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Cultivation-Dependent Analysis of Microorganisms Associated with Various Hydraulic Fracturing Fluids

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CULTIVATION-DEPENDENT ANALYSIS OF MICROORGANISMS ASSOCIATED WITH VARIOUS HYDRAULIC FRACTURING FLUIDS

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ABSTRACT

Cultivation-Dependent Analysis of Microorganisms Associated with Various Hydraulic Fracturing Fluids

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The overarching goal of this study is to gain more insight to the microbial interactions associated with hydraulic fracturing by studying the culturable microorganisms present in various types of hydraulic fracturing fluids. Extraction of natural gas and other unconventional resources using hydraulic fracturing has increased in the last decade and very few studies have been conducted on the microorganisms associated with the various water types used in the process. From the very few published studies, the only cultivation-dependent method used involved determining the most probable number (MPN) of various metabolic groups, however the researchers failed to ask more in-depth questions regarding whether the microorganisms found are capable of metabolizing hydrocarbons or if they produce biosurfactants/bioemulsifiers. This study we diverge from the previous work by using a culture-

dependent approach to characterize unique colony types of culturable aerobic-heterotrophic microorganisms associated with hydraulic fracturing fluids and to answer more in-depth questions, which can only be answered by growth of the microbes under various conditions.

The first objective of this research focuses on isolating and identifying unique colony types of culturable microorganisms from several hydraulic fracturing fluid samples from different oil-bearing formations. Identifying the microorganisms present in the various fluids can help uncover potential microbiological problems that affect the efficiency of oil and natural gas extraction. Currently, deleterious microorganisms affecting the petroleum industry include sulfate-reducing bacteria (SRB), acid-producing bacteria (APB) and microorganisms that make extracellular polymeric substances (EPS). Sulfate-reducing bacteria are well-known for their role in corrosion, well fouling, and toxicity to human health. APB also contribute to corrosion of steel and concrete while EPS producing bacteria promote the formation of biofilms that further contributes to biocorrosion. Comparing the microorganisms present in the different hydraulic fracturing water samples will help identify potential microbiological problems from the study sites and define where the most potential for microbial problems exist.

In order to address the other objectives of this study, culturable microorganisms are required; therefore, limitations due to isolation and low isolate number must be acknowledged. Cultivation-independent analysis describe the bacterial community associated with a sample whether living or dead and without concern for the origin of the bacteria within, whereas cultivation-dependent analysis provides isolates with which to further explore potential interactions of microbes and the petroleum.

In the second objective of this research we characterize the culturable isolates found from the various fluid samples by investigating their response to various ranges of salinity, pH, and temperature, including those understood to be present in the subsurface environment. Data from previous published studies have inferred that the microorganisms found in flowback and produce waters are more adapted to harsh conditions based on geochemical data collected from the samples. A recently published study by Kelvin Gregory at Carnegie Mellon University (2014) found more genes associated with stress tolerance in produce water compared to source water using a metagenomic survey. This study will determine if the inferences from the previous metagenomic survey are valid from the perspective of viable and culturable isolates.

The third objective will determine if the isolated microorganisms can create emulsions with petroleum oils and if they can use petroleum oils as their sole source of carbon and energy. Many microorganisms can use hydrocarbons as a source of carbon and energy by utilizing various metabolic pathways with beta-oxidation being the primary pathway (Alvarez, 2003; Atlas, 1981). Some research has shown that certain microbes produce biosurfactants (BS) or bioemulsifiers (BE) in the presence of hydrocarbons to aid in their utilization of hydrocarbons (Atlas, 1981). The purpose of this research objective will be to further expand on biosurfactant or bioemulsifier production and its relationship to hydrocarbon utilization, along with determining whether wastewaters associated with hydraulic fracturing could serve as a source to find novel BS/BE-producing isolates.

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CHAPTER 1

INTRODUCTION

Hydraulic fracturing describes a petroleum engineering technique of well-stimulation used in the extraction of petroleum oil and natural gas. This technique combines the principles of fluid mechanics and petroleum geology to assist in the extraction of petroleum from the subsurface. Hydraulic fracturing uses the theories of fluid mechanics to provide practical applications involving fluids and the forces that act upon them, while petroleum geology concerns the formation, exploration, movement, and origin of petroleum in the subsurface. In this case, the term petroleum includes any gas, liquid or solid hydrocarbon compounds found in sedimentary rocks (Speight, 2014).

The exploration of petroleum involves identifying subsurface rocks that either produce or are able to produce petroleum. These are called *source rocks* and the subsurface rocks that accumulate the petroleum are called *reservoir rocks*. Petroleum usually accumulates in *reservoir rocks* that are porous and permeable, but in some cases the petroleum can remain stuck in the source rock if it has low permeability. Porosity describes a rock medium's ability to hold fluids (void space within rock structure) and is expressed as a percentage in comparison to the total volume of a particular type of rock. Permeability describes the resistance a fluid encounters as it moves through a rock medium and is expressed as Darcy unit (D), usually as milliDarcy (mD). Reservoir rocks usually have interconnected pores with porosity above 10% and permeability above 1 mD to be classified as reservoir rocks (Broadhead, 2002; Tissot & Welte, 1984). Petroleum produced from the source rocks migrates (primary migration) away

from the source rock and travels through various rocks of different porosities and permeability's (secondary migration) until it reaches an impermeable layer (seal) where accumulation occurs (trap) (Broadhead, 2002; Tissot & Welte, 1984). *Oil reservoirs* refer to entrapped accumulations of petroleum, while an *oil field* refers to a series of reservoirs within a common rock structure or neighboring formations, and *oil basins/provinces* refer to a group of fields found in a single geological environment (Speight, 2014).

Petroleum exploration incorporates a systematic process of investigation and identification consisting of four levels which occur in succeeding order with the first level being the identification of a potential oil basin (also referred to as sedimentary basins) (Magoon & Dow, 1994). Once a potential sedimentary basin has been identified, classification of that particular basin's petroleum system follows. This entails finding the spatial, geological, historical, and quantitative relationship between the source rock and resulting petroleum contained within that basin. After classification, the exploration process then includes determining the economic potential by investigating the series of traps containing accumulated petroleum called *plays* (Magoon & Dow, 1994). The last step in petroleum exploration entails a conceptual term called *prospect*, which describes the process of drilling a trap to determine if it contains commercial quantities of petroleum. The last step is considered conceptual because if a prospect turns out to be successful then the site is called a gas/oil field but if the drilled site is unsuccessful the term prospect disappears (Magoon & Dow, 1994). The first two levels of exploration are independent of any economic consideration but instead are dependent on the geological history and geochemical composition within a potential basin, while the last two steps are highly dependent on economics.

Petroleum occurring in a reservoir that allows the crude material to be recovered by conventional pumping methods defines the term *conventional resources* (Speight, 2014). *Unconventional resources* are all other types of petroleum that do not necessarily occur in or have a reservoir. This includes various types of petroleum-bearing source rock that fail to produce economically favorable rates of hydrocarbon production without stimulation of the well (Passey, Q.R., Bohacs, K.M., Esch, W.L., Klimentidis, R. and Sinha, S., 2010). These unconventional petroleum-bearing source rocks typically have low permeability (less than 1 mD) and include tight-gas sandstone, gas hydrates, shale gas, oil shale and coal bed methane. Unconventional resources are more abundant than conventional resources, but extraction requires additional processes that make them economically unfavorable. Hydraulic Fracturing or fracking, represents one of the additional processes used on unconventional resources where water mixed with various additives, including sand, is pumped into a petroleum formation at a high enough pressure necessary to create fractures. These fractures increase the permeability of the rock which increases the flow rate of gas and oil to the wellbore. This technique was developed in the U.S during the 1940's and has become industrialized within the last decade because of the development of another process, directional drilling, where a well's direction can be changed with depth in directions other than vertical (usually horizontal) (Q. Wang, Chen, Jha, & Rogers, 2014). These additional processes have made the extraction of unconventional resources economically favorable.

The process of hydraulic fracturing consists of many steps, the first step of which is drilling, casing and perforating of the well. Once the well has been drilled and cased (the addition of a protective layer of cement surrounding the well pipe), a perforating gun is

lowered into the well where a discharge will occur and create holes (perforations) in the well and casing. After perfing the well, hydraulic fracturing (frack job) takes place where up to a million gallons of fresh water, called source water, is trucked to the site with large quantities of chemical additives and other equipment such as blenders/mixers and pumper trucks. The chemicals used in hydraulic fracturing are listed in Table 1. The amount of chemicals used for a typical frack job only accounts for less than 10% of the total composition of the fluids, with the majority of the 10% consisting of proppant. These chemicals are added to the fresh water before pumping it down the well.

Type of Chemicals Used	Function
Acids	Dissolve any cement debris and carbonates in formation
Biocides	Inhibit microbial growth
Breakers	Breaks down the gellant/polymer (depolymerization)
Clay Stabilizers	Prevents the mobilization of clays
Corrosion Inhibitors	Prevents rusting of pipes
Cross-linkers	Thickens the fluids (polymerization)
Foamers	Reduces the amount of fluid required for a frack job
Defoamers	Removes foam to allow trapped natural gas to escape
Friction Reducers	Creates slick water & minimizes friction
Gellants	Increases the viscosity of fluid
pH Stabilizers	Stabilizes pH
Proppants	Sand or ceramic beads that 'prop' open the fractures
Scale Inhibitors	Inhibits scale build-up in pipes

Table 1. Chemicals used in a typical hydraulic fracturing operation.

The chemical composition of the fracturing fluids varies between wells and in many cases is proprietary, limiting public disclosure about its chemical contents. The pumper trucks first inject diluted acid under high pressure into the well to dissolve cement debris in the well. Next, the fracturing fluid, also called slick water, which consists of source water mixed with the various chemicals minus the proppant and breaker, is created in a slurry blender that feeds into the pumper trucks. The fracturing fluids are injected into the well at sufficiently high pressures to fracture the unconventional petroleum reservoir structure. After this process is finished, the proppant is pumped into the formation to hold open the newly created fractures. The last part of the process involves the addition of the breaker which starts the flowback process, where excess fluids come back to the surface. The excess fluid is called flowback water and it comes up to the surface for up to a month after the fracturing process. After the flowback period, the well brings petroleum products to the surface and is then "in production". Periodically, throughout the life of the well, subsurface brine will migrate into the fracturing zone and come up to the surface. These fluids are called production or produced water and are classified as hazardous waste, along with flowback water, which is usually injected into disposal wells located deep in the earth.

Bacteria are known to cause problems in pipes and wells associated with the extraction and transportation of crude oil products through a process called biocorrosion, also known as microbially-influenced corrosion (MIC). This is where biofilms of key metabolic groups of microorganisms deteriorate metallic material (Sheng, Ting, & Pehkonen, 2007). Microorganisms existing in biofilms associated with MIC include acid-producing bacteria (APB), iron-oxidizing bacteria (IOB), iron-reducing bacteria (IRB), sulfur-oxidizing bacteria (SOB),

sulfate-reducing bacteria (SRB) and extracellular polymeric substance (EPS) producing bacteria. The most detrimental group in the biofilm is the sulfate-reducing bacteria (SRB); these use sulfate as a terminal electron acceptor and reduce it to toxic and corrosive hydrogen sulfide (H_2S) (Guidotti, 1994; Sheng et al., 2007). The H_2S can react with metals in pipes forming metal sulfide precipitates and atomic hydrogen, which weakens the integrity of the metal through a process called sulfide stress corrosion cracking (Javaherdashti, Raman, Panter, & Pereloma, 2006). Equipment associated with the extraction, storage, and transportation of petroleum products has been known to be affected by MIC. Biocides are constantly used in hydraulic fracturing to prevent or control MIC and biogenic H_2S production.

The recent industrialization of hydraulic fracturing has provided microbiologists with an opportunity to study the bacterial communities associated with these fluids. Less than ten manuscripts have been published to date, starting in 2011, that have analyzed bacterial communities associated with hydraulic fracturing fluids (Davis, Struchtemeyer, & Elshahed, 2012; Mohan, Bibby, Hartsock, Hammack, Vidic, & Gregory, 2013; Mohan, Hartsock, Hammack, Vidic, & Gregory, 2013; Mohan, Bibby, Lipus, Hammack, & Gregory, 2014; C. G. Struchtemeyer, Davis, & Elshahed, 2011; C. G. Struchtemeyer & Elshahed, 2012; Wuchter, Banning, Mincer, Drenzek, & Coolen, 2013). These studies all used culture-independent methods to analyze the bacterial communities with one study incorporating a culture-dependent method in the form of a most probable number (MPN) assay (C. G. Struchtemeyer & Elshahed, 2012). A culture-independent method involves extracting the total DNA from a sample and sequencing either a gene or the total DNA. This method can identify and provide ecological data from a sample independent of culturing techniques and can identify microorganisms never cultured in a

laboratory (Rappe 2003). Culture-dependent methods involve culturing microorganisms from samples and isolating individuals into pure cultures. This method suffers in that it can only identify and analyze what can be grown but it allows researchers to analyze microorganisms in more genetic detail.

Microbial communities in fracturing fluids (produced, source and flowback waters) associated with the Barnett Shale Play in Texas were the subject of recent research (C. G. Struchtemeyer & Elshahed, 2012). The researchers investigated the microbial communities using culture independent analysis of total DNA to determine how the hydraulic fracturing process affected the communities. Using most probable number (MPN) assays, they found that the addition of biocides failed to completely kill all the microorganisms in the flowback water and that the flowback water became dominated by spore-forming members belonging to the phylum Firmicutes, which was probably due to both the extreme conditions of the subsurface formation and the hydraulic fracturing process. The produced water consisted of halotolerant organisms from the phyla Firmicutes and Proteobacteria. Overall, the researchers found that the microbial community in each type of hydraulic fracturing fluid sample was distinctly unique from each other.

Three additional studies described the microbial communities in all the hydraulic fracturing fluids from wells located in the Marcellus Shale Play in Pennsylvania (Mohan et al., 2013a; Mohan, Hartsock, Hammack et al., 2013b). The researchers determined that the source water was dominated by aerobic microorganisms belonging to the class *Alphaproteobacteria* and *Gammaproteobacteria*, while the produced water eventually became dominated by

bacteria belonging to the class *Clostridia* six months after well completion (Mohan et al., 2013a). The authors investigated three flowback water impoundment ponds spatially as well as under three conditions: untreated (no biocide added), amended (biocide added for three weeks), and pretreated (suspended solids removal and aeration for 80 days with the purpose of reuse) (Mohan, Hartsock, Hammack et al., 2013b). The diversity increased with depth for both the untreated and amended ponds while the pretreated pond became dominated by bacteria belonging to the genus *Roseovarius*. They also performed a quantitative PCR (qPCR) assay along with 4',6-diamidino-2-phenylindole (DAPI) cell staining microscopy to measure bacterial abundances in all the samples; they found that the untreated and amended ponds had the highest abundance. The researchers conducted a shotgun metagenomic survey on source and produced water with the goal of determining the metabolic potential and function in each sample (Mohan, A.M., Bibby, K.J., Lipus, D., Hammack, R.W. and Gregory, K.B., 2014). The result of the bioinformatic analysis was that produced water had a higher abundance of genes responsible for carbohydrate metabolism, respiration, sporulation, dormancy, iron acquisition, iron metabolism, stress response and sulfur metabolism when compared to the source water. The researchers suggested that the metabolic potential, metabolic function and microbial community shifted in response to the increased stress conditions caused by the hydraulic fracturing process and environmental conditions within the subsurface shale. This was the first published study that characterized the microbes in source and produced water from natural gas wells in the Marcellus Shale Play.

The published literature shows a common trend of finding halotolerant/halophilic organisms belonging to the phyla Firmicutes, Proteobacteria and Bacteriodetes in produced

water samples (Davis et al., 2012; Mohan et al., 2013a; Mohan, Hartsock, Hammack et al., 2013b; Mohan et al., 2014; C. G. Struchtemeyer et al., 2011; C. G. Struchtemeyer & Elshahed, 2012; Wuchter et al., 2013). The metagenomics approach provides answers about "what" the microbes in the produced water can do while providing some insight into "how" they are performing these functions (Mohan, Bibby, Lipus, Hammack, & Gregory, 2014). The culture-dependent approach differs by addressing other important questions. For example which microbes are actually capable of growth from the various water types? To what extent can these microbes perform certain functions? Determining the microorganisms' response to various abiotic variables such as salinity, temperature, and pH, along with other characterization experiments provides more insight into the microbial response to this novel environment and possibly provides more supporting evidence for inferences to the metabolic capabilities of microbes in the these fluids. This study will diverge from the previous research approaches and use a culture-dependent method to obtain and characterize the microorganisms present in the various fluids capable of growth under laboratory conditions. Determining how these microorganisms respond to various abiotic factors, similar to the conditions experienced in both the fluids and subsurface formation, will help us learn more about the bacterial communities present in these fluids and possibly the subsurface. An additional aspect of this research is to screen the culturable isolates for their ability to utilize and/or emulsify petroleum oils, with the goal of finding novel isolates with potential biotechnological applications, something that cannot be done using strictly molecular approaches.

CHAPTER 2

ISOLATION, IDENTIFICATION, AND CHARACTERIZATION OF CULTURABLE MICROORGANSIMS ISOLATED FROM VARIOUS HYDRAULIC FRACTURING FLUIDS

Abstract

The increase in hydraulic fracturing operations occurring in the U.S.A. has provided microbial ecologists with a novel anthropogenic environment to investigate. The few published studies investigating the microbial communities associated with hydraulic fracturing fluids have all used a culture-independent, approach along with geochemical data to shed light on the subject. This study differs by using a culture-dependent approach to investigate unique colony types of culturable microorganisms associated with hydraulic fracturing fluids. The limitations in culturing the various metabolic groups have narrowed this study to focus only on the aerobic, heterotrophic, culturable microorganisms associated with source, flowback, and produce waters collected from oil and gas wells from the Bakken Shale in North Dakota. Flowback water from the Niobrara Shale in Colorado and produced water from the Niobrara-Mowry Shale in Wyoming were also collected and analyzed from oil and natural gas wells. Forty unique aerobic-heterotrophic microorganisms were isolated from various hydraulic fracturing waters associated with the Bakken, Niobrara, and Niobrara-Mowry shale plays. This culture-dependent approach provided an opportunity to individually characterize the culturable microorganisms with the goal of understanding how these organisms respond to various abiotic variables similar to the environments from which they were isolated. The characterization data

provided evidence in support of a previous observation that microorganisms found in produced waters are more adapted to grow in stress conditions as evidenced by their response to various temperature, salinity, and pH conditions. In addition, the culturable microorganisms' ability to utilize hydrocarbons as a source of carbon and energy and whether they were capable of creating emulsions with petroleum oils was also investigated. The majority of hydrocarbon utilizing microorganisms were surprisingly isolated from the source water compared to the flowback and produced water samples. The results from this survey have identified several novel biosurfactant/bioemulsifier-producing microorganisms that warrant further investigation, and demonstrate how hydraulic fracturing waters could possibly serve as a source of novel isolates with potential biotechnological applications.

Introduction

Hydraulic fracturing (fracking) has been around since the 1940's but research investigating the microbial communities involved in its operation has not been made public, that is, until the last decade, because of its increased practice in the U.S. The development of horizontal drilling and hydraulic fracturing has led to a more efficient method of extracting crude oil and natural gas from low-permeability petroleum reservoirs, once considered to be uneconomical. These advances have resulted in a massive increase in the practice of hydraulic fracturing in the U.S, initially with the Barnett Shale Play in the late 1990's, followed by the Marcellus and Bakken Shale Plays in the mid-late 2000's (Q. Wang et al., 2014). The economic success from fracking these shale plays has resulted in increased drilling in other low-permeability petroleum-bearing shale formations located throughout the U.S. This recent

expansion of hydraulic fracturing has provided microbial ecologists with an opportunity to investigate a novel anthropogenically-impacted environment. The published research investigating only the microbial communities associated with various hydraulic fracturing waters has concentrated on operations extracting petroleum products from the Barnett, Marcellus, and Antrim Shale Plays (Davis et al., 2012; Mohan et al., 2013a; Mohan, Hartsock, Hammack et al., 2013b; C. G. Struchtemeyer & Elshahed, 2012; Wuchter et al., 2013).

This study will diverge from the other published research approaches by using a classical isolation approach along with 16S rRNA gene analysis for identifying the culturable microorganisms found in the various fluids associated with hydraulic fracturing of the Bakken, Niobrara and Niobrara-Mowry formations. The geochemical characteristics of flowback and produced water varies within and between shale plays but published data shows that produced waters have high salinity, slightly acidic to neutral pH (pH 5-7), and come from wells with down-hole temperatures ranging from 65°C - 150°C (Shaffer et al., 2013; C. G. Struchtemeyer et al., 2011; D. Wang, 2012). Characterizing the culturable aerobic-heterotrophic microorganisms found in various hydraulic fracturing fluids, and analyzing their response to these variables, will provide more insight about the microbiology associated with hydraulic fracturing. In addition, this research project aims to provide groundwork in the screening of biosurfactant and bioemulsifier producing microorganisms found in these fluids. In the past, microorganisms that produce biosurfactants (BS) and or bioemulsifiers (BE) have been screened from environments in the presence of hydrocarbons or contaminated by hydrocarbons. The goal of screening the culturable microorganisms from hydraulic fracturing fluids for BS/BE production will determine

if potential biotechnological applications of these compounds can be provided from this novel environment.

Materials and Methods

Sample Collection

Samples were collected from oil and gas operations at the Bakken Shale Play located in North Dakota which include: source water (NDS), flowback water (NDF) and produced water (NDP). Additional samples of flowback water from the Niobrara Shale in Colorado (CF) and produced water from the Niobrara-Mowry Shale in Wyoming (WP) were also collected from oil and natural gas wells. Samples were collected in sterile containers and stored at 4°C, then immediately transported to the University of Nevada, Las Vegas (UNLV) campus (Table 1 & Figure 1).

Water Source	Location	Date Received	Shale Play
Flowback	North Dakota	11/29/2011	Bakken
Produced	North Dakota	11/29/2011	Bakken
Flowback	Colorado	09/19/2012	Niobrara
Flowback	North Dakota	01/30/2013	Bakken
Source	North Dakota	02/27/2013	Bakken
Produced	Wyoming	02/27/2013	Niobrara-Mowry

Table 1. Hydraulic fracturing water types tested, origin of samples, and the date the sample was receive for analysis.

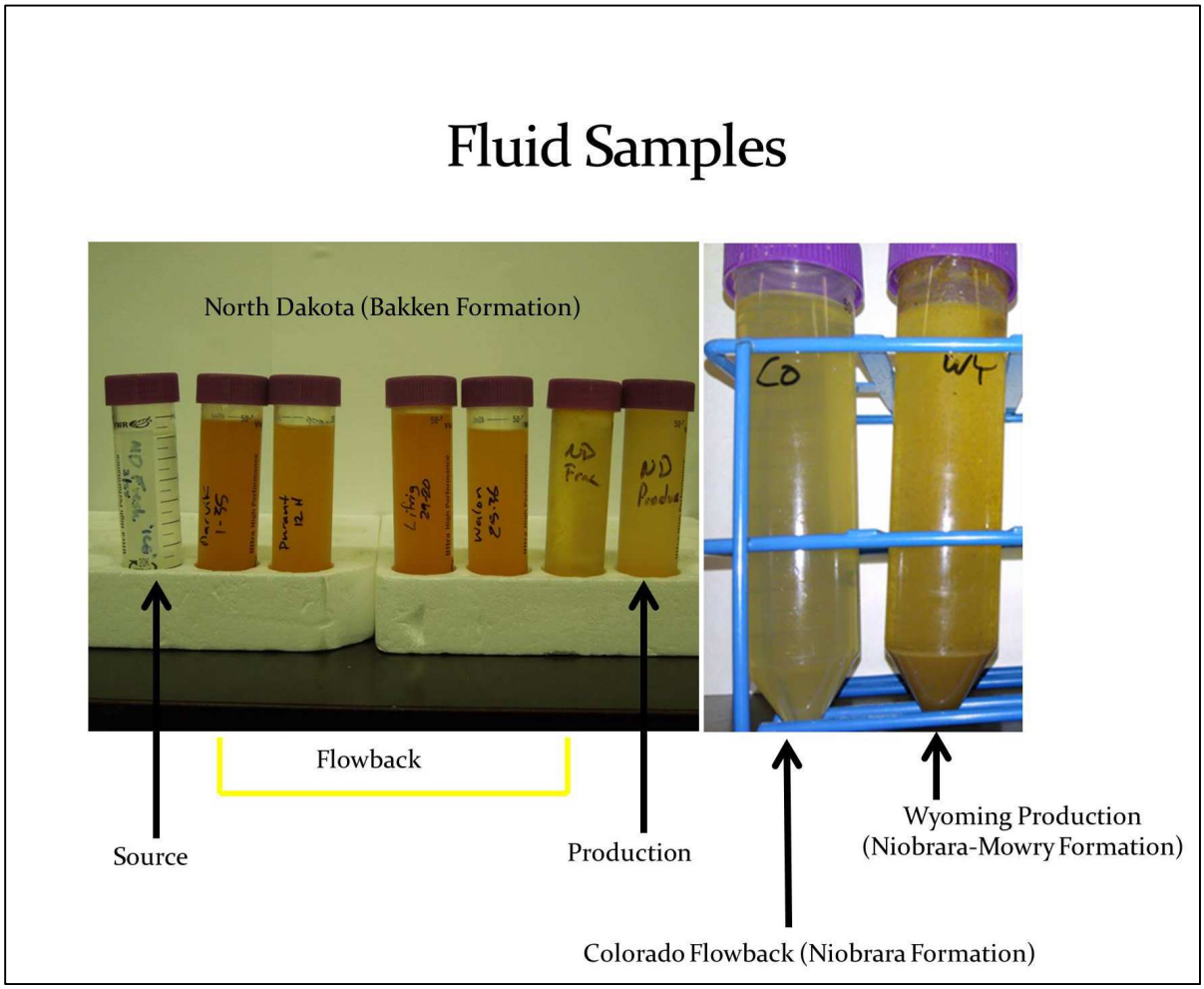

Figure 1. Hydraulic fracturing water samples from North Dakota, Colorado, and Wyoming.

Media and Cultivation

All samples were serially diluted in Reasoner's 2A broth (R2B) to 10^{-6} and plated using the spread plate technique on in Reasoner's 2A agar (R2A) at various salinities (Reasoner, Blannon, & Geldreich, 1979). To obtain various salinities in either agar or broth, NaCl was added to create: 3%, 5%, 6%, 9%, and 12%. All media were sterilized by autoclaving at 121°C for 30 minutes.

All plating's were incubated at 28°C and observed daily until growth was observed (NapCo E Series Model 303 Incubator, Jouan Inc., USA). Isolates were picked with a sterile inoculating needle, transferred to a new R2A agar plate of the same salinity and streaked for isolation. Isolates were then given identification codes, successively transferred at least three times, and Gram stained to confirm that a pure isolate was obtained. The isolates were then streaked onto R2A (with no additional NaCl) to determine if they required salinity for growth. Isolates were transferred to labeled 8 mL screw-cap culture tubes containing 3mL R2B and incubated at 28°C for 3-5 d (VWR International, USA). Frozen stocks of all isolates were made by mixing 0.75 mL of fresh culture with 0.75 mL of sterile 30% glycerol stock solution in sterile 1.5 mL microfuge tubes (Eppendorf® microtubes 3810X, Fischer Scientific, USA). The microfuge tubes were frozen and stored in at -80°C (VWR 3-Cubic Foot Ultra Low -86°C Chest Freezer, VWR International, USA).

Master Plate Stocks

Cultured isolates from all water sources were moved from individual R2A plates to a master plate containing 20 isolates each. Master plates were maintained on 0% and 3% R2A and incubated at 28°C for 2-3 d until noticeable growth was observed. Thereafter, new R2A master plates were stored at 4°C for a period of no more than two weeks. Various physiochemical analyses were performed using the master plate isolates for inoculation. Fresh master plates were prepared for each experiment below.

Salinity Growth Range

Isolates were transferred to R2A with the following salinities: 0%, 3%, 6%, 9%, 12%, 15% and 18% of NaCl. These plates were incubated at 28°C for 14 d total with periodic observation of growth every 2 d. Each isolate's growth was observed and compared to its growth on R2A, or in the case of isolates 3-2, 11AY, and P1L, R2A containing 3% NaCl. All subsequent experiments involving 3-2, 11AY and P1L were done using 3% NaCl containing media.

pH Growth Range

Isolates were transferred to R2A with the following pH values: 3, 4, 5, 6, 8, 9, 10, 11, 12, and 13. These were incubated at 28°C for 14 d total with periodic observation of growth every 2 d. Each isolate's growth was observed and compared to its growth on R2A. Stability of pH was checked by periodically measuring R2B at the low and high pH values, which did not change after a week of testing.

Temperature Growth Range

Each isolate was transferred from R2A master plates to labeled 8 mL screw-cap culture tubes containing 3mL R2B (VWR International, USA). Tubes were incubated in an orbital shaker (Barnstead International MaxQ™ 4000 Benchtop Orbital Shaker, ThermoScientific, USA) at 28°C and 100 rpm for 7 d. A volume of 0.1 mL was used to inoculate six labeled 3 mL R2B tubes. The optical density at the 600 nm wavelength (OD_{600}) was measured at time zero (T_0) by placing the culture tube into a spectrophotometer containing a modified sample holder (Beckman DU 530 Life Science UV/Vis Spectrophotometer, Beckman Coulter, Pasadena, CA). Two tubes of each isolate were placed in a test-tube rack and incubated at the following temperatures: 28°C, 37°C, and 45°C. Optical density readings were recorded periodically up to 30 d to record growth. Growth was determined by plotting the difference in absorbance values (absorbance of each time point – absorbance at T_0) over time.

Oxidative-Fermentation Test

Each isolate was transferred from R2A master plates to labeled 8 mL screw-cap culture tubes containing 3mL R2B. Tubes were incubated in an orbital shaker at 28°C and 100 rpm for 3 d. The culture tubes were used to inoculate two sterile Oxidative-Fermentation tubes (O-F) using an inoculating needle (HUGH, R. and LEIFSON, E., 1953). One inoculated O-F tube from each pair was overlaid with 0.5 mL sterile mineral oil to create anaerobic conditions. The tubes were incubated for 4 d at 28°C and the results were recorded to determine which isolates can ferment and/or oxidize glucose.

E_{24} Test

Each isolate was transferred from R2A master plates to labeled 8 mL screw-cap culture tubes containing 3mL R2B. Tubes were incubated in an orbital shaker at 28°C and 100 rpm for 7 d. These were used as seed cultures for 25 mL R2B in 50 mL polypropylene centrifuge tubes (VWR® SuperClear™ Ultra-High Performance Centrifuge Tubes, VWR International, USA). Tests were prepared in triplicate and incubated in an orbital shaker set at 28°C and 100 rpm for 7 d, with the exception of 1A8 and 3-3 orange which were incubated for 14 d. After the incubation period, cultures were pulse-vortex mixed (5 sec pulses) until a homogenous mixture was observed and a modified E24 assay was performed to determine each isolate's emulsification index (Neufeld, R.J. and Zajic, J.E., 1984). Two mL was sterilely removed from each 25 mL R2B culture and placed in a labeled 8 mL screw-cap culture tube containing 2 mL of kerosene (Klean–strip 1K heater fuel kerosene, W.M Barr, USA) and capped. The culture tube was then agitated on a Vortex mixer set at 3200 rpm (VWR vortex genie 2, VWR International, USA) for 2 min and placed in a test tube rack undisturbed for 24 hours. The emulsification index was determined by measuring the height of the emulsion compared with the total height of all phases using a ruler. The emulsification index for each culture tube was determined using the formula: Emulsification Index (EI) = $\frac{height\ of\ emulsion\ (mm)}{Total\ height\ of\ liquids\ (mm)}\ x\ 100$.

Six replicates from each 25 mL culture tube were performed.

Bacterial Adhesion to Hydrocarbon (BATH) assay

Each isolate was transferred from a master plate to a 250 mL shaker flask with deep baffles (VWR International, USA) containing 100 mL R2B and incubated in an orbital shaker at 28°C and 100 rpm for 7 d. After 7 d, 40 mL of the culture was placed in a labeled 50 mL polypropylene centrifuge tube and the cells pelleted for 10 minutes at 4,000 rpm (Beckman J2-HS, Beckman Coulter, USA). The supernatant was discarded and the process was repeated until all of the cells were harvested. The bacterial pellet was suspended in sterile phosphate buffer saline (PBS) with the following composition: 8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.44 L^{-1} Na_2HPO_4, and 0.24 L^{-1} KH_2PO_4, with a final pH between 7.2-7.4, by the addition of sterile PBS and gentle vortex mixing to obtain a homogenized solution. The PBS-washed cells were centrifuged for 10 minutes at 4000 rpm. The supernatant was discarded and the process was repeated one more time prior to the modified BATH assay being performed (Saini, 2010). The washed bacterial pellet was suspended in sterile PBS, dilutions were made until an optical density between 0.4 – 0.6 was obtained. The OD_{600} of each PBS cell suspension was measured by spectrophotometrically. The optical density of each tube was recorded as the initial value. A volume of 0.5 mL of kerosene was placed in each tube followed by gentle vortex mixing for 1 min. The tubes were left undisturbed for 30 min. The OD_{600} of each tube was recorded as the final value and used to determine each isolates cell surface hydrophobicity (%H) based on the formula: $\frac{OD600\ initial - OD600\ final}{OD600\ intial}\ x\ 100$. Isolates with a %H above 0.100 were consider to produce BS/BE at the surface of its cell instead of secreting it into the culture medium.

Hydrocarbon Utilization Assay

Each isolate was transferred from R2A master plates to labeled 8 mL screw-cap culture tubes containing 3mL R2B. Tubes were incubated at 28°C and 100 rpm for 7 d. These were used as seed cultures to inoculate modified M9 minimal medium broth lacking any organic carbon source (M9+NC), M9 supplemented with 1 % (w/v) glucose (M9+G), M9 supplemented with 20 µL n-hexadecane (M9+H), and M9 supplemented with 20 µl kerosene (M9+K). The total volume for each M9 medium used was 4 mL in sterile 8 mL screw-cap culture tubes. Each isolate (100 µl) was inoculated in triplicate under each condition from the seed cultures. Negative controls were inoculated with sterile water. The optical density at the 420 and 600 nm wavelengths (OD_{420} and OD_{600}) was measured to record time zero (T_0). The tubes were placed in an orbital shaker set at 28°C and 100 rpm. Optical density readings were recorded periodically up to 30 d for growth. Growth was determined by plotting the difference in absorbance values (absorbance of each time point – absorbance at T_0) over time.

Biocide Resistance Test

Labeled tubes containing 4 mL R2B were inoculated from master plates, and incubated in an orbital shaker set at 28°C and 100 rpm for 1 d. A volume of 0.5 mL was used to inoculate six labeled 8 mL screw-cap culture tubes containing 3 mL R2B, where three tubes served as a positive control (no glutaraldehyde) and the other 3 tubes as the experimental treatment. The optical density measured at the 600 nm wavelength (OD_{600}) was recorded for time zero (T_0). The OD_{600} was recorded at 1 and 3 hours after initial inoculation to confirm growth. After 3hr, a volume of 0.5 mL of 800 ppm glutaraldehyde solution was added to the three experimental

tubes (100 ppm final concentration); 0.5 mL of sterile DI water was added to the controls. The OD_{600} values were recorded at 1, 3 and 24 hours after biocide addition. Efficiency of the biocide treatment was determined by plating 20 µL from each biocide treated tube onto R2A or 3% salinity R2A. The plates were incubated at 28°C for 2-3 d and monitored for growth. The experiment was repeated again with a volume of 0.5 mL glutaraldehyde solution to create a final biocide concentration of 200 ppm. Bactericidal was defined as lack of growth on plates and no increase in OD_{600} after biocide addition. Ineffective biocide concentration or resistance was defined as growth on plates and increase in OD_{600} after addition of biocide.

Amplification of 16S rRNA genes of cultured microorganisms by Polymerase Chain Reaction (PCR)

Pure culture isolates were inoculated into R2B and incubated at 28°C for 3-7 d with the exception of isolate 3-2, P1L and 11AY which were grown in 3% NaCl R2B. Once turbidity was observed, a modified whole cell direct PCR was performed because of its high success rate in amplifying DNA (Saris, Paulin, & Uhlén, 1990). A total volume of 1.5 mL of pure culture broth was placed in a sterile 1.5 mL microfuge tube. The cells were pelleted by centrifugation at 10,000 rpm for 1 minute at 4°C (Beckman Coulter Microfuge® 22R Centrifuge, Beckman Coulter, Pasadena, CA). The supernatant was discarded and 0.5 mL of sterile phosphate buffered saline (PBS) with the following composition: 8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.44 L^{-1} Na_2HPO_4, and 0.24 L^{-1} KH_2PO_4, with a final pH between 7.2-7.4, was added to the bacterial cell pellet. The pellet was resuspended using 5 s pulses with a Vortex mixer set at 3200 rpm (Vortex-Genie® 2 Untimed Mixer, VWR International, USA). The cells were pelleted again under the same centrifugation

conditions as above. The supernatant was discarded and the process was repeated once more for a total of three times. After the second PBS wash, 0.5 mL of sterile distilled H_2O was added to the bacterial pellet and it was resuspended by pulse vortex mixing at 3500 rpm. The centrifugation was repeated once more. The supernatant was discarded and 200-250 µL of sterile distilled H_20 was added to the tube. The pellet was again resuspended by vortex mixing, incubated in a standard heatblock set at 95°C for 3-5 min, and immediately placed in a -80°C freezer for 10 min (VWR Heatblock, VWR International, USA). After 10 min, the tubes were left at room temperature to thaw completely and mixed with a Vortex mixer set at 3200 rpm for 5 s followed by centrifugation at 4,000 rpm for 60 s. The supernatant from each tube provided template DNA for PCR. Isolated bacterial 16S rDNA was amplified by PCR using universal primer set 8F (5'AGAGTTTGATCCTGGCTCAG 3' Tm = 55.2°C) and modified 1492R (5'ACGGCTACCTTGTTACGACTT3' Tm = 57.4°C) (IDT, Coralville, IA) (Weisburg, Barns, Pelletier, & Lane, 1991). The PCR mixture contained: 25 µL of Midas™ Mix (Monserate Biotechnology Group, San Diego, CA), 1 µM of each primer, 5 µl of template DNA, and 10 µL of nuclease-free water (Promega, Madison, WI, USA) to make a final volume of 50 µL. The PCR amplification was performed in a GeneAmp PCR System 2400 machine (PerkinElmer, Inc, Akron, OH) with the following conditions: initial melt cycle (94°C for 2 min) followed by 30 cycles of melting (94°C for 30 s), annealing (53°C for 45 s), extension (72°C for 90 s), and a final extension (72°C for 5 min). Confirmation of proper amplification was determined on a 1% agarose gel containing 1X GelRed™ (PhenixResearch, Candler, NC) with 6 µL of GelPilot 1kb Plus ladder (Qiagen, Germantown, MD) in multiple wells. Running conditions were 90 V for 1.5-2.0 hr. The gel was

visualized in a UVP Gel-Doc Imager using 302 nm excitation wavelength and amplified products were confirmed if they had a molecular weight around 1.5 kb. Amplified PCR products were purified using an E.Z.N.A Cycle Pure Kit (Omega Bio-Tek, Norcross, GA) according to the manufacturer's instructions and collected into labeled, sterile 1.5 mL microfuge tubes. The purified 16S rRNA gene products from the various isolates were quantified for their purity and concentration using a NanoVue™ 4282 V1.7.3 spectrophotometer (GE Healthcare Bio-Sciences, Pittsburgh, PA). PCR amplified 16S rRNA gene products with an A_{260}/A_{280} ratio between 1.8 - 2.0 and a DNA concentration above15 µg/µL were saved for sequencing.

Partial sequencing of amplified 16S rDNA

Amplified 16S rDNA products from all culturable isolates were submitted for sequencing to the UNLV Genomics Center where the sequencing was performed using an Applied Biosystem® 3130 DNA Analyzer with ABI BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies™, USA). Each isolate's amplified DNA was placed in a labeled PCR tube (Fisherbrand™ 0.2mL flat-cap PCR tube, Fisher Scientific, USA) with a final volume of 10 µL containing: 10 – 40 ng template DNA, 3.2-10 µM sequencing primer, and if applicable, nuclease-free water for dilution. The average read lengths provided from UNLV Genomics Center were around 900 bp and the 8F primer was used for sequencing. This theoretically, yields sequence data from position 9 to position 908 on the 16S rDNA gene. This partial sequencing method covers the 16S rRNA gene variable region V1-V4 and has been shown to be sufficient for identification of many bacterial isolates to the genus level (Kim, Morrison, & Yu, 2011; Woo et al., 2003).

Analysis of sequence data

The sequencing data were analyzed and trimmed manually using Sequence Scanner Software v1.0 (Applied Biosystems® by Life Technologies, Foster City, CA). Trimmed sequences had an average contiguous read length of 750 bp with Quality Values (QV's) > 20. The trimmed sequences were then analyzed using the Basic Local Alignment Search Tool (BLAST®) nucleotide algorithm (BLASTn) from the NCBI database and webserver (Altschul, Gish, Miller, Myers, & Lipman, 1990). All isolates were identified to the genus level from the sequence data. Isolates with the same BLASTn results were collapsed so that one isolate represented that particular organism. All isolates with unique sequences were checked for chimeras using the short sequences algorithm from the DECIPHER web-server (Wright, Yilmaz, & Noguera, 2012). Unique sequences were then submitted to Genbank® and assigned accession numbers KM219076-KM219112 and KM357910-KM357912. The unique sequences were aligned against the SILVA database (release 119) using Mothur v.1.33.0 (Quast et al., 2013; Schloss et al., 2009). The aligned sequences were then manually curated using Bioedit and MEGA6 (MEGA) (Hall, 1999; Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Aligned, curated sequences of all unique isolates, along with a bacterial outgroup were used to construct a phylogenetic tree in order to make inferences of evolutionary relationships. This was done using the maximum likelihood method in MEGA6. Taxonomic classification of each isolate based on its BLASTn results was determined using the NCBI Taxonomy database and the List of Prokaryotic Names with Standing in Nomenclature (LPSN) database, available at *http://www.bacterio.net* (Euzeby, 1997; Parte, 2014; Sayers et al., 2011). Verification of each unique isolate taxonomic

classification was verified using the RDP Naive Bayesian rRNA Classifier (version 2.10, October 2014) with a confidence threshold set at 95% (Q. Wang, Garrity, Tiedje, & Cole, 2007).

Results and Discussion

16S rRNA gene analysis

The partial sequence analysis of the 16S rRNA gene for each isolate of this study has identified 40 unique bacterial taxa as shown in Table 2. Phylogenic analysis of all isolates revealed that 50% belong to γ-Proteobacteria, 15% belong to Bacilli, 12.5% belong to α-Proteobacteria, 10% belong to Actinobacteria, 5% belong to β-Proteobacteria, 5% belong to Cytophagia, and 2.5% belong to Flavobacteriia. As can be seen in the phylogenetic tree (Figure 2), isolates *Bacillus sp.*_NDF_Durant1 and *Bacillus sp.*_NDF_Durant3 might be the same organism since their clustering has a statistical supporting value of 100%. The blast results of these isolates' partial 16S genes show that they are related to the *Bacillus anthracis*, *Bacillus cereus*, or *Bacillus thuringiensis* group, which is at the center of a debate as to whether or not they are actually the same species (Helgason et al., 2000; Ticknor et al., 2001). Because they came from the same water sample, they might be considered as the same, however, additional testing demonstrated differences between the isolates. *Pseudomonas sp*._NDS_0-5 and *Pseudomonas sp*._CF_LR12 also appear to be the same organism because their clustering has a statistical supporting value of 83% and equal branch lengths. *Pseudomonas sp*._CF_LR8, *Pseudomonas sp.*_ NDS_5-4 and *Pseudomonas sp.*_ NDS_0-2 appear to be the same organism because their clustering has a statistical supporting value of 93%, but only *Pseudomonas*

sp._CF_LR8 and *Pseudomonas sp.*_ NDS_0-2 had equal branch lengths while *Pseudomonas sp.*_ NDS_5-4 had a slight longer branch length. This indicates that *Pseudomonas sp.*_ NDS_5-4 is closely related to *Pseudomonas sp*._CF_LR8 & *Pseudomonas sp.*_ NDS_0-2 but different from the pair. Although *Pseudomonas sp*._CF_LR8 & *Pseudomonas sp.*_ NDS_0-2 show such close relatedness, they were isolated from not only different locations, North Dakota and Colorado, but they were also isolated from different water types, source and flowback. It is, therefore, unlikely that they are the same strain even if they are the same species. Since we used a partial sequence of the 16S rRNA gene, these pairs of isolates appear to be the same using the maximum likelihood method, thus showing the limitations of using a partial sequence of the 16S rRNA gene for analysis. The phylogenetic results would bring the number of unique isolates found in this study to 37, but a decision a was made to consider the pairs *Pseudomonas sp*._CF_LR8 & *Pseudomonas sp*._NDS_0-2 and *Pseudomonas sp*._CF_LR12 & *Pseudomonas sp*._NDS_ 0-5, as unique organisms since they were isolated from different water samples that are not associated with the same shale play. Isolates *Bacillus sp.*_NDF_Durant1 and *Bacillus sp.*_NDF_Durant3 were isolated from the same water sample but they displayed different growth patterns to pH, salinity and temperature that suggest that they are not the same organism.

Accession #	Sample	ID	Phylum	Class	Genus
KM219076	NDS	0-1	Bacteroidetes	Cytophagia	*Algoriphagus*
KM219105	NDS	0-2	Proteobacteria	Gammaproteobacteria	*Pseudomonas*
KM219077	NDS	0-3	Proteobacteria	Alphaproteobacteria	*Sphingobium*
KM219107	NDS	0-4	Proteobacteria	Gammaproteobacteria	*Pseudomonas*
KM219078	NDS	0-5	Proteobacteria	Gammaproteobacteria	*Pseudomonas*
KM219079	NDS	0-7	Bacteroidetes	Flavobacteriia	*Flavobacterium*
KM219080	NDS	0-8	Proteobacteria	Gammaproteobacteria	*Pseudomonas*
KM219081	NDS	0-11	Proteobacteria	Betaproteobacteria	*Hydrogenophaga*
KM219082	NDS	0-12	Proteobacteria	Alphaproteobacteria	*Porphyrobacter*
KM219083	NDS	1/2-2	Proteobacteria	Alphaproteobacteria	*Brevundimonas*
KM357910	NDS	1/2-4	Proteobacteria	Gammaproteobacteria	*Pseudomonas*
KM219084	NDS	1/2-5	Proteobacteria	Gammaproteobacteria	*Pseudomonas*
KM219108	NDS	1/2-6	Proteobacteria	Alphaproteobacteria	*Agrobacterium*
KM357911	NDS	5-1	Proteobacteria	Gammaproteobacteria	*Rheinheimera*
KM219085	NDS	5-4	Proteobacteria	Gammaproteobacteria	*Pseudomonas*
KM357912	NDS	5-5	Proteobacteria	Gammaproteobacteria	*Pseudomonas*
KM219103	NDF	Durant 1	Firmicutes	Bacilli	*Bacillus*
KM219109	NDF	Durant 2	Firmicutes	Bacilli	*Paenibacillus*
KM219086	NDF	Durant 3	Firmicutes	Bacilli	*Bacillus*
KM219090	NDF	3-2	Proteobacteria	Gammaproteobacteria	*Chromohalobacter*
KM219112	NDP	P1L	Firmicutes	Bacilli	*Bacillus*
KM219102	NDP	11AY	Actinobacteria	Actinobacteria	*Cellulomonas*
KM219088	NDP	PYT	Actinobacteria	Actinobacteria	*Microbacterium*
KM219104	NDP	AP	Actinobacteria	Actinobacteria	*Curtobacterium*
KM219087	NDP	PYO	Actinobacteria	Actinobacteria	*Micrococcus*
KM219089	NDP	3-3 Orange	Proteobacteria	Alphaproteobacteria	*Paracoccus*
KM219091	CF	1A1	Proteobacteria	Gammaproteobacteria	*Klebsiella*
KM219111	CF	2-1	Proteobacteria	Gammaproteobacteria	*Raoultella*
KM219097	CF	Kero 2	Proteobacteria	Gammaproteobacteria	*Enterobacter*
KM219093	CF	3A1	Proteobacteria	Gammaproteobacteria	*Proteus*
KM219110	CF	LR12	Proteobacteria	Gammaproteobacteria	*Pseudomonas*
KM219096	CF	LR14	Proteobacteria	Gammaproteobacteria	*Pseudomonas*
KM219092	CF	1C1	Proteobacteria	Gammaproteobacteria	*Shewanella*
KM219099	CF	LR15	Bacteroidetes	Cytophagia	*Pontibacter*
KM219095	CF	LR5	Firmicutes	Bacilli	*Bacillus*
KM219101	CF	3-1	Proteobacteria	Betaproteobacteria	*Achromobacter*
KM219100	CF	LR8	Proteobacteria	Gammaproteobacteria	*Pseudomonas*
KM219098	CF	Kero 1-1	Proteobacteria	Gammaproteobacteria	*Shewanella*
KM219094	CF	LR2	Proteobacteria	Gammaproteobacteria	*Shewanella*
KM219106	WP	Wy	Firmicutes	Bacilli	*Bacillus*

Table 2. Taxonomic classification of cultured isolates phylum, class and genus. Accession numbers were assigned for each isolates partial 16S rRNA gene sequence and are stored in the NCBI database.

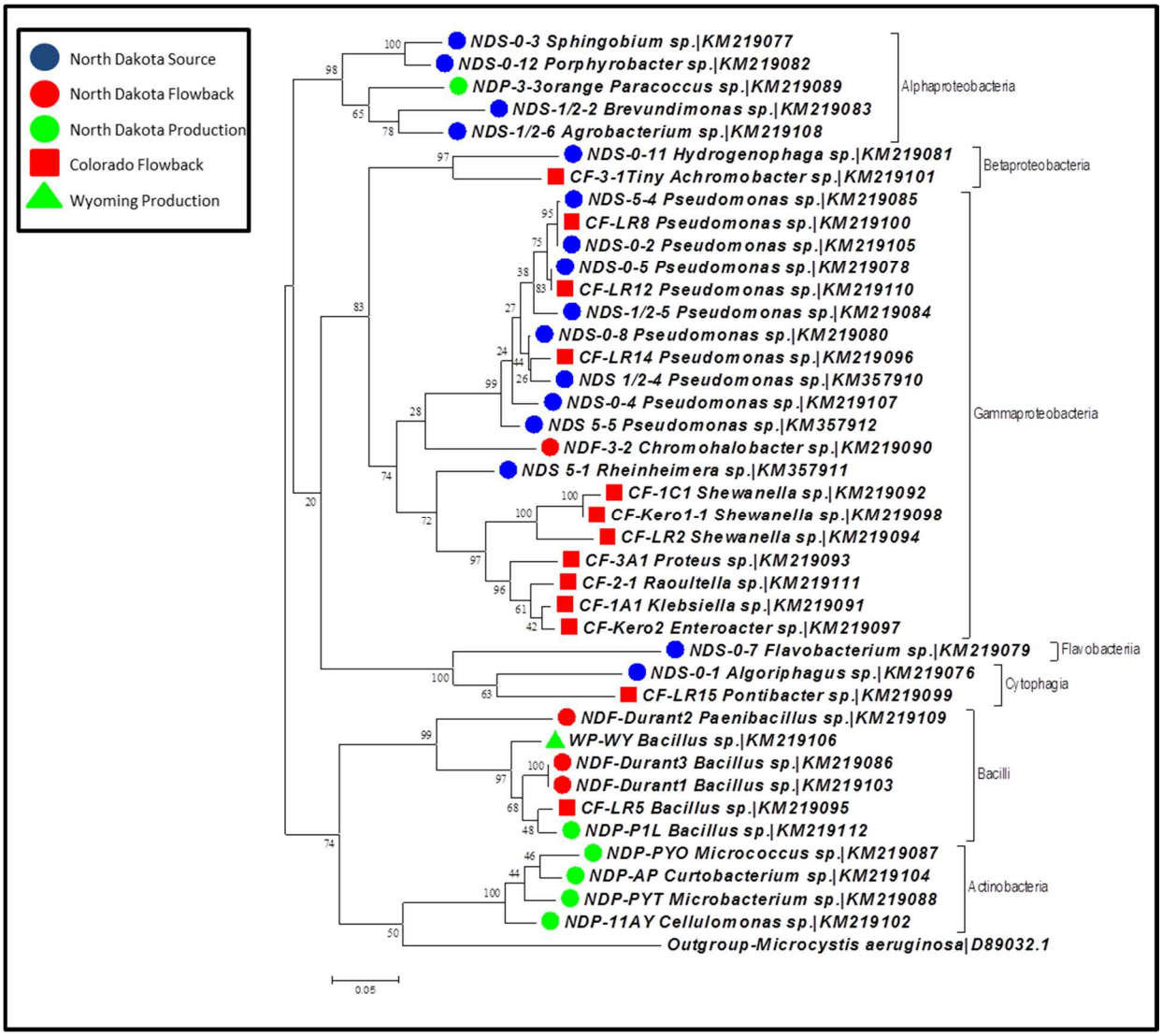

Figure 2. Molecular phylogenetic analysis by the maximum likelihood method.
The evolutionary history was inferred by using the maximum likelihood method based on the general time reversible model. The percentage in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the neighbor-Joining method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6.

The bacterial genera isolated from each sample along with their abundances are shown in Figure 3. This graph portrays the microbial similarities and differences between the samples. The majority of the isolates from NDS belong to the phylum Proteobacteria (87.5%), with the remaining belonging to phylum Bacteriodetes (12.5%). Firmicutes accounted for the majority of isolates found in NDF (75%), while Proteobacteria only accounted for a quarter (25%). Actinobacteria were only found in NDP and accounted for the majority of its isolates (66.7%), while Firmicutes (16.7%) and Proteobacteria (16.7%) accounted for less than a quarter each. Proteobacteria accounted for the majority of isolates found in CF (84.6%) while Firmicutes (7.7%) and Bacteriodetes (7.7%) accounted for less than 10% each. WP only had one isolate and it belonged to the phylum Firmicutes (100%).

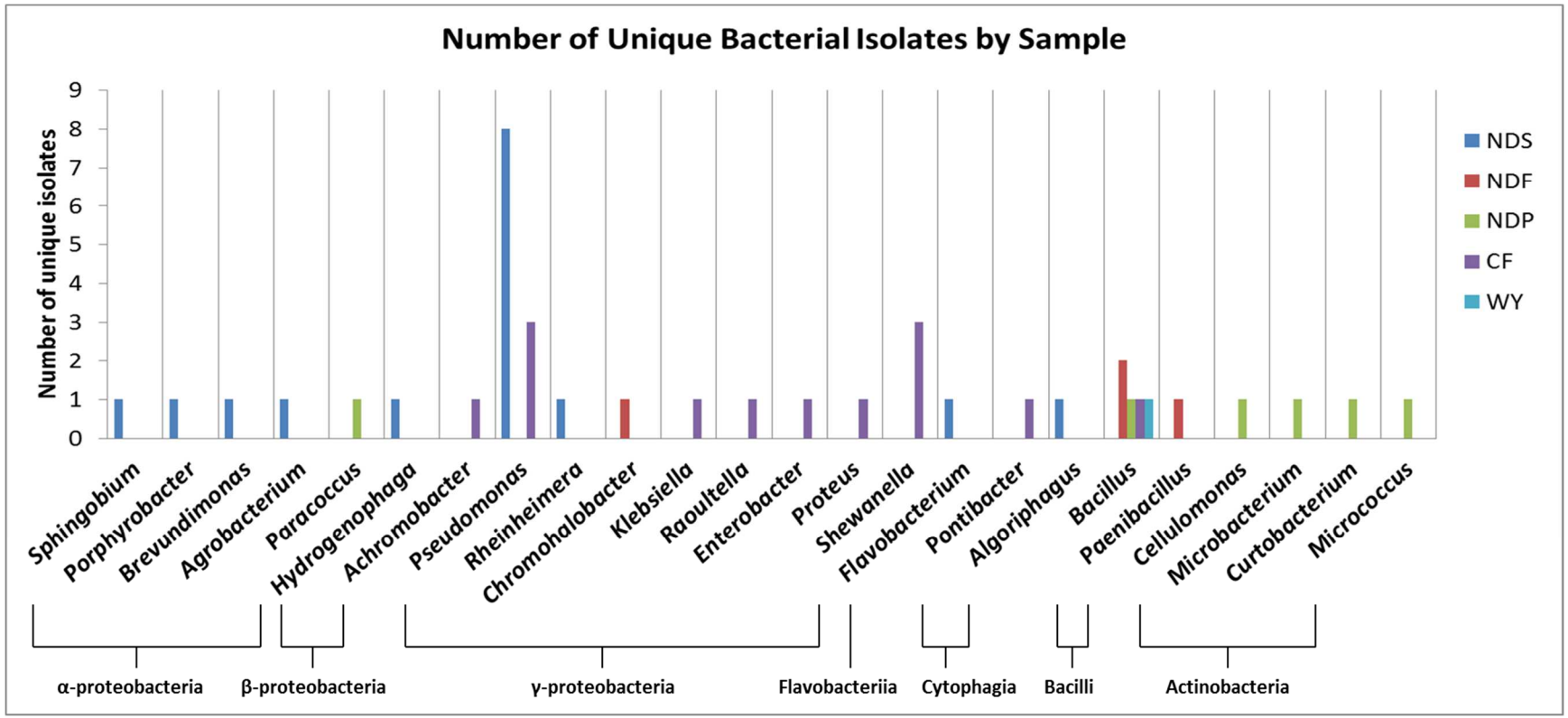

Figure 3. Number of unique isolates from the various samples identified to the genus level and grouped by class**.**

When comparing only the North Dakota samples (Figure 3), the culturable aerobic-heterotrophic community shifts from being dominated by γ-Proteobacteria in NDS to a community dominated by Bacilli in NDF which then shifts again to a community dominated by Actinobacteria in NDP. The bacterial community of the hydraulic fracturing fluid undergoes drastic changes throughout the process of extracting oil and natural gas. The initial fracturing water creates an unfavorable environment for microbes because of the addition of many chemicals, including biocides, to the source water. Biocides are consistently used in the extraction and transportation of oil and natural gas to prevent biocorrosion and biofouling caused by bacteria. This, coupled with the high pressures involved with injection and fracturing, changes the environment that microbes encounter considerably. The unfavorable environment of the fracturing water helps explain the low number of culturable isolates found in the flowback water sample and the change in culturable types; both Bacilli and Actinobacteria are endospore-forming bacteria that could be present in their resistant form.

Produced water contains brine from the petroleum formation that mixes with fracturing fluids in the subsurface and comes to the surface periodically throughout the life of the well. The chemicals in the fracturing fluids and the harsh formation conditions present many challenges for survival and growth of the microbial community associated with produced water. It is, therefore, difficult to determine if the organisms in produced water samples are indigenous to the petroleum formation, were introduced during the extraction process from the deep subsurface, or introduced from the surface. Interestingly, the culturable isolates found in the North Dakota produced water were more abundant compared to the flowback water, which suggests that the environment became more favorable at some point in time after

the fracturing process and/or subsurface bacteria were washed out of the formation. Another interesting observation was that all the Actinobacteria isolated in this study were found in NDP. Whether these isolates were indigenous to the subsurface or introduced during the collection of the water remains unknown but the data imply that the Actinobacteria were not introduced from the source water because no members of that taxonomic class were found in the source water, and it is unlikely that all Actinobacteria would have been destroyed by the fracturing process or chemical additions since Actinobacteria are endospore formers.

Salinity and Temperature

Results of salinity testing are shown in Table 3. All isolates grew at 0% salinity with the exception of isolate, *Chromohalobacter sp*._NDF_3-2, which only grew with added NaCl, requiring NaCl for growth. During salinity testing, 100% of the isolates in NDS, NDP, and CF were able to grow on 3% salinity R2A; one NDF isolate *Paenibacillus sp.*_NDF_Durant2, could not. This observation indicated that the isolate was not active in the flowback water and may have been present in its dormant endospore form. In 6% and 9% salinity R2A, 37% of NDS, 75% of NDF, and 83% of NDP isolates could grow under higher salinity conditions. CF showed a different trend where 85% of its isolates could grow under 6% salinity and 62% could grow under 9% salinity. No isolate from NDS could grow at salinities above 9%, as might be expected from microorganisms found in fresh water. Approximately 50% of NDF and NDP isolates could grow under 12% salinity and only 46% of CF isolates could, thus supporting the observation of the metagenomic study (Mohan, A.M., Bibby, K.J., Lipus, D., Hammack, R.W. and Gregory, K.B., 2014) where microbes found in produced water had a higher abundance of genes related to stress tolerance compared to source water microbes. Six isolates from flowback and produced

water samples, *Chromohalobacter sp.*_NDF_3-2, *Microbacterium sp*._NDP_PYT, *Micrococcus sp*._NDP_PYO, *Klebsiella sp*._CF_1A1, *Bacillus sp*._CF_LR5 and *Bacillus sp*._WP_WY, were able to grow on both 15% and 18% salinity, while isolate, *Paracoccus sp.*_NDP_3-3Orange, was able to grow at 15% but not 18% salinity. It can be inferred for the NDF and NDP samples that isolates *Paenibacillus sp.*_NDF_Durant2 and *Bacillus sp*._NDP_P1L were probably not vegetative cells but rather present in their dormant spore state because the Paenibacillus isolate could not grow in even 3% salinity and the Bacillus isolate could only grow in salinities of 0% and 3%.

Table 4 provides the temperature growth responses for each of the isolates. More isolates from NDP (50%), WP (100%), and CF (46%) grew at 45°C compared to NDS (12.5%) and NDF (0%) isolates. These results indicate that isolates found in produced waters are more adapted to stress conditions in the form of increased temperature compared to isolates found in source water. The temperature of a fracture well will be cooler compared to the surrounding subsurface formation because of water‘s high specific heat capacity. The waters used in hydraulic fracturing are not transformed into steam when injected into the formation, which indicates that the temperature must be below boiling, and therefore, could harbor growth of microorganisms. Wells extracting from high temperature petroleum formations still experience microbial influenced corrosion (MIC) and biofouling, indicating microbial activity occurring at elevated temperatures (Struchtemeyer, C.G., Davis, J.P. and Elshahed, M.S., 2011).

Organism	0%	3%	6%	9%	12%	15%	18%
*Algoriphagus sp.*_NDS_0-1	++	++	+	+	-	-	-
*Pseudomonas sp.*_NDS_0-2	+++	+++	-	-	-	-	-
*Sphingobium sp.*_NDS_0-3	+++	+++	-	-	-	-	-
*Pseudomonas sp.*_NDS_0-4	+++	+++	-	-	-	-	-
*Pseudomonas sp.*_NDS_0-5	+++	+++	-	-	-	-	-
*Flavobacterium sp.*_NDS_0-7	+++	+++	-	-	-	-	-
*Pseudomonas sp.*_NDS_0-8	+++	+++	+++	+	-	-	-
*Hydrogenophaga sp.*_NDS_0-11	+++	+++	-	-	-	-	-
*Porphyrobacter sp.*_NDS_0-12	++	++	-	-	-	-	-
*Brevundimonas sp.*_NDS_1/2-2	+++	+++	-	-	-	-	-
*Pseudomonas sp.*_NDS_1/2-4	+++	+++	+++	+	-	-	-
*Pseudomonas sp.*_NDS_1/2-5	+++	+++	+++	+	-	-	-
*Agrobacterium sp.*_NDS_1/2-6	+++	+++	+	+	-	-	-
*Rheinheimera sp.*_NDS_5-1	+++	+++	+	+	-	-	-
Pseudomonas sp._NDS_5-4	+++	+++	-	-	-	-	-
Pseudomonas sp.._NDS_5-5	+++	+++	-	-	-	-	-
*Bacillus sp.*_NDF_Durant1	+++	+++	++	+	+	-	-
*Paenibacillus sp.*_NDF_Durant2	+++	-	-	-	-	-	-
Bacillus sp._NDF_Durant3	+++	+++	++	+	-	-	-
Chromohalobacter sp._NDF_3-2	-	+	+++	+++	+++	+++	+++
*Bacillus sp.*_NDP_P1L	+++	+++	-	-	-	-	-
*Cellulomonas sp.*_NDP_11AY	+++	+++	++	+	-	-	-
*Microbacterium sp.*_NDP_PYT	+++	+++	++	+	+	+	+
*Curtobacterium sp.*_NDP_AP	+++	+++	++	+	-		
*Micrococcus sp.*_NDP_PYO	+++	+++	+++	+++	+++	++	+
*Paracoccus sp.*_NDP_3-3Orange	+++	+++	++	+	+	+	-
Klebsiella sp._CF_1A1	+++	+++	++	++	+	+	+
Raoultella sp._*CF_2-1*	+++	+++	++	++	+	-	-
*Enterobacter sp.*_CFKERO2	+++	+++	++	++	+	-	-
*Proteus sp.*_CF_3A1	+++	+++	++	++	+	-	-
*Pseudomonas sp.*_CF_LR12	+++	+++	++	-	-	-	-
*Pseudomonas sp.*_CF_LR14	+++	+++	++	-	-	-	-
*Shewanella sp.*_CF_1C1	+++	+++	-	-	-	-	-
*Pontibacter sp.*_CF_LR15	+++	+++	+	+	-	-	-
*Bacillus sp.*_CF_LR5	+++	+++	+++	+++	+++	++	+
*Achromobacter sp.*_CF_3-1	+++	+++	++	++	-	-	-
*Pseudomonas sp.*_CF_LR8	+	++	-	-	-	-	-
*Shewanella sp.*_CF_KERO1-1	+++	+++	++	-	-	-	-
Shewanella sp._CF_LR2	+++	+++	++	++	++	-	-
*Bacillus sp.*_WP_WY	+++	+++	++	++	++	+	+

Table 3. Growth response to salinity conditions. Growth Key: (-) = no growth, (+) = little growth, (++) moderate growth, (+++) = robust growth.

Organism	28°C	37°C	45°C
*Algoriphagus sp.*_NDS_0-1	+	+	-
*Pseudomonas sp.*_NDS_0-2	+	+	-
*Sphingobium sp.*_NDS_0-3	+	+	-
*Pseudomonas sp.*_NDS_0-4	+	+	-
*Pseudomonas sp.*_NDS_0-5	+	-	-
*Flavobacterium sp.*_NDS_0-7	+	+	-
*Pseudomonas sp.*_NDS_0-8	+	+	-
*Hydrogenophaga sp.*_NDS_0-11	+	-	-
*Porphyrobacter sp.*_NDS_0-12	+	+	-
*Brevundimonas sp.*_NDS_1/2-2	+	+	+
*Pseudomonas sp.*_NDS_1/2-4	+	+	-
*Pseudomonas sp.*_NDS_1/2-5	+	+	-
*Agrobacterium sp.*_NDS_1/2-6	+	+	+
*Rheinheimera sp.*_NDS_5-1	+	+	-
Pseudomonas sp._NDS_5-4	+	-	-
*Pseudomonas sp.*_NDS_5-5	+	+	-
*Bacillus sp.*_NDF_Durant1	+	-	-
*Paenibacillus sp.*_NDF_Durant2	+	-	-
Bacillus sp._NDF_Durant3	+	+	-
Chromohalobacter sp_NDF_3-2	+	+	-
*Bacillus sp.*_NDP_P1L	+	+	+
*Cellulomonas sp.*_NDP_11AY	+	+	+
*Microbacterium sp.*_NDP_PYT	+	-	-
*Curtobacterium sp.*_NDP_AP	+	+	-
*Micrococcus sp.*_NDP_PYO	+	+	+
*Paracoccus sp.*_NDP_3-3Orange	+	-	-
Klebsiella sp._CF_1A1	+	+	+
Raoultella sp.*_CF_2-1*	+	+	+
*Enterobacter sp.*_CF_KERO2	+	+	+
*Proteus sp.*_CF_3A1	+	+	+
*Pseudomonas sp.*_CF_LR12	+	+	+
*Pseudomonas sp.*_CF_LR14	+	+	-
*Shewanella sp.*_CF_1C1	+	+	-
*Pontibacter sp.*_CF_LR15	+	+	-
*Bacillus sp.*_CF_LR5	+	+	+
*Achromobacter sp.*_CF_3-1	+	+	-
*Pseudomonas sp.*_CF_LR8	+	+	-
*Shewanella sp.*_CF_KERO1-1	+	+	-
Shewanella sp._CF_LR2	+	+	-
*Bacillus sp.*_WP_WY	+	+	+

Table 4. Temperature growth range results. Growth Key: (-) = no growth, (+) = growth.

When considering the NDF isolates' ability to grow at high salinity and elevated temperature, only two isolates meet the likely criteria for survival in flowback water, i.e., *Chromohalobacter sp.*_NDF_3-2 and *Bacillus sp*._NDF_Durant3. This suggests that these isolates may have originated from the petroleum formation and not the source water. The other two NDF isolates' origins remain debatable but the evidence suggests that isolate, *Paenibacillus sp*._NDF_Durant2, probably originated from the source water/surface because it cannot grow at 37°C, 45°C, or salinities above 3%. *Bacillus sp*._NDF_Durant1 also likely did not originate from the petroleum formation because it could not grow at 37°C or 45°C even though it could grow at salinities above 3%.

The data collected from NDP suggest that isolates *Cellulomonas sp*._NDP_11AY and *Micrococcus* sp._NDP_PYO are adapted to survive in produced water conditions and likely originated from the petroleum formation because they can grow up to 18% salinity and 45°C. *Microbacterium sp*._NDP_PYT, *Curtobacterium sp*._NDP_AP and *Paracoccus sp*._NDP_3-3Orange could grow up to 9% salinity and 28°C, which suggests that they could have originated in the produced water at a later time when the conditions became more favorable, but likely either were dormant in the petroleum formation or introduced during the movement of the water to the surface. The characterization data suggest that *Bacillus sp*._NDP_P1L was present in its dormant endospore form and likely not from the petroleum formation because it failed to grow under high salinity conditions typically experienced in the formation. It can be hypothesized that the harsh conditions encountered in the petroleum formation selects for certain microorganisms such as members from the class Actinobacteria. The NDP characterization data supports other published research showing that the majority of the

microorganisms from produced waters are adapted to the harsh conditions and stress (Mohan et al., 2014).

pH and Oxidative-Fermentation Test

Results of the pH testing (Table 5) reveal that all isolates could grow on pH 5 – 11 agar plates, which is within the pH range of fracturing water previously reported (Mohan et al., 2013b; Struchtemeyer, C.G. and Elshahed, M.S., 2012). These results suggest that changes in the pH of the fracturing fluids alone during the fracturing process, and mixing of brine with the fracturing fluids in the subsurface, most likely have little or no negative impact on microbial growth and/or survival. Comparison of the hydraulic fracturing fluids reveals that WP (100%), NDP (67%), and CF (54%) isolates could grow under acidic conditions (pH 3) when compared to NDS (0%) & NDF (25%) isolates which demonstrated far less ability to cope with high salt content. These results provide more supporting evidence that microorganisms found in produced water tend to be adapted to survive harsh conditions.

The oxidative-fermentation test determines which isolates can produce acidic by-products from oxidative metabolism, fermentation or both. The majority of WP (100%), CF (85%), and NDF (75%) could oxidize glucose into acidic by-products while a significant percentage of isolates from NDP (50%), and NDS (44%) could as well (Table 6). More isolates from WP (100%), CF (62%) and NDF (50%) could ferment glucose into acidic by-products, while fewer isolates from NDP (33%) and NDS (6%) could so. Isolates capable of producing acidic by-products through both oxidative metabolism and fermentation are; *Bacillus sp._NDF*_Durant1, *Bacillus sp._NDF*_Durant3, *Cellulomonas sp.*_NDP_11AY, *Curtobacterium sp._NDP*_AP,

*Klebsiella sp.*_CF_1A1, *Raoultella sp.*_CF_2-1, *Enterobacter sp.*_CF_KERO2, *Proteus sp.*_CF_3A1, *Pontibacter sp.*_CF_LR15, *Bacillus sp.*_CF_LR5, *Shewanella sp.*_CF_KERO1-1, *Shewanella sp.*_CF_LR2, and Bacillus sp._WP_WY. These observations indicate that deleterious microorganisms associated with MIC appear to be selected for at some point after the injection of the fracturing fluids. All the samples had a significant percentage of isolates capable of producing acidic by-products oxidatively, therefore, caution should be taken when reusing flowback or produced water for further hydraulic fracturing. The results also stress the importance of well maintenance for preventing MIC because a significant percentage of isolates from produced water samples are capable of fermentative production of acid.

Biofilm formation plays a vital role in MIC and research has shown that within biofilms, APB can create an acidic environment in the biofilm that differs from the surrounding fluid by a factor of 2 pH units (Vroom et al., 1999). The pH results here revealed that a higher percentage of isolates from flowback and produced water samples were able to grow at pH < 4 compared to the source water sample. The O-F test also showed a similar trend where a higher percentage of isolates from flowback and produced water samples were able to produce acidic by-products anaerobically. The majority of studies related to MIC neglect to focus on the influence APB alone may have in the corroding pipes, but a recent publication using theoretical modeling suggests that APB alone can be just as detrimental as SRBs in pipe corrosion (Gu, 2014). The results of the pH and O-F tests show that a significant number of isolates in flowback and produced waters are APB and that those isolates are more tolerant to acidic pH. It can be hypothesized that those APB could survive deep in biofilms that are anaerobic and acidic, thus presenting a corrosion threat to the oil industry. This indicates that more research

relating to microorganisms found in hydraulic fracturing waters should be directed towards APB and their role in MIC.

Organism	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10	pH 11	pH 12	pH 13
*Algoriphagus sp.*_NDS_0-1	-	-	+	++	++	++	+	+	+	+	-
*Pseudomonas sp.*_NDS_0-2	-	+	++	++	++	++	++	++	++	++	+
*Sphingobium sp.*_NDS_0-3	-	++	++	++	++	++	++	++	++	+	-
*Pseudomonas sp.*_NDS_0-4	-	+	++	++	++	++	++	++	++	++	+
*Pseudomonas sp.*_NDS_0-5	-	+	+	++	++	++	++	++	++	++	-
*Flavobacterium sp.*_NDS_0-7	-	+	++	++	++	++	++	++	++	++	-
*Pseudomonas sp.*_NDS_0-8	-	++	++	++	++	++	++	++	++	++	++
*Hydrogenophaga sp.*_NDS_0-11	-	+	++	++	++	++	++	++	++	++	+
*Porphyrobacter sp.*_NDS_0-12	-	++	++	++	++	++	++	++	++	++	-
*Brevundimonas sp.*_NDS_1/2-2	-	++	++	++	++	++	++	++	++	++	-
*Pseudomonas sp.*_NDS_1/2-4	-	++	++	++	++	++	++	++	++	++	-
*Pseudomonas sp.*_NDS_1/2-5	-	++	++	++	++	++	++	++	++	++	++
*Agrobacterium sp.*_NDS_1/2-6	-	++	++	++	++	++	++	++	++	++	++
*Rheinheimera sp.*_NDS_5-1	-	-	+	++	++	++	++	++	++	++	++
Pseudomonas sp._NDS_5-4	-	+	++	++	++	++	++	++	++	++	++
*Pseudomonas sp.*_NDS_5-5	-	+	+	++	++	++	++	++	++	-	-
*Bacillus sp.*_NDF_Durant1	-	-	-	+	++	++	++	++	++	++	-
*Paenibacillus sp.*_NDF_Durant2	-	-	++	++	++	++	++	++	++	-	-
Bacillus sp._NDF_Durant3	+	++	++	++	++	++	++	++	++	++	++
Chromohalobacter sp_NDF_3-2	-	-	++	++	++	++	++	++	++	++	-
*Bacillus sp.*_NDP_P1L	+	+	++	++	++	++	++	++	++	++	+
*Cellulomonas sp.*_NDP_11AY	-	+	++	++	++	++	++	++	++	++	-
*Microbacterium sp.*_NDP_PYT	++	++	++	++	++	++	++	++	++	++	++
*Curtobacterium sp.*_NDP_AP	++	++	++	++	++	++	++	++	++	++	-
*Micrococcus sp.*_NDP_PYO	+	++	++	++	++	++	++	++	++	++	++
*Paracoccus sp.*_NDP_3-3Orange	-	++	++	++	++	++	++	++	++	+	-
Klebsiella sp._CF_1A1	++	++	++	++	++	++	++	++	++	++	+
Raoultella sp.*_CF_2-1*	++	++	++	++	++	++	++	++	++	++	+
*Enterobacter sp.*_CF_KERO2	++	++	++	++	++	++	++	++	++	++	+
*Proteus sp.*_CF_3A1	+	++	++	++	++	++	++	++	++	++	++
*Pseudomonas sp.*_CF_LR12	++	++	++	++	++	++	++	++	++	++	++
*Pseudomonas sp.*_CF_LR14	-	++	++	++	++	++	++	++	++	+	-
*Shewanella sp.*_CF_1C1	-	++	++	++	++	++	++	++	++	++	-
*Pontibacter sp.*_CF_LR15	-	-	-	+	++	++	++	++	++	++	-
*Bacillus sp.*_CF_LR5	++	++	++	++	++	++	++	++	++	++	-
*Achromobacter sp.*_CF_3-1	++	++	++	++	++	++	++	++	++	++	++
*Pseudomonas sp.*_CF_LR8	-	+	++	++	++	++	++	++	++	+	-
*Shewanella sp.*_CF_KERO1-1	-	++	++	++	++	++	++	++	++	++	-
Shewanella sp._CF_LR2	-	++	++	++	++	++	++	++	++	++	-
*Bacillus sp.*_WP_WY	++	++	++	++	++	++	++	++	++	++	++

Table 5. pH growth range. Growth Key: (-) = no growth, (+) = growth, (++) robust growth.

Organism	Oxidize	Ferment
*Algoriphagus sp.*_NDS_0-1	-	-
*Pseudomonas sp.*_NDS_0-2	+	-
*Sphingobium sp.*_NDS_0-3	+	-
*Pseudomonas sp.*_NDS_0-4	-	-
*Pseudomonas sp.*_NDS_0-5	-	-
*Flavobacterium sp.*_NDS_0-7	-	+
*Pseudomonas sp.*_NDS_0-8	+	-
*Hydrogenophaga sp.*_NDS_0-11	-	-
*Porphyrobacter sp.*_NDS_0-12	-	-
*Brevundimonas sp.*_NDS_1/2-2	+	-
*Pseudomonas sp.*_NDS_1/2-4	+	-
*Pseudomonas sp.*_NDS_1/2-5	+	-
*Agrobacterium sp.*_NDS_1/2-6	+	-
*Rheinheimera sp.*_NDS_5-1	-	-
Pseudomonas sp._NDS_5-4	-	-
*Pseudomonas sp.*_NDS_5-5	-	-
*Bacillus sp.*_NDF_Durant1	+	+
*Paenibacillus sp.*_NDF_Durant2	-	-
Bacillus sp._NDF_Durant3	+	+
Chromohalobacter sp_NDF_3-2	+	-
*Bacillus sp.*_NDP_P1L	-	-
*Cellulomonas sp.*_NDP_11AY	+	+
*Microbacterium sp.*_NDP_PYT	+	-
*Curtobacterium sp.*_NDP_AP	+	+
*Micrococcus sp.*_NDP_PYO	-	-
*Paracoccus sp.*_NDP_3-3Orange	-	-
Klebsiella sp._CF_1A1	+	+
Raoultella sp.*_CF_2-1*	+	+
*Enterobacter sp.*_CF_KERO2	+	+
*Proteus sp.*_CF_3A1	+	+
*Pseudomonas sp.*_CF_LR12	+	-
*Pseudomonas sp.*_CF_LR14	+	-
*Shewanella sp.*_CF_1C1	-	-
*Pontibacter sp.*_CF_LR15	+	+
*Bacillus sp.*_CF_LR5	+	+
*Achromobacter sp.*_CF_3-1	-	-
*Pseudomonas sp.*_CF_LR8	+	-
*Shewanella sp.*_CF_KERO1-1	+	+
Shewanella sp._CF_LR2	+	+
*Bacillus sp.*_WP_WY	+	+

Table 6. Oxidative-Fermentation Testing. Key: (+) positive, (-) negative.

Hydrocarbon utilization, E24 and BATH screenings for Biosurfactants and Bioemulsifiers

All isolates were tested for their ability to utilize hydrocarbons (n-hexadecane and kerosene) as their sole carbon and energy (Table 7, Figure A.2-A.3). From source water (NDS), 37% of the isolates were able to utilize n-hexadecane while 31% could utilize kerosene as a sole source of carbon and energy. Flowback water samples yielded 50% (NDF) and 15% (CF) isolates capable of utilizing n-hexadecane and none of the isolates able to utilize kerosene. None of the isolates in produced water (NDP and WP) could utilize n-hexadecane while a few isolates from NDP (33%) could utilize kerosene. Subsurface hydrocarbons are a ready source of carbon and energy for formation microorganisms and surprisingly, very few of the microorganisms isolated from flowback and produced water samples with the exception of NDF were able to grow on the two hydrocarbons. These results support what oil industry researchers know about indigenous freshwater and marine microorganisms, i.e., they often have the ability to utilize and/or degrade hydrocarbons, which is one of the many reasons for biocide usage in hydraulic fracturing (Hazen, T.C., Dubinsky, E.A., DeSantis, T.Z., Andersen, G.L., Piceno, Y.M., Singh, N., Jansson, J.K., Probst, A., Borglin, S.E., Fortney, J.L., Stringfellow, W.T., Bill, M., Conrad, M.E., Tom, L.M., Chavarria, K.L., Alusi, T.R., Lamendella, R., Joyner, D.C., Spier, C., Baelum, J., Auer, M., Zemla, M.L., Chakraborty, R., Sonnenthal, E.L., D'haeseleer, P., Holman, H.Y., Osman, S., Lu, Z., Van Nostrand, J.D., Deng, Y., Zhou, J. and Mason, O.U., 2010; Kostka, J.E., Prakash, O., Overholt, W.A., Green, S.J., Freyer, G., Canion, A., Delgardio, J., Norton, N., Hazen, T.C. and Huettel, M., 2011). .

Various methods have been used to screen environmental isolates that produce biosurfactants (BS) and bioemulsifiers (BE), particularly from environments in the presence of hydrocarbons or contaminated by hydrocarbons (Batista, S.B., Mounteer, A.H., Amorim, F.R. and Tótola, M.R., 2006; Bodour, A.A., Drees, K.P. and Maier,R.M., 2003; Hommel, 1990; Neufeld, R.J. and Zajic, J.E., 1984; Satpute, S.K., Banpurkar, A.G., Dhakephalkar, P.K., Banat, I.M. and Chopade, B.A., 2010). The results from this study indicate that the E_{24} assay would serve well as the primary screening method for BS/BE producing microorganisms because this test detects BS and BE by the creation of visible emulsions. Table 8 shows each isolate's emulsification index listed from highest to lowest with isolates *Pseudomonas sp.*_NDS_0-2, *Pseudomonas sp.*_NDS_5-4, *Pseudomonas sp.*_NDS_5-5, and *Klebsiella sp.*_CF_1A1, and *Bacillus sp*._CF_LR5 providing the top five values. Approximately 77% of CF, 69% of NDS, and 50% of NDF isolates are potential BS/BE producing candidates, using an arbitrary EI cutoff value of 0.30. Only 17% of NDP and 0% of WP isolates are potential candidates indicating that produced water samples are not good sources of these potential candidates. There is no pattern indicating that a type of fracturing water yields more microbes capable of emulsion production, in fact, it appears from the isolates tested that more surface-associated (source and flowback water samples) isolates are capable of emulsion production, a trait most often associated with hydrocarbon utilization.

The BATH assay determines the cell surface hydrophobicity of microorganisms and can indicate which isolates produce biosurfactants or bioemulsifiers at the surface of their cells, compared to secreting them into their surrounding environment. A few isolates had negative %H values, which is hypothesized to be the result of cell lysis due to the presence of

hydrocarbons (Saini, 2010). The results of the E_{24} and BATH tests shown in Table 7 & 8 have identified a few novel BS/BE producing microorganisms: Algoriphagus *sp*._NDS_0-1, *Sphingobium sp*._NDS_0-3, *Flavobacterium sp*._NDS_0-7, *Hydrogenophaga sp*._NDS_0-11, *Brevundimonas sp*._NDS_1/2-2, *Chromohalobacter sp*._NDF_3-2, *Pontibacter sp*._CF_LR15, *Bacillus sp*._CF_LR5, *Shewanella sp*._CF_KERO1-1, *Shewanella sp*._CF_LR2 and *Bacillus sp*._WP_WY. These are novel because there are no published data regarding the compounds these microorganisms produce. Isolates that likely produce these compounds at the surface of their cells include, Algoriphagus *sp*._NDS_0-1, *Sphingobium sp*._NDS_0-3, *Pseudomonas sp*._NDS_1/2-4, *Pseudomonas sp.*_NDS_1/2-5, *Raoultella sp.*_CF_2-1, and *Enterobacter sp.*_CF_KERO2, based on their high %H values. Known BS/BE-producing isolates include *Bacillus sp*._WP_WY, *Raoultella sp.*_CF_2-1, and *Enterobacter sp.*_CF_KERO2, which are the subject of publications acknowledging similar species from the same genera capable of creating emulsions with petroleum oils (Calvo, Toledo, & González-López, 2004; Pérez-Armendáriz, Mauricio-Gutiérrez, Jiménez-Salgado, Tapia-Hernández, & Santiesteban-López, 2013; Sarafzadeh, Hezave, Ravanbakhsh, Niazi, & Ayatollahi, 2013). The results of the two screening tests indicate that hydraulic fracturing waters can serve as a source for novel microorganisms with biotechnological applications pertaining to petroleum degradation and BS/BE production.

Organism	HC-H	Growth $O.D_{420\ nm}$[a]	HC-K	Growth $O.D_{420\ nm}$[b]	BATH %H	% SD
Algoriphagus sp._NDS_0-1	N	0.005	Y	0.030	11.8	2.3
Pseudomonas sp._NDS_0-2	Y	0.399	Y	0.136	4.3	2.5
Sphingobium sp._NDS_0-3	N	-0.003	N	-0.016	18.3	1.9
Pseudomonas sp._NDS_0-4	Y	0.043	N	-0.005	4.1	1.4
Pseudomonas sp._NDS_0-5	Y	0.16	Y	0.014	2.7	0.8
Flavobacterium sp._NDS_0-7	N	0.001	Y	0.189	8.8	2.4
Pseudomonas sp._NDS_0-8	N	0.007	N	-0.002	1.8	0.4
Hydrogenophaga sp._NDS_0-11	Y	0.124	Y	0.023	0.3	0.2
Porphyrobacter sp._NDS_0-12	N	0.003	N	-0.016	5.1	2.3
Brevundimonas sp._NDS_1/2-2	Y	0.094	N	-0.017	7.0	0.6
Pseudomonas sp._NDS_1/2-4	N	-0.002	N	-0.002	1.8	1.0
Pseudomonas sp._NDS_1/2-5	N	0.004	N	-0.014	35.2	4.2
Agrobacterium sp._NDS_1/2-6	N	-0.002	N	-0.011	15.1	3.9
Rheinheimera sp._NDS_5-1	N	-0.007	N	-0.013	43.2	4.9
Pseudomonas sp._NDS_5-4	Y	0.123	Y	0.015	-1.3	1.9
Pseudomonas sp._NDS_5-5	N	0.005	N	-0.007	1.9	0.3
Bacillus sp._NDF_Durant1	N	-0.006	N	-0.026	4.8	2.1
Paenibacillus sp._NDF_Durant2	N	0.006	N	-0.009	12.2	4.9
Bacillus sp._NDF_Durant3	Y	0.182	N	-0.018	16.2	5.8
Chromohalobacter sp_NDF_3-2	Y	0.010	N	-0.001	9.8	0.5
Bacillus sp._NDP_P1L	N	-0.002	N	-0.009	2.2	1.4
Cellulomonas sp._NDP_11AY	N	0.000	Y	0.051	5.0	1.0
Microbacterium sp._NDP_PYT	N	0.002	Y	0.011	1.2	3.7
Curtobacterium sp._NDP_AP	N	0.000	N	-0.016	-13.3	2.4
Micrococcus sp._NDP_PYO	N	0.000	N	-0.017	56.5	5.6
Paracoccus sp._NDP_3-3Orange	N	0.004	N	-0.040	59.9	8.3
Klebsiella sp._CF_1A1	N	-0.003	N	-0.015	2.2	1.5
Raoultella sp.*_CF_2-1*	Y	0.019	N	-0.011	10.8	3.6
Enterobacter sp._CF_KERO2	N	-0.005	N	-0.009	35.6	12.1
Proteus sp._CF_3A1	N	-0.008	N	-0.016	3.2	1.2
Pseudomonas sp._CF_LR12	N	0.016	N	-0.010	1.9	0.8
Pseudomonas sp._CF_LR14	N	0.002	N	-0.015	15.4	3.0
Shewanella sp._CF_1C1	N	-0.002	N	-0.016	3.9	1.7
Pontibacter sp._CF_LR15	N	0.000	N	-0.019	-11.2	6.4
Bacillus sp._CF_LR5	N	0.014	N	0.006	-2.7	1.3
Achromobacter sp._CF_3-1	N	-0.006	N	-0.018	7.5	1.9
Pseudomonas sp._CF_LR8	Y	0.216	N	-0.018	-2.4	2.0
Shewanella sp._CF_KERO1-1	N	0.005	N	-0.010	6.3	1.6
Shewanella sp._CF_LR2	N	0.003	N	-0.017	1.3	0.9
Bacillus sp._WP_WY	N	0.002	N	0.006	0.6	0.1

Table 7. Hydrocarbon utilization and bacterial adhesion to hydrocarbons (BATH) results. (n = 4 for BATH, and n = 3 for hydrocarbon utilization). HC-H represents n-hexadecane, HC-K represents kerosene. [a] Average difference in absorbance between no carbon and n-hexadecane at day 35. [b] Average difference in absorbance between no carbon and kerosene at day 30.

Organism	Average EI %	% Std. Deviation
Klebsiella sp._CF_1A1	57.4	3.2
***Pseudomonas sp._*NDS_0-2**	55.3	8.2
***Pseudomonas sp._*NDS_5-5**	55.1	9.7
Pseudomonas sp._NDS_5-4	54.8	9.4
***Bacillus sp._*CF_LR5**	53.8	2.5
***Enterobacter sp._*CF_KERO2**	52.8	3.9
***Pseudomonas sp._*CF_LR8**	51.4	12.4
***Shewanella sp._*CF_KERO1-1**	47.6	7.3
Raoultella sp.*_CF_2-1*	45.4	3.0
***Agrobacterium sp._*NDS_1/2-6**	45.1	11.9
***Proteus sp._*CF_3A1**	44.6	8.2
***Flavobacterium sp._*NDS_0-7**	44.3	6.4
***Brevundimonas sp._*NDS_1/2-2**	42.2	14.1
***Pseudomonas sp._*NDS_0-5**	40.4	12.1
***Pseudomonas sp._*NDS_1/2-5**	39.8	14.9
***Algoriphagus sp._*NDS_0-1**	39.6	5.9
***Achromobacter sp._*CF_3-1**	38.8	14.8
***Bacillus sp._*NDF_Durant1**	37.8	19.6
***Sphingobium sp._*NDS_0-3**	36.7	4.5
***Pseudomonas sp._*CF_LR12**	36.1	7.6
***Hydrogenophaga sp._*NDS_0-11**	35.4	17.9
Chromohalobacter sp_NDF_3-2	35.2	7.5
***Pontibacter sp._*CF_LR15**	31.3	4.9
***Paracoccus sp._*NDP_3-3Orange**	30.0	8.6
***Pseudomonas sp._*NDS_0-4**	24.5	15.8
Shewanella sp._CF_LR2	21.9	19.4
***Bacillus sp._*WP_WY**	20.5	19.6
***Shewanella sp._*CF_1C1**	16.0	5.1
***Pseudomonas sp._*NDS_0-8**	14.7	3.7
***Pseudomonas sp._*CF_LR14**	12.2	14.6
***Porphyrobacter sp._*NDS_0-12**	11.7	14.0
***Cellulomonas sp._*NDP_11AY**	10.0	0.0
***Microbacterium sp._*NDP_PYT**	10.0	11.5
***Pseudomonas sp._*NDS_1/2-4**	8.9	13.6
***Paenibacillus sp._*NDF_Durant2**	1.8	4.4
***Rheinheimera sp._*NDS_5-1**	1.7	4.1
Bacillus sp._NDF_Durant3	0.0	0.0
***Bacillus sp._*NDP_P1L**	0.0	0.0
***Curtobacterium sp._*NDP_AP**	0.0	0.0
***Micrococcus sp._*NDP_PYO**	0.0	0.0

Table 8. Emulsification Index values for all culturable isolates.

Biocide Resistance Assay

Bactericidal testing in glutaraldehyde solutions (contact time of 24 hours) revealed that 100 ppm biocide concentration exhibited no bactericidal effect on 37.5% of NDS, 50% of NDF & NDP, 61% of CF and 100% of WP, i.e., viable cells survived the treatment (Table 9). These results indicate that isolates from flowback and produced water are more resistant to biocides and that some isolates from the source water are naturally unaffected by this particular biocide at a 100 ppm concentration. A more concentrated glutaraldehyde concentration (200 ppm) was shown to be bactericidal to all isolates with the exception of *Klebsiella sp.*_CF_1A1, where one out of three tubes showed growth when plated on R2B after a 24 hr contact time with the biocide. The 200 ppm glutaraldehyde concentration is the same as the minimum inhibitory concentration (MIC) determined previously by Struchtemeyer et al. (2012). These are the first data for efficiency of glutaraldehyde on individual culturable microorganisms isolated from hydraulic fracturing waters.

Taken all together, only three isolates, all Pseudomonas sp. from NDS (*Pseudomonas* sp._NDS_0-8, *Pseudomonas* sp._NDS_1/2-4, and *Pseudomonas* sp._NDS_1/2-5) would probably survive the chemical additives encountered in flowback water because they are resistant to 100 ppm glutaraldehyde, can grow at 9% salinity and 37°C. The fact that the only organisms from the source water sample able to grow under the conditions of the flowback and produce water are from the genus *Pseudomonas,* and that no members from that genus were recovered from NDF or NDP, indicated that an effective biocide application was used in the North Dakota fracturing operation.

The CF and WP samples allow comparison between flowback and produced water isolates from different geographies and shale plays. The large number of isolates recovered from CF indicates that either the biocide application was insufficient in controlling microbial growth or that the conditions in the shale formation were not harsh. Interestingly, 31% of the isolates from CF belong to the family Enterobacteriaceae, which might suggest some interaction with black water or agricultural wastewater (possibly runoff from cattle farms because of associated odor). The lack of geochemical data from CF obstructs our assessment of conditions encountered in the flowback water. The microbial characterization of *Klebsiella sp.*_CF_1A1 and *Bacillus sp*._CF_LR5 suggests that they were able to survive the harsh conditions associated with a petroleum formation because they could grow at pH 4-5, 18% salinity and 37°C. The presence of CF isolates from the families Pseudomonadaceae and Enterobacteriaceae, and their characterization data, makes it probable that they originated from the source water and not the subsurface. WP only yielded one culturable isolate, *Bacillus sp*._WP_WY, which was obtained from a high dilution (needed to dilute the biocide in the sample before growth could occur) which resulted in a low CFU count (< 10). Low cell counts and the necessity for a high dilution before seeing colony growth, indicates that proper biocide application was practiced and/or that the conditions of the petroleum formation were harsh and unfavorable for survival. The WP isolate could survive in the petroleum formation in that it was able to grow at all temperatures, pH values and salinity values tested and supports other observations regarding the presence of members from the phylum Firmicutes which are likely to be associated with produced waters and petroleum formations.

Organism	Bactericidal (100 ppm)	Bactericidal (200 ppm)
***Algoriphagus sp.*_NDS_0-1**	Yes	Yes
***Pseudomonas sp.*_NDS_0-2**	Yes	Yes
***Sphingobium sp.*_NDS_0-3**	No (1)	Yes
***Pseudomonas sp.*_NDS_0-4**	Yes	Yes
***Pseudomonas sp.*_NDS_0-5**	Yes	Yes
***Flavobacterium sp.*_NDS_0-7**	Yes	Yes
***Pseudomonas sp.*_NDS_0-8**	No (2)	Yes
***Hydrogenophaga sp.*_NDS_0-11**	Yes	Yes
***Porphyrobacter sp.*_NDS_0-12**	No (3)	Yes
***Brevundimonas sp.*_NDS_1/2-2**	No (3)	Yes
***Pseudomonas sp.*_NDS_1/2-4**	No (3)	Yes
***Pseudomonas sp.*_NDS_1/2-5**	No (1)	Yes
***Agrobacterium sp.*_NDS_1/2-6**	Yes	Yes
***Rheinheimera sp.*_NDS_5-1**	Yes	Yes
Pseudomonas sp._NDS_5-4	Yes	Yes
***Pseudomonas sp.*_NDS_5-5**	Yes	Yes
***Bacillus sp.*_NDF_Durant1**	Yes	Yes
***Paenibacillus sp.*_NDF_Durant2**	Yes	Yes
Bacillus sp._NDF_Durant3	No (3)	Yes
Chromohalobacter sp_NDF_3-2	No (3)	Yes
***Bacillus sp.*_NDP_P1L**	No (1)	Yes
***Cellulomonas sp.*_NDP_11AY**	Yes	Yes
***Microbacterium sp.*_NDP_PYT**	Yes	Yes
***Curtobacterium sp.*_NDP_AP**	No (2)	Yes
***Micrococcus sp.*_NDP_PYO**	No (3)	Yes
***Paracoccus sp.*_NDP_3-3Orange**	Yes	Yes
Klebsiella sp._CF_1A1	No (3)	No (1)
Raoultella sp.*_CF_2-1*	No (3)	Yes
***Enterobacter sp.*_CF_KERO2**	No (3)	Yes
***Proteus sp.*_CF_3A1**	No (3)	Yes
***Pseudomonas sp.*_CF_LR12**	Yes	Yes
***Pseudomonas sp.*_CF_LR14**	No (3)	Yes
***Shewanella sp.*_CF_1C1**	No (3)	Yes
***Pontibacter sp.*_CF_LR15**	No (2)	Yes
***Bacillus sp.*_CF_LR5**	No (2)	Yes
***Achromobacter sp.*_CF_3-1**	Yes	Yes
***Pseudomonas sp.*_CF_LR8**	Yes	Yes
***Shewanella sp.*_CF_KERO1-1**	Yes	Yes
Shewanella sp._CF_LR2	Yes	Yes
***Bacillus sp.*_WP_WY**	No (3)	Yes

Table 9. Biocide testing of hydraulic fracturing isolates. No 200 ppm treated tubes showed growth with the exception for isolate Klebsiella sp._CF_1A1, where one out of three tubes was positive when plated. A Yes report indicates that no growth (effective biocide) resulted following the treatment.

Conclusion

In this study, we investigated the microbiology associated with various fluids involved with a hydraulic fracturing operation, with the exception of drilling mud. Approximately 40 unique culturable bacteria were isolated from five different hydraulic fracturing water samples from three geographical locations within the U. S. Data from the source and flowback water isolates from North Dakota suggest that the hydraulic fracturing process (addition of chemicals, specifically biocides, coupled with the high injection pressures) along with the extreme environmental conditions of the petroleum formation, reduces the viable number of microorganisms introduced into the subsurface from the source water. The small number of culturable isolates found in the flowback water compared to the number found in source water, along with their characterization data, provided evidence to support this claim.

The various screening tests for biosurfactant and bioemulsifier (BS/BE) production indicate the importance of cultivation-dependent analysis and suggest that hydraulic fracturing water samples might serve as a source for novel BS/BE producing bacteria. The E_{24} test was the best screening method because it provides direct observation of emulsion formation. Results from the E_{24} and BATH tests have identified a few novel BS/BE producing microorganisms, Algoriphagus *sp*._NDS_0-1, *Sphingobium sp*._NDS_0-3, *Flavobacterium sp*._NDS_0-7, *Hydrogenophaga sp*._NDS_0-11, *Brevundimonas sp*._NDS_1/2-2, *Chromohalobacter sp*._NDF_3-2, *Pontibacter sp*._CF_LR15, *Bacillus sp*._CF_LR5, *Shewanella sp*._CF_KERO1-1, and *Shewanella sp*._CF_LR2 and *Bacillus sp*._WP_WY, that have no published data regarding the production of BS/BE compounds. The results from the BS/BE screening indicate that hydraulic fracturing

waters can serve as a source for novel microorganisms with biotechnological potential pertaining to petroleum degradation and BS/BE production.

The pH, temperature, and salinity data provide direct evidence that NDP isolates are able to grow in adverse environmental conditions compared to NDS isolates, and suggests that these culturable microorganisms are likely active and adapted to survive in the extreme environment. Results presented here support metagenomic observations regarding microorganisms from produced water having a higher abundance of genes related to stress response conditions (Mohan, A.M., Bibby, K.J., Lipus, D., Hammack, R.W. and Gregory, K.B., 2014). The metagenomic study provided data from produced and source water samples from the Marcellus Shale Play, while we provided data from produced water samples from the Bakken and Niobrara-Mowry Shale Plays and source water from the Bakken Shale Play. Both of these reports support a trend regarding microorganism growth under stressed conditions when the microorganisms come from produced water in the hydraulic fracturing process independent of geographic location.

The recent expansion and projected increase in hydraulic fracturing practices in the United States has provided scientists with an opportunity to study the effects this process has on microorganisms present in the fracturing fluids and to determine the effects these microorganisms may have on the process, but only on a limited basis. Lack of disclosure regarding the chemical composition of the fracturing fluids in what hydraulic fracturing companies consider to be a trade secret, along with the unwillingness of these companies to provide fluid samples for research purposes, marginalizes the advancement of research in this

field. We were fortunate to receive water samples involved with hydraulic fracturing operations from North Dakota, Colorado and Wyoming that provided an opportunity to contribute to understanding the microbiology associated with hydraulic fracturing.

APPENDIX

Figure A.1. Enrichment cultures of SRB grown in Postgate Medium C (6.0 g L^{-1} Sodium lactate, 44.5 g L^{-1} Na_2SO_4, 1.0 g L^{-1} NH4Cl, 1.0 g L^{-1} Yeast extract, 0.5 g L^{-1} KH2PO4, 0.3 g L^{-1} Sodium citrate·$2H_2O$, 0.06 g L^{-1} $CaCl_2·6H_2O$, 0.06 g L^{-1} $MgSO_4·7H_2O$, 0.004 g L^{-1} $FeSO_4·7H_2O$, with a final pH between 7.3 – 7.7). Attempts to isolate pure cultures were unsuccessful.

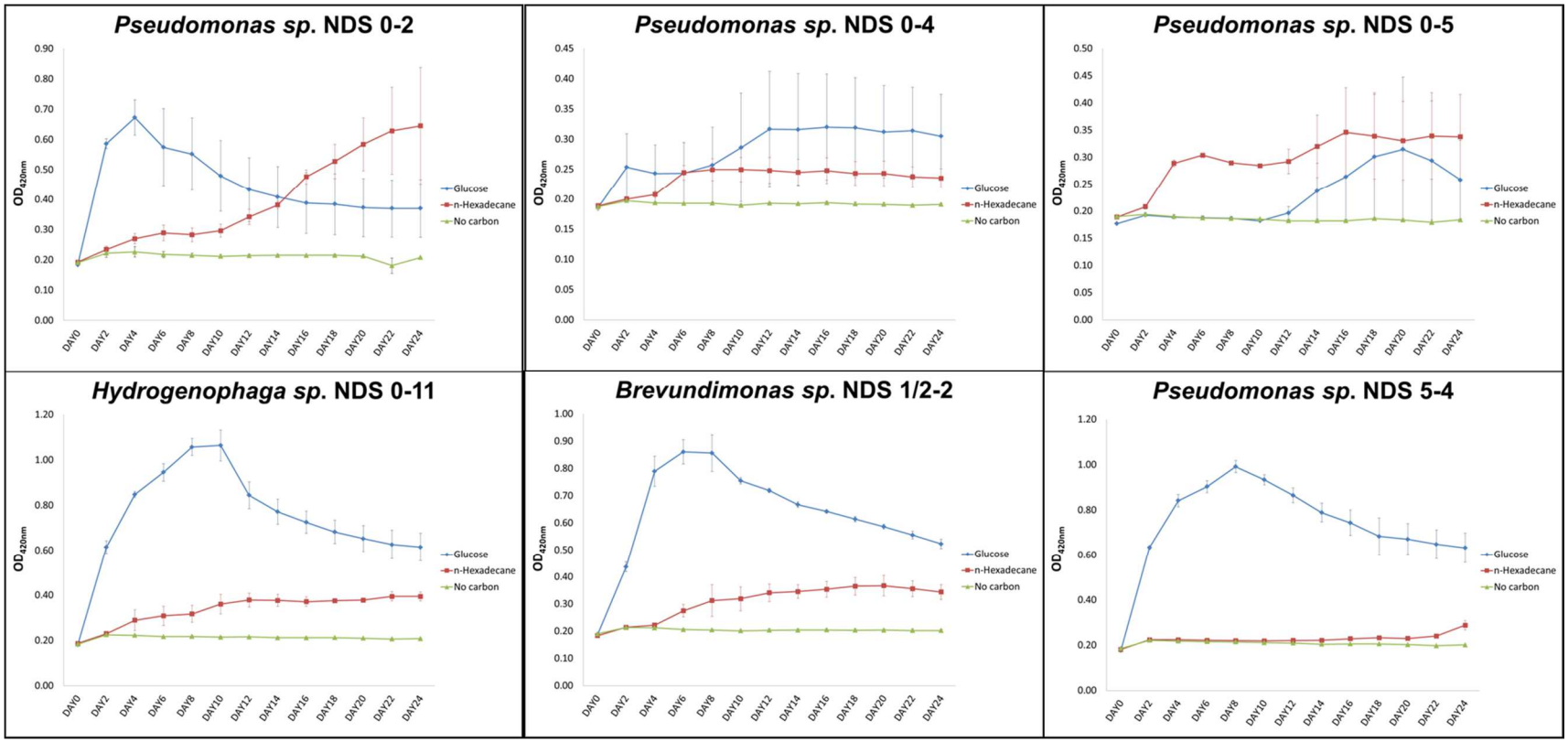

Figure A.2. Hydrocarbon utilization growth curves for source water isolates. Optical density was measured at 420nm, n = 3, and error bars are standard error values.

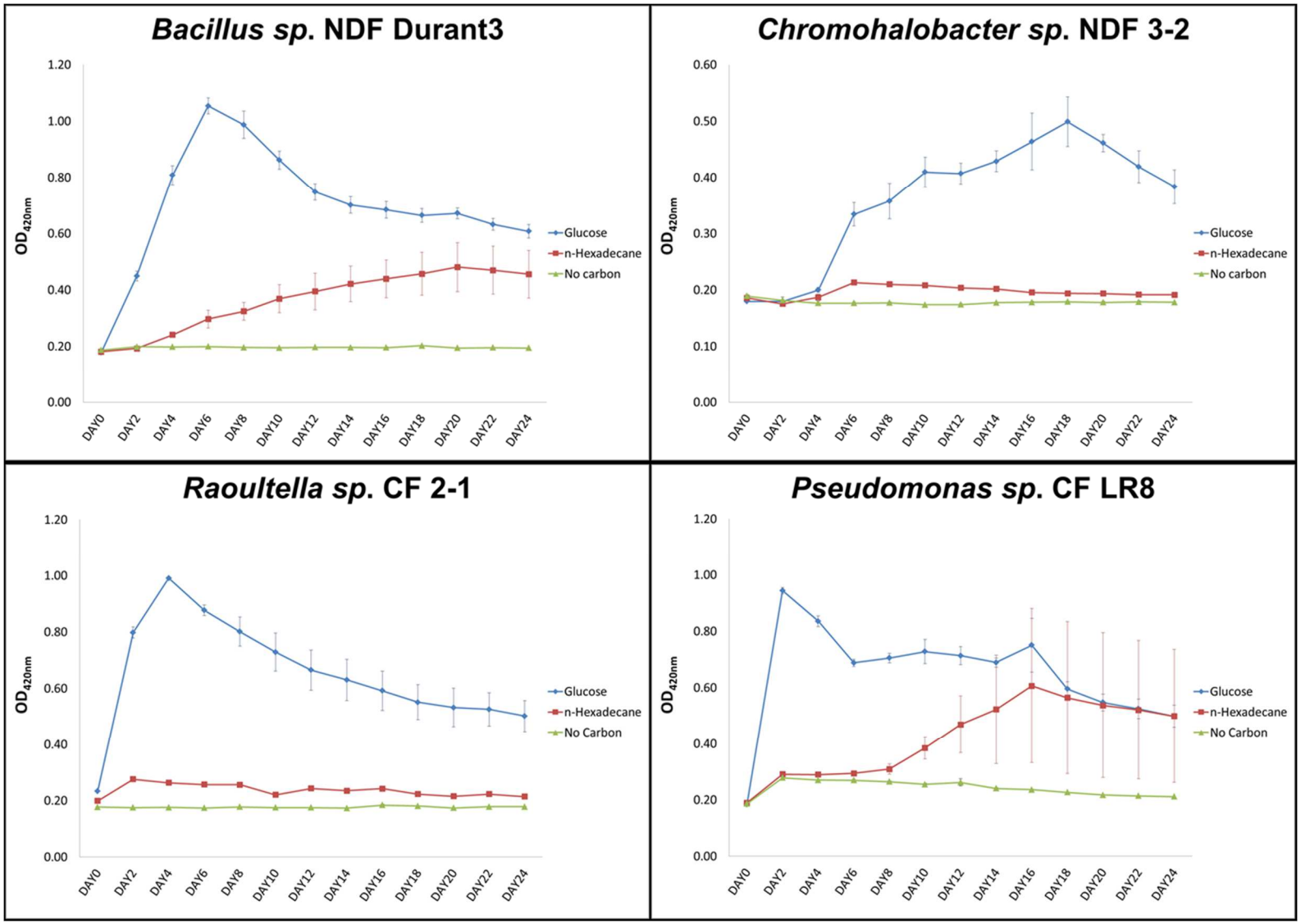

Figure A.3. Hydrocarbon utilization growth curves for flowback water isolates. Optical density was measured at 420nm, n = 3, and error bars are standard error values

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CURRICULUM VITAE

Anthony Harrington

Education

- M.S in Biology, University of Nevada, Las Vegas, August 2015, 3.90 GPA
- B.A in Chemistry, University of Nevada, Las Vegas, June 2012, 3.01 GPA
- B.S in Biology, University of Nevada, Las Vegas, August 2008, 3.02 GPA

Research Experience

- **University of Nevada, Las Vegas (August 2012 – present)**

Cultivation of various microorganisms from various hydraulic fracturing water samples (mostly aerobic-heterotrophic microorganisms and a few anaerobic sulfate-reducing microorganisms). Identification of isolated microorganisms to the genus level with some species level identification by partial sequencing of the 16S rRNA gene (V1-V4 variable region). Created clone libraries of sulfate-reducing bacteria (SRB) by PCR amplifying the dissimilatory sulfate reductase (DSR) gene and cloning the amplified products into pGEM-T vectors which were sequenced to identify the various SRB's contained in a mixed culture. Differentiated 9 closely related *Pseudomonas sp*. by PCR amplifying their repetitive extragenic palindromic elements (rep-PCR) and comparing their fingerprinting pattern. Extensive characterization of 39 unique isolated microorganisms which includes: measuring emulsification indices, salinity range, temperature range, carbon source preference, pH range, and oxidation-fermentation capabilities. Collected preliminary data relating to the isolation and characterization of biosurfactants and bioemulsifiers from microbial isolates that make emulsions with petroleum-based oils (total carbohydrate content, FTIR, MS, H-NMR and C-NMR analysis of solvent extracted compounds).

- **NSF Environmental Microbiology REU Program, University of Nevada, Las Vegas (Summer 2011)**

Identifying the different microorganisms present in a commercial inoculant, Fritzyme, using molecular biology techniques under the guidance of Dr. Penny S. Amy. Measured metabolic activities of Nitrifying bacteria using colorimetric test strips. Identified various microorganisms in Fritzyme by sequencing clone libraries that were created by the cloning of a multiplex-PCR containing amplified product of the 16S rRNA and Nitrifier-specific genes.

- **NSF Bioenergy REU Program, University of Nebraska, Lincoln (Summer 2010)**

Conducted independent research project under the guidance of Dr. Karrie A. Weber, studying the adsorption of T4 bacteriophages to synthetic ferrihydrite with a goal of determining the role bacteriophages may have in the sequestering of organic carbon in aquatic environments.

Work Experience

- **Pure Essences Labs (November 2011 – June 2012)**

  Lab Technician:

  Duties include, following good manufacturing practice, monitoring equipment to ensure proper functioning (daily suitability testing of scales), calibration of instruments (pipettes), identification of amino acids and minerals using FTIR, identification of raw materials using Thin Layer Chromatography, quantifying nutraceutical compounds using UV-VIS, assist chemist and lab director with sample prep (microwave and hot block digestion for ICP-MS), Gas Chromatography analysis for 1,4-dioxane, octyl methoxycinnamate, methylparaben, propylparaben, and butylated hydroxytoluene in cosmetic products such as lotions, shampoos and conditioners performed as contract work for an outside company.

- **Nevada Conservation Corps (May 2008-June 2009)**

  Crew Member:

  Assist project partners (USFW, NPS, BLM, USFS, and UNLV researchers) in restoration projects such as decommissioning of ATV roads in Gold Butte, NV (Area of Environmental Concern), constructing road blocks of trails leading to various petroglyphs, creating road-block barriers protecting cryptobiotic soil, and invasive species control/removal. Provided assistance to the Ash Meadows National Wildlife Refuge staff over the summer of 2008 with population counts of the Armargosa pupfish (*Cyprinodon nevadensis mionectes*) along with trapping and eradicating invasive fish and amphibian species that threaten the endangered pupfish. Assisted NPS personnel in Death Valley with trail maintenance of Wild Rose peak trial along with invasive weed removal of Russian Thistle (Salsola kali) on the valley floor.

Teaching Experience

- **University of Nevada, Las Vegas (August 2012 – 2015)**

  Teaching Assistant

  Biology 189 lab: Fundamentals of Life Science (5 semesters)

  Biology 251 lab: General Microbiology (1 semester)