Microbial Community Dynamics in the Hydraulic Fracturing Water Cycle from Two Newly Fractured Shale Gas Wells in the Duvernay Formation, Alberta

by

Cheng Zhong

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Department of Earth and Atmospheric Sciences

University of Alberta

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Abstract

The microbial ecology of the hydraulic fracturing water cycle may influence the efficiency of shale gas production and strategies for water reuse and treatment. In this study, microbial community dynamics were tracked by sequencing of 16S rRNA genes coupled with enumeration of live/dead cells in flowback and produced water (FPW) from two newly fractured Duvernay shale oil and gas wells (112-115°C). For both wells sampled, I found that numbers of total cells, microbial diversity and richness were considerably reduced, the highly diverse initial freshwater communities rapidly shifted to become dominated by halotolerant genus *Halanaerobium*, and subsequently DNA was insufficient for sequencing. Moreover, lower cell viability, microbial diversity, and faster enrichment in *Halanaerobium* were observed in the early period of FPW from the well that used recycled produced water (RPW). Furthermore, I discovered in a separate experiment that adding 10% RPW in freshwater quickly enriched *Halanaerobium*, and fostered other heterotrophic genera affiliated to the class *Alphaproteobacteria* before injection. My results have implication of microbial ecology in high temperature brine may not consistent with low temperature brine, the predominance members of *Halanaerobium* may pose a risk of detrimental impact from microorganisms downhole, and undesirable bacteria may alter original freshwater communities before fracturing due to FPW recycling.

Preface

The technical methods referred to the untargeted organic analysis (chapter 2) in this thesis were designed by Dr. Chenxin Sun (Professor Jonathan Martin's lab), and the technical apparatus referred to the inorganic analysis of cations (chapter 2) in this thesis were designed by Dr. Shannon Flynn and Mr. Konstantin von Gunten. All the data analyses relevant to the microbiology and concluding analyses in chapters 2-7 are my original work, as well as the literature review in chapter 1.

Acknowledgements

I thank all the professors and friends who have contribution to my research, especially Professor Daniel S. Alessi, my direct supervisor, for offering me this very interesting project. I sincerely appreciate the academic knowledge, instructions, and financial support that I received from Dr. Alessi. My sincere acknowledgements are also delivered to my co-supervisor, Professor Brian Lanoil, and my committee member, Professor Greg Goss; Dr. Lanoil provided me with many helpful advices on this thesis in the aspect of Microbiology and has carefully reviewed my thesis; Dr. Goss offered me his excellent lab equipment to conduct my experiments, and also put many professional and useful comments on this thesis. I also thank Professor Karlis Muehlenbachs as my committee chair who forced me to think about the geochemistry changes in the hydraulic fracturing water cycle, which as a result that improved the quality of this thesis. Besides, I could not handle this project without the technical support from Joy Li and Dr. Camilla Nesbø. Moreover, Dr. Shannon Flynn and Mr. Konstantin von Gunten, my senior colleagues and friends, helped me to learn the ICPMS-QQQ, generated the inorganic data for this research, and offered me many suggestions on this thesis. Dr. Chenxin Sun in Professor Jonathan Martin's lab helped me a lot with the analysis of organic chemistry.

I am grateful for the research funding from Encana Corporation and Natural Sciences and Engineering Research Council (NSERC). Encana Corporation offered me useful profiles for the horizontally fractured wells sampled in this research. Finally, I sincerely thank my family who always stand behind me, and support me.

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Chapter 1: Introduction

1.1 Unconventional Oil and Gas Development

1.1.1 Global Shale Oil and Gas Development

Natural gas, an important energy source, is primarily comprised of methane and smaller fractions of butane, ethane, propane, and other gases (Arthur and Langhus 2008). Shale gas has been suggested to have a great exploration potential; the proved reserves of total natural gas are approximately 205 trillion m^3 globally, but the estimation of shale gas that produced from hydrocarbon-rich shale formations are 716 trillion m^3, which are over three times higher than the proved reserves (The World Factbook; Boyer et al. 2011; Vengosh et al. 2014). In addition to shale gas, condensed oil is also often produced along shale gas production. In the past decade, unconventional hydrocarbon exploration, including shale gas, has rapidly increased throughout North America (Rivard et al. 2014). The estimated global demand of natural gas is expected to grow, and unconventional oil and gas exploration is likely to be launched in shale gas reservoirs in China, South America, South Africa, Europe, and Australia (Gregory et al. 2011; Boyer et al. 2011).

1.1.2 Canadian Shale Oil and Gas Development

Canada is one of the world's largest natural gas producers in 2014, after United States, Russia, Iran, and Qatar (US Energy Information Administration). While natural gas reserves are across Canada, but approximately 75% of the current production is concentrated in British Columbia (BC) and Alberta (Council of Canadian Academies

2014). The large fraction of natural gas production in western Canada because the Montney and Duvernay shales in BC and Alberta are the most active shale gas plays with an estimated 25 trillion m^3 natural gas in place, which consist approximately 37% of the estimated total Canadian shale gas reserves (Goss et al. 2015; EIA 2015). In the past few years, factors such as the decline of conventional oil and gas production along with growth in shale gas exploration has resulted in an increasing contribution of shale gas to total hydrocarbon production in Canada (Rivard et al. 2014; Natural Resources Canada 2016). Between November 2011 and March 2014, there were at least 4917 oil and gas wells that have been hydraulically fractured in Alberta and BC alone (Alessi et al. 2017). However, Canadian shale gas fields in such as those found in New Brunswick, Nova Scotia, and Quebec are not active at present because the recent local laws put moratorium on hydraulic fracturing in these areas (http://www.pennstateshalelaw.com, global shale law compendium: shale governance in Canada, June 30, 2017). Addtionally, in many cases, production volumes of liquid hydrocarbons and natural gas are variable, and production of dry gas without liquid hydrocarbons is currently difficult to achieve at a profit (Rivard et al. 2014), Therefore, making shale gas exploration in New Brunswick, Nova Scotia, and Quebec feasible will require not only improved technologies, but also the better characterization and assessment of the hydraulic fracturing water cycle. More recently, declines in oil and gas prices since 2015 have, to some degree, impeded the unconventional oil and gas development in Alberta and BC. Many fewer unconventional oil and gas wells have been drilled since 2015, compared to previous years during which when the oil and gas prices were higher (personal communication, Encana Corporation).

1.2 Water Management in Shale Oil and Gas Exploration

1.2.1 Hydraulic Fracturing Technology

The technology of hydraulic fracturing in combination with horizontal drilling has created a global energy revolution. This technology has been used to enhance hydrocarbon production since the 1940s. However, shale gas formations are characterized by low permeability, and lateral reservoir zones may be thin (Arthur 2009; Heffernan 2013; Rivard et al. 2014). Thus, the traditional technology of hydraulic fracturing as applied in vertical boreholes is not sufficiently effective in recovering hydrocarbons from shale oil and gas reservoirs. In recent years, horizontal drilling has been combined with multiple-stage hydraulic fracturing to commercially exploit shale oil and gas formations. The combination of these two older technologies allows for the creation of extensive fracture networks in the target formation, and the recovery of economic quantities of oil and gas (Gregory et al. 2011).

1.2.2 Water Use in Hydraulic Fracturing

While hydraulic fracturing has allowed for access to huge energy resources, it has also raised considerable water-related concerns including environmental, cultural, and political issues (Mauter et al. 2014). Hydraulic fracturing involves a water cycle that connects deep geologic formations with water derived from near-surface sources. Source water for a hydraulically fractured well may be derived from single source or a combination of fresh surface water, fresh ground water, saline ground water, or recycled/treated flowback and produced water (FPW) (Alessi et al. 2017). The volume of

water used for fracturing is considerably large; approximately 11,000-15,000 m^3 of water is required for a single horizontal drilling and hydraulic fracturing operation (Arthur 2009), which is approximately equivalent to the water volume of 4-6 Olympic-sized swimming pools hold. Base-fluids including guar-based fluids, slickwater fluids, viscoelastic surfactant-based fluids, and energized fluids are used for hydraulic fracturing operations in unconventional reservoirs (Barati and Liang 2014). Not only are the types of base-fluids used variable, but the compositions of injected fracturing fluids are also complex, and their chemical additives may vary with individual shale oil and gas operations to adapt to heterogeneous formation geology found across fields and plays (The U.S. Environmental Protection Agency 2015). In general, the classes of chemical additives include cross-linkers, breakers, acid, friction reducers, surfactants, potassium chloride, scale inhibitors, pH adjusting agents, iron control agents, corrosion inhibitors, and biocides, These chemical additives are used in injected fracturing fluids is to optimize the performance of the fracturing process and resource extraction (Barati and Liang 2014).

1.2.3 Flowback and Produced Water (FPW)

Once fracturing is complete, pressure is released from the wellhead, resulting in the return of considerable volumes of FPW to the surface. The chemistry of this water varies considerably as a function of return time or volume produced. In fact, it is difficult to precisely distinguish the boundary between flowback and produced water, and the distinction often depends on their chemical compositions of the return water (Alessi et al. 2017). In general, flowback has higher fractions of compounds from the original

fracturing fluids, and produced water has higher fractions of compounds from the shale formation. Therefore, FPW typically contains high concentrations of salts and metals derived from the target shale formation, in-situ hydrocarbons, and soluble organic constituents originally found in the hydraulic fracturing fluids (Arthur 2009; Gregory et al. 2011; Goss et al. 2015; Alessi et al. 2017). Recently, there is emerging evidence that chemical compounds of concern in hydraulic fracturing related fluids are often missed in standard water analyses, and that many secondary reaction products are generated by downhole reactions (Stringfellow et al. 2014; Gagnon et al. 2016; He et al. 2017).

1.2.4 Management of FPW in Pilot Areas

Storage, treatment, and disposal of FPW, which contains a complex mixture of constituents, is challenging and costly to industry. Firstly, the cumulative volume of FPW generated from hydraulic fracturing sites is considerably large as compared to wastewater generation from conventional oil and gas operations. For example, in the Marcellus play, total cumulative FPW was 5,370,000 m^3 in 2014, and the volume of FPW accumulated is approximately equivalent to 0.06% of the total volume of surface water in Pennsylvania (Lutz et al. 2013; Charles et al. 2008). Furthermore, transport and disposal of FPW containing metals, volatile organics, and radioactive compounds found in FPW may also pose risk to ecosystems (Small et al. 2014; Vengosh et al. 2014; Warner et al. 2012; He et al. 2017; Yao et al. 2015; Gaucher et al. 2014), which will be discussed in detail in section 1.2.5.

To handle FPW generated from hydraulic fracturing, traditional disposal methods include: (1) discharging FPW to surface water (2) injecting FPW into the deep subsurface

using disposal wells, (3) transporting FPW to commercial water treatment plants, and (4) reusing FPW for subsequent hydraulic fracturing (Gregory et al. 2011; Goss et al. 2015; Alessi et al. 2017). For oil and gas industry, management of FPW generated from hydraulic fracturing is variable from place to place; for example, in the U.S. and Canada, directly discharging FPW to surface water is either strictly regulated or completely prohibited (Goss et al. 2015); recycling and deep well injection are two primary FPW management strategies in the Marcellus play, U.S. (Barbot et al. 2013); recycling and deep well injection are also the primary choices for FPW management in the Barnett play in the U.S., but reuse of FPW in Barnett was only approximately 5% in 2011 (Nicot and Scanlon 2012).

In Alberta and BC, Canada, FPW can be disposed of only in approved disposal wells, or recycled after being treated in the water treatment facilities (Rokosh et al. 2012; OGC 2014). Recycling of FPW is supposed to reduce the volume of freshwater use, so increasing recycling of FPW in Alberta may benefit to the environment and cost for operators. The quality of FPW recycling varies from one operation to another, and the recycling and reuse of FPW may cause unintended detrimental impacts on oil and gas production. To effectively recycle and reuse of FPW on a new fracturing job, there are many issues that must be considered, including: the dilution ratio of RPW to freshwater, pre-filtration to remove turbidity and total suspended solids, and treatment of the many ions in FPW such as calcium, magnesium, barium, iron, and sulfates, and the impact of bacteria in FPW (Mantell 2011).

1.2.5 FPW Spills in Transport

In addition to the potentially high costs of disposal and treatment, the handling of large amounts of FPW also results in various environmental concerns. One of the problems and has been confirmed to regularly occur are accidental spills of fracturing fluids and FPW at the surface. The frequency of the FPW spills is high; a study of 31,481 unconventional oil and gas wells drilled in Pennsylvania, North Dakota, New Mexico, and Colorado from 2005 – 2014 revealed that between 2-16% of wells reported a spill in every single year (Patterson et al. 2017). Since many spills often occur during shale oil and gas production, the environmental impacts and potential toxicity of FPW becomes important, and many unknowns remain. However, only a few toxicity studies have been conducted that used real FPW in recent years. The difficulty of getting real FPW samples from oil and gas operators, combined with the limitation of analytical techniques, have undoubtedly impeded the assessment of FPW toxicity. Recent research, for example, has shown that fish may be detrimentally impacted by FPW diluted more than 200 times in water (He et al. 2017). Furthermore, the microbial communities in a stream in West Virginia have been shown to be influenced by unconventional oil and gas waste disposal operations (Fahrenfeld et al. 2017), suggesting the need for further characterization and assessment of FPW chemistry and toxicity, and the potential effects of FPW spills on aquatic and terrestrial organisms, including microbiota.

1.3 Oil and Gas Exploration in Duvernay Formation

1.3.1 Geological Setting of the Duvernay Formation

The Duvernay Formation (Upper Devonian), which is the fractured formation from which hydraulic fracturing water cycle samples were collected for this study, is located in west-central Alberta and is divided into East Shale Basin and West Shale Basin. The Duvernay Formation is an emerging unconventional hydrocarbon field with great reserves of shale oil and gas. At present, the Duvernay shale is the second largest shale gas field after the Montney shale in Canada with an estimated 2.1-6.4 trillion m^3 recoverable gas. The lower limit of the Duvernay shale reserve is equivalent to Barnett Formation, and the average volume of recoverable gas reserve of Duvernay Formation is approximately 1/3 of that of the Marcellus Formation (Goss et al. 2015). The source rock in the Duvernay Formation contains moderate quantities of total organic carbon (TOC) with an average value of 4.5%, and the TOC content is generally higher in the East Shale Basin carbonate rocks (Dunn et al. 2014). Compared to other shale gas formations in Canada such as the Montney Formation, thermal maturity of hydrocarbons in the Duvernay Formation is variable, and so the formation produces natural gas with an increasing portion of liquids from the west to the east side (Rokosh et al. 2012).

Like many other shale gas formations such as the Montney and Marcellus Formations, carbonates are important components of the lithology of the Duvernay Formation. The East Shale Basin is primarily comprised of organic-rich shale lime-mudstone; Within the West Shale Basin, the rock gradually grades to less calcareous and more shale-rich toward the west side of the formation (Rokosh et al. 2012). The reservoir

characterization of the Duvernay Formation indicates that it has low porosity (average 6.5%) and permeability (average 394 nD), which is similar to other typical shale gas reservoirs around the world (Dunn et al. 2014). The Duvernay Formation is deep compared to some relatively shallow shale formations such as Marcellus and Barnett Formation; depending on geographic location, depths to the Duvernay range from 2,500 m to 4,000 m, and the thickness ranges between 10-70 m (Packers Plus Inc. 2014). The reservoir temperature of the formation has been estimated between 104-121°C (Smith 2013), and more precisely to be between 112-115°C for the two wells studied here (personal communication, Encana Corporation). Compared to the reservoir temperature of the Marcellus Formation (54-77°C) and the Barnett Formation (65-82°C) (Bowker 2007; Rodgerson, Ruegamer, and Snider 2005; Smith 2013), the estimated reservoir temperature of Duvernay Formation is considerably higher.

1.3.2 Water Management in the Duvernay Play

Slickwater and energized slickwater are water-based fluid and proppant combination, which are commonly applied to fracture the Duvernay Formation (Wasylishen and Fulton 2012). There were at least 1,923 hydraulically fractured wells in Duvernay Formation between November 2011 and March 2014 (Goss et al. 2015; Alessi et al. 2017). However, the reported volume of water used to fracture a single well in the Duvernay is not consistent among previous studies. CSUR (2013) reported an estimated of 10,000-60,000 m^3 of water used to fracture a single well, but more recently Alessi et al. (2017) and Goss et al. (2015) recently suggested the average volume of water used per well was less than 10,000 m^3 per well. According to geoSCOUT, there are 13,361

disposal wells in Alberta and BC combined, and the majority of the FPW from Duvernay has been handled via deep well injection, but there is a significant shift towards recycling and reuse in recent years (Goss et al. 2015). Regardless of the exact volume numbers of FPW, there is a considerable volume of FPW transport and disposal during unconventional oil and gas exploration in Alberta and in other, more developed shale gas plays in North America.

1.4 Environmental Microbiology in the Hydraulic Fracturing Water Cycle

1.4.1 Detrimental Microbial Impacts on Hydrocarbon Production

Understanding the microbiology and biogeochemistry of hydraulic fracturing operations may lead to both economic and environmental benefits. Microbially-induced problems often occur during hydrocarbon production, including gas souring events, facility corrosion, small fracture plugging, and chemical additives consumption, which in turn influences shale gas production and hydraulic fracturing water management strategies (Gieg et al. 2011; Van Hamme et al. 2003; Kermani and Harrop 1996; Elliott 2015). Biocides, as one of the chemical additives in injected fracturing fluids, are used to prevent detrimental impacts that may be induced by microbes. Single or multiple commercial biocides may be used at a well, including glutaraldehyde, 2,2-dibromo-3-nitrilopropionamide (DBNPA), alkyl dimethyl benzyl ammonium chloride (ADBAC), tetrakis (hydroxymethyl) phosphonium sulfate (THPS), 2-bromo-2-nitropropane-1,3-diol (Bronopol), dimethyloxazolidline (DMO), 1-(3-chloroallyl)-3,5,7-triaza-1-azoniaadamantane chloride (CTAC), and tris (hydroxymethyl) nitromethane (THNM) (Dow Microbial Control). However, many biocides are easily degraded, are likely to

transform into persistent and potentially hazardous compounds, and the fate of biocides under downhole conditions have not yet been well-examined (Kahrilas et al. 2015). Furthermore, their efficacy at inhibiting microbial growth and activity have *is situ* been called into question (Struchtemeyer and Elshahed 2012).

1.4.2 Microorganisms in the Hydraulic Fracturing Water Cycle

Microbial community dynamics have been studied in only a small fraction of shale oil and gas exploration projects worldwide, and the majority of the pilot-scale shale oil and gas microbiology studies are located in the U.S. (Mouser et al. 2016). However, reports of whether microbes can inhabitant in the subsurface usually vary from one studied site to another. The composition of microbial communities recovered in FPW shift dramatically from those detected in injected fracturing fluids. In particular, halotolerant bacteria, especially the bacterial genus *Halanaerobium*, are progressively enriched in relative abundance as well flowback proceeds. For example, studies conducted in the Barnett shale in Texas have shown that microbial communities in FPW differ considerably from those found in injected fracturing fluids prior to injection (Davis et al. 2012; Struchtemeyer and Elshahed 2012). More recently, studies on wells drilled into the Marcellus Formation in Pennsylvania have shown a transition to dominance by halotolerant microbial communities that are progressively enriched as the well proceeds from initial flowback into the later produced water stage (Mohan et al. 2013; Cluff et al. 2014). Other microorganisms such sulfate reducing, fermenting, and methanogenic bacteria have also been directly detected in or cultured from FPW (Strong et al. 2013; Akob et al. 2015). For the important microbial community in the hydraulic fracturing

water cycle, Daly et al. (2016) observed that halotolerant bacteria were detected in injected fracturing fluids at low abundance, and surmised that their persistence in FPW was likely supported by glycine betaine, which is one of the residual organic constituents in injected fracturing fluids. For FPW samples containing undetectable levels of DNA, *in situ* high temperatures in formation are likely an important restriction in inhibiting microbial growth (Elliott 2015; Gaspar et al. 2016). For example, a recent study on wells fractured >4 km in the Montney Formation that are located near Kobes, BC showed an increase in the relative abundance of the halophilic genus *Halomonas* in early FPW, but subsequently insufficient DNA was isolated for sequencing (Elliott 2015).

Identifying the sources and substrates supporting microbial growth in fluids in the hydraulic fracturing water cycle is challenging. The most likely sources of microbial communities in FPW, especially in hotter, lower porosity plays, is input fracturing fluids, but the composition of these communities is modified by the subsurface environmental conditions (e.g. salinity, temperature) as well as the fluid composition (e.g. organics, biocides). This observation is supported by the halotolerant bacteria that persist in FPW and have been detected in the input fluids at low abundances (Daly et al. 2016). Moreover, it has been hypothesized that microbes may be introduced via drilling fluids. Previous studies indicate that freshwater communities shift to predominantly *Firmicutes* and *Gammaproteobacteria* during the water-based drilling formulation processes; in contrast, there was insufficient biomass to detect by PCR amplicons that were absent in the synthetic, oil-based drilling fluids (Struchtemeyer et al. 2011; Mohan et al. 2013). Furthermore, microbial communities may also derived *in situ*, from the formation itself (Onstott et al. 1998). However, thermogenic shale gas formations typically have

temperatures higher than the known upper temperature limit for life (121°), and this then precludes *in situ* microbial inhabitants (Jones and Lineweaver 2010; Montgomery et al. 2005; Kashefi 2003; Wilhelms et al. 2001). Nanodarcy permeability and the extremely small and limited pore-space of shale formations are also likely to constrain on the activity and survival of microbes (Fredrickson et al. 1997). Another factor that may influence the identification of microbial substrates in the hydraulic fracturing water cycle is that FPW recycling is commonly used in making up the injected fracturing fluids. The microbial communities found in FPW that is stored in surface impoundments may biologically contaminate subsequent hydraulic fracturing operations during the FPW recycling process, and this recycling may also lead to the development of microbial communities that are adapted to the FPW conditions and resistant to efforts to control them (Murali Mohan et al. 2013).

1.5 Problem Statement and Research Objectives

The biogeochemistry of the hydraulic fracturing water cycle is poorly understood, and there is no investigation of microbial communities in the water cycle of fractured wells in the Duvernay Formation, the among largest unconventional plays in western Canada. My primary research goal is to characterize the microbial community dynamics in hydraulically fractured wells in the Duvernay shale gas play, to better understand hydraulic fracturing on the microbial ecology. Since previous studies suggested that FPW recycling may seed undesirable bacteria to the injected fracturing fluids, I also aim to investigate the effect of FPW recycling on microbial community dynamics, and determine the timing of shifts in microbial communities before injection.

The hypothesis of my study is that increased salinity and temperature in the hydraulic fracturing water cycle of the deep shale reservoir quickly alter the microbial community composition of injected fracturing fluids, and recycling of FPW influences that shift. To test my hypothsis and conduct the research agenda, specific tasks I completed include:

I. I participated in the chemical analyses of FPW samples, including measurement of TDS and major elements, and identification and quantification of unknown organics.

II. I counted the cellular biomass of live and dead cells under epifluorescence microscope for source water (SW), recycled produced water (RPW), and FPW samples, and determine the microbial community composition using non-culture based method (16S rRNA sequencing).

III. I conducted a separate batch experiment in laboratory by mixing freshwater with RPW to simulate FPW recycling, using the method mentioned in objective II to determine biomass, cell viability, and microbial community abundance in mixed fluids.

The results of the total cell number, survival ratio, microbial richness and diversity, and microbial community abundance were used to describe the microbial community dynamics in the hydraulic fracturing water cycle. Changes in salinity, temperature, and concentration of organic constituents in FPW were used to interpret the potential influence of ambient environments on the microbial communities. My study contributes to optimizing FPW management including handling, treatment, recycling and reuse in the

hydraulic fracturing water cycle, which directly links to reducing water cycle environmental impacts and achieving overall economic benefits for oil and gas operators.

Chapter 2: Methodology

2.1 Research Overall Strategy

In this research, I considered two newly horizontally fractured shale oil and gas wells, which have geographically close and geologically similar. To complete a full investigation of the hydraulic fracturing water cycle from the Duvernay shale play, I sampled including source water (SW), recycled produced water (RPW), flowback and produced water (FPW), and oil-based drilling fluids from the very beginning of the drilling operation until over 100 days after oil and gas production, and these water samples were also used for my batch experiment in laboratory to make mixed fluid as part of a FPW recycling simulation. To improve the overall reliability of microbial community dynamics throughout over 100 days of FPW, I used a high-density sampling approach, similar to the sampling strategy used in Elliott (2015), but different from the majority of previous relevant studies. The overall workflow and experimental equipment used for my study are shown in Figure 1. The analysis of inorganic chemistry was assisted by Dr. Shannon Flynn and Mr. Konstantin von Gunten, and analysis of organic chemistry was assisted by Dr. Chenxin Sun (Prof. Jonathan W. Martin's lab).

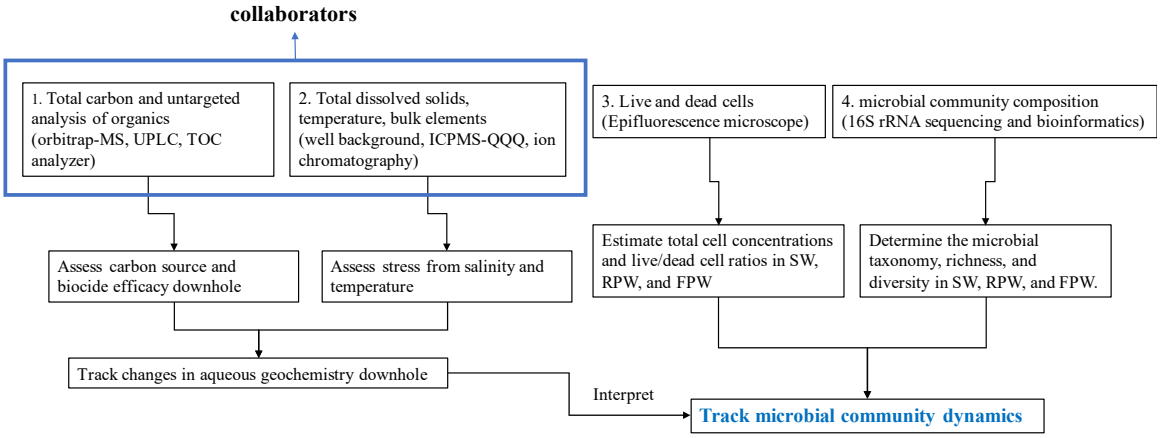

Figure 1. Workflow chart showing the overall strategies and available experimental equipment

2.2 Sampling

2.2.1 Sampling Strategies in the Hydraulic Fracturing Water Cycle

Samples were received from well 1 (Well ID: 103/01-12-063-21W5) and well 2 (Well ID: 100/12-30-063-21W5), which are located near Fox Creek, Alberta, Canada (Figure 2). Both wells were horizontally drilled into the Duvernay Formation at vertical depths of over 3km. Unfortunately, the chemistry of the injected fracturing fluids used for wells 1 and 2 is proprietary information that belongs to a service corporation and was thus unavailable. The major difference between two wells is that FPW was reused for well 1 to supplement freshwater in the mixing of injected fracturing fluids, while well 2 only used freshwater. FPW samples from well 1 were collected between August 2016 and January 2017, and FPW from well 2 was collected between December 2016 and March 2017 (Table S1). Generally, FPW samples were collected more frequently on hourly and daily timescales, and then were collected monthly thereafter. Oil-based drilling fluid and

RPW sample were collected from well 1 (RPW sample was sourced from nearby fracturing operations in the Duvernay shale gas play).

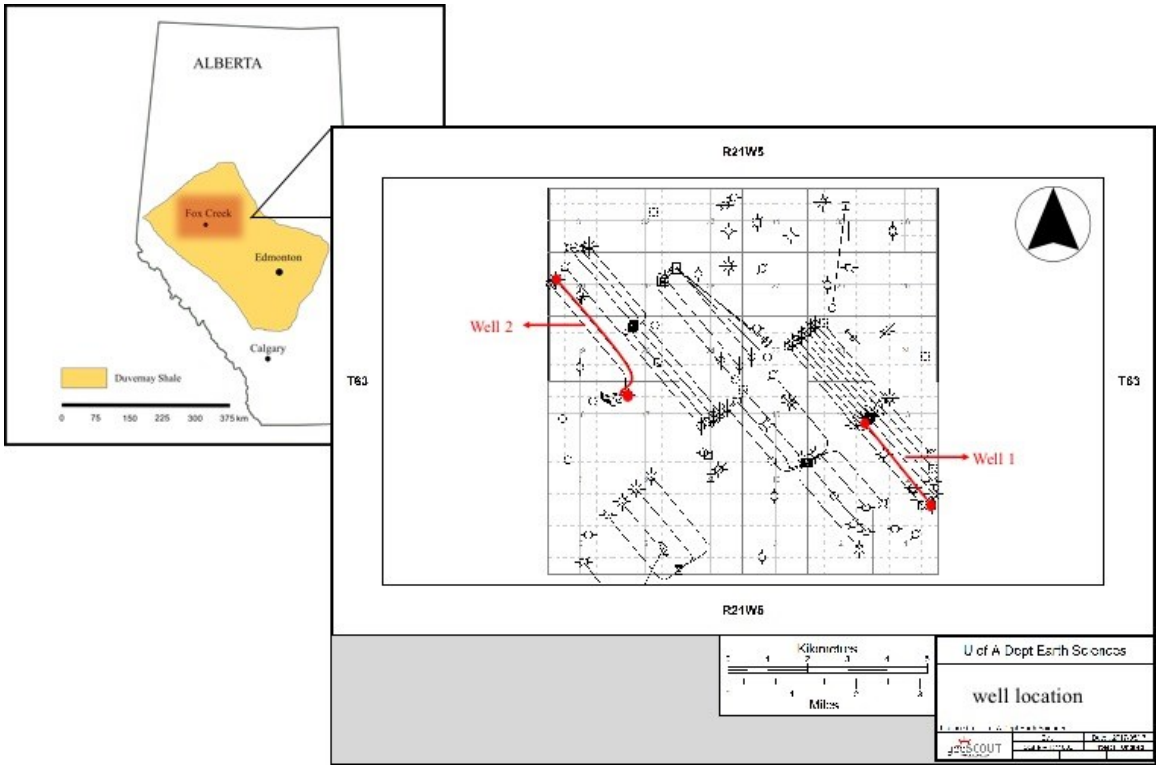

Figure 2. Locations of Wells 1 and 2 (modified from Rokosh et al. 2012 and geoSCOUT)

Figure 3 illustrates the placement of fluids sampled in the overall hydraulic fracturing water cycle. SW samples were collected at water storage ponds near wells 1 and 2, and from the North Saskatchewan River (NSR). Well 1 FPW samples were collected after the oil/water separator. FPW samples were collected before the separator, because multiple wells were flowing into the same oil/water separator at well 2; in this way, I could ensure that all collected return fluids originated from well 2. Samples were couriered to the University of Alberta in sealed 20 L polypropylene buckets. To prevent the contaminant from inside containers, all the buckets were risen by FPW first before

collection. When FPW samples collected at well 2 were received in the laboratory, liquid hydrocarbons were removed using a separatory funnel.DNA isolation and cell enumeration were performed in sterile environments within 24 hours of sample arrival. The resulting DNA isolates were stored at -80°C until further processing.

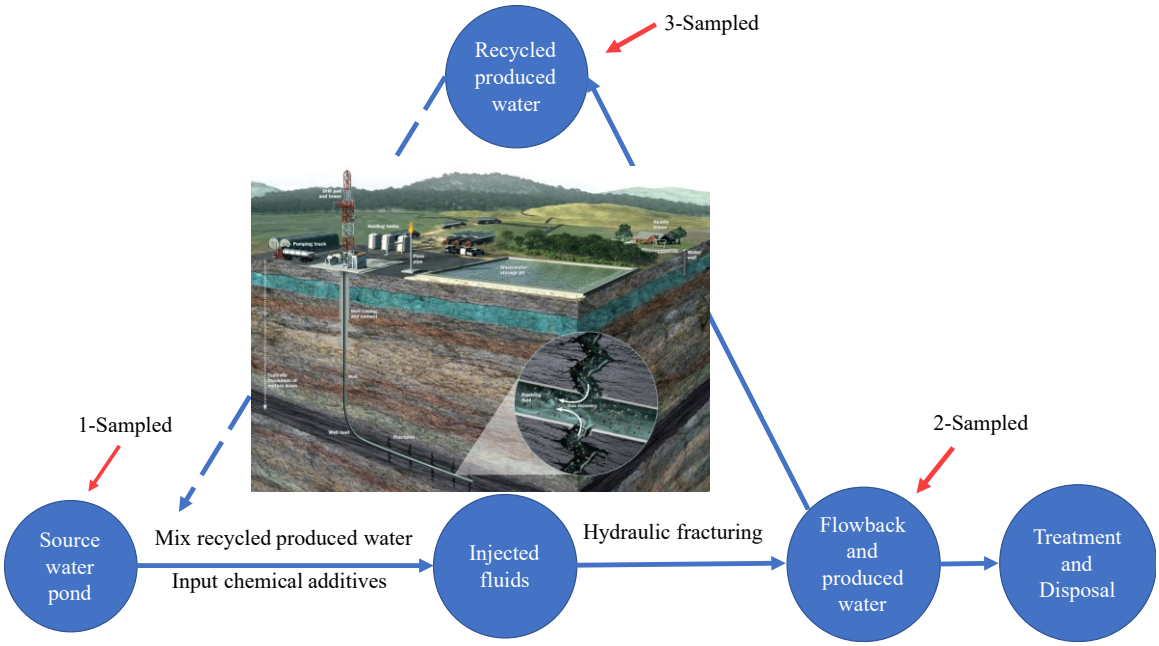

Figure 3. Sampling strategies and locations (image source: http://www.geologypage.com/)

2.2.2 Subsampling Strategies for FPW Recycling Simulation

In order to further investigate the microbiological effects of reusing FPW on making injected fracturing fluids, RPW was mixed with SW collected from well 1 and NSR water in the laboratory at a 1:9 ratio, a ratio that is practical for recycling by oil and gas operators (Mantell 2011;Cluff et al. 2014). As controls for cell counting, SW from well 1 and the NSR water sample were each mixed with sterile deionized (DI) water at the same ratio. The mixed fluids were incubated in closed high density polyethylene

containers in the laboratory at room temperature without shaking. Mixed fluids were taken from the mixture of well 1 SW-RPW for cell enumeration at eight time points after: 1 h, 6 h, 1 day, 3 days, 7 days, 17 days, 25 days, and 40 days. Ten time points were sampled for the NSR-RPW incubation, after: 0 h, 1 h, 6 h, 1 day, 3 days, 7 days, 17 days, 25 days, 45 days, and 60 days. Three of these time points were selected for 16S rRNA sequencing for each mixture system: 1, 7, and 40 days incubation for well 1 SW-RPW, and 7, 17, and 60 days incubation for NSR-RPW.

2.3 Aqueous Geochemistry Analyses

2.3.1 Filtration

Prior to analysis, water samples were filtered through 0.2 μm nylon membranes (Agilent Technologies) before doing anion cation, and TOC measurements, and were pre-filtered through 0.45 μm PTFE membranes (Agilent Technologies) before characterizing organic compounds.

2.3.2 Inorganic Chemistry Analyses

Dr. Shannon Flynn and Mr. Konstantin von Gunten conducted the total dissolved solids (TDS), cation, and bromide measurements. TDS were estimated from the measurement of specific gravity. For the cation and bromide measurements, where sample dilution was necessary, 18MΩ ultrapure water was used. Samples were acidified at a rate of 6 μL per 10 mL using trace grade 2% nitric acid and 0.5% hydrochloric acid. All analyses were for cations and bromide were performed using an Agilent 8800 Triple Quadrupole ICP-MS (ICP-QQQ) with a RF power of 1550 W and a RF reflected power

of 18 W. The ICP-QQQ was operated with a microMist nebulizer and nickel/copper cones. Several unique features of the ICP-QQQ were utilized during analysis, including high matrix mode for high total dissolved solids (TDS) samples with 8 mL/min argon dilution (HMM), MS/MS mode for greater mass resolution, and the gas reaction cell with O_2 gas (10% max flow) and H_2 gas. An inline internal standard system was employed to add a solution of 0.5 ppm indium to each sample, which was used to correct for instrument drift. Additionally, a standard solution of known concentration was analyzed at the beginning, middle and end of the run as an additional QA/QC check of the instrumental drift and to assess the instrumental precision and accuracy. To determine the concentrations of major elements, SW, RPW, and FPW samples were analyzed for anions (Cl^-, SO_4^{2-}) at the Natural Resources Analytical Lab (NRAL) of the University of Alberta using an ion chromatograph (Dionex Corporation DX 600) with an AS9-HC 4 mm analytical column and a AG9-HC guard column, and a 4 mm ASRS Ultra suppressor.

2.3.3 Organic Chemistry Analyses

TOC was measured as non-purgeable organic carbon at the NRAL using a TOC-V CHS/CSN Model TOC Analyzer (Shimadzu Corporation). The organic chemistry analyses were conducted by Dr. Chenxin Sun. Ultra-high performance liquid chromatography (UPLC; ThermoFisher Scientific) and ultra-high resolution Orbitrap mass spectrometry (Orbitrap-MS; ThermoFisher Scientific) were used for the untargeted analysis of aqueous phase organic compounds, because the profiles of organic compounds in FPW are complex and the structures of individual organic molecules in FPW are largely unknown (Ferrer and Thurman 2015b; Elsner and Hoelzer 2016). Due to

the complexity of unknown organics in the FPW, the identification strategy for the organic compounds in FPW was to isolate the organic constituents with relatively strong signals. Then, these organic constituents having strong peaks were identified and classified, and then compared to changes microbial community composition to infer potential linkages to organic constituents.

The organic compounds in FPW with insufficient concentrations were concentrated by liquid−liquid extraction. To do so, 200 mL of filtered late period of FPW was extracted three times with 30 mL of dichloromethane. The dichloromethane phases were then combined and evaporated to approximately 5 mL using a rotary evaporator, then reduced to dryness under a flow of nitrogen (99.998% purity). The organic extract was dissolved in 1 mL of methanol for instrumental analysis. The filtered early period of FPW was used for organic analysis without further extraction due to the much higher concentrations of organic compounds. 5 μL of the organic extract of late FPW (less organics) or 50 μL of filtered early FPW (more organics) was injected on a Hypersil Gold C18 analytical column (50 × 2.1 mm, 1.9 μm particle size; Thermo Fisher Scientific). The mobile phase, flowed at 0.4 mL/min, was composed of (A) 15 mM ammonium acetate in water and (B) methanol. The gradient was as follows: 0-5 min, 1% B; 5-35 min, linear gradient to 100% B; 35-37 min, linear gradient back to 1% B and hold for 5 min. For FPW samples, the first 3 min of eluent was discarded through a six port, two position valves, due to the high sample salinity. The Orbitrap-MS was operated with an ESI source in ion-positive mode. The ionization potential was set at 4 kV, while the sheath, aux, and sweep gas flows were set to 35, 15 and 2 (arbitrary units), respectively. Vaporizer and capillary temperature were at 325 °C and 300 °C, respectively. Acquisition

was performed in full scan mode (*m/z* 100 to 1000) at 2.3 Hz with resolving power set to a nominal value of 120,000 at full width half-maximum at *m/z* 400. The tandem mass spectrometry analyses were performed using data dependent mode with collision-induced dissociate (CID) at 20, 25 and 35 eV and also higher-energy collision dissociation (HCD) at 50eV and 80 eV. Thermo Xcalibur 2.2 software was used for data acquisition and analysis.

2.3.4 Quality Control of Chemical Analyses Data

Sample availability of hydraulic fracturing related fluids was often influenced by the ongoing oil and gas operations, which results in a lack of replicate samples in most relevant studies (Mouser et al. 2016). In addition to sampling from two separate wells, to optimize my data quality and reliability, well 1 samples were measured 8 times by ICP-QQQ (first sampling location in this study). Based on the high precision of the results from the analyses for well1, and considering overall experimental efficiency, the high cost of processing large numbers of samples by high-density sampling, and the lack of sampling availability on site, samples for well 2 were analyzed by ICP-QQQ four times. For the analysis of pH, anions, and TOC, samples from well 1 and well 2 were shipped to NRAL and analyzed using quality control standards established for that laboratory. All results were reported with instrumental standard deviation (SD) as applicable.

2.4 Enumeration of Live and Dead Cells

For the bacterial viability test, the Live/Dead BacLight Viability kit (Molecular Probes Corporation) was used according to the manufacturer's instruction. The kits

utilized a mixture of SYTO 9 green-fluorescent nucleic acid and propidium iodide red-fluorescent nucleic acid to distinguish relatively healthy cells with intact membranes from dead cells with broken membranes. To prepare cell counting, 250 μL of water samples were mixed with 250 μL staining dye that consisted of 6 μM SYTO 9 stain and 30 μM propidium iodide. Samples were stained and incubated in the dark for 15 min at room temperature, and then were filtered onto 0.2 μm black polycarbonate membranes (GVS, Life Sciences). Whatman glass microfiber filters (GE Healthcare, Life Sciences) were used as membrane support filters. My method to estimate total cell concentrations modified from Weinbauer et al. (1998); for each sample, average of total cells per field of 15 fields (n=15) were selected at random counted on LEICA DMRXA epifluorescence microscope with 358× magnification, which were used to estimate the cell concentrations per mL (the average total cell numbers per field were equal to the sum of live and dead cells in the same field). The live/dead cell ratios were calculated by the division of accumulated live and dead cell numbers in a total of 15 fields.

2.5 16S ribosomal RNA (16S rRNA) Sequencing

To recover DNA from the SW, RPW, and FPW, 500-1500 mL of SW, RPW, and FPW samples were passed through 0.22 μm pore size hydrophilic polypropylene membranes (Pall, Life Sciences). To recover DNA from samples collected in the separate batch experiments, 50-300 mL of mixed fluids (incubation water samples from the mixture fluids of well 1 SW-RPW and NSR-RPW) were passed through 0.22 μm pore size hydrophilic polypropylene membranes (Pall, Life Sciences), followed by DNA extraction using the FastDNA spin kit for soil (MP Biomedicals, Solon, OH). DNA was

also extracted from 0.5 g wet weight of oil-based drilling fluids. 16S rRNA gene fragments were amplified using the universal bacterial and archaeal primer sets F515 (5′-GTG CCA GCM GCC GCG GTA A-3′) and R806 (5′- GGA CTA CHV GGG TWT CTA AT-3′) with Illumina adapters, covering the V4 region. The 25 μL PCR mixture contained 2.5 μL microbial DNA, 5 μL amplicon PCR forward primer (1 μM), 5 μL amplicon PCR reverse primer (1 μM), and 12.5 μL 2× KAPA HiFi HotStart Ready Mix. The PCR reaction began with a 3 min initial denaturation (95 ^{o}C) followed by 35 cycles of 30 sec denaturation (95 ^{o}C), 30 sec primer annealing (55 ^{o}C), and 30 sec extension (72 ^{o}C) and a final 5 min extension (72 ^{o}C). For SW, FPW and RPW samples that had low DNA concentrations, 500 mL of each water samples was first concentrated onto 0.22 μm hydrophilic polypropylene membranes (Pall, Life Sciences), following DNA extraction and PCR amplification. If no PCR product band was observed after gel electrophoresis, I extracted DNA from a 1-2 L aliquot of the same water sample. If DNA was not recovered following this procedure and after an additional duplicate extraction was performed, the total DNA concentration was regarded as below the detection limit. For samples in which DNA was successfully recovered, duplicate PCR reactions of each sample were pooled into a sterile 1.5 mL tube. Library preparation and paired ends sequencing were conducted on an Illumina Miseq System at the Applied Genomics Core (TAGC) Sequencing Facility at University of Alberta.

2.6 Bioinformatics and Statistical Analyses

Raw sequences for each sample from Illumina Miseq were combined, quality filtered (sequences are longer than 350 bp, primer and barcode mismatches,

homopolymers longer than 8 bp), and aligned to a combined bacterial and archaeal SILVA database (version 128) using Mothur software (Schloss et al. 2009; Schloss, Gevers, and Westcott 2011). Sequences were further improved according to the Mothur SOP (Pruesse et al. 2007; Huse et al. 2010; Schloss 2010; Schloss et al. 2011) and then checked for chimeras using UCHIME (Edgar et al. 2011). This process resulted in 14,909 unique sequences of the total 96,446 sequences that were used for the further analyses. Sequences sharing ≥ 97% similarity in identity were clustered into operational taxonomic units (OTUs). OTUs were then classified according to the highest taxonomic resolution possible using the Ribosomal Database Project classifier (trainset 16) with an 80% confidence threshold (Wang et al. 2007). Sequences corresponding to chloroplast, mitochondria, or Eukaryota were removed, and the remaining sequences were binned to phylotypes according to the taxonomic classification. Classification of binned OTUs into the class level were performed using R software with the Phyloseq package (Mcmurdie and Holmes 2013). Processed sequences were subsampled to 3154 sequences (smallest library size) per sample, and used for further alpha and beta diversity analyses (Gihring et al. 2012). All the alpha and beta diversity analyses were based on the phylotype-based approach. The coverage, Chao1 richness, non-parametric Shannon diversity, and inverse of the Simpson diversity index as implemented in Mothur were used to analyze the microbial community coverage, richness, and diversity of each sample (Shannon 1948; Simpson 1949; Chao 2017). Nonmetric multidimensional scaling (NMDS) was calculated on Bray-Curtis dissimilarity indices, and was visualized using 20 runs of real data using R software with the Phyloseq package (Mcmurdie and Holmes 2013). In the NMDS plot, I examined the spatial separations between distinct groups in the ordination using the

AMOVA test. The AMOVA test with 1000 runs of real data was used to test whether the separations in the ordination were statistically significant (Excoffier et al. 1992); p values < 0.05 were considered to represent statistically different microbial communities. To determine whether there were statistically significant differences between OTU abundances among distinct groups in the ordination, non-parametric t-tests were employed, using the metastats command implemented in Mothur (White et al. 2009); p values < 0.05 were considered to represent a significant influence. Spearman rank coefficients were also calculated in Mothur software to describe the relationship between the water geochemistry (e.g. pH, salinity, and temperature) and OTUs in the ordination; p values < 0.05 were considered to represent a significant influence on the direction of the distinct groups in the ordination.

Chapter 3: Results

3.1 Aqueous Geochemistry

3.1.1 pH

Changes in pH for wells 1 and 2 in 20 days of flowback and produced water (FPW) were shown in Figure 4. For well 1, FPW samples were more acidic than was SW. pH quickly decreased from 8.10 of SW to 6.82 of the initial FPW, and further decreased to 5.43 by day 8 h, then became relatively consistent between 5-6, and increased to 6.11 by day 19 of FPW. Recycled produced water (RPW) had lowest value of 4.7. For well 2, pH increased from 7.44 of SW to 7.61 of the initial FPW, then considerably decreased to the range between 5-6, indicating FPW from well 2 were also acidic, which was consistent with the observation of FPW from well 1.

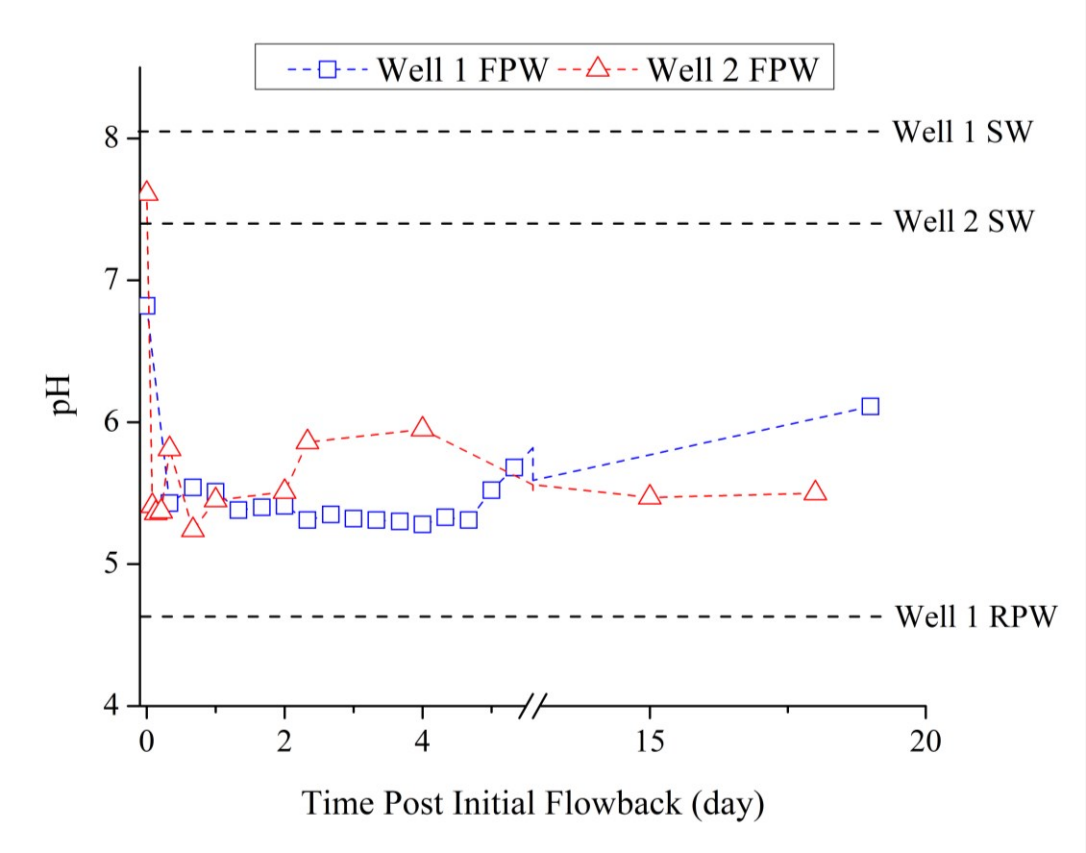

Figure 4. Temporal changes in FPW pH; well 1 RPW and wells 1 and 2 SW are included for reference and did not change over time.

3.1.2 Total Dissolved Solids (TDS) and Ions

For both wells 1 and 2, the TDS dramatically increased once FPW commenced (Figure 5). For well 1, the TDS increased from 5.31×10^3 ppm at 0 h to 1.68×10^5 ppm at 8 h, The relatively rapid changes in the TDS occurred were within a day of FPW, gradually climbed to the peak concentration of 2.19×10^5 ppm by day 53, then slightly dropped afterwards. For well 2, the TDS increased from 288 ppm at 0 h to 6.42×10^3 ppm at 20 min, further increased to 1.45×10^5 ppm by 1.33 h, and reached to the peak of 2.68×10^5 ppm at day 115. Afterward, the TDS dropped such that trends in the TDS in FPW

samples from well 2 were also consistent with those observed for well 1. However, the TDS of the initial FPW from well 1 was much higher than the TDS of the initial FPW from well 2, most likely because FPW was recycled at well 1, but not at well 2.

The concentrations of each element measured in this research are presented in Tables S3 and S4 (Appendix). Results of distribution in concentrations of elements measured in samples from wells 1 and 2 were consistent with each other. In general, compared to SW, bulk elements Na, Ca, and K were elevated in FPW and RPW; concentrations of more rare metal ions such as Sr, Mg, and B were also elevated. Other cations that were considered in this research, including Pb, Cu, As, Cd, and Al were below the detection limit. The dominant anion was Cl in both FPW and RPW, and concentrations of Br were hundreds of ppm. Figure 6 shows changes in bulk sulfur in FPW from wells 1 and 2. For well 1, total S concentrations dramatically increased from 2 ppm in SW to 80 ppm in the initial FPW, further increased to the peak of 82 ppm by 16 h of FPW, then dropped to 40 ppm by day 91, and subsequently climbed to 59 ppm by day 120. For well 2, total sulfur concentration was 87 ppm by 1.33 h of FPW, increased to 100 ppm by 4.8 h of FPW, then fluctuated in the range between 89-102 ppm in the first 6 days. Subsequently, S concentrations from day 9.04 to a low of 32 ppm at day 17.5 of FPW, then climbed to over 50 ppm by day 6. Thus, for both wells 1 and 2, a general trend for changes in concentrations of bulk sulfur was observed as dramatically increasing first, decreased subsequently, and then climbing again. Changes in the ratio of S/Cl in FPW for both wells are shown in Figure 7. For well 1, the S/Cl ratio for RPW was in the magnitude of 10^{-4}; the S/Cl ratio for SW was 0.46, then declined to approximately 10^{-3} for the initial FPW, then dramatically decreased and became relatively stable at

approximately 10^{-4} throughout of the rest of well FPW. For well 2, the total S concentrations were in the order of 10^{-3} during the first 3 days of FPW, then dropped and became relatively consistent at approximately 10^{-4} by day 5.67 of FPW, which the observation was consistent with the decreasing trend that was observed in FPW from well 1.

3.1.3 Flow temperature

Flow temperatures were recorded from the wellhead as well FPW proceeded. Different from the trend of rapidly increasing TDS over the course of hours, flow temperatures were observed to reach a plateau a few days following the initiation of FPW for both wells (Figure 5). For well 1, the flow temperature of FPW gradually increased from 20°C in SW after the initial FPW (20°C) proceeded, and subsequently reached the maximum value of 77°C by day 4 of FPW, then decreased from 76°C by day 6 to 53°C by day 19, and finally became relatively consistent in the range between 46-60°C after 20 days of FPW. For well 2, the flow temperature began at -25°C in the initial FPW (due to a large fraction of methanol in the sample), rose to 60°C by day 2, further increased to 70°C by day 5, and peaked at 71°C by day 10; afterward, the temperature dropped from 71°C by day 15 to 55°C by day 18, and became relatively consistent at approximately 55°C afterwards. The overall changes in flow temperatures between two wells were relatively consistent with each other. However, there was a considerable difference of the flow temperature between the initial FPW from well 1, and that from well 2.

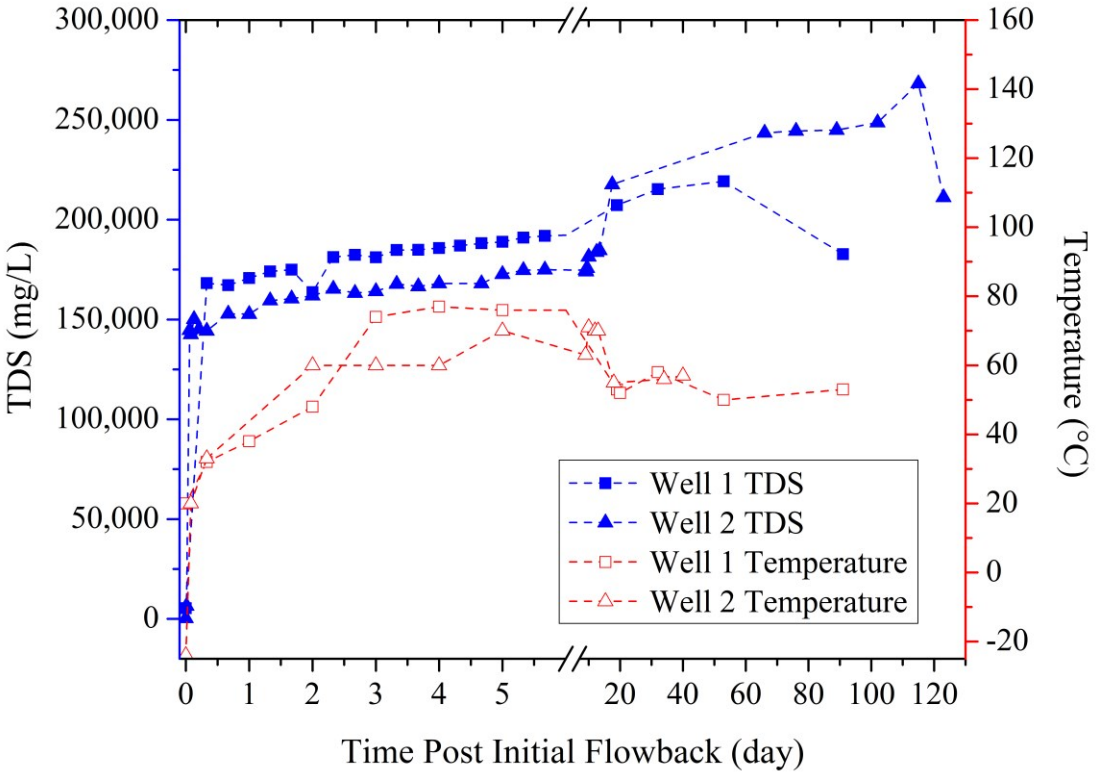

Figure 5. Temporal changes in FPW TDS (data source: Dr. Shannon Flynn) and flow temperature (data source: Encana Corporation).

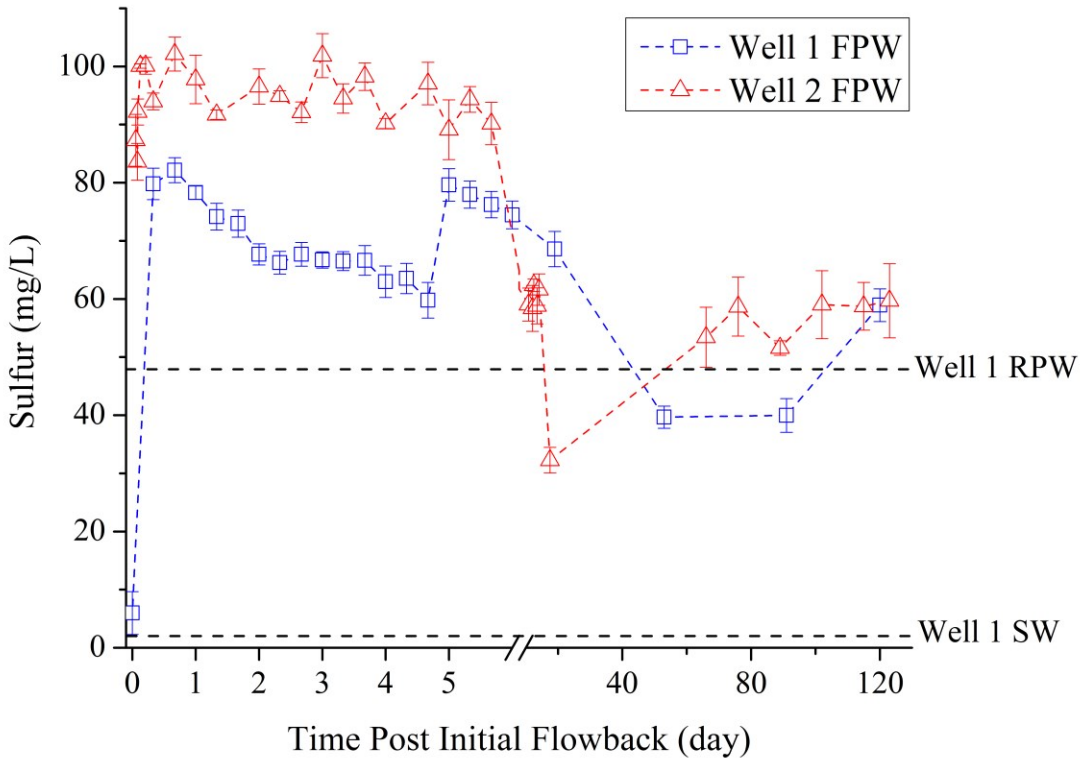

Figure 6. Temporal changes in total S concentrations of FPW (data source: Dr. Shannon Flynn); well 1 RPW and SW total S concentrations are included as references. Error bars are instrumental standard deviations (SD) for well 1 (n=8) and well 2 (n=4).

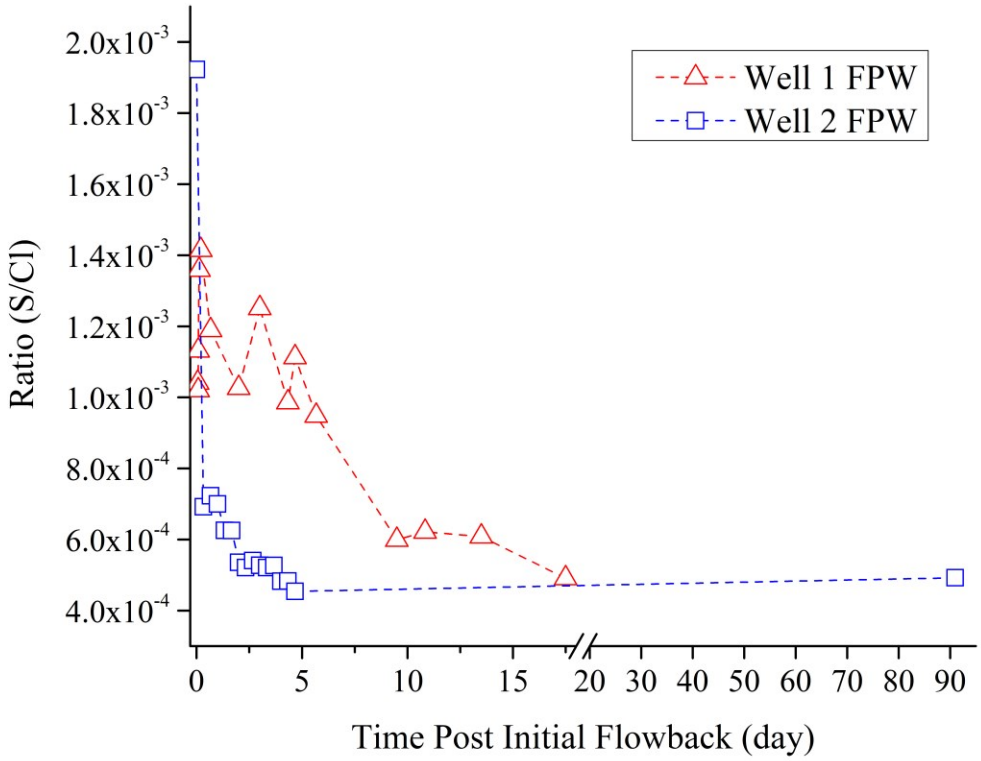

Figure 7. Temporal changes in S/Cl ratio of FPW (data source: Dr. Shannon Flynn)

3.1.4 Organic Constituents in FPW

The total organic carbon (TOC) concentration for well 1 SW was 43.7 ppm. The average value of TOC for the well 1 FPW samples was 283 ppm, with a minimum value of 212 ppm by day 4.67 of FPW, and the average value for well 2 FPW samples was 334 ppm, with a minimum value of 271 ppm by day 5 of FPW. TOC values were initially high in both wells, but rapidly decreased during the early period of FPW for both wells. For well 1, the initial FPW contained 1.18×10^4 ppm TOC, decreased dramatically to 402 ppm by 8 h after the start of flowback, and then became relatively consistent. For well 2, the TOC was 4,710 ppm at 2 h of FPW, decreased to 1,346 ppm at 2.5 h, 711 ppm at 3 h,

and 302 ppm at 4 h, and then became relatively consistent. For well 2, a large volume of residual MeOH at 0 h and 0.33 h of FPW samples precluded the measurement of TOC in those samples.

Figure 8 shows the range of organic compounds that were detected in the FPW samples at 1.44 h, 3.12 h and 3 day from well 2. The most dominant group of peaks, eluting between 6 and 13 min, were identified as a series of polyethylene glycol (PEG) compounds, which are polymers of ethylene oxides (EO) ranging between monomers of 4 and 15. The accurate mass measurement (mass error < 2 ppm) of protonated, ammonium adducts and sodium adducts of PEG, together with MS/MS mass fragmentation spectra, confirmed these identifications. PEG was added into fracturing fluids as surfactants, and this group of polymers have also been observed in hydraulic fracturing water samples collected from other shale gas plays (e.g., Thurman et al. 2014). As shown in the MS spectra (Figure 8), the overall signal and therefore the abundance of PEGs and other organic constituents quickly decreases as a function of FPW return time and volume.

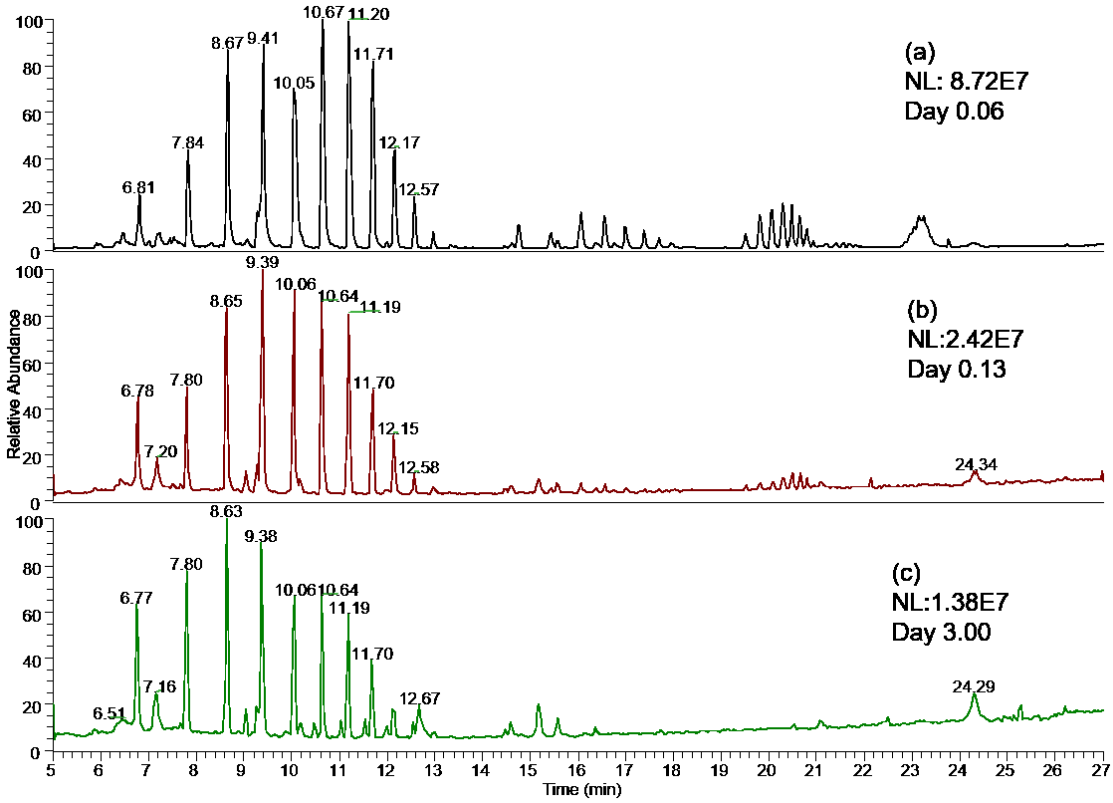

Figure 8. Total ion chromatogram of HPLC/ESI(+) orbitrap MS analysis of FPW Day 0.06 (1.44 h), 0.13 (3.12 h), 3 (3 day) from well 2 (data source: Dr. Chenxin Sun).

Besides the surfactant PEG, a series of alkyl dimethyl benzyl ammonium chloride (ADBAC) homologues ranging from C8 to C14 were also detected. ADBAC was added into the hydraulic fracturing fluids as biocides in order to prevent bacterial growth (Kahrilas et al. 2015). The extracted ion chromatography of ADBAC-C8 ($C_{17}H_{30}N^+$), ADBAC-C10 ($C_{19}H_{34}N^+$), ADBAC-C12 ($C_{21}H_{38}N^+$), ADBAC-C14 ($C_{23}H_{42}N^+$) (Figure 9a) showed the increased retention time of these homologues on the C18 column used, which is consistent with the increased chain length from C8 to C14. The MS/MS spectrum of $C_{17}H_{30}N^+$ at *m/z* 248.2372 (Figure 9b) showed the product ion $C_{10}H_{22}N^+$ at *m/z* 156.1744 from the fragmentation at benzylic amine bond and also the tropylium ion $C_7H_7^+$ at *m/z* 91.0539, supporting the structural identification of ADBAC-C8. The same

fragmentation pattern of ADBAC was also observed in another study by Ferrer and Thurman (2015a). To further confirm the identification, MS/MS analysis was also performed on a commercially available standard ADBAC-C8. Using the calibration curve of the ADBAC-C8 standard, the concentration of ADBAC (C8-C14) in FPW at 1.44 h was estimated at 25 ppb, but concentration of this group of compounds rapidly decreased until it was below the detection limit of 0.5 ppb by day 3 of FPW.

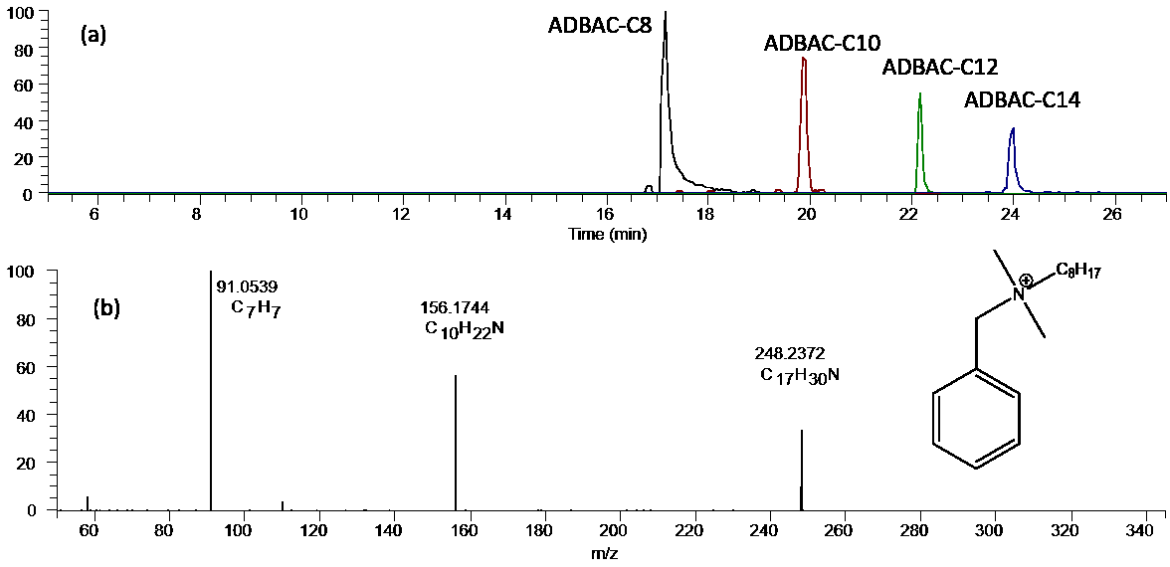

Figure 9. (a) extracted ion chromatograms of ADBAC-C8, C10, C12 and C14; (b) MS/MS spectrum of ADBAC-C8 ion at m/z 248.2372 under CID 25eV. The structure of ADBAC-C8 is illustrated in the figure (data source: Dr. Chenxin Sun).

Another group of compounds eluting at 24.3 min that may be related to bacterial activities were identified as octylphenol ethoxylates that are composed of EO between 7 and 11 (see Figure 10c for general structure). Octylphenol ethoxylates are commonly used as surfactants in hydraulic fracturing fluids and readily degraded to octylphenol, and endocrine disruptor, by microbial activity (Orem et al. 2014). The average mass

difference between the ions shown in Figure 10a was 44.0261, indicating these ions are a homologous series of ethoxylates differing in the in the number of EO units. Taking the ion at *m/z* 620.4365 as an example, the empirical formula of this parent ion was assigned as $C_{32}H_{62}O_{10}N^+$ based on accurate mass measurement (mass error 0.48 ppm). The neutral loss of ammonia yielded the product ion at *m/z* 603.4108 (Figure 10b), indicating the parent ion at *m/z* 620.4365 was an ammonium adduct $[C_{32}H_{58}O_{10}+NH_4]^+$. The product ion at *m/z* 491.2857 was probably generated from the loss of 112.1251 mass units from the ion at *m/z* 603.4108. Based on the accurate mass measurement, the loss of 112.1251 mass units was assigned as C8H16. This characteristic loss of C8H16 was also observed in the MS/MS spectra of other parent ions at *m/z* 532.3842, 576.4104, 664.4628 and 708.4889 in Figure 10a. A series of mass losses of 44.0261 mass units resulting in diagnostic ions at *m/z* 89.0698, 133.0860 and 177.1123, also supported the PEG structure. The tropylium ion $C_7H_7^+$ at *m/z* 91.0539 was observed in the MS/MS spectrum of the parent ion at m/z 620.4365 under HCD 80 eV (Figure 10c), supporting the speculation of a benzene ring structure. Compared with the MS/MS spectrum of the octylphenol ethoxylates (EO=9) standard further confirmed the identification.

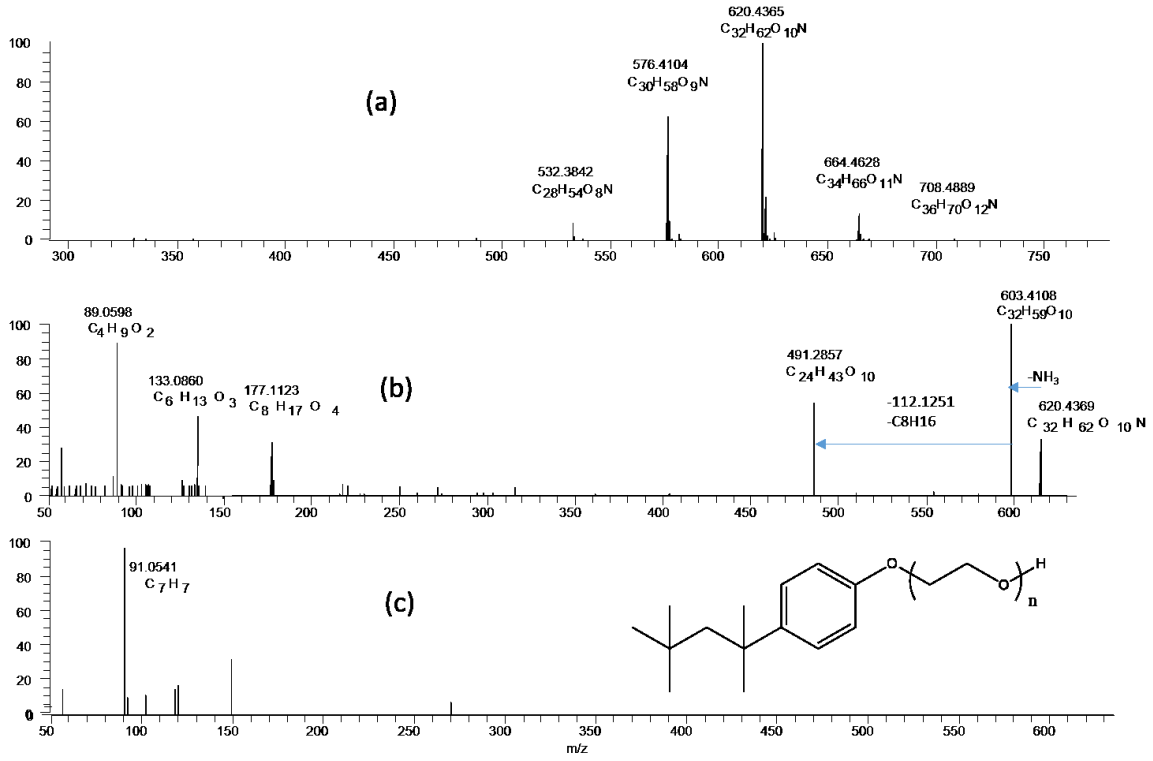

Figure 10. (a) mass spectrum of peak at 24.3 min in Figure 8a; (b) MS/MS spectrum of the ion at m/z 603.4108 under CID 35 eV; (c) MS/MS spectrum of the ion at m/z 603.4108 under HCD 80 eV. The structure of octylphenol ethoxylates is illustrated in the figure (data source: Dr. Chenxin Sun).

3.2 Microbial Community Dynamics in FPW from Wells 1 and 2

3.2.1 Total Cells and Cell Viability in SW, RPW, and FPW

Figures 11 and 12 show changes in numbers of total cells coupled with live/dead cell ratios (cell survival ratios) in FPW for both wells. For well 1, total cells of $2.58(\pm0.10)\times10^{6}$ cells mL^{-1} (cell survival ratio: 0.87) in SW dramatically decreased to $7.93(\pm1.92)\times10^{5}$ cells mL^{-1} (cell survival ratio: 0.60) in the initial FPW, then dropped to $9.31(\pm7.22)\times10^{4}$ cells mL^{-1} (cell survival ratio: 0.34) by day 4 of FPW, and further

declined to $7.08(\pm3.63)\times10^4$ cells mL^{-1} (cell survival ratio: 0.87) by day 20 of FPW, and thereafter became almost undetectable (SD>average cells counted per field). RPW from well 1 had $5.03(\pm3.39)\times10^4$ cells mL^{-1} (cell survival ratio: 1.17). For well 2, a total of $1.04(\pm0.30)\times10^6$ cells mL^{-1} (cell survival ratio: 0.30) in SW was also reduced to $4.82(\pm1.40)\times10^5$ cells mL^{-1} (cell survival ratio: 0.67) in the initial FPW, which was consistent with the considerable reduction of cellular biomass from SW to FPW as observed in FPW from well 1. Then, total cells further decreased to $5.25(\pm4.98)\times10^4$ cells mL^{-1} (cell survival ratio: 0.36) by 2 h of FPW, and the rate of decrease was faster than for well 1. There was a considerable recovery in total cells to $7.22(\pm2.12)\times10^5$ cells mL^{-1} (cell survival ratio: 0.75) by day 1 of FPW, and cell viability was recovered within a day, which was not observed in the early period of FPW from well 1. Subsequently, total cells dropped to almost undetectable levels by day 34 and thereafter. Based on the observation of the two wells studied here, the numbers of total cells dramatically decreased from levels measured in SW the flowback process began, becoming almost undetectable during the later period of fluid returned.

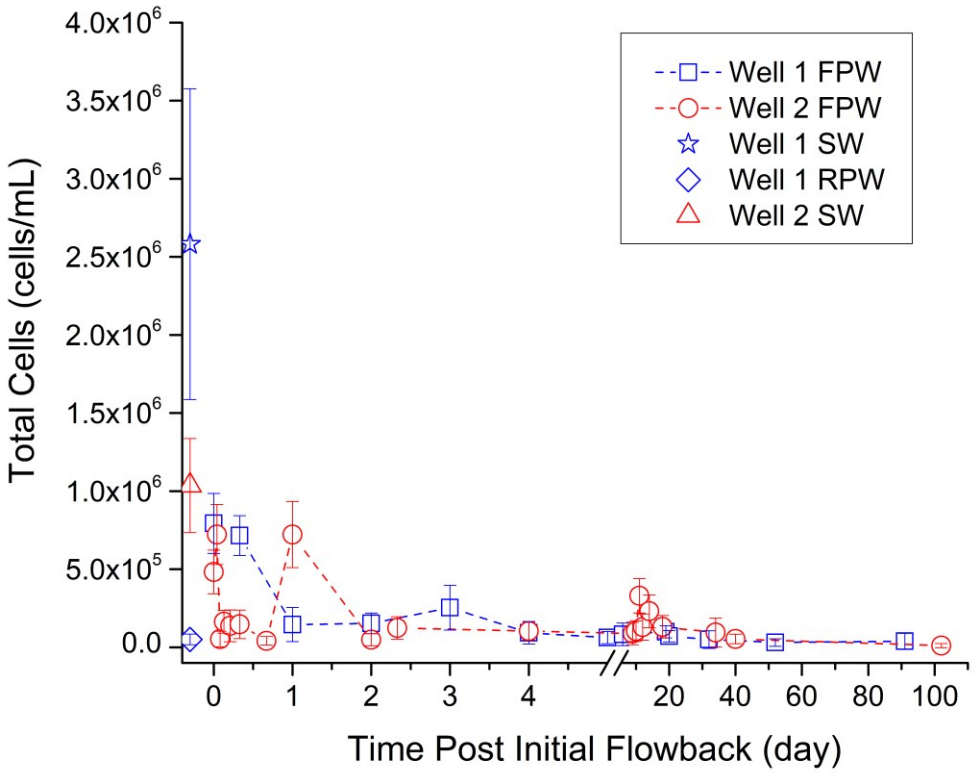

Figure 11. Temporal changes in total cells from SW, RPW, and FPW samples.

Error bars are SD from 15 fields (n=15)

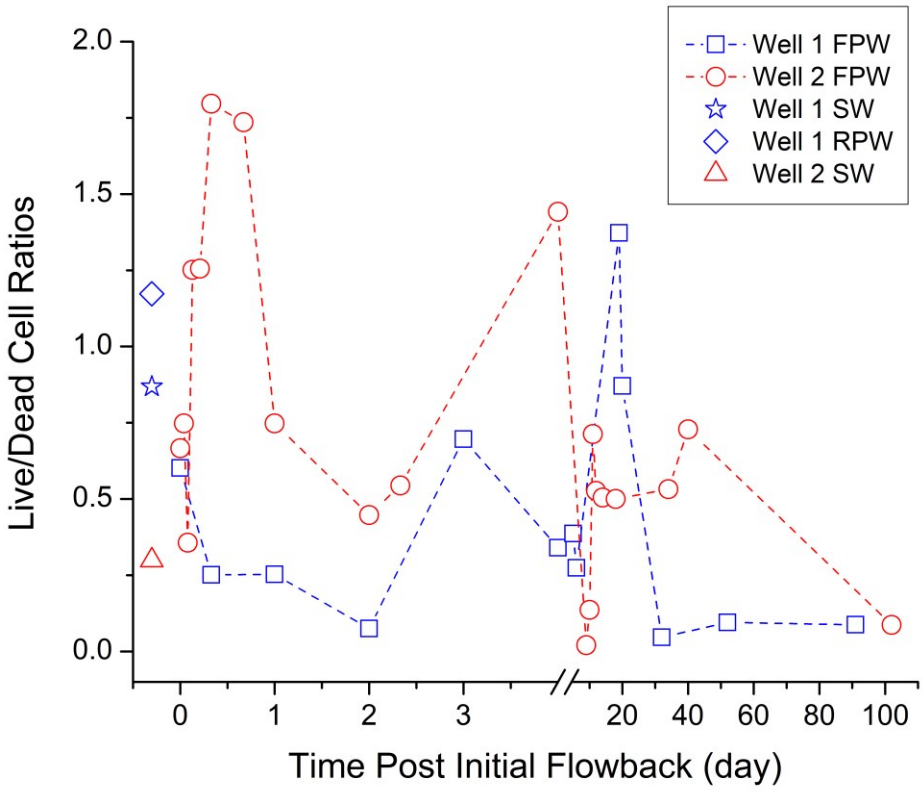

Figure 12. Temporary changes in the ratio of live/dead cells in SW, RPW, and FPW samples

3.2.2 DNA in Drilling Fluids, SW, RPW, and FPW

The overall quantity of DNA recovered from FPW and RPW was low, correlated with the low biomass observed via live/dead staining. I was not able to amplify 16S rRNA genes from RPW and oil-based drilling fluids as there were little or no detectable genomic DNA and very low or undetectable microbial biomass. To verify that the components of oil-based drilling fluid did not inhibit the extraction of DNA, I injected a known amount of *E. coli* into the oil-based drilling fluid (cultured overnight at 37°C); I was able to extract amplifiable genomic DNA from this control. The concentrations of

DNA in FPW from wells 1 and 2 quickly dropped after the early period of FPW, based on electrophoresis experiments (Figures 13 and 14), and I was not able to amplify 16S rRNA genes after day 1 of FPW for well 1, and day 18 of FPW for well 2.

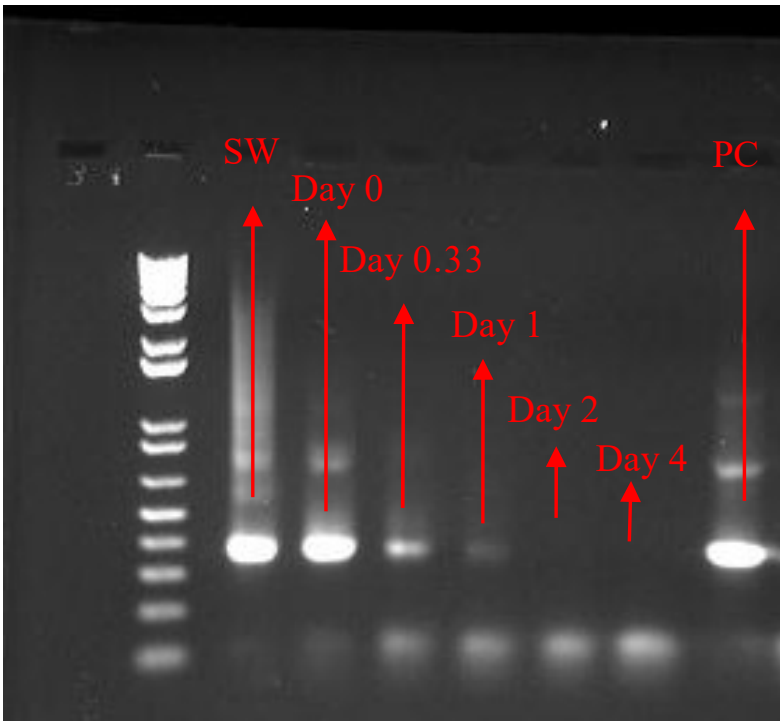

Figure 13. Gel image for PCR amplicons from well 1 before clean up and optimization

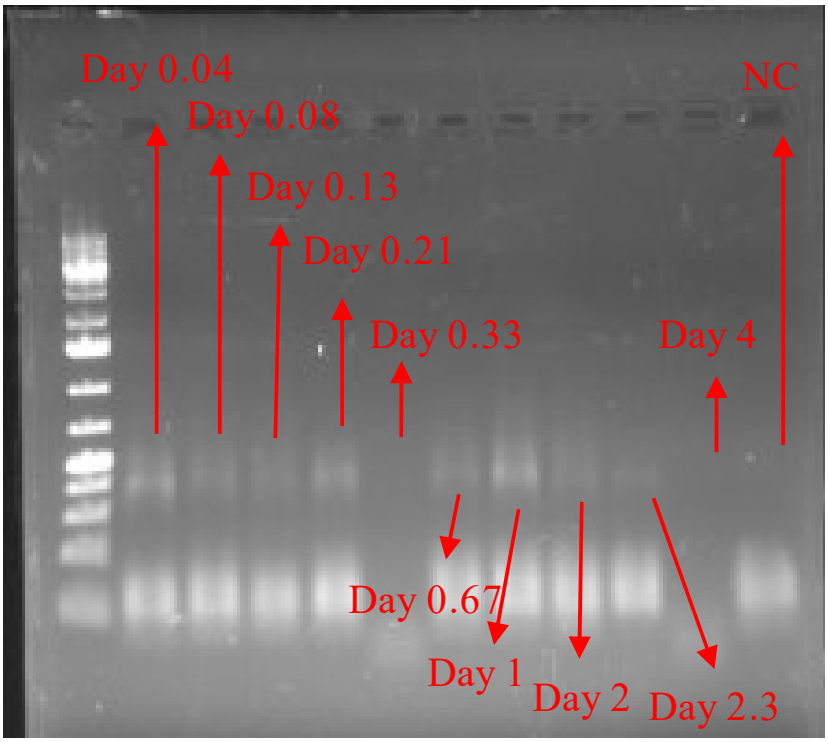

Figure 14. Gel image for PCR amplicons from well 2 before clean up and optimization

3.2.3 Microbial Community Abundance, Richness, and Diversity in SW and FPW

16S rRNA sequencing was conducted on samples for which sufficient genomic material was recovered. I found that Bacteria dominated in both the SW and FPW samples for both wells, while Archaea were found at lower levels. In general, the Shannon diversity index, Simpson evenness, and Chao1 richness index showed that microbial diversity and richness were the highest in the SW and declined in FPW as fluid returned for both wells (Table 2). However, temporary increases in richness at day 0 and 0.04 of flowback were observed for well 2. In contrast to the gradual decrease in richness

and diversity observed from SW to FPW in well 2, the microbial community was less diverse in FPW samples from well 1 from the very beginning.

Table 1 Coverage, richness, and diversity of microbial community for SW and FPW samples

Location	Sample	Coverage Percentage	Observed OTU	Chao1	Inverse Simpson	Nonparametric Shannon
Well 1	SW	99	120	148	20.05	3.56
	Day 0	99	40	62	2.30	1.12
	Day 0.33	100	15	23	1.46	0.64
	Day 1	100	16	37	1.46	0.66
Well 2	SW	99	116	148	21.36	3.60
	Day 0	98	173	246	15.76	3.58
	Day 0.04	98	142	190	2.34	1.96
	Day 1	99	86	109	1.90	1.46
	Day 10	100	47	58	1.65	1.03
	Day 18	100	21	23	1.16	0.39

The abundance of microbial communities in FPW (class level) for both wells 1 and 2 is illustrated in Figure 15. For well 1, abundant sequences in SW were correlated to a diverse microbial community including bacterial classes *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Flavobacteriia*, *Planctomycetacia*, *Actinobacteria*, and *Sphingobacteriia*. At the genus level, the most abundant sequences were similar to the unclassified *Planctomycetaceae* (13.22% of the total sequences), within the class *Planctomycetacia*, phylum *Planctomycetes*, followed by 8.89% of the total sequences that were highly matched to the unclassified Actinomycetales, within the class *Actinobacteria*, phylum *Actinobacteria*, and 6.49% of the total sequences that were similar to the unclassified Bacteroidetes, within the phylum *Bacteroidetes*. The most abundant sequences that were classified at the genus level, were matched to the

Flavobacterium (4.53% of the total sequences) within the class *Flavobacteriia*, phylum *Bacteroidetes*, and *Hydrogenophaga* (3.10% of the total sequences) within the class *Betaproteobacteria*, phylum *Proteobacteria*, and *Prosthecobacter* (2.63% of the total sequences) within the class *Verrucomicrobiae*, phylum *Verrucomicrobia*, and *Polynucleobacter* (2.47% of the total sequences) within the class *Betaproteobacteria*, phylum *Proteobacteria*. Less abundant, but still regularly detected, sequences were matched to the genera including *Sphingorhabdus* (1.63% of the total sequences) within the class *Alphaproteobacteria*, phylum *Proteobacteria*, and *Sphingobium* (1.52% of the total sequences) within the class *Alphaproteobacteria*, phylum *Proteobacteria*, and *Legionella* (1.52% of the total sequences) within the class *Gammaproteobacteria*, phylum *Proteobacteria*. Other genera were represented by <1% of the total sequences. There was a shift in microbial communities from SW to the initial FPW (Figure 15). In the initial FPW, sequences matching to *Clostridia* affiliated with the phylum *Firmicutes* became the dominant bacterial class of the entire microbial community. Within the class *Clostridia*, sequences of the total sequences were highly similar to the halotolerant/halophilic genera *Halanaerobium* (51.84% of the total sequences), *Selenihalanaerobacter* (40.49% of the total sequences), and *Orenia* (3.44% of the total sequences)(Oremland et al. 2017; Eder et al. 2001; Switzer et al. 2001). Other genera were <1% of the total sequences. Similar to the findings for the initial FPW, sequences highly similar to *Clostridia* formed the dominant bacterial classes at 8 h and day 1 of FPW. Compared to the initial FPW, the relative abundance of the sequences matching to *Halanaerobium* relatives increased to 81.80% by 8 h of FPW, further increased to 81.35% by day 1 of FPW. The relative abundance of the sequences correlated to

Selenihalanaerobacter decreased to 12.08% by 8 h, and 14.25% by day 1 of FPW. The relative abundance of the sequences related to *Orenia* increased to 5.50% by 8 h of FPW, then declined to 2.24% by day 1 of FPW. Additionally, 1.06% of the total sequences were matched to the genus *Duganella* by day 1 of FPW. This genus is a member of the *Oxalobacteraceae* family, which is often associated with plants and plant-associated soils and have been cited as potential PCR contaminants (Tanner et al. 1998).

The abundance of microbial communities discovered in SW from well 2 was similar to that of the SW used to fracture well 1. Sequences correlated to bacterial classes such as *Acidimicrobiia*, *Alphaproteobacteria*, *Betaproteobacteria*, *Flavobacteriia*, *Actinobacteria*, and *Sphingobacteriia* formed the dominant components of the microbial communities. At the genus level of these bacterial classes, the most abundant sequences were similar to the unclassified *Actinomycetales* (12.06% of the total sequences) within the class *Actinobacteria*, phylum *Actinobacteria*. Within the class *Betaproteobacteria*, sequences were classified into the genera *Polynucleobacter* (3.42% of the total sequences), *Rhodoferax* (1.59% of the total sequences), and *Comamonas* (1.43% of the total sequences). Within the class *Flavobacteriia*, sequences were correlated to the genera *Flavobacterium* (7.21% of the total sequences) and *Fluviicola* (1.01% of the total sequences). Besides, 3.81% of the total sequences were *Armatimona* within the class *Armatimonadia*, phylum *Armatimonadetes*. Other genera represented <1% of the total sequences. In contrast of the initial FPW from well 1 that was dominated by *Clostridia*, the initial FPW from well 2 contained a greater fraction of bacterial classes such as *Betaproteobacteria, Gammaproteobacteria, Actinobacteria*, and *Flavobacteriia* that were detected in the corresponding SW (Figure 15). In the initial FPW, 34.59% of the total

sequences were highly similar to the classes *Clostridia* and *Bacilli*. Within the class *Clostridia*, sequences similar to the genera *Halanaerobium* (5.30% of the total sequences) and *Orenia* (7.88% of the total sequences) were prevalent. Within the *Bacilli*, sequences related to the genus *Trichococcus* were predominant (15.39% of the total sequences). Less abundant of the genera *Bacteroides* (6.27% of the total sequences) within the class *Bacteroidia*, phylum *Bacteroidetes*, and *Aquabacterium* (7.91% of the total sequences) affiliated with the class *Betaproteobacteria*, phylum *Proteobacteria*. Other genera such as *Cloacibacterium*, *Dietzia*, *Tepidiphilus*, *Shewanella*, *Acinetobacter*, and *Polynucleobacter* were detected at between 1-3% of the total sequences. However, there was no continuous increases in these genera in the initial FPW, while the fraction of sequences correlating to the class *Betaproteobacteria* increased as well flowback proceeded, eventually forming the dominant bacterial classes in samples at 1 h, day 1, and day 10 of FPW. Within this class, the dominant sequences were related to the genus *Ralstonia*, and the sequences relative to the total sequences increased from 64.43% at 1 h, 74.47% at day 1, to 76.52% by day 10. Similarly, the genus *Burkholderia* within the class *Betaproteobacteria* increased from 8.90% by 1 h, 12.50% by day 1, to 13.42% by day 10. Both *Ralstonia* and *Burkholderia* have been identified previously as potential PCR contaminants (Salter et al. 2014). Subsequently, a shift in sequences matching to *Clostridia* by day 18 of FPW became predominant. Within the class *Clostridia*, the majority of sequences were highly similar to the *Halanaerobium* (92.64% of the total sequences). Sequences related to the *Ralstonia* declined to 4.79% of the total sequences by day 18.

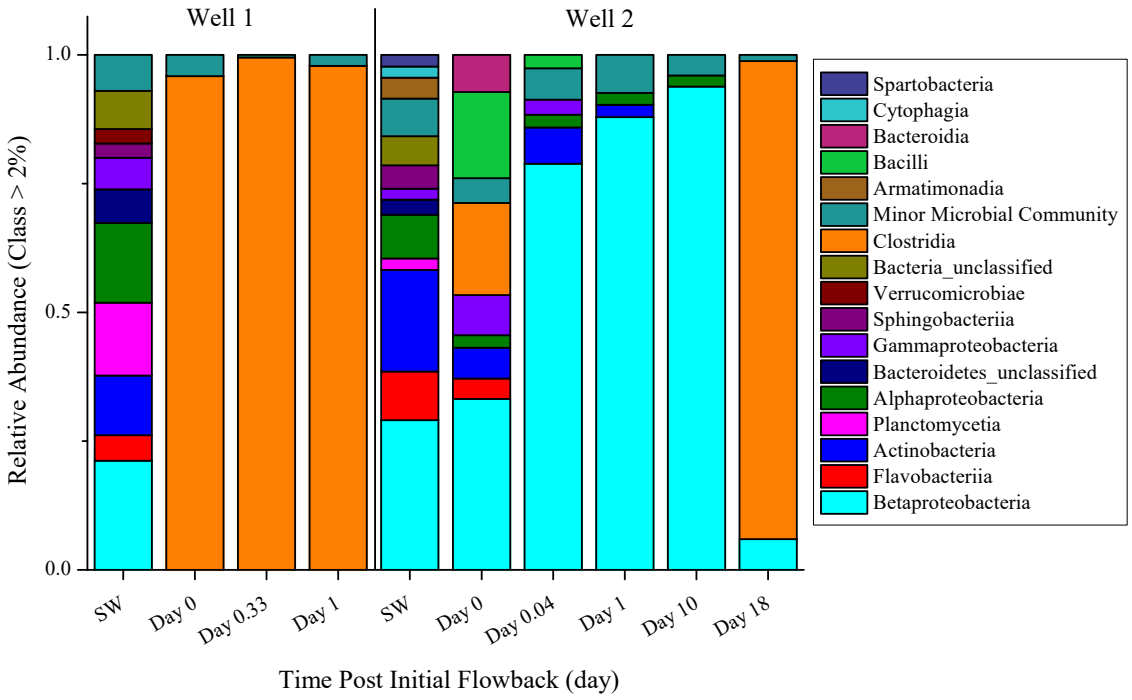

Figure 15. Class-level taxonomy of sequences obtained from SW and FPW for wells 1 and 2.

3.3 Microbial Community Dynamics in Base-fluids with 10% RPW

3.3.1 Biomass and Cell Viability Influenced by 10% RPW

The influences on cellular biomass and cell viability after the addition of 10% RPW to 90% surface water, to simulate the use of RPW in a field fracturing operation (without blending chemical additives), were studied in separate batch experiments. Influences on total cells numbers and cell survival ratios are shown in Figures 16 and 17. For the mixture of SW collected from well 1with 10% RPW, the total cell numbers were not significantly influenced by mixing, but cell survival ratios were considerably depressed (0.07 at 1 h, 0.22 at 6 h, 0.20 at 1 day, and 0.06 at 3 day) compared to the

corresponding control groups. However, the depression of cell viability was temporary, recovering by 7 days after the start of incubation, and reaching a peak by 40 days of incubation. For the mixed fluids containing 90% NSR water with 10% RPW, the addition of 10% RPW was also observed to have no significant influence on the numbers of total cells, but did reduce the cell viability at 0 h (0.05), 1 h (0.07), 6 h (0.07), and 1 day (0.76) as compared to the corresponding control groups. This observation was relatively consistent with the results of adding 10% RPW to 90% well 1 SW.

Consistent with the depression of cell viability, intact cells were heavily inhibited by the addition of 10% RPW (Figure 18 and 19). For the mixed fluids of well 1 SW with 10% RPW, the numbers of intact cells in treatment groups were significantly ($p<0.05$) lower than the control groups in the first 3 days of incubation. However, numbers of intact cells recovered by 3 days of incubation, and increased to the peak of $8.73(\pm2.85)\times10^5$ cells mL^{-1} by 40 days of incubation, at which time intact cell numbers were significantly ($p<0.05$) higher than were found in parallel reference solutions. For the mixed fluids containing 90% NSR water with 10% RPW, the numbers of intact cells in treatment groups were significantly ($p<0.05$) lower than the control groups within 1 day of incubation, then numbers of intact cells recovered by day 1, and further climbed to the peak of $9.77(\pm3.45)\times10^5$ cells mL^{-1} by day 45. This is consistent with the observation of the mixed fluids of 90% well 1 SW with 10% RPW, that intact cell numbers were significantly ($p<0.05$) higher than were found in parallel control solutions.

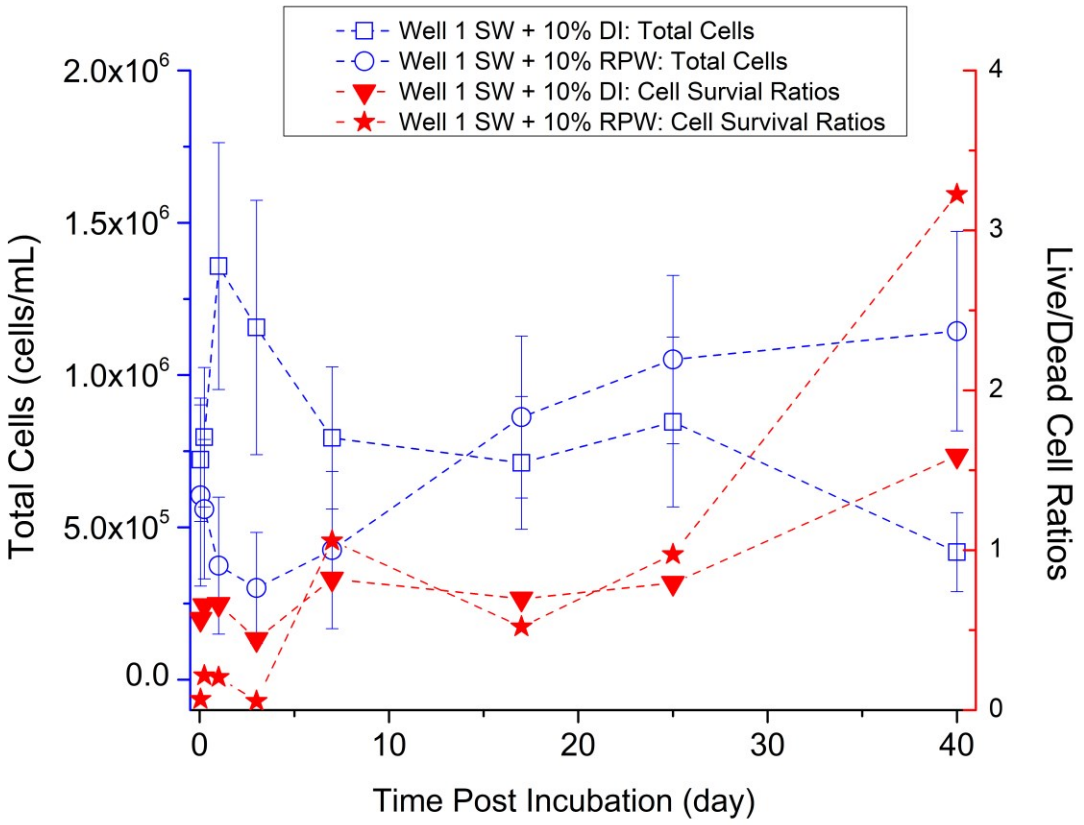

Figure 16. Influence of mixing RPW with well 1 SW freshwater on total cells and cell survival ratios. Error bars are SD from 15 fields (n=15).

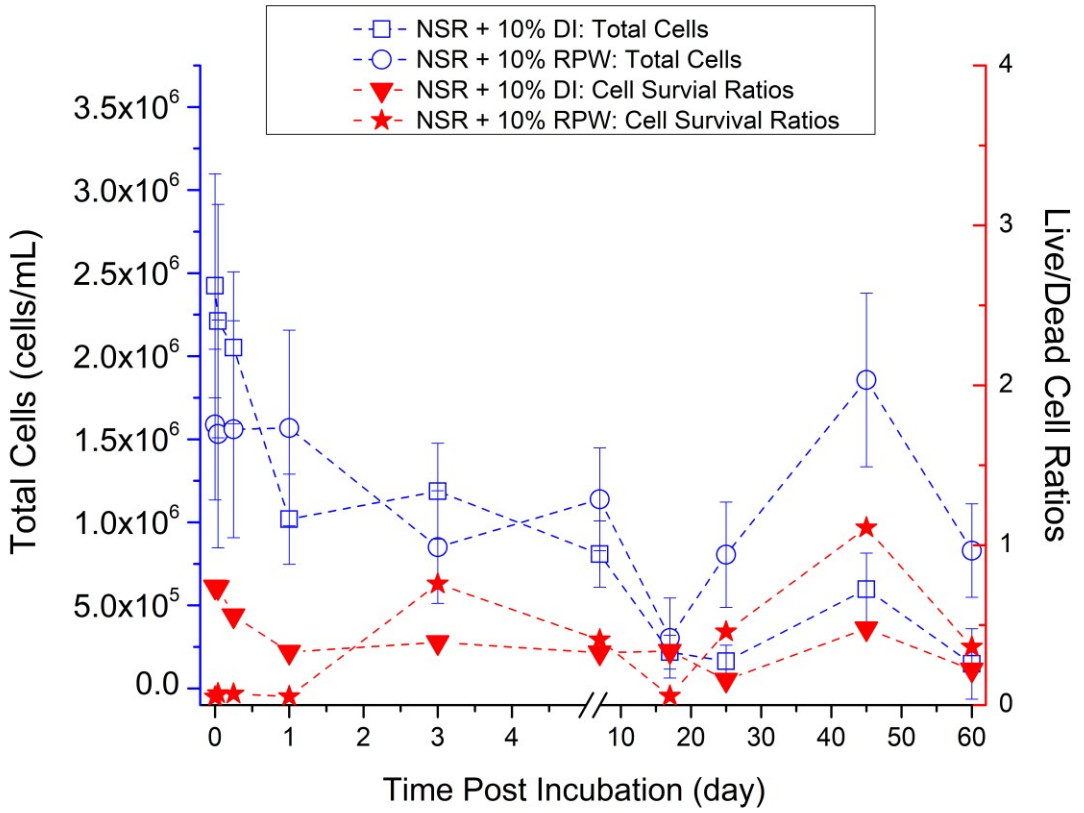

Figure 17. Influence of mixing RPW with NSR freshwater on total cells and cell survival ratios. Error bars are SD from 15 fields (n=15).

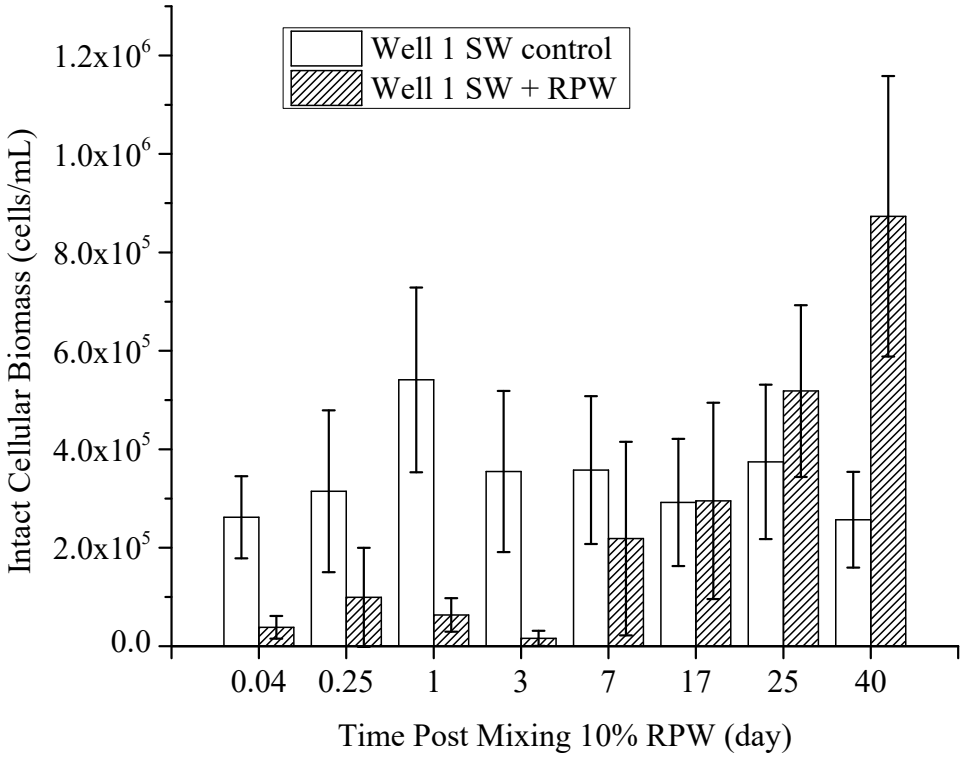

Figure 18. Influence of mixing RPW with SW freshwater from well 1 on intact cell numbers. Error bars are SD from 15 fields (n=15).

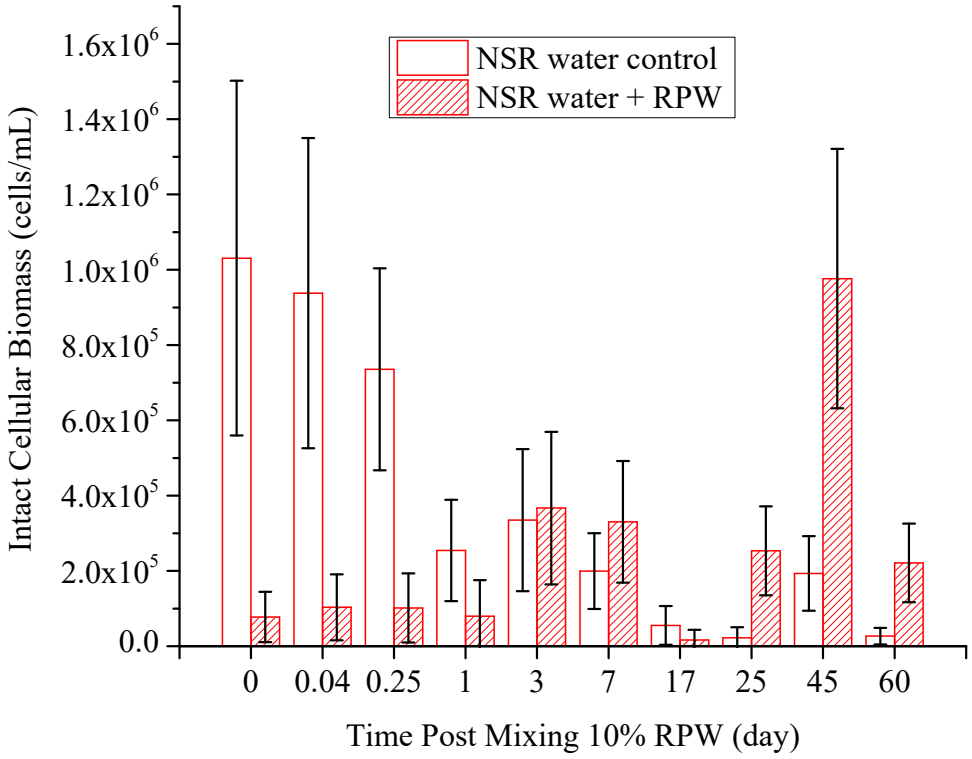

Figure 19. Influence of mixing RPW with NSR freshwater on intact cell numbers. Error bars are SD from 15 fields (n=15).

3.3.2 Changes in Abundance, Richness, and Diversity of Microbial Communities after Adding 10% RPW

The coverage, richness and diversity index of microbial communities after the addition of 10% RPW are shown in Table 2. The results showed microbial community diversity and richness were temporarily depressed by the addition of 10% RPW. For the mixed fluid of well 1 SW mixed with 10% RPW, richness and diversity of microbial communities were slightly increased from the original SW to 1 day of incubation, then both gradually decreased from 1 day of incubation to 17 and 40 days of incubation. For

the mixed fluid of NSR water with 10% RPW, the richness and diversity of microbial communities by 7 days of incubation were approximately two fold less than in original NSR water, while both recovered by 60 days of incubation.

Table 2 Coverage, richness, and diversity of microbial after freshwater (well 1 SW and NSR water) mixing 10% RPW

Mixed Fluids	Sample	Coverage Percentage	Observed OTU	Chao1	Inverse Simpson	Non-parametric Shannon
Well 1 SW - RPW	SW	99	120	148	20.05	3.56
	Day 1	99	124	159	17.18	3.57
	Day 17	99	106	143	11.98	3.25
	Day 40	99	88	124	13.66	3.11
NSR water - RPW	NSR water	98	109	189	7.14	2.67
	Day 7	99	50	72	2.28	1.56
	Day 17	100	55	67	4.29	2.02
	Day 60	99	77	108	10.30	2.93

Changes in the abundance of microbial communities (class level) after adding 10% RPW for both mixed fluids are shown in Figure 20. For the mixed fluids of well 1 SW with 10% RPW, sequences related to the bacterial classes *Betaproteobacteria*, *Flavobacteriia*, *Planctomycetacia*, and *Actinobacteria* all decreased after 1, 7, and 40 days of incubation. Sequences classified into the class *Alphaproteobacteria* continuously increased at 1 day (27.62% of the total sequences) and 7 days (37.86% of the total sequences) of incubation, and eventually became the predominant bacterial class by 40 days of incubation (70.58% of the total sequences). Within the class *Alphaproteobacteria*, sequences similar to the genera including unclassified *Reyranella* (5.88% of the total sequences), *Sphingorhabdus* (2.81% of the total sequences), unclassified *Sphingomonadaceae* (2.19% of the total sequences), unclassified *Rhodobacteraceae*

(1.44% of the total sequences), and *Brevundimonas* (1.02% of the total sequences) were observed by 1 day of incubation. By 7 days of incubation, sequences correlated to the genera affiliated with *Alphaproteobacteria*, were unclassified *Sphingomonadaceae* (10.36% of the total sequences), unclassified *Rhizobiaceae* (4.71% of the total sequences), *Brevundimonas* (4.57% of the total sequences), *Sphingobium* (3.99% of the total sequences), unclassified *Rhodobacteraceae* (2.31% of the total sequences), *Rhizobium* (2.08% of the total sequences), *Phenylobacterium* (1.62% of the total sequences), *Sphingorhabdus* (1.50% of the total sequences), and *Gemmobacter* (1.05% of the total sequences). Sequences matched to the similar genera within *Alphaproteobacteria*, but at increasing fractions were detected by 40 days of incubation, including *Brevundimonas* (14.81% of the total sequences), unclassified *Rhizobiaceae* (11.68% of the total sequences), *Phenylobacterium* (10.14% of the total sequences), unclassified *Sphingomonadaceae* (9.47% of the total sequences), unclassified *Rhodobacteraceae* (7.50% of the total sequences), *Rhizobium* (3.36% of the total sequences), *Blastomonas* (2.43% of the total sequences), and *Sphingomonas* (2.39% of the total sequences). Remarkably, sequences matching to *Halanaerobium* that were absent in the SW from well 1, increased to 4.20% of the total sequences by 1 day of incubation, and were consistently detected thereafter by 7 days of incubation (5.58% of the total sequences), and 40 days of incubation (4.92% of the total sequences).

For the mixed fluid of NSR with 10% RPW, the percentage of the sequences matching with *Alphaproteobacteria* increased from 0.7% in NSR water to 68% by 60 days of incubation. By 7 days of incubation, the most abundant sequences were similar to the genus *Pseudomonas* (64.99% of the total sequences) affiliated with the class

Gammaproteobacteria, phylum *Proteobacteria*. 2.44% of the total sequences were similar to unclassified *Rhizobiaceae* within the class *Alphaproteobacteria*, phylum *Proteobacteria*. By 17 days of incubation, sequences similar to *Pseudomonas* decreased to 32.36%, sequences matching to unclassified *Methylophilaceae* increased to 33.93%, and sequences correlated to unclassified *Rhizobiaceae* were 2.73%. After that, the majority of the sequences were highly similar to *Alphaproteobacteria* by 60 days of incubation, including genera *Rhizobium* (19.06% of the total sequences), unclassified *Rhizobiaceae* (16.51% of the total sequences), *Hyphomonas* (9.69% of the total sequences), *Devosia* (3.06% of the total sequences), *Blastomonas* (2.66% of the total sequences), and *Sphingomonas* (2.05% of the total sequences). Consistent with the observation from well 1 with 10% RPW, the sequences similar to *Halanaerobium*, which were also absent in the NSR water, were detected at low abundance after 7 days (0.05% of the total sequences), 17 days (0.10% of the total sequences) and 60 days (0.78% of the total sequences) of incubation.

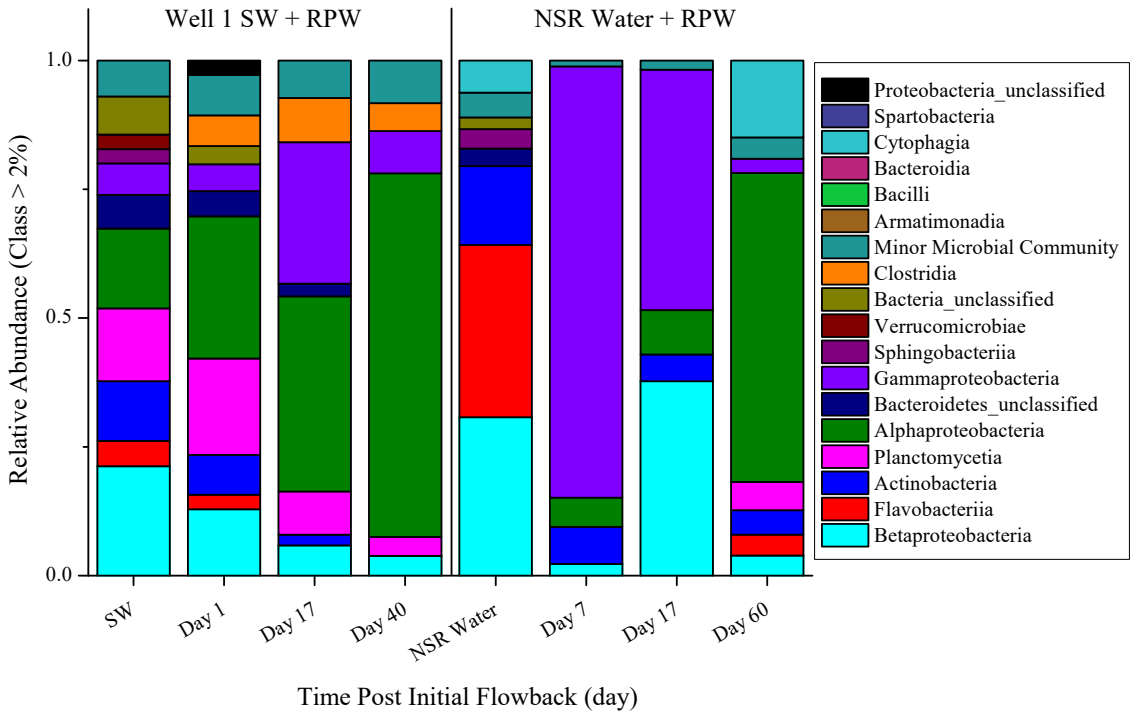

Figure 20. Class-level taxonomy of sequences obtained from the mixed fluids that 10% RPW mixed with 90% freshwater (Well 1 SW and NSR water).

3.4 Beta-Diversity of Microbial Communities in the Hydraulic Fracturing Water Cycle

The similarity of water samples (SW, FPW, and mixed fluids) at different stages in the hydraulic fracturing water cycle are shown on the Bray-Curtis Nonmetric multidimensional scaling (NMDS) supported by AMOVA tests. The ordination (stress 0.11) demonstrated the statistically significant spatial separation between SW and FPW microbial communities ($p<0.05$). The ordination revealed two different pathways of microbial shift between the two fractured wells (Figure 21). Sequences matching to genera *Polynucleobacter*, *Halanaerobium*, *Orenia*, *Burkholderia*, *Ralstonia*, *Opitutus*,

and *Brevundimonas* in FPW were identified to significantly ($p<0.05$) represent differentiation from the microbial communities in SW. Moreover, sequences similar to the genera *Polynucleobacter*, *Halanaerobium*, *Microbacterium*, *Gemmobacter*, and *Blastomonas* in mixed fluids after the addition of 10% RPW were identified to significantly ($p<0.05$) differ from microbial communities in SW, an exercise which explored the pathway that the microbial communities take during FPW recycling at the surface. Based on the ordination, I identified two stages for FPW from both wells 1 and 2: "Early Stage" and "Late Stage", in which the spatial similarity was statistically different ($p<0.05$). The ordination suggested an "Early Stage" of the shift of microbial communities in FPW was absent in well 1. The AMOVA test showed that the spatial separation between "Late Stage" and SW was significant ($p<0.05$), and that the difference between the "Early Stage" and SW was not significant. Therefore, "Early Stage" samples resembled the SW, but "Late Stage" samples differed significantly, and none of these groups clearly resembled the community found in my experiment designed to simulate FPW recycling. In the ordination, the relatively abundant sequences similar to the genera *Flavobacterum*, *Brevundimonas*, and *Phenylobacterium* were significantly ($p<0.05$) correlated to the SW stages. On the contrary, genera *Halanaerobium* and *Selenihalanaerobacter* related sequences were significantly ($p<0.05$) correlated to the "Late Stage". The dominant genera *Ralstonia* and *Burkholderia* related sequences were significantly ($p<0.05$) correlated to "Early Stage". Sequences similar to the genera *Halanaerobium* and *Selenihalanaerobacter* were the only genera that significantly ($p<0.05$) increased from "Early Stage" to "Late Stage". Sequences similar to genera *Rhizobium* and *Pseudomonas* were significantly ($p<0.05$) correlated to incubation

samples of mixed fluids from FPW recycling. The Spearman Rank Correlation index showed that increasing TDS significantly ($p<0.05$) correlated to shifts toward the genera *Halanaerobium* and *Selenihalanaerobacter*, and that temperature and pH had no significant influence on this shift.

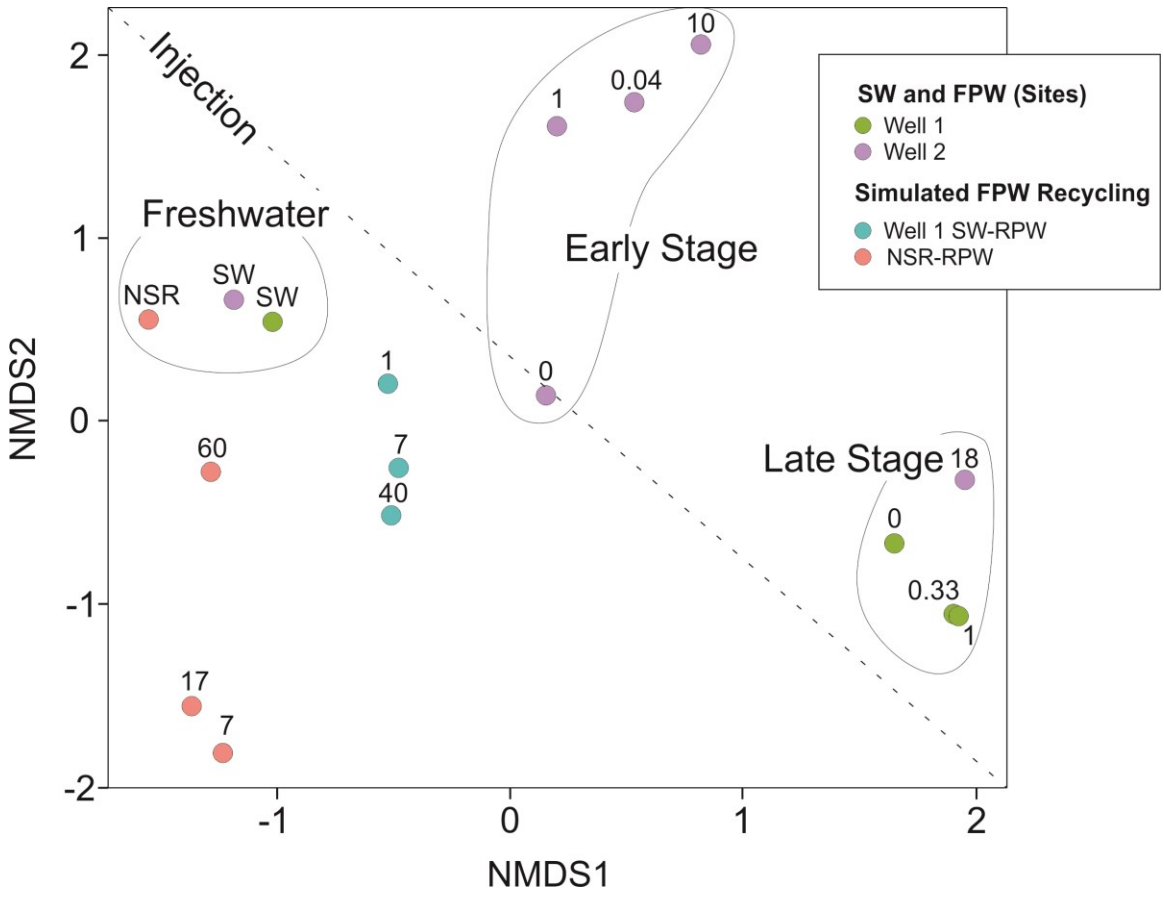

Figure 21. NMDS plot (stress: 0.11) showing dissimilarity distances between each sequencing point; the labels on dots are number of days post the initial flowback or incubation days after freshwater mixing 10% RPW.

Chapter 4: Discussion

4.1 Microbial Community Dynamics in High Temperature Brine

Total cells and cell viability in source water (SW) used for well 2 were much lower than in SW used for well 1 (Figures 11 and 12), which was probably caused by the locally cold weather during operations at well 2 (-1 to 2°C average at the time of fracturing; http://www.accuweather.com). In general, the continuous decline in cellular biomass concentration coupled with the overall low ratios of live/dead cells at both wells 1 and 2, are likely to indicate decreasing microbial biomass downhole (Figures 11 and 12). The difficulty in counting cells in samples having low concentrations under the microscope and the potential for colloidal interference due to high clay content may result in overestimates of the cell numbers (Pepper et al. 2015). Therefore, the cell concentrations estimated in flowback and produced water (FPW) and recycled produced water (RPW) from wells 1 and 2 are possibly even lower than estimated in this research. This result suggests that total cell numbers, within standard deviations, was consistent with the result from Akob et al. (2015) that observed low cell numbers (10 to 10^4 cells mL^{-1}) in FPW from the Marcellus and Burket Shale in Pennsylvania, for which DNA was insufficient for sequencing without enrichment.

The decrease in biomass likely reflects the elevated living stress for the microbial communities in the deep formation, and the primary stress is most likely from increased salinity; the sequences were similar to a microbial community dominated by (in the case of well 1) or containing high levels of (in the case of well 2) *Halanaerobium*, a halotolerant bacterial genus whose presence indicates a salinity-driven stress to the

freshwater communities initially present in injected fracturing fluids. This study demonstrates *Halanaerobium* may dominate in the initial FPW, and the shift was positively correlated to the rapidly increasing TDS in the initial stages of FPW in the wells I studied (Figure 22; Cluff et al. 2014; Murali Mohan et al. 2013). The shift to predominantly sequences related to *Halanaerobium* was delayed relative to well 1 in well 2, in which *Ralstonia* was found to be transiently predominant during the first few days of FPW samples. Members of the genus *Ralstonia* are known to have the capacity to grow on diesel fuel in saline environments and to aerobically degrade various hydrocarbons (Stapleton et al. 2000; Widada et al. 2002; Kleinsteuber et al. 2006). Consistent with this observation, members of the second most abundant genus at that sampling time, *Burkholderia*, were also found to aerobically degrade polyaromatic hydrocarbons in oil-contaminated soil (Kim et al. 2003). These shifts matched my observation of a large fraction of residual drilling fluid components and condensate oil in early FPW from well 2, since FPW samples were collected before the oil and gas separator. The large fraction of hydrocarbon components in the early period of FPW from well 2 potentially served as nutrients for *Ralstonia* and *Burkholderia*. Moreover, the early period of FPW may have contained relatively higher concentrations of dissolved oxygen, which were carried into the subsurface through injected fracturing fluids, and so in favor of the growth of aerobic strains in *Ralstonia* and *Burkholderia*, while restricting the growth of the obligate anaerobe *Halanaerobium*. However, it is possible that *Ralstonia* were present as PCR contaminants (Salter et al. 2014), which became relatively dominant due to the originally low biomass in FPW.

Consistent with the observation that the DNA quickly decreased to below the detection limit in the FPW from Montney fractured wells (Elliott 2015), DNA in the late period of FPW from my studied Duvernay wells was also lower than the detection limit. High flowback temperatures, reaching to nearly 80°C, were recorded in flowback at the wellheads, consistent with the reported down hole temperatures of the Duvernay reservoir in the targeted region (112-115°C). The temperature of FPW cools as it moves from the reservoir through colder stratigraphic layers to reach the surface, so the recorded surface temperature represents a minimum for the reservoir temperature. If it is assumed that the injected water temperature at the bottom eventually warms to near the reservoir temperature, this temperature will considerably exceed those favorable for the growth of a microbial community dominated by *Halanaerobium*, where characterized isolates are known to grow between 20°C and 51°C (Bhupathiraju et al. 2017; Ravot et al. 1997). This, combined with the lack of sequences similar to typical theomorphic lineages, may cause the overall low microbial biomass and undetectable DNA in FPW from both wells. Moreover, FPW contained various chemicals that can inhibit the PCR process, such as excess salts and input chemicals may also inhibit the PCR reaction (Bessetti 2007). This, combined with the low biomass in FPW samples, may have led to DNA being undetectable in later periods of fluids returned, even after PCR reaction.

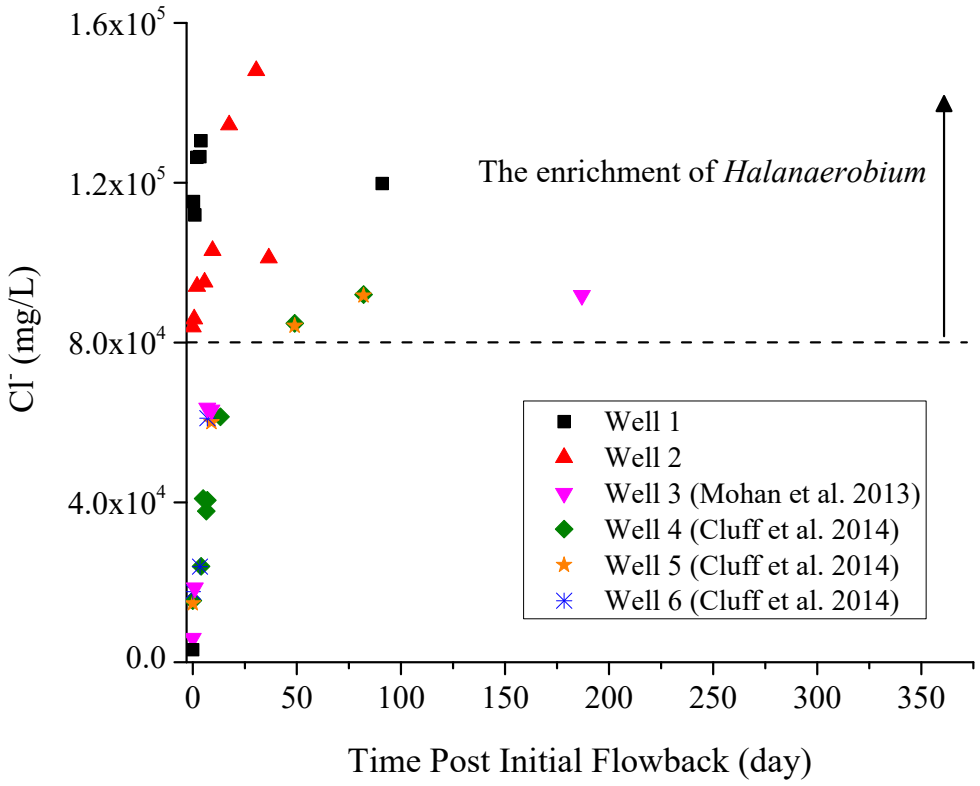

Figure 22. Changes in FPW salinity (based on Cl^- concentrations) between Duvernay Formation (wells 1 and 2) and Marcellus Formation (wells 3, 4, 5, and 6)

4.2 *Halanaerobium*

Halanaerobium is a halotolerant bacterial genus that has previously been found to be gradually enriched in FPW across shale gas formations in North America (Mouser et al. 2016). Although two pathways of microbial community change were observed in samples from wells 1 versus well 2, seuqnces matching to *Halanaerobium* became the predominant in FPW from both wells until DNA was undetectable. The growth of *Halanaerobium* in FPW is likely to cause detrimental impact on the subsurface infrastructure and natural gas quality, since, for example, one of the *Halanaerobium*

strains, *H. congolense*, can ferment a wide variety of carbohydrates and reduce thiosulfate and sulfur to sulfides (Ravot et al. 1997). While DNA was not detectable in later FPW samples, early FPW samples likely came from shallower regions of the vertical section of the well, having a lower temperature. Thus, the thermal regime, relative to bottom hole temperatures, may be more conductive to bacterial growth. In this study, none of the sequences in SW samples from wells 1 and 2, and North Saskatchewan river (NSR) water samples were similar to the *Halanaerobium*, which either indicated the absence of halotolerant bacteria in the freshwater environments or that their cell concentrations were below the detection limit. DNA was insufficient in the RPW and in the majority of the late period of FPW samples from both wells. This, to some degree, impeded the identification of the substrate for *Halanaerobium*, but it may imply that the *in situ* conditions of Duvernay Formation were not a favorable habitat for *Halanaerobium* and other halotolerant bacteria. Additionally, the result that sequences matching to *Halanaerobium* were measured after the addition of 10% RPW showed the fact that *Halanaerobium* can largely regenerate in the hydraulic fracturing water cycle whenever the ambient environments become suitable for them to grow, and the factor that may drive the growth of *Halanaerobium* was likely the FPW compositions. Thus, my result was in partly consistent with that of Daly et al. (2016), that hydraulic fracturing created habitat favorable for halotolerant bacteria including *Halanaerobium*, but my study additionally suggests that deep shale reservoirs such as the Duvernay may not as favorable for halotolerant bacteria as those shallower ones with lower down hole temperatures. This study also indicates that genomes of halotolerant bacteria detected in

low abundance in input fracturing fluids are also likely introduced by the recycling of FPW.

4.3 Nutrients and Inhibitors in FPW for Microorganisms in FPW

Compared to the influences from salinity and temperature, the biocide appears to be less efficient in inhibiting microbial growth in FPW. There were approximately 10^5 intact cells mL^{-1} in the initial FPW for both wells. For well 2, the viable cell numbers were not significantly different from those in the corresponding SW, and ratios of live/dead cells in the early period of flowback returned were higher than in SW (Figure 12). Moreover, the concentration of the biocide ADBAC dramatically and quickly decreased 50 times as flowback proceeded; this rapid reduction in biocide concentration may mean that for the majority of the FPW period, the dose of biocide is insufficient to inhibit microbial growth.

The identification and quantification of unknown organics revealed that FPW contains nutrients that may support microbial growth. Decreases in TOC concentrations as well flowback proceeded indicates that the concentration of potential carbon sources in initial FPW was much higher than for the rest of the flowback period, and so fracturing fluids were responsible for transporting large quantities of carbon into the formation. In terms of the organic constituents identified in this study, PEGs, depending on their structures, are one of the molecules that may serve as carbon sources for bacteria (Kumari et al. 2009; Huang et al. 2005; Kawai 2002). Octylphenol, a biodegradation product of octylphenol ethoxylates, was not detected in this study. This result further supported the observation of decreased total cell numbers and low cell viability, to indicate low

biomass downhole. The total S concentrations may imply the presence of potential electron acceptor for *Halanaerobium* to use in its metabolism; however, in this study, I could not further confirm the speciation of the sulfur, which impeded further assessment the availability of sulfur sources for *Halanaerobium*.

4.4 Influence on the Microbial Community by FPW Recycling

The recycling of FPW was found to have a significant influence on the microbial ecology associated with the hydraulic fracturing water cycle. This may be in part due to the elevated salinity of injected fracturing fluids that are made up with RPW, as in the example of well 1 (Figure 5), which in turn favors the growth of halotolerant bacteria earlier in the hydraulic fracturing water cycle. In the wells I studied, sequences related to *Halanaerobium* dominated from the beginning of well 1 FPW. However, in well 2 where RPW was not used, the shift towards *Halanaerobiu* was slower, likely because it was seeded with the genus from cells in the RPW used in well 1. Furthermore, the drop in cell survival ratios, combined with decreases in the lineages of *Flavobacteriia*, *Actinobacteria*, and *Sphingobacteriia* that commonly are detected in freshwater environments following RPW addition, also confirms stress from elevated salinity in the process of FPW recycling (Aizenberg-Gershtein et al. 2012; Rosenberg et al. 2012). My results also show that recycling of FPW not only leads to the increases in the relative abundance of halotolerant bacteria, but that other heterotrophic genera such as *Sphingomonas*, *Brevundimonas*, and *Rhizobium* increased after adding 10% RPW. Many members of these genera plus *Halanaerobium* are known to degrade a variety of organics (e.g. polynuclear aromatic hydrocarbons, organophosphorus compounds, and polysaccharides)

(Deshpande et al. 2004; Ye et al. 1996; Liang et al. 2016; Chen et al. 2004). Based on this observation, the shift from freshwater communities to bacteria capable of biodegradation of downhole organic chemicals may influence the organic additives used in fracturing fluids, and through that, impact hydraulic fracturing infrastructures and the overall performance of a well.

4.5 Implication for FPW Treatment and Recycling

The results confirm my hypothesis that the microbial community dynamics may be heavily influenced by downhole salinity, and possibly by temperature. The microbial ecology of high temperature shale brines is almost certainly not the same as lower temperature shale brines (e.g. the Marcellus shale; Cluff et al. 2014). This study suggests that injected fracturing fluids in deep shale formations may have extremely low viable cellular biomass, and this restriction may result from complex selective forces in the subsurface, corresponding to physical and chemical changes in FPW. For example, I detected decreases in nutrient supply and dissolved oxygen content, but these factors may not be perfect indicators as the fracturing technology used at each hydraulic fracturing operation may vary. In contrast, salinity and temperature are likely two primary variables in constraining viable biomass in the two wells studied, and may influence the long-term structure of microbial communities during shale oil and gas exploration. These two variables are not easily influenced by details of the fracturing job, and are to some degree predictable if the reservoir characteristics are known. In this study, I observed that halotolerant microbial communities were relatively enriched at the expense of the original-freshwater community. After that, increased temperature probably became the

primary inhibitor of the growth of halotolerant bacteria that survived after the salinity selection. Thus, this case study of Duvernay Formation may benefit the evaluation of the downhole microbial community abundance, richness, and diversity in different stages of production for other shale oil and gas reservoirs that may have differing geologic conditions. A better understanding of microbes in the hydraulic fracturing water cycle may then lead to improvements in water treatment strategies and potentially reduce unnecessary cost for chemical additives such as biocides, and perhaps reduce the toxicity of returning fluids. My results suggest that FPW recycling may seed the dominant bacterial genomes that are detected in FPW, consistent with the faster enrichment of halotolerant bacteria in well 1 that in well 2. Moreover, following the addition of RPW, the microbial communities may shift to ones that utilize organic components in the made-up fracturing fluids. A primary challenge is in overcoming the difficulty in generating sufficient DNA for microbial community analyses from FPW samples containing low biomass. Furthermore, my results suggest that the biocides used in these two wells do not seem to inhibit cells capable of utilizing fracturing chemical additives, which may increase the complexity of FPW treatment and reuse strategies.

Chapter 5: Experimental Drawbacks and Improvement

Due to sampling limitation, inherent at working wellsites, a lack of replicate samples and sampling locations may, to some degree, influence the accuracy of the results. Further studies to monitor the microbial community dynamics, over longer production periods, may result in a better understanding of the microbial ecology in the hydraulic fracturing water cycle. In this study, I have considered the flowback and produced water (FPW) for over 100 days. However, it may require an even longer time for microbes to adapt to the subsurface environments. Therefore, I propose to continue to sample produced water from both wells sampled over a longer period of production, in case a microbial community eventually adapts to downhole conditions. In this research, I only have considered one mixing ratio (1:9) to investigate the microbial effects from reusing FPW as a pilot-scale study. However, the ratio of mixing recycled produced water and freshwater varies from one hydraulic fracturing operation to another, and so carrying out the experiment of FPW recycling at more mixing ratios may benefit the understanding of the microbial community effects of FPW reuse. Moreover, the influence of microbial metabolisms on the chemicals in injected fluids, and an optimized strategy that identifies specific factors in microbial growth are critical for understanding the implications of these microbial shifts for fracturing processes. This step relies on the further analyses into the composition of FPW, which will be aided by the development of analytical methods for quantifying unknown compounds. Finally, metagenomics and/or enrichment cultures could be conducted to complement existing 16S rRNA analyses, to determine the microbial metabolisms of key bacteria in the hydraulic fracturing water cycle.

Chapter 6: Conclusions

Hydraulic fracturing is a technology that often uses large volumes of freshwater, and produces similarly large volumes of flowback and produced water (FPW). In the water cycle in hydraulic fracturing, microorganisms may grow and consequently influence shale oil and gas production and water treatment and reuse strategies. In this study, the microbial communities measured in the source water used to make up the injected hydraulic fracturing fluids was inhibited by the hypersaline and very likely high temperature environments found downhole in the fractured Duvernay Formation, and increasing salinity was significantly correlated to the shift of sequences related to microbial community dominating with members of *Halanaerobium*. Additionally, the biocide treatment used was not efficient in restricting cellular biomass and preventing enrichment of halotolerant bacteria. Concomitant with the decreasing biomass, microbial diversity and richness were also reduced. My study demonstrated that halotolerant bacteria consistent with *Halanaerobium* could rapidly become the predominant bacterial genus downhole, but which ultimately were no longer detected in late period of FPW. By conducting a separate experiment in laboratory, 10% recycled produced water (RPW) was found to quickly cause loss of cell viability and decreases in relative abundance in microbes originally derived from freshwater environments. However, after the decimation of the original microbial community, I observed the growth of halotolerant/heterotrophic genera including *Halanaerobium*, which have been found to biodegrade a wide range of organics. My study suggested microbial ecology in high temperature brine may be different from that in low temperature brine, but the temporary predominance of sequences matching to *Halanaerobium* in FPW may pose the risk of

detrimental impact from microorganisms, and the undesirable bacteria may alter the original freshwater microbial communities prior to fracturing due to FPW recycling, and through that influence the surface and subsurface infrastructures.

Chapter 7: Bulleted Suggestions for Hydraulic Fracturing Process Improvements

Based on my results on wells 1 and 2, I have the following assessments and suggestions for the industrial partner Encana Corporation:

- In the deep reservoir, hypersaline environments combined high subsurface temperature may naturally restrict the microbial biomass, richness, and diversity.
- The efficacy of the biocide used in the two operations (wells 1 and 2) to control downhole biomass seems low; in the future, a biocide that targets halotolerant bacteria may be more beneficial.
- Recycling of FPW may seed halotolerant bacteria, and alter the freshwater microbial communities to bacteria that can exist in the presence of or even use fracturing organic compounds. This then poses a risk to the performance of fracturing fluids and infrastructure in fracturing jobs.
- Reducing the preparation time of making injected fracturing fluids reduce the growth of undesirable microbes during FPW recycling.

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Appendices

Table S1 Samples in hydraulic fracturing water cycles received from Fox Creek, Alberta for 16S rRNA sequencing

Well	Sample	Time Post Initial Flowback
well 1	Oil-based drilling fluids	NA
	Source water	NA
	Recycled Produced Water	NA
	FPW	Day 0
	FPW	Day 0-8 h
	FPW	Day 1
	FPW	Day 2
	FPW	Day 3
	FPW	Day 4
	FPW	Day 5
	FPW	Day 6
	FPW	Day 19
	FPW	Day 20
	FPW	Day 32
	FPW	Day 53
	FPW	Day 91
	FPW	Day 120
well 2	Source Water	NA
	FPW	Day 0
	FPW	Day 0-1 h
	FPW	Day 0-2 h
	FPW	Day 0-3 h
	FPW	Day 0-5 h
	FPW	Day 0-8 h
	FPW	Day 0-16 h
	FPW	Day 1
	FPW	Day 1-8 h
	FPW	Day 2
	FPW	Day 2-8 h
	FPW	Day 3
	FPW	Day 4
	FPW	Day 5

	FPW	Day 9
	FPW	Day 10
	FPW	Day 11
	FPW	Day 12
	FPW	Day 13
	FPW	Day 14
	FPW	Day 15
	FPW	Day 18
	FPW	Day 34
	FPW	Day 40
	FPW	Day 102

Table S2 Full operation conditions for each analysis run by ICPMS-QQQ

Element	**Dilution Factors**	**Standard Matrix**	**Gas Mode**
Li 7	80	2200 ppm NaCl in 2% HNO_3 and ½% HCl	No Gas HMM
B 11	80		No Gas HMM
Na 23	10		No Gas HMM
Mg 24	80		No Gas HMM
Al 27	80		No Gas HMM
Si 28	80		No Gas HMM
S32-48	80		10% O2 HMM
K39	80		No Gas HMM
Ca 40	80		H_2 HHM
Mn 55	80		No Gas HMM
Fe 56	80		No Gas HMM
Co 59	80		No Gas HMM
Ni 60	80		No Gas HMM
Cu 63	80		No Gas HMM
Zn 66	80		No Gas HMM
As 75-91	80		10% O2 HMM
Br 79	80		No Gas HMM
Sr 88	80		No Gas HMM
Cd 114	80		No Gas HMM
Ba 137	80		No Gas HMM
Pb 208	80		No Gas

Table S3 Major inorganic components for well 1 SW, RPW, and FPW (data source: Dr. Shannon Flynn)

Time (day)	Na (ppm)	B (ppm)	Mg (ppm)	K (ppm)	Ca (ppm)	Fe (ppm)	Sr (ppm)	Br (ppm)	Cl (ppm)	S (ppm)
SW	9.5	0.0	7.3	0.8	32.5	0.1	0.2	BDL	3.8	1.7
RPW	68176.2	76.1	942.0	2114.8	12295.5	7.2	1202.0	306.1	139820.2	48.6
0	1396.6	3.3	23.6	52.2	197.8	0.1	19.0	5.5	3108.1	6.0
0.33	54509.7	77.2	737.9	1929.6	8799.0	134.7	893.2	237.1	115283.7	79.8
0.67	53595.8	78.8	731.2	1923.7	8685.4	150.6	893.2	240.4	113655.6	82.1
1	54256.9	79.1	737.7	1928.5	8625.9	150.6	904.0	241.7	111907.5	78.3
2	56821.5	81.0	768.4	2030.7	8503.4	49.5	947.3	245.7	126340.3	67.7
3	56368.1	82.5	805.6	2098.3	8975.5	84.0	1008.5	257.1	126539.2	66.7
4	55373.5	80.8	813.5	2120.6	9052.2	76.2	1037.4	269.8	130501.1	63.0
5	55605.3	78.0	829.7	2005.4	9761.9	57.6	1048.8	350.3	NA	79.6
6	56163.8	79.1	834.5	1985.9	9604.3	52.7	1066.8	276.5	NA	74.5
19	60314.8	81.9	923.0	2097.5	10770.6	NA	1227.8	310.3	NA	68.6
32	65164.4	82.3	808.3	2077.2	11333.1	NA	1233.5	319.0	NA	39.7
53	67762.3	89.6	853.1	2162.7	11566.5	924.5	1290.3	331.3	NA	40.0
91	56858.5	80.2	869.0	2065.8	10093.6	139.6	984.9	258.5	119779.7	58.9

Note: below detection limit (BDL)

Table S4 Major inorganic components for well 2 FPW (data source: Dr. Shannon Flynn)

Time (day)	Na (ppm)	B (ppm)	Mg (ppm)	K (ppm)	Ca (ppm)	Fe (ppm)	Sr (ppm)	Br (ppm)	Cl (ppm)	S (ppm)
0.06	47382.3	66.9	614.4	1574.1	6759.1	185.0	672.9	228.3	83882.4	87.3
0.08	44180.7	68.9	578.6	1469.3	6209.5	153.9	632.0	220.2	81964.4	83.6
0.13	47484.1	74.8	656.5	1597.6	6936.2	167.0	723.5	259.5	73666.0	100.1
0.21	46198.7	72.3	628.9	1536.1	6684.7	166.6	684.7	236.0	70757.5	100.1
0.33	44576.8	72.6	623.4	1530.2	6183.8	73.1	679.0	232.5	NA	94.0
0.67	47877.5	74.4	665.6	1612.9	6984.2	106.7	724.8	246.3	85910.0	102.1
1	46811.8	73.1	674.3	1615.2	7162.9	69.1	741.3	250.9	NA	97.8
2	50734.0	79.0	718.0	1678.0	7291.6	64.7	786.7	264.4	94091.2	96.5
3	50248.6	76.0	744.4	1734.7	7718.4	54.3	826.7	270.3	81424.4	101.9
4	50489.2	75.6	736.7	1690.9	7818.1	61.1	821.6	268.5	NA	90.2
5	52029.8	78.5	775.9	1759.7	8010.6	69.6	867.0	270.9	NA	89.1

5.67	46707.0	84.6	830.6	1879.6	8530.3	84.8	938.9	298.7	95105.6	90.2
9.38	53655.7	67.6	793.3	1976.4	8875.0	81.3	863.9	258.6	107222.1	66.3
9.5	54121.9	84.8	799.9	1940.7	9057.8	68.2	880.8	230.9	103053.3	61.7
10	55445.1	87.6	830.9	2016.2	9645.7	57.5	917.4	260.2	NA	62.7
10.83	55349.3	84.6	822.5	1987.6	9889.8	43.3	907.2	253.8	94961.6	59.0
11.75	56844.4	73.9	835.3	2106.3	9718.4	53.2	926.3	244.5	102533.8	58.9
12.08	56631.8	83.7	836.8	2021.5	9749.7	20.5	921.6	262.3	NA	58.4
13.5	58836.3	80.0	868.8	2097.2	9711.0	77.9	976.1	253.7	99948.4	60.8
14.08	57872.1	80.0	866.1	2088.2	10216.7	72.7	973.6	256.5	NA	61.6
17.5	66262.7	90.3	988.6	2633.9	12989.3	100.3	1307.3	312.0	134486.7	32.3
30.5	NA	NA	NA	NA	NA	NA	NA	NA	148066.0	NA
36.5	NA	NA	NA	NA	NA	NA	NA	NA	101154.6	NA

Table S5 AMOVA tests in different stages of operations as I observed in the ordination

AMOVA

p-value	SW	Early	Late	SW-RPW Early	SW-RPW Late	NSR-RPW Early	NSR-RPW Late
SW	1	0.074	0.031	0.213	0.243	0.208	0.113
Early		1	0.033	0.076	0.181	0.187	0.066
Late			1	0.06	0.197	0.1	0.078
SW-RPW Early				1	0.682	0.317	0.352
SW-RPW Late					1	NA	NA
NSR-RPW Early						1	0.66

NSR-RPW Late	1

Experiment-wise error rate: 0.05

Pair-wise error rate (Bonferroni): 0.005

Table S6 AMOVA tests in different types of water samples (SW, FPW, and mixed fluids) in the hydraulic fracturing water cycle

AMOVA			
p-value	SW	FPW	Mixed Fluids
SW	1	0.018	0.056
FPW		1	<0.001*
Recycled Mixed Fluids			1