	1.7
	-GA +YE	2.2	1.3	4.4	1.6
	-GA -YE	2.2	1.0	3.5	1.7
3	+GA -YE	2.4	0.5	2.4	1.3
	+GA +YE	2.4	0.4	1.2	1.7
	-GA +YE	2.2	1.3	4.4	1.6
	-GA -YE	4.8	0.5	-0.6	1.5
Mean	+GA -YE	2.1	0.3	2.1	1.1
	+GA +YE	2.2	0.1	-2.2	0.8
	-GA +YE	2.0	0.4	2.0	1.8
	-GA -YE	3.0	0.4	3.0	0.9

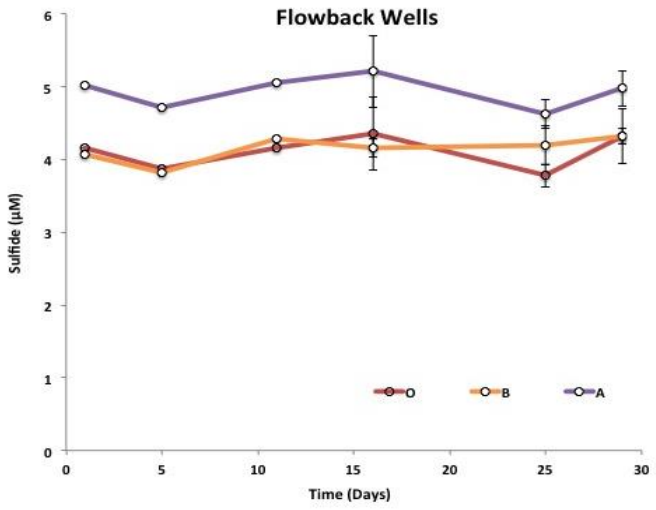

Figure 10: The average of sulfide concentrations in Flowback water samples from well A, B and O showed no change in sulfide concentration and no difference between the treatments.

2.3.2 Microbial community composition

Generally, the Phylum-level diversity in all injection fluids (IF) (Figure 11) is greater than that of produced fluids (PF) (Figure 14). Microbial community profiles from IF included more than nine principle phyla in the original fluid (prior to incubation) and 3-4 phyla in each treated fluid after the five weeks of incubation (Figure 11). However, microbial community profiles from PF included only 5 major phyla (Figure 14). Injection fluids were initially dominated by bacteria related to the phyla Actinobacteria, Acidobacteria, Epsilonbacteraeota, Verrucomicrobia, Proteobacteria, Planctomycetes, Gemmatimonadetes, Firmicutes, Bacteroidetes, Patescibacteria, as well as some unclassified bacteria (Figure 11). A shift in the microbial community profile occurred when samples received only YE, both YE and GA, or only GA after the period of

incubation (Figure 11). The addition of only YE increased the relative abundance of Bacteroidetes and Firmicutes in all triplicate fluids, while the relative abundance of Proteobacteria and Verrucomicrobia decreased (Figure 11). Meanwhile, adding only GA led to a variable increase in the relative abundance of Bacteroidetes, in treatment IF1-GA+YE in particular, and Proteobacteria in treatments IF2-GA+YE and IF3-GA+YE (Figure 11). Actinobacteria decreased across all triplicate treatments (Figure 11). Amending fluids with both YE and GA increased the relative abundance of Firmicutes and Proteobacterial phyla (Figure 11).

The most abundant OTUs in the injection fluids prior to incubation belong to unclassified genera of Burkholderiaceae and Methylophilaceae, as well as Pseudomonas, Sphingorhabdus, Perlucidibaca, and Sediminibacterium.

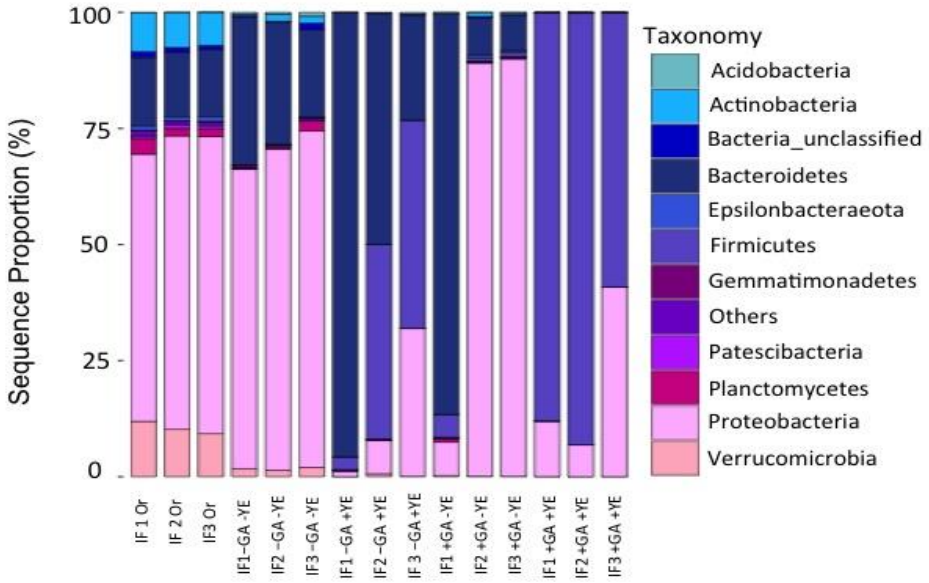

Figure 11: The distribution of 16S rRNA reads per phylum for injection fluids (IF). The

number of reads per phylum is displayed as a percentage of the total reads for each sample.

After 30 days of incubation, the genera in the injection fluids remained the same implying a relatively limited bottle effect (Figure 12). When injection fluids were amended with yeast extract, some genera disappeared or decreased in relative abundance such as Pseudomonas, the unclassified Burkholderiaceae, and Sediminibacterium. Some genera also increased in relative abundance including unclassidied Bacteroidales, Desulfosporosinus, Arcticibacter, and unclassified Clostridiales (Figure 12). Injection fluids only amended with glutaraldehyde led to an increase in unclassified Bacteroidia in one sample as well as unclassified Methylophilaceae and Burkholderiaceae. Injection fluids amended with both glutaraldehyde and yeast extract had increases in the Desulfosporosinus, Sedimentibacter, Pseudomonas, and Trichococcus.

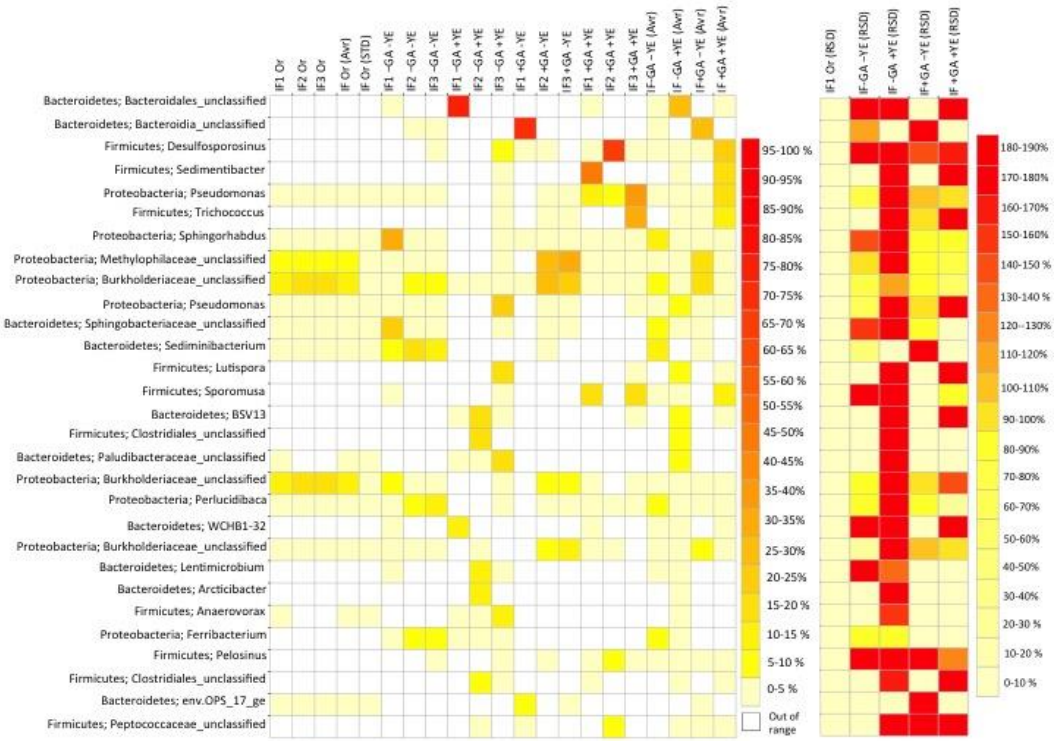

Figure 12: A heatmap of the top most abundant operational taxonomic units (OTUs) at 97% similarity (genus level) in the initial and incubated injection fluids (IF). Additions are indicated by annotation GA for glutaraldehyde and YE for yeast extract. "+" refers to the addition of GA or YE, "-" to no addition, Avr refers to the average, RSD refers to the Relative Standard Deviation. Dark red squares represent the most abundant OTUs (highest % reads), and light-yellow squares represent the lowest abundance OTUs (lowest % reads). White square represent out of the range >0.02 OTU relative abundance (means very low OTU detected). Inner scale-bar represents the percent of the total reads for each sample, while the outer scale-bar corresponds to RSDs. Data was visualized as a heatmap using JColorGrid.

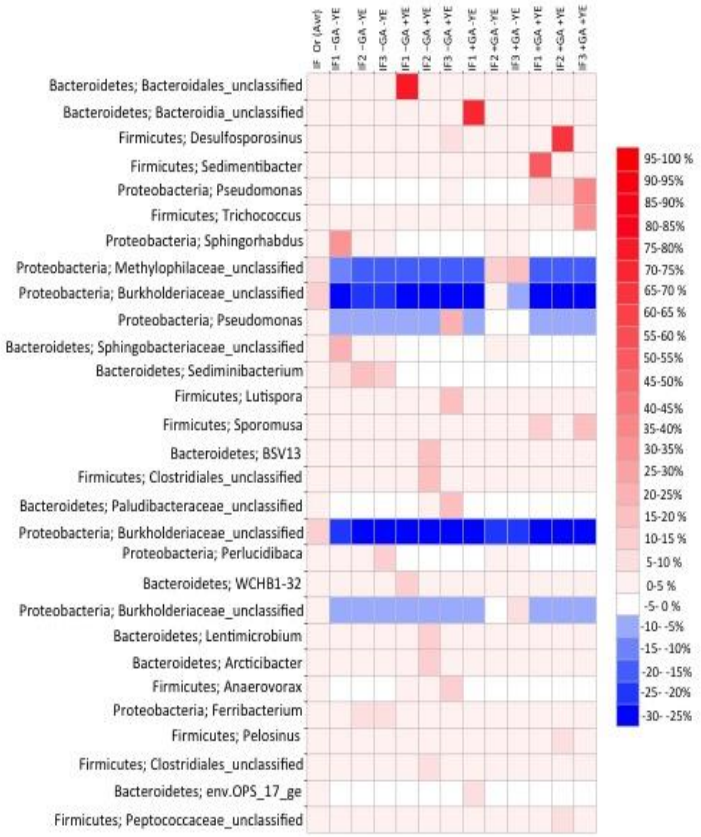

Figure 13: A heatmap illustrating dynamics in the relative abundance of OTUs in injection fluids over the course of the incubation period. Changes in relative abundance were calculated by subtracting the relative abundance of particular OTU at the beginning of the experiment by its relative abundance at the end of the experiment. Red squares represent an increase the relative abundance of an OTU (higher % reads), while blue squares represent a decrease of OTUs (lower % reads). White squares represent no change).

The microbial community in produced fluids (PF) was initially dominated by bacteria related to phylum Firmicutes, Halanaerobiaeota, Epsilonbacteraeota, Bacteroidetes, and Proteobacteria (Figure 14). Most of the PF community members belong to the

Proteobacteria (96-99%), with a small component of Bacteroidetes across all fluids (Figure 14). Relative abundances of the Epsilonbacteraeota and Halanaerobiaeota phyla increased in all incubated produced fluids (Figure 14).

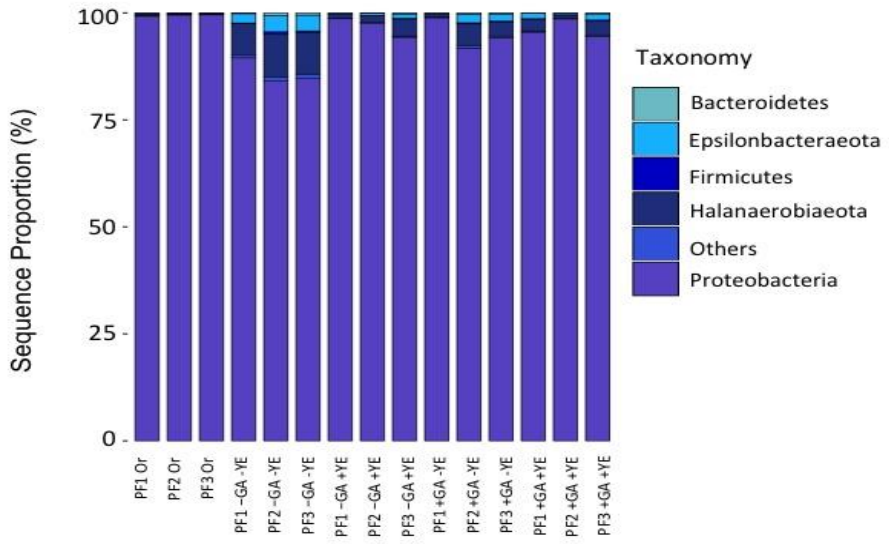

Figure 14: The distribution of 16S rRNA reads per phylum for each produced fluids (PF). The number of reads per phylum is calculated as a percentage of the total reads for each sample.

The most abundant OTUs at the genus level in produced fluids (PF) comprise (55-98%) *Cobetia*, (0.3-9%) *Hanaaerobium*, and (0.2-9%) *Halomonas* across all samples (Figure 15). Regardless of adding GA, YE or both to the fluids, the community composition did not change appreciably over the course of the incubations (Figure 14). Three different genera of Arcobacter belonging to the Epsilonbacteraeota were detected across all amended fluids (Figure 14). A number of taxa related to known sulphate

reducers were also detected including unclassified Gammaproteobacteria, Sulfurospirillum, Desulfuromonadales, and Dethiosulfatibacter (Figure 14). Most of the detected genera slightly increase in abundance over the course of the incubation (Figure 16). At the same time, the genus Cobetia decreased across all treatments (Figure 16)

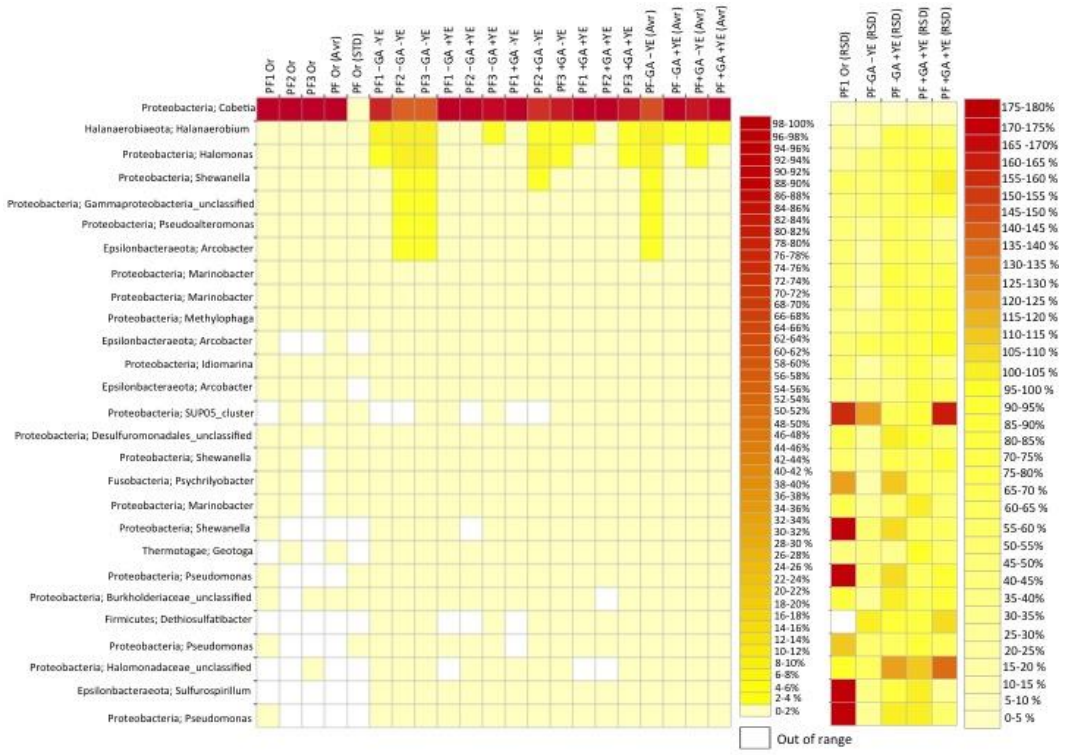

Figure 15: A heatmap of the top most abundant operational taxonomic units (OTUs) at 97% similarity (genus level) in the initial and incubated produced fluids (IF). Additions are indicated by annotation GA for glutaraldehyde and YE for yeast extract. "+" refers to the addition of GA or YE, "-" to no addition, Avr refers to the average, RSD refers to the Relative Standard Deviation. Dark red squares represent the most abundant OTUs (highest % reads), and light-yellow squares represent the lowest abundance OTUs (lowest % reads). White square represent out of the range >0.02 OTU relative abundance (means

very low OTU detected). Inner scale-bar represents the percent of the total reads for each sample, while the outer scale-bar corresponds to RSDs. Data was visualized as a heatmap using JColorGrid.

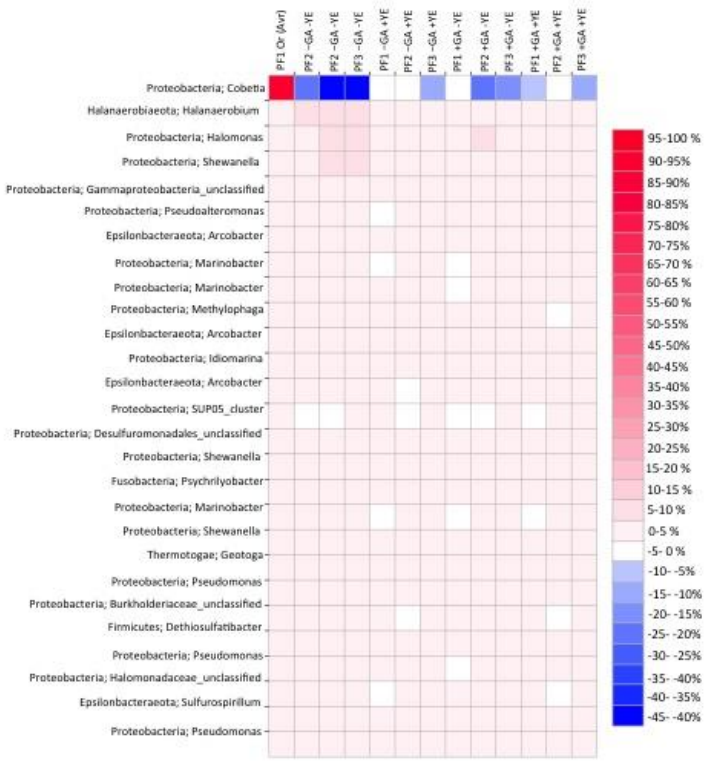

Figure 16: A heatmap illustrating dynamics in the relative abundance of OTUs in produced fluids over the course of the incubation period. Changes in relative abundance were calculated by subtracting the relative abundance of particular OTU at the beginning of the experiment by its relative abundance at the end of the experiment. Red squares represent an increase the relative abundance of an OTU (higher % reads), while blue squares represent a decrease of OTUs (lower % reads). White squares represent no change).

Discussion

2.4.1 Sulfate reduction in unamended fluids

The rate of sulfate reduction in unamended injection and production fluids was low. In unamended injection fluids, sulfide did not accumulate appreciably, whereas sulphide production in unamended produced fluids was significant. The microbial community in the unamended injection fluids remained largely constant, except for a decrease in the Verrucomicrobia and Actinobacteria phyla and a slight increase in the Bacteroidetes. Microbial communities in unamended produced fluids remained constant with the exception of declining relative abundances of Cobetia. The lack of appreciable sulphate reduction in the unammended injection and produced fluids, given abundant sulphate, implies lack of suitable electron donor, an absence of sulphate reducing bacteria, or inhibition by some fluid property like the presence of heavy metals or biocide. Sulfide production in produced fluids in the absence of sulphate reduction, implies alternative pathways of sulphide production, possibly using alternative sulphur species like elemental sulphur or thiosulfate.

2.4.2 Effect of yeast extract

To test for electron donor limitation, I amended injection and production fluids with yeast extract, which provides fermentative bacteria substrates for the ultimate production of electron donors for respiration, including sulphate reduction. Amendment of injection waters with yeast extract induced appreciable reduction of sulphate and

production of hydrogen sulphide (Figures 4 and 5). This implies that injection fluids host sulphate reducing and fermentative bacteria that are generally limited by organic electron donor availability. Amendment with yeast extract indeed caused increases in the relative abundance of phyla Bacteroidetes and Firmicutes containing many taxa with fermentative metabolic potential. While taxa related to known sulphate reducing bacteria were not immediately apparent, a more detailed analysis of the amplicon sequence data yielded a number of relatively low abundance taxa related to organisms known to grow through sulphate reduction in the lab (Table 7). A conspicuous example is a member of the *Desulfosporosinus* genus that represented up to 70% of the total community in injection fluid incubations amended with both glutaraldehyde and yeast extract (see below).

Amendment with yeast extract did not stimulate sulphate reduction or sulphide production in produced fluids implying that sulphate reduction was not electron donor limited. The microbial community in production fluids amended with yeast extract was relatively constant in comparison to the shifts observed in the injection fluids, implying a lack of response from the fermentative organisms present. Many of the taxa present are related to known fermenters, and thus fermentation in these fluids, and ultimately respiration, may be inhibited by some fluid property such as salinity, which is a key difference between the production fluids and the injection fluids.

2.4.3 Effects of glutaraldehyde

To test the effects of the common biocide glutaraldehyde on microbial sulphate reduction and sulphide production, I amended injection and produced fluids with

glutaraldehyde. Since amendment with yeast extract stimulated sulphate reduction and sulphide production in injection waters, I also co-amended injection and produced fluids with glutaraldehyde and yeast extract. Rates of sulfide production and sulphate reduction were comparable between unamended injection fluids and injection fluids amended solely with glutaraldehyde. This is not surprising given the apparent limitation of sulphate reduction and sulphide production by lack of electron donor. Rates of sulphide production and sulphate reduction were lower in injection fluids co-amended with yeast extract and glutaraldehyde that those solely amended with yeast extract alone. This suggests that glutaraldehyde is at least partially effective at inhibiting the combination of fermentation and sulphate reduction, though the current experimental setup does not allow us to discriminate between the two. Many of the taxa that were enriched in injection fluids under the sole amendment with yeast extract were also enriched in response to co-amendment with glutaraldehyde and yeast extract, implying that glutaraldehyde was not effective in precluding ingrowth of these taxa, though quantitative information on cell abundances would be required to make this conclusion more definitely. Nevertheless, glutaraldehyde is only partly effective at inhibiting sulphide production when organic matter is readily available. As noted above, members of the *Desulfosporosinus* genus represented up to 70% of the total community in injection fluid incubations amended with both glutaraldehyde and yeast extract. Members of the *Desulfosporosinus* genus are known as obligate anaerobic sulphate reducers, and their ingrowth the form a numerically abundant component of injection fluids amended with glutaraldehyde and yeast extract implies their resistance to glutaraldehyde in our experiments. *Desulfosporosinus* have been identified in other shale gas systems (Table 6)

and our findings also implicate *Desulfosporosinus* in hydrogen sulphide production and well souring in fracing systems in the Ft. St. John area of Northern BC. This should be tested through further experiments.

Sulfate reduction in co-amended produced fluids was negligible, as it was in unamended produced fluids, and produced fluids amended solely with yeast extract. Sulfide production rates, on the other hand were unaffected by glutaraldehyde and in fact were low but comparable across all treatments. This implies that, like yeast extract, glutaraldehyde has no effect on microorganisms that might be involved in sulphide production.

Table 6: 16S rRNA analysis classified of sulfate-reducing bacteria in previous studies [34,56,68,130-137].

genus	Metabolism	Source
Caminicella sp.	Reduces sulfur and thiosulfate	Alain et al., 2002
Halanaerobium sp.	Reduces sulfur and thiosulfate	Liang et al., 2016
Desulfobacter sp.	Reduces sulfate	Mori K, (2010)
Desulfovibrio sp.	Reduces sulfate	Mori K, (2010)
Dethiosulfovibrio sp.	Reduces sulfur and thiosulfate	Surkov et al., 2001
Geotoga sp.	Reduces sulfur and thiosulfate	Youssef et al., 2009

Desulfohalobium sp.	Reduces sulfate	Ollivier et al., 1991; Jakobsen et al., 2006
Desulfovermiculus sp.	Reduces sulfate	Belyakova et al., 2006
Desulfomonas sp.	Reduces sulfate	cluff et al., 2014
Desulfomicrobium sp.	Reduces sulfate	cluff et al., 2014
Desulfotomaculum sp.	Reduces sulfate	Struchtemeyer et al., 2012
Desulfosporosinus sp.	Reduces sulfate	Struchtemeyer et al., 2012
Sulfurospirillum sp.	Reduces sulfur and thiosulfate	Kodama and Watanabe, 2007

This is consistent with the lack of changes to the production fluid microbial community in response to glutaraldehyde amendment, with the exception of the decline in the relative abundance of Cobetia across all treatments.

2.4.4 Sulfur reducing bacteria in fracing systems

Sulfur-metabolizing bacteria are of primary concern to the natural gas sector because of the deleterious impacts of hydrogen sulphide on gas production[67,76]. Prior work has identified a number of sulfur-reducing bacteria in fracing fluids (table 6). These sulfidogenic bacteria can be broadly divided into two principal groups[22].The first group are the obligate respiratory sulfate, sulfur, and thiosulfate reducers that use these

compounds as a terminal electron acceptor in anaerobic respiration[22]. The second group are bacteria that primarily grow fermentatively, with capacity for facultative of production of sulfide from sulfur and thiosulfate[22]. Several of the sulfate-reduing bacteria found here (Table 7 and 8) were identified in previous investigations of microbial communities from different shale formations and production facilities (Table 6).

The detailed 16S rRNA gene analyses revealed multiple members of the microbial communities within amended samples that are related to those observed in prior studies of various natural gas wells and production facilities. Examples include organisms that are affiliated with the genera *Dethiosulfatibacter*, *Sulfurospirillum*, *Desulfobacter*, *Dethiosulfovibrio*, *Desulfosporosinus* and *Halanaerobium* (table 6 &7). These genera have previously been isolated or detected via 16S rRNA gene sequencing from different shale gas wells (table 6). Notably, the injection fluids that received yeast extract only had a high rate of sulfide reduction up to 16 μM/d after 30 days, at the same time the number of OTUs of genus *Desulfosporosinus* increased in these fluids (table 4). Moreover, glutaraldehyde appeared to promote the growth of *Desulfosporosinus* relative to other taxa(figure 12). Future studies may focus on better constraining the potential role of *Desulfosporosinus* in well souring and its control through application of alternative biocides.

Table 7: The 16S rRNA analysis classified of genus level of sulfate-reducing bacteria in injection fluids (IF). The number of reads per phylum is calculated as a percentage of the total reads in each sample.

Phylum	***Genus***	**IF1 OR**	**IF2 OR**	**IF3 OR**	**IF1 -GA -YE**	**IF2 -GA -YE**	**IF3 -GA -YE**	**IF1 -GA +YE**	**IF2 -GA +YE**	**IF3 -GA +YE**	**IF1 +GA -YE**	**IF2 +GA -YE**	**IF3 +GA -YE**	**IF1 +GA +YE**	**IF2 +GA +YE**	**IF3 +GA +YE**
Firmicutes	*Desulfosporosinus*	0	0	0	0	0	0.02	0	0	6	0.001	0.01	0	2	70	23
Epsilonbacteraeota	*Sulfurospirillum*	0	0	0	0	0	0	0	0	0	0.003	0	0	0	0	0
Epsilonbacteraeota	*Sulfuricurvum*	1	0.8	0.9	0.03	0	0	0.003	0	0	0	0.6	0.3	0	0.01	0
Epsilonbacteraeota	*Sulfurospirillum*	0.002	0	0	0	0	0	0.007	0.003	0	0	0	0	0	0	0.001
Epsilonbacteraeota	*Sulfurimonas*	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Proteobacteria	*Desulfomicrobium*	0	0	0	0.001	0	0	0	0	0	0	0	0	0	0	0
Synergistetes	*Dethiosulfovibrio*	0	0	0	0	0	0	0	0	0	0.02	0	0	0	0	0

Table 8: 16S rRNA analysis classified of genus level of sulfate-reducing bacteria in injection fluids (IF). The number of reads per phylum is calculated as a percentage of the total reads in each sample.

Phylum	***Genus***	**IF1 OR**	**IF2 OR**	**IF3 OR**	**IF1 -GA -YE**	**IF2 -GA -YE**	**IF3 -GA -YE**	**IF1 -GA +YE**	**IF2 -GA +YE**	**IF3 -GA +YE**	**IF1 +GA -YE**	**IF2 +GA -YE**	**IF3 +GA -YE**	**IF1 +GA +YE**	**IF2 +GA +YE**	**IF3 +GA +YE**
Halanaerobiaeota	*Halanaerobium*	04	0.2	0.2	7	10	10	0.9	2	4	0.6	5	3	3	0.8	3
Proteobacteria	*Desulfuromonadals*	0.04	0.02	0.01	0.2	0.4	0.3	0.04	0.06	0.2	0.03	0.2	0.09	0.07	0.04	0.1
Thermotogae	*Geotoga*	0.004	0.01	0.004	0.1	0.2	0.3	0.02	0.04	0.02	0.01	0.1	0.03	0.04	0.01	0.04
Firmicutes	*Dethiosulfatibacter*	0	0	0	0.01	0.2	0.07	0.001	0.005	0.01	0	0.05	0.05	0.006	0.01	0.06
Epsilonbacteraeota	*Sulfurospirillum*	0.004	0	0	0.06	0.1	0.1	0.006	0.01	0.03	0.01	0.08	0.03	0.02	0.02	0.01
Epsilonbacteraeota	*Helicobacter*	0	0	0	0	0.003	0	0	0.002	0	0	0	0	0	0	0
Epsilonbacteraeota	*Sulfuricurvum*	0	0	0	0	0.006	0.004	0	0	0	0	0.004	0.003	0.001	0	0.004
Epsilonbacteraeota	*Sulfurospirillum*	0	0	0	0.007	0.01	0.01	0.002	0.002	0.004	0.002	0.008	0.003	0.001	0.004	0.014
Firmicutes	*Desulfotomaculum*	0.001	0	0	0.001	0	0	0	0	0	0	0	0	0.002	0	0
Synergistetes	*Dethiosulfovibrio*	0.001	0.01	0	0.005	0.02	0.004	0	0.002	0.02	0	0.01	0.02	0.005	0	0.004

Conclusion

Overall, my thesis demonstrates that evaluation of the effects of biocides on frac fluid microbial communities is tractable through laboratory incubation studies. Results from a first set of incubation studies demonstrate that reactive organic matter, like yeast extract, can stimulate fermentation and sulphate reduction in injection fluids. This is accompanied by shifts in microbial community composition. Stimulation of sulphate reduction by organic matter can be partially inhibited by the application of glutaraldehyde. Sulfide production in produced fluids is decoupled from sulphate reduction and is not stimulated by the addition of organic matter, nor is it controlled by amendment with glutaraldehyde. The microbial community in produced fluids is much less diverse than that in injection fluids and it is resistant to changes due to amendments. Further experiments should be designed to more broadly examine the diversity of fluid compositions that develop in fracing operations, and to explore the full range of potential biocides.

Outlook

Findings from this research advance the current understanding of the efficacy of glutaraldehyde on sulfur reduction by microbial communitird in fracing fluids, providing new insight into microbial responses to biocides (glutaraldehyde). Nevertheless, future research is recommended to further evaluate and confirm observations made here. Efforts should target the efficacy of other biocides in fracing fluids. It is important also to conduct further work on sulfate-reducers in fracing fluids and to isolate them in pure lab cultures to test the impact of diverse additives to fracing fluids. Microbial community

analyses made here focused on profiling of taxonomic compositions using gene 16S rRNA sequencing but future work could address microbial community metabolic through metagenomic analyses. Metagenomic analyses could provide more information on the specific roles (metabolic capabilities) of detected microbes and could confirm their metabolic capacity for sulfide production in shale gas systems. Further analyses using metagenomics, could target Halanaerobium dominated fluids given their potential abundance and importance across diverse fracing fluids and systems. Research efforts should also focus on developing an effective biocide treatment to control sulfur-reducing organisms, in particular, of the genus Halanaerobium given the limited data currently available on the efficacy of biocides against the genus Halanaerobium. Thus, exposure of Halanaerobium isolates to different biocide combinations may help to identify treatments for effective control of Halanaerobium populations in fracing fluids. Collectively, more research will further enhance the current knowledge of microbial activity in fracing systems facilitating the development and application of new strategies to improve control on microbial activities and reduce corrosion, biofouling, and souring.

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