MICROBIOLOGY OF HYDRAULIC FRACTURING WASTEWATER

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The extraction of natural gas and oil from shale formations using hydraulic fracturing generates large volumes of wastewater, often termed produced water. One of the biggest challenges associated with produced water management is microbial activity. Microorganisms growing in produced water may have the ability to form biofilms and produce acids and sulfides, which can contribute to biocorrosion and gas souring. This dissertation investigates the microbial ecology of microorganisms living in produced water by studying their community structure and metabolic potential as well as the active, genetic response of *Pseudomonas* biofilms to the biocide sodium hypochlorite to inform microbial control. First, storage guidelines for hydraulic fracturing produced waters intended for microbiological analysis were developed. Results suggested microbial communities in produced water samples to remain stable when stored at 4^{o}C for three days or less. Next, the microbial ecology of 42 Marcellus Shale produced water samples was analyzed. Samples were dominated by the taxa *Halanaerobiales*, specifically the genus *Halanaerobium*. Subsequently, metagenomic sequencing and binning allowed the recovery and annotation of a *Halanaerobium* draft genome. Annotation results suggested *Halanaerobium* to have the metabolic potential for acid production and sulfide production through thiosulfate reduction. Microbiological assessment of produced waters from 18 Bakken Shale wells, sampled across a six-month time frame, confirmed the presence of *Halanaerobium* in produced water.

However, the microbial community structure was found to change temporally, and the majority of the samples were dominated by the order *Bacillales*. Finally, the active, genetic response to the broad-spectrum biocide sodium hypochlorite, which is also used for microbial control in hydraulic fracturing operations, was assessed. *Pseudomonas fluorescens* biofilms were exposed to sublethal concentrations of sodium hypochlorite and differential genes expression was analyzed. Results suggested genes involved in oxidative stress response pathways and multidrug efflux mechanisms to be upregulated, demonstrating genetic components to be involved in sodium hypochlorite resistance. Ultimately, findings from this dissertation enhance the current understanding of microbial community dynamics in produced water and may help to limit corrosion, control fouling and souring issues, protect well infrastructure, and minimize unnecessary biocide application.

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1.0 INTRODUCTION

This dissertation investigates the microbial ecology in hydraulic fracturing produced water from the Marcellus and Bakken Shale regions using 16S rRNA and shotgun metagenome sequencing studies, evaluates the metabolic potential of native produced water organisms by recovering and annotating a *Halanaerobium* draft genome, and analyzes genetic hypochlorite response mechanisms in *Pseudomonas* biofilms via differential gene expression analysis.

1.1 MOTIVATION AND OBJECTIVE

1.1.1 Advances in hydraulic fracturing and importance of produced water management

Improvements in high-volume hydraulic fracturing and horizontal drilling techniques have led to an increase in oil and gas production from unconventional reservoirs, changing the current and future energy landscape [1]. As a result, natural gas and oil from shale regions has emerged as one of the leading energy sources in the United States. Hydraulic fracturing also has a global impact, as hydraulic fracturing operations have increased in, for example, Canada and the United Kingdom [2-4]. Shale gas and oil from unconventional reservoirs are extracted from the ground through a process termed hydraulic fracturing (‘fracking’), a technology that uses

pressurized liquids, sand and chemicals that create micro fractures to release hydrocarbons from underground formations [5, 6]. In high-volume hydraulic fracturing operations, large quantities (15-20 million liters) of fracture fluid are injected into the well, fracturing the target formation and thus stimulate reservoir permeability [6-9]. After the hydraulic fracturing process is complete, between 5% and 100% of fracking fluid, together with subsurface brines, return to the wellhead, where the liquid must be collected and managed. This wastewater, which is often termed "produced water", returns to the surface for the rest of the well's lifetime and represents a major management challenge [5, 10]. Produced water is generally saline to hypersaline and characterized by total dissolved solids (TDS) concentrations as high as 350,000 mg/l, and high concentrations of inorganic ions such as sodium, calcium, barium, strontium, and chloride, making conventional treatment difficult and appropriate produced water management one of the utmost operational and environmental concerns associated with hydraulic fracturing [5, 11]. The most common produced water management approach is deep well injection, a process that is critically viewed as it has been associated with seismic activity [5, 12]. Recently, reuse of produced water in hydraulic fracturing operations has emerged as a desirable management approach. Produced water reuse reduces environmental and logistical concerns associated with produced water disposal and minimizes freshwater requirements for fracturing fluid [5]. Holding ponds are commonly used to store produced water for up to several months before reinjection.

1.1.2 Microbial activity in hydraulic fracturing systems and its role in produced water management

One of the major concerns associated with produced water management is biological activity. Microorganisms have been shown to live in hydraulic fracturing produced water and in

the hydraulic fracturing infrastructure [10, 13, 14]. Microbial processes in hydraulic fracturing systems can negatively impact oil and gas production and recovery, minimize the potential for produced water recycling, and increase the risk for environmental contamination [5, 15, 16]. Examples for biological activity, causing issues in produced water and infrastructure, are microorganisms producing hydrogen sulfides (through the reduction of either sulfates or thiosulfate), acids (through anaerobic respiration), and biofilm formation, which can foul the subsurface fractures [16, 17]. These processes can also cause corrosion of infrastructure equipment and pipelines, and lead to souring of natural gas, both of which may lead to additional operating costs and environmental concerns. Microbial activity in hydraulic fracturing produced water and the hydraulic fracturing infrastructure has been identified and investigated by several studies, however additional work on microbial populations living in these systems and their biological activity would improve the current understanding of microbial community dynamics. In addition, previous observations have suggested operational parameters, such as well age and biocide application, to impact the microbial ecology in produced waters. However, few attempts have been made to investigate parameters potentially driving microbial populations in hydraulic fracturing systems.

1.1.3 Economic impact of microbial activity on the oil and gas industry

Microorganisms growing in hydraulic fracturing oil and gas wells and the hydraulic fracturing infrastructure, such as the upper well casing, piping, the separator, or storage equipment, can contribute to corrosion and well souring. Corrosion related failures represent more than 25% of failures experienced in the oil and gas industry [18]. The NACE (National Association of Corrosion Engineers) estimates that approximately 20% of all corrosion in the oil

and gas industry is caused by microbes [19, 20]. As of 2016 the oil and gas industry spent approximately 7 billion dollars annually on direct corrosion repair and control and also invested 1.4 billion dollars to protect and restore production and exploration infrastructure and equipment [21]. A different source reports costs of several hundred million dollar due to microbial induced corrosion in the production, transport, and storage of oil and gas [22]. For example, evaluation of microbial influenced corrosion in a hydraulically fractured California oilfield revealed the presence of pinhole leaks in the oil-water gathering system and also identified elevated numbers of acid and sulfide producing bacteria. The damage was attributed to corrosion and resulted in costs of 1.8 million dollars to replace and modify facilities [22, 23]. These observations and numbers suggest microbial activity contributing to corrosion to cost operators across the United States between several million of up to several billion dollars every year.

Similarly, operators have to mitigate the production of hydrogen sulfide (H_2S) in natural gas and oil, a process called souring. Souring in gas and oilfield systems can occur thermo-chemically, but is also often attributed to the action of bacteria reducing various sulfur compounds [24]. Different methods can be used to remove hydrogen sulfide from natural gas. Liquid H_2S scavengers systems (e.g. triazine based) are one commonly used approach, however operating costs from just the chemical consumption can be between $8 to $10 per pound of H_2S removed, resulting in large overall costs for treatment [25]. For example, a Haynesville play operator estimated a cost of $14 million annually just to purchase the scavenger chemical needed for gas sweetening [25, 26]. Extraction of H_2S from the hydrocarbon stream early in the process has been recommended, as this practice can extend the operating life of midstream and downstream equipment [27, 28]. Remediation of damaged oil and gas wells (through for example excessive sulfide contamination) also represents an additional cost. Davies et al. reports

US states have spent approximately $319 million in recent decades to plug and remediate more than 70,000 oil and gas wells [29].

This data suggests microbial activity in hydraulic fracturing to not only interfere with operations and represent an environmental issue, but to also have a large economic impact. Therefore, improved understanding of the microbial ecology and optimized biocide treatment may help to minimize costs for corrosion repair and hydrocarbon treatment.

1.1.4 The importance of biocide efficacy during produced water management

Attempting to control microbial growth, hydraulic fracturing well operators typically apply biocides. Glutaraldehyde and DBNPA (2,2-dibromo-3-nitrilopropionamide) are two of the most commonly applied biocides added to hydraulic fracturing produced water; however, more than 20 are used in the industry [15]. Anecdotal reports have suggested current biocide application strategies only successfully eliminate approximately 60% of microbes in the fracturing fluid, which is also supported by high bacterial concentrations in produced water following the hydraulic fracturing process [10, 15]. In addition, there is concern about the ecological impact of biocides due to inadvertent release [15]. Several studies have observed resistance of produced water organisms to glutaraldehyde, DBNPA, and other biocides in vitro [17, 30]. Furthermore, recent investigations have suggested hypersaline produced water to cause increased resistance to glutaraldehyde in produced water microorganisms and identified genetic pathways that may contribute to resistance [31, 32]. Genetic resistance to commonly used hydraulic fracturing biocides, therefore, needs to be taken into consideration when evaluating produced water management strategies. Despite these findings, genetic resistance mechanisms in

produced water organisms remain poorly understood, as the current understanding is based only on a few microorganisms and a small number of biocides.

The research presented in this dissertation investigates the microbial diversity and community in hydraulic fracturing produced waters from two different unconventional reservoirs. Furthermore, biological activity in these systems is explored by looking at metabolic pathways of produced water microorganisms. Finally, genetic biocide resistance mechanisms in the genus *Pseudomonas* (a microorganism commonly detected in produced water) are evaluated.

1.2 DISSERTATION ORGANIZATION

The objectives of this dissertation have been addressed in five research projects, which represent three manuscripts for journal publication. The dissertation research is presented in the following chapters.

Chapter 2: A review of the microbial ecology in hydraulic fracturing produced water and use of biocides during hydraulic fracturing operations.

This chapter reviews the current understanding of the microbial ecology in hydraulic fracturing produced water. Results from previous studies that have evaluated the microbial ecology of produced waters from different shale gas regions across the United States are summarized and analyzed. Additionally, the importance and limitations of the literature currently available are highlighted. Overall, twelve different studies evaluating the microbial ecology of Marcellus, Barnett, Antrim, and Bakken Shale produced waters were considered for this review. Results identified the genus *Halanaerobium* as one of the most abundant taxa in hydraulic

fracturing produced waters, independent of geographical region. Previous work revealed a shift in microbial community structure from early flowback water to produced waters obtained from older wells. Furthermore, the literature review investigates the current research on biocide efficacy and resistance in produced water microorganisms.

Chapter 3: Evaluating the influence of storage conditions of hydraulic fracturing produced water samples intended for microbiological analysis.

This chapter evaluates the effects of storage conditions of produced water samples intended for microbiological analysis. Improper storage of water samples can lead to undesired changes in community structure, invalidating potential results and interpretation of data. Three types of produced water were sampled and a true baseline sample was preserved at the time of sampling. Produced water samples were then exposed to two different storage conditions (4^{o}C and room temperature) and sampled for a 7 day period. 16S rRNA sequencing was used to evaluate changes in community structure throughout the time of storage. Results suggested the microbial community structure in samples stored at room temperature to change within 24 hours, as the community structure in samples stored at 4^{o}C remained similar for 3 days. These findings show the importance of proper storage at 4^{o}C to preserve the original community structure and allow the development of storage guidelines for produced water samples from hydraulic fracturing intended for microbiological analysis.

Chapter 4: Predominance and metabolic potential of *Halanaerobium* in produced water from hydraulically fractured Marcellus Shale wells.

This chapter focuses on evaluating the microbial community structure 42 Marcellus Shale produced water samples and investigates the role of *Halanaerobium* in hydraulic fracturing produced water by recovery and annotation of a *Halanaerobium* draft genome from a Marcellus Shale produced water metagenome. Prior to this study, no study had evaluated samples from more than three well sites and across a time frame of more than six months [10, 13, 14]. Data from this study provides insights into the microbial ecology of produced water samples from 42 Marcellus Shale wells, representing 18 well sites, with well ages between six months and five years. Furthermore, this study is the first to identify statistical correlations between ecological data such as community structure and diversity, and operational parameters such as total dissolved solids (TDS), well age, and biocide treatment combination. Large-scale studies as these are necessary to gain a better understanding of microbial activity in produced waters and the hydraulic fracturing infrastructure to identify the factors impacting microbial growth and improve produced water management. Evaluation of the community structure using 16S rRNA sequencing confirmed the dominance of *Halanaerobium* across all samples, supporting its role as one of the most abundant and wide-spread produced water taxa. Statistical analysis suggested a correlation between the abundance of the taxa *Halanaerobiales* and biocide treatment combination. Furthermore, we were able to recover a *Halanaerobium* draft genome from produced water metagenomic data. Annotation and analysis of annotation results allowed the evaluation of metabolic pathways potentially interfering with hydraulic fracturing operations. Analysis particularly focuses on acid production, sulfide reduction, biofilm formation, and stress resistance mechanisms. Data analysis also allowed phylogenetic and functional comparison of produced water *Halanaerobium* populations to other *Halanaerobium* species and closely related taxa living in similar environments. Findings from this task reveal *Halanaerobium* as one of the

potentially major contributors to acid and sulfide production in Marcellus Shale produced water. The draft genome, recovered and annotated as part of this task, represents one of the first genomes recovered from hydraulic fracturing produced water.

Chapter 5: Microbiological evaluation of hydraulic fracturing produced water from 17 Bakken Region wells.

In this chapter, the microbial ecology and chemistry of produced waters from 17 Bakken formation and Three Forks formation hydraulic fracturing wells are investigated across a six month time frame. Unlike for the Marcellus Shale, little data on the microbiology and chemistry of produced waters from Bakken Shale region is currently available. As the Bakken Shale region has different geological and operational characteristics than the Marcellus Shale, separate analysis is necessary to understand the dynamics of microbial populations occurring in these environments. Findings will help to develop appropriate produced water management strategies and reduce the risk for microbially influenced corrosion, microbial biofouling, and biological sulfide production in the hydraulic fracturing infrastructure. Produced waters were sampled four times across a six month time frame from at 17 different well sites. The microbial community structure was analyzed using 16S rRNA sequencing and microbial abundance was assessed using quantitative PCR. Furthermore, basic geochemical data (TDS, DOC, pH) was collected. Data analysis suggested Bakken Shale produced waters to be characterized by high TDS concentrations and low overall microbial abundance. The community structure was found to be similar in diversity to produced waters from other regions, but unique in composition. *Bacillales*, *Halanaerobiales*, and *Pseudomonadales* were found to be the most dominant taxa across all samples.

Chapter 6: Peroxide scavenging and multidrug efflux highlight *Pseudomonas fluorescens* biofilm resistance to the broad spectrum antimicrobial sodium hypochlorite.

In this chapter, the active, genetic response, potentially contributing to biocide resistance, to the biocide sodium hypochlorite is investigated by analyzing differential gene expression in biofilms of the model organisms *Pseudomonas fluorescens*. We exposed 48-hour biofilms of *Pseudomonas fluorescens* to a range of sodium hypochlorite concentrations to determine a sublethal exposure dose. Then we evaluated the genetic response at this dose using a transcriptome analysis, looking at differential gene expression. Results suggested 0.6 mg/L sodium hypochlorite to be a sublethal dose for 48-hour biofilms of *Pseudomonas fluorescens*. RNA-seq analysis suggested genes involved in oxidative stress response, in particular peroxide scavenging, and multidrug efflux to be upregulated. Specifically, genes encoding the organic hydroperoxide resistance protein Ohr, the alkyl hydroperoxide reductase subunits AhpC and AhpF, and the multidrug efflux pump subunit MexE were induced. Furthermore, several genes encoding proteins involved in amino acid synthesis and energy metabolism pathways were downregulated. This task provides useful insights on how microorganisms respond to stresses induced by sodium hypochlorite. Results may be useful to improve biocide selection and application strategies in industrial settings, including hydraulic fracturing operations.

Chapter 7: Summary and conclusion

Chapter 7 summarizes the main findings from the research presented in this dissertation. Major conclusions, implications, and limitations are highlighted.

2.0 A REVIEW OF THE MICROBIOLOGY ASSOCIATED WITH HYDRAULIC FRACTURING PRODUCED WATER

2.1 INTRODUCTION

Recent improvements in hydraulic fracturing technology and horizontal drilling have led to a significant increase of unconventional hydrocarbon resources within the last decade [33]. The rising number of hydraulic fracturing wells across the United States has also resulted in large volumes of produced water, a wastewater generated as a byproduct of gas and oil production [5, 34]. Produced water is not only characterized by high TDS concentrations, but also harbors microbial populations, which may have the capability to interfere with hydraulic fracturing operations and cause long term damage to the well and hydraulic fracturing infrastructure [5]. As management of microbial activity in these large quantities of wastewaters represents one of the major challenges associated with hydraulic fracturing operations, multiple research efforts have been made to evaluate the microbial community structure and microbial activity in produced water from hydraulic fracturing operations (Table 2-1). This summary aims to present the primary findings of these studies and assess the current understanding of hydraulic fracturing produced water microbiology.

2.2 MICROBIAL COMMUNITY STRUCTURE AND BIOLOGICAL ACTIVITY IN PRODUCED WATER FROM THE MARCELLUS SHALE REGION

The majority of data on hydraulic fracturing produced water microbial ecology is based on samples from the Marcellus Shale formation. Several studies have used 16S rRNA and metagenomic sequencing to evaluate the microbial community structure and the metabolic potential of hydraulic fracturing produced water microorganisms from this region.

Mohan et al. evaluated the bacterial load and microbial community structure of hydraulic fracturing fluid, flowback water from a hydraulic fracturing wellhead, across a nine day period post-fracture, and produced water from the gas-water separator 187 days post-fracture [10]. Bacterial loads were found to be similar in the hydraulic fracturing fluid and early flowback water (~10^7 16S rRNA gene copies per ml) and lower in the separator samples taken after 187 days (~10^4 16S rRNA gene copies per ml). Taxonomic analysis revealed similar community structure in early flowback water and hydraulic fracturing fluid, dominated by the orders *Rhodobacterales* and *Pseudomonadales*. A change in community structure was observed in day 7 and day 9 samples, with most sequences affiliated with the orders *Vibrionales* and *Alteromonadales.* The produced water sample taken a half-year later was almost exclusively characterized by sequences classified as the order *Halanaerobiales*. This observation supports a shift from an aerobic microbial community in early flowback water to an anaerobic microbial community in later stage produced water. Mohan et al. presented not only one of the first efforts to evaluate the microbial ecology of hydraulic fracturing produced water using culture-independent methods, but also remains one of the few studies to this date to evaluate wellhead samples.

To gain insights into the metabolic potential of produced water microbial populations Mohan et al. also evaluated the source water, produced water day 1, and produced water day 9 samples using shotgun metagenomic sequencing [35]. Metagenomic taxonomy data confirmed the 16S rRNA sequencing results from the previous study. Functional annotation using the SEED subsystem database suggested microbial populations in day 9 produced water samples have a higher relative abundance of carbohydrate, iron acquisition, and stress response genes, likely due to the presence of hydrocarbons and increased levels of inorganics and organics. Furthermore, functional mapping revealed little evidence for classical sulfate reduction. These findings represented the first insights into the metabolic potential of hydraulic fracturing produced water microbial populations, suggest that microorganisms in these systems respond to changes in hydrocarbon content, and suggest produced water microbial populations to have the genetic ability to respond to the stresses present in hydraulic fracturing produced water.

Produced water from hydraulic fracturing operations is often kept in impoundments for long-term storage. This management strategy has been suggested to promote microbial growth. Mohan et al. evaluated the microbial ecology of untreated, biocide treated, and aerated Marcellus Shale flowback water impoundments at three sampling depths [36]. Cell counts suggested aeration and biocide treatment do not affect the overall microbial abundance. Community structure analysis using bacterial and archaeal 16S rRNA primers suggested bacterial populations exposed to glutaraldehyde biocide treatment change with depth and are dominated by the bacterial taxa *Clostridia*. Similar observations were made for the untreated impoundments, with the majority of surface microorganisms belonging to the genus *Roseovarius.* The taxa *Marinobacterium* and *Clostridia* were found to be more abundant at the middle and bottom depths. Communities from the aerated impoundments were found to be uniform across the

sampling depth and characterized by aerobic taxa such as *Roseovarius*. Microbial abundance and activity in flowback/produced water holding ponds is of particular interest, as produced water is often recycled, and microorganisms growing in holding ponds may get reinjected into wells as part of fracturing fluids. Data from this study represents one of the first attempts to assess the microbial burden of produced water stored in holding ponds and suggests management strategies, such as aeration or biocide treatment, can alter the microbial community structure, but have little impact on the overall bacterial load.

Cluff et al. evaluated injected fluids and produced waters from three hydraulic fracturing wells at several time points [13]. Injected fluids were sampled at all three wells, early flowback waters (days 3 to 13 post fracture) were also collected at all three wells, and produced waters at days 49, 82, and 329 post fracture were collected at two wells. Community structure analysis using 16S rRNA sequencing suggested injected fluids to be dominated by aerobic organisms; however some variation in community structure was observed depended on the amount of produced water recycled in the fracturing fluid. The microbial community structure in early flowback waters from was found to change rapidly within the first two weeks post-fracture. Samples were characterized by high abundances of *Burkholderia*, *Halolactibacillus*, *Arcobacter Marinobacter*, *Thermococcus*, and *Vibrio*. The microbial community structure in later produced water was found to be more stable, and dominated by the taxa *Halanaerobium* and *Halomonadaceae*, both halophilic, anaerobic organisms.

The microbial community structure observed by Cluff et al. is similar to that of previously evaluated produced water, characterized by a shift from an aerobic early flowback community to a community dominated by anaerobic, halophilic organisms in later stage produced water. The authors also try to correlate the microbial ecology across the samples with

measured chemical parameters, finding community shifts to be correlated with a decrease in alkalinity, inorganic and organic carbon concentrations, and an increase in ionic content.

Cluff et al. presented one of the most detailed hydraulic fracturing produced water studies available at the time of publication, confirming the shift from an aerobic to an anaerobic community structure across the first 6 month. However, shifts in community structure, in some cases in less than 24 hours, suggest potential sampling challenges.

Akob et al. evaluated the microbiology of produced water from 13 different Pennsylvania shale gas wells, 12 Marcellus Shale wells and one Burket Shale well [37]. While the number of wells evaluated was greater than in previous studies, microbiological analysis consisted of culture-based tests for anaerobic fermenters, methanogens, and hydrogen sulfide producing bacteria. Anaerobic fermenters were found to be present in produced waters from all wells. Tests also suggested methanogens to be present in produced waters from five different wells, and hydrogen sulfide producing bacteria to be present in produced waters from seven different wells. The Burket well was evaluated for its microbial community structure using 16S rRNA sequencing, and most identified OTUs were affiliated with the genus *Halanaerobium*. The Burket well was in production for 5 months at the time of sampling, and sampled produced water was characterized by TDS concentrations of over 150,000 mg/L. Work by Akob et al. presents evidence for sulfide, acid production, and methanogenic activity in Marcellus Shale produced water and further supports the importance of the genus *Halanaerobium* in later stage, hypersaline produced waters.

Table 2-1: Table summarizing previous studies having analyzed the microbial ecology of hydraulic fracturing produced water.

Authors	Year	Region	Number of prod. water samples	Source	Well/pond ages at time of sampling	Method
Mohan et al.	2013	Marcellus Shale	1 well, 4 samples	Wellhead (3 samples), separator (1 sample)	Days 1, 7, 9, and 187	16S rRNA, qPCR
Mohan et al.	2013	Marcellus Shale	9 total samples (3 impoundments at 3 depth)	Flowback water impoundment	Approx. 80 days stored prior to sampling	16S rRNA, MPN
Cluff et al.	2014	Marcellus Shale	16 total samples (3 wells sampled periodically)	Wellheads (10 samples), separators (6 samples)	3.5 to 328 days	16S rRNA
Akob et al.	2015	Marcellus Shale, Burket Shale	12 Marcellus wells, 1 Burket well	Separator tanks	5 month (Burket), 10 – 38 month (Marcellus)	Enrichment culture (12), 16S rRNA (1)
Mohan et al.	2014	Marcellus Shale	One well sampled two times	Wellhead	Days 1 and 9	Metagenome
Daly et al.	2016	Marcellus Shale	1 well, 4 samples	Wellhead (2 samples), and separator (2 samples)	Days 7, 13, 82, and 328	Metagenomic binning
Vikram et al.	2016	Marcellus Shale	3 samples	Holding pond (2), hauling truck (1)	N/A	16S rRNA, metatranscriptome
Struchtemeyer et al.	2011	Barnett Shale	2 wells, 4 samples	2 Separator samples, 2 frac pond samples	24 hours and 2 month	16S rRNA, MPN
Davis et al.	2012	Barnett Shale	17 total samples (2 wells, up to 6 time points per well)	2 separators, 2 storage tanks,	Newly drilled	16S rRNA
Liang et al.	2016	Barnett Shale	4 comingled produced water samples from 6 wells, two time points	Separator	N/A	16S rRNA, MPN, enrichment and isolation
Wuchter et al.	2013	Antrim Shale	3 wells	Wellhead	Approx. 5 month	16S rRNA
Strong et al.	2013	Bakken Shale, Marcellus Shale	1 Marcellus Shale samples, 2 Bakken Shale samples	Wellhead (Marcellus, 1 Bakken), Separator (1 Bakken)	18 month (Marcellus), no data on Bakken samples	16S rRNA

As microbial activity in hydraulic fracturing operations can lead to issues with microbially influenced corrosion, gas souring, and biofilm formation, research in this area also focused on metabolic pathways contributing to these processes. One of the most detailed studies, evaluating the metabolic potential in produced waters from the Marcellus Shale region and building upon previous work, was recent work by Daly et al. [16]. Using DNA from the same produced water samples previously evaluated by Cluff et al. with 16S rRNA sequencing, Daly et al. reconstructed "persisting shale genomes", a term used by the authors for draft genomes from abundant produced water microorganisms, and analyzed their microbial metabolism [16]. Overall, six *Halanaerobium*, two *Halomonadaceae*, four *Marinobacter*, one *Methanohalophilus*, one *Methanolobus*, and two unidentified *Halobacteriaceae* bins were recovered from the metagenomic data. The *Halobacteriaceae* bin was novel in genome composition, named Candidatus Frackibacter, and suggested to be unique to the shale environment. The authors' metabolic analysis of recovered genomes revealed genes involved in glycine and betaine uptake and synthesis, and methanogenesis pathways driven by methylamine and methanol utilization in *Methanohalophilus*. In addition, sucrose respiration pathways in *Pseudomonas* and *Marinobacter*, aerobic hydrocarbon degradation pathways in *Marinobacter*, *Halomonadaceae*, and *Pseudomonas*, and sulfide production mechanisms in *Halomonadaceae* were identified. The study specifically highlighted the metabolic potential of Candidatus Frackibacter, suggesting these organisms to produce acetate via glycine betaine fermentation, homoacetogenesis, and sugar fermentation. Daly et al. also investigated the functional potential of the dominant produced water genus *Halanaerobium*, suggesting this taxa has the ability to ferment amino acids, ethylene glycol, and the sugars sucrose, fructose, glucose, and maltose. In addition,

analysis revealed potential for biofilm formation, motility, acid production, and thiosulfate reduction.

Data obtained in this study contributes to the current understanding of the microbial ecology in hydraulic fracturing produced water, confirming the roles of common produced water organisms such as *Halanaerobium* and *Marinobacter*, but also revealing new organisms of interest such as Candidatus Frackibacter. Metagenomic analysis also suggested *Halomonadaceae* can contribute to sulfide and acid production.

To date, one study has evaluated microbial activity in hydraulic fracturing produced water using transcriptomic tools. This approach offers the opportunity to evaluate the active community in a sample and identifies active metabolic pathways. Vikram et al. analyzed active microbial communities in three different Marcellus Shale produced water samples using a metatranscriptome approach [38]. Two different storage pond samples and one sample obtained from a produced water hauling truck were examined. Taxonomy analysis revealed differences in community structure between 16S rRNA sequencing results and metatranscriptome data (active microbial community), demonstrating the importance of taking these differences into account when developing microbial control strategies. Analysis of active communities suggested the *Enterobacteriaceae*, *Vibrionaceae*, and *Bacillaceae* to be most abundant in the truck samples. Pond samples contained active communities of *Enterobacteriaceae*, *Pseudomonadaceae*, and *Burkholderiaceae*. Furthermore, 15% of the active community in the truck sample was found to be Archaea, suggesting Archaea play a more important role in produced water than previously assumed. The authors name different salinities (higher in truck samples) and produced water/well ages (pond produced water had been stored, truck samples was newly delivered) as possible explanations for different community structures and varying diversities.

Analysis of microbial activity revealed the expression of biofilm genes in truck samples and the expression of sulfate reduction genes in pond and truck samples, suggesting these processes were actively occurring. One notable observation was the identification of an acetoclastic methanogenesis pathway in the truck sample, suggesting active methane production in the evaluated produced waters. Finally, the authors identify a variety of active stress resistance mechanisms, including osmotic, oxidative, and periplasmic stress response genes.

Investigations by Vikram et al. showed why it is important to evaluate microbial activity with different approaches. Results suggested significant differences between inactive and active microbial communities in hydraulic fracturing produced water. Furthermore, this study was the first to identify active biofilm formation, sulfate production, and methane production pathways in produced waters from hydraulic fracturing.

Preliminary conclusions on microbial community dynamics in Marcellus Shale produced waters can be drawn based on existing studies. When analyzing the microbial community structure in produced waters it is necessary to differentiate between early produced water or flowback water (returning to the surface within the first few days and weeks after the hydraulic fracturing job), late produced water (returning to the surface month or years later), and flowback/produced water sitting in open storage ponds or impoundments. The summarized studies suggested early and impoundment produced water to be dominated by aerobic microorganisms, often abundant in taxa such as *Marinobacter*, *Pseudomonas*, *Vibrio*, *Roseovarius*, and *Arcobacter*. Later stage produced water was dominated by halophilic, anaerobic organisms, particularly the taxa *Halanaerobium* and *Halomonas*. Furthermore, a previously undiscovered organism, potentially unique to the produced water environment and named Candidatus *Frackibacter*, was identified. Archaea such as the taxa *Methanohalophilus*

and *Methanolobus* were also discovered in later stage Marcellus Shale produced water, and potentially contribute to methane production.

2.3 MICROORGANISMS IN PRODUCED WATERS FROM BARNETT SHALE WELLS

While Marcellus Shale hydraulic fracturing produced waters have been best characterized microbiologically, several studies have also investigated bacterial communities in hydraulic fracturing produced waters and hydraulic fracturing facilities from other shale gas and oil regions. Previous efforts investigated bacterial communities in hydraulic fracturing fluids, produced waters, and production facilities in the Barnett Shale in north central Texas [14, 17, 39]. The Barnett Shale is, together with the Eagle Ford and the Haynesville Plays, one of the leading shale gas producers in the southern United States [14, 39]. Furthermore, it is considered one of the deepest shale gas plays, with a depth of up to 8,000 feet and sub-surface temperatures of up to 82^{o}C.

In a study published in 2011, Struchtemeyer et al. evaluated frac pond (also often referred as storage pond or impoundment) and separator flowback water samples and blender hydraulic fracturing fluid samples from two wells (24 hours and 2 month post fracture) [14]. All samples were analyzed using MPN (most probable number) enumeration studies, and frac pond and separator flowback water samples were evaluated using 16S rRNA pyrosequencing. 16S rRNA data revealed frac pond communities to be dominated by a diverse microbial community, including *Actinobacteria*, *Firmicutes*, *Bacteroides*, *Betaproteobacteria*, and *Cyanobacteria*. In

contrast, separator flowback water samples were dominated by facultative anaerobes, in particular the taxa *Bacillaceae*, *Clostridiaceae*, *Planococcaceae*, and *Halanaerobiaceae*. In addition, the authors report the identification of sequences associated with the sulfate reducing taxa *Desulfotomaculum* and *Desulfosporosinus*. Results from this study suggest microbial community dynamics in Barnett Shale produced waters to be similar to those observed in the Marcellus Shale, with aerobic taxa dominating pond samples and facultative anaerobes in separator flowback/produced waters. Results also support the presence of classical sulfate reducing taxa and thiosulfate reducing taxa in produced waters from the Barnett Shale.

Hydraulic fracturing operations utilize an infrastructure of production lines, pipes, tanks, and other equipment, all of which can be colonized by microorganisms and potentially damaged by microbial activity. To better understand microbial community dynamics throughout these locations, Davis et al. evaluated the microbiology of natural gas well production facilities in the Barnett Shale using 16S rRNA sequencing [39]. Both tank and separator samples were evaluated from two well sites across a six month time frame [39]. Microbial communities in produced water tanks were found to be more diverse than separator samples. Tank samples were characterized by high abundances of Beta-, Gamma-, and Epsilonproteobacteria, in particular the taxa *Marinobacter*, *Arcobacter*, and *Pseudomonas*. Separator samples were dominated by Firmicutes, especially the order *Halanaerobiales*. Within each location communities had high levels of similarity across the evaluated time frame. Results suggest microbial community structure to change between different hydraulic fracturing infrastructure locations, in this case the separator and the storage tank. Due to the higher abundance of anaerobic, fermentative microorganisms in the separator, this and closely located areas may be more susceptible to microbial influenced corrosion (MIC).

Halanaerobium is one of the most abundant microorganisms in hydraulic fracturing produced water, and may be considered as one of the major contributors to acid and sulfide production in hydraulic fracturing systems. One published study has successfully isolated *Halanaerobium* from hydraulic fracturing produced water. Liang et al. describe the analysis of two Barnett Shale produced water samples using 16S rRNA sequencing and the subsequent isolation of a *Halanaerobium* strain from one of the samples [17]. Samples were taken from the same well within a three-month timeframe. Both samples were characterized by relatively high *Halanaerobiales* abundances, with most sequences affiliated with the genera *Orenia* and *Halanaerobium*. They also report an observed relative abundance of 5% for the sulfate reducing order *Desulfovibrionales*, suggesting classical microbial sulfate reduction to be factor in sulfide production in the Barnett Shale. Isolation and characterization of a *Halanaerobium* isolate (strain DL-01) revealed a close phylogenetic relationship to the species *Halanaerobium kushneri*, and suggested *Halanaerobium* DL-01 to degrade guar gum, produce acetate and sulfide when using thiosulfate as an election acceptor, and to be unable to utilize sulfate.

This study also represents one of the only attempts to expose a produced water isolate to biocide treatments. A QAC (quaternary ammonium compound) was found to effectively inhibit sulfide and acetate production of *Halanaerobium* DL-01 at 13.5 mg/L, while glutaraldehyde and Tetrakis-(Hydroxymethyl)-Phosphonium Sulfate (THPS) were required at higher concentrations (~400-500 mg/L) [17]. The authors propose the presence of thiosulfate as a potential reason for the limited effectiveness of THPS, but do not propose a mechanism, other than produced water interactions, for glutaraldehyde resistance.

Data from this study provides evidence that *Halanaerobium* can contribute to acid production and sulfide production in hydraulic fracturing produced water systems. Furthermore,

Liang et al. showed that glutaraldehyde is not effective at inhibiting *Halanaerobium* growth, and recommends QAC compounds as the preferred antimicrobial treatment against *Halanaerobium*.

Work done on the microbial ecology of Barnett Shale flowback and produced water reveals a microbial system similar to that observed in the Marcellus Shale. Microbial community structures were found to be similar in composition and abundance. These studies further confirmed the role of the Firmicutes, in particular the anaerobic, fermentative taxa *Halanaerobiales* and *Bacillales* in produced water, and the role of more aerobic taxa such as *Marinobacter* and *Pseudomonas* in produced water storage locations. Results also suggest Barnett Shale produced waters to harbor greater numbers of classical sulfate reducing bacteria than observed in the Marcellus Shale. The second study also evaluated microbial activity and microbial interference in piping, storage tanks, and other operational equipment. Available Barnett Shale work also highlights the role of *Halanaerobium* and its contribution to acid and sulfide production in produced water environments and its potential for biocide resistance.

2.4 MICROBIAL ECOLOGY IN PRODUCED WATERS FROM THE ANTRIM SHALE GAS REGION

The Antrim Shale is a natural gas formation in the Michigan Basin, and has been suggested to contain biogenic gas, which represents evidence for microbial activity in the subsurface [40]. In a 2013 study, Wuchter et al. investigated the microbial diversity and methanogenic activity in Antrim Shale formation waters [41]. Three gas-producing wells in the Michigan Basin were sampled for produced waters at the wellheads. *Bacteroidetes*,

Proteobacteria, and *Firmicutes* were found to be the most dominant taxa across all three samples, and included fermentative, anaerobic, sulfate, iron, and nitrate reducing organisms. Identified taxa included *Halanaerobium*, *Halocella*, *Orenia*, *Cytophaga*, *Desulfuromonas*, and *Arcobacter*. Despite differences in well production ages (5 to 24 months) *Halanaerobium* abundance was similar across all three samples. These findings suggest a diverse bacterial community structure, characterized by both fermentative and sulfate and thiosulfate reducing taxa, to exist in Antrim Shale produced waters. The abundance of the genus *Halanaerobium* across all samples supports the important role of this taxon in later stage produced waters, independent of location.

Archaeal abundances were low across all samples and detected archaeal sequences were found to have the closest similarity to the methylotrophic methanogens *Methanolobus* and *Methanohalophilus* and the hydrogenotrophic methanogens *Methancalculus* and *Methanoplanus*. The discovery of *Methanohalophilus* agrees with observations from the Marcellus Shale region, where a *Methanohalophilus* draft genome could be recovered from produced water and methanogen activity was observed in produced water holding ponds [38, 42]. The identification of methanogens in Antrim Shale produced waters supports the theory of biogenic gas production in the Antrim formation; however, observed abundances were not higher than previously identified in produced waters from other shale gas regions.

2.5 MICROBIAL ECOLOGY IN PRODUCED WATERS FROM THE BAKKEN SHALE REGION

While multiple studies have evaluated the microbial ecology in Marcellus Shale produced water, little is currently known about microorganisms in produced waters from the Bakken Shale region. Strong et al. evaluated two Bakken Shale produced water samples. One sample was taken from the wellhead and one sample was taken from the oil water separation tank; both samples were obtained from wells producing oil [43]. The community structure in the wellhead sample was similar to that detected in other studies, as it was characterized by high abundances of *Halanaerobium* and *Marinobacterium*. The separator tank sample was highly abundant in *Pseudomonas* sequences, suggesting a more aerobic environment, previously observed in storage tanks and ponds. The wellhead sample was also characterized by 100 times higher salinity than the separator tank sample, supporting the hypothesis that *Halanaerobium* particularly thrives in hypersaline environments. While Strong et al. does not report qPCR or MPN data, overall microbial abundances in Bakken Shale produced waters are likely low, due to high subsurface temperatures of more than 200^{o}F [44]. A study attempting to investigate biological contribution to sulfide production was not able to report any data on microbial abundances and community structure due to low biomass [44].

Overall, the microbial ecology in Bakken Shale produced waters was found to be very similar to that observed in the Marcellus Shale and Barnett Shale, with both halophilic, aerobic, and anaerobic taxa being abundant, depending on the sample source (e.g. separator tank or wellhead), sample production age, and salinity. Bakken data also showed that fermentative and

sulfate reducing taxa can be abundant in produced waters, confirming trends previously observed in Barnett Shale produced waters.

2.6 USE OF BIOCIDES TO CONTROL MICROBIAL ACTIVITY IN PRODUCED WATERS

Microbial activity during oil and gas production and microbial activity in produced waters and the hydraulic fracturing infrastructure can lead to issues with reservoir souring, biocorrosion, and biofouling. As previously discussed, produced water samples from multiple oil and gas regions around the United States have been shown to harbor various microbial populations, which can contribute to these processes. Currently, operators utilize different types of biocides to control microbial growth; however, application strategies have been suggested to be unspecific and occur with limited efficiency. Only a few studies have analyzed the usage, application strategies, and efficacy of biocides in hydraulic fracturing, and an even smaller number of studies attempted to investigate microbial resistance mechanisms in produced waters. Here, current literature on these two topics will be briefly summarized and analyzed.

Kahrilas et al. reviewed current usage of biocides in hydraulic fracturing operations in a 2014 paper [45]. The electrophilic biocide glutaraldehyde was determined to be the most frequently used biocide (based on data from Fracfocus) with a frequency of 27%, followed by the also electrophilic biocide 2,2-Dibromo-2-cyanoacetamide (DBNPA) at 24%. The authors also state the use of biocides represents a danger for environmental contamination, through for example surface spills during transportation. Furthermore, produced waters may be treated using

industrial or municipal wastewater treatment methods and released into the environment. The authors point out that the release of sub-lethal biocide concentrations can cause adaption and resistance in surviving microorganisms [46]. Kahrilas et al. also discuss current research on mobility of biocides, stating current data does not support the migration of biocides from fracturing fluids or produced water to aquifers. These findings suggest groundwater contamination through biocides to be unlikely, but environmental contamination may occur through inadvertent release.

One of the potentially biggest challenges for operators is the degradation, and especially biodegradation, of biocides. This is especially an issue for the commonly used biocide glutaraldehyde, which auto-polymerizes, especially under high pH conditions, thereby losing its efficacy. Kahrilas et al. also evaluated the potential for biodegradation [45]. Glutaraldehyde, DBNPA, and tetrakis (hydroxymethyl) phosphonium sulfate (THPS) have been reported to be susceptible to biodegradation; however, for many other biocides no data on potential biodegradation is available. The authors also note the lack of data available on the fate of biocides under downhole conditions, which represent a unique (high pressure, high temperature) environment and may lead to additional degradation mechanisms, which have not previously been recognized. Based on these characteristics glutaraldehyde and DBNPA are not ideal biocides for microbial control during hydraulic operations.

Finally, Kahrilas et al. evaluated the toxicity of the biocides currently used in hydraulic fracturing, concluding most of them to be only low to moderately toxic, however, some degradation products may be more toxic and persistent. One of the advantages of glutaraldehyde is its low environmental toxicity, which is one of the reasons it is used so frequently in the hydraulic fracturing industry.

Overall, this study offers insights into current biocide application strategies, analyzing issues and risks associated with the usage of different biocides. Some of the most commonly used biocides such as glutaraldehyde and DBNPA were also found to be most susceptible to degradation, which could affect their efficacy. As efficacy is one of the most important characteristics in a biocide, these findings are relevant to current produced water management strategies. Moreover, efficient biocides can be applied at lower concentrations, and thus represent a smaller environmental concern.

Two studies have evaluated the efficacy of biocides during the hydraulic fracturing process. Fichter et al. evaluated biocide application strategies in the Barnet Shale and tested several common biocides for their efficacy [47]. The biocides were tested on produced water samples with aerobic, fermentative, and sulfate reducing bacteria. Subsequently, six different types of biocides tetrakis (hydroxymethyl) phosphonium sulfate (THPS), glutaraldehyde, glutaraldehyde/QAC blend, DBNPA, isothiazolin, and solid bronopol), ranging in concentration between 30 ppm and 300 ppm, were tested for their ability to reduce acid and sulfide producing bacteria. Results suggested THPS concentrations of 50 ppm or higher and glutaraldehyde/QAC blend concentrations of 100 ppm or higher to be most efficient. A glutaraldehyde concentration of 100 ppm was found to lead to a 2-log reduction (30%) in cell counts and a 4-log reduction (60%) was observed, when a concentration of 200 ppm was used. DBNPA concentrations as high as 100 ppm did not result in any reduction of acid or sulfide producing bacteria. These findings suggested THPS is one of the most efficient biocides when attempting to control acid and sulfide producing bacteria.

In a similar study, Struchtemeyer et al. evaluated seven biocides, including glutaraldehyde, sodium hypochlorite, tetrakis (hydroxymethyl) phosphonium sulfate (THPS),

and didecyldimethylammonium chloride (DDAC) for their efficacy to treat *Desulfovibrio desulfuricans* and a sulfate reducing enrichment culture from a Barnett Shale produced water storage pond [47]. Planktonic cultures and biofilms were exposed to biocide concentrations between 12.5 ppm and 400 ppm, and minimum inhibitory concentrations (MIC) for each treatment were determined. Biofilm MICs were found to be higher than planktonic culture MICs, an observation that agrees with previous observations suggesting biofilms are more resistant to antimicrobial treatments. Differences in determined MICs were particularly observed for biofilms, as DDAC concentrations as low as ~20 ppm and THPS concentrations as much as ~40 ppm were enough for inactivation of both *Desulfovibrio desulfuricans* and the sulfate reducing enrichment culture. On the other hand, concentrations of ~200 ppm glutaraldehyde and ~100 ppm sodium hypochlorite were necessary to achieve inhibition in both culture and biofilm. These findings suggest biofilms to be more resistant to the evaluated biocides than planktonic cultures and suggest DDAC and THPS to be more efficient than glutaraldehyde and sodium hypochlorite.

Struchtemeyer et al. also evaluated the efficacy of biocides in the presence of organic loads (in this study humic acid was used as an organic load, which also refers to organic carbon concentrations or organic matter). DDAC was found to be the only biocide not affected by organic loads that were added to the cultures. For all other evaluated biocides, MICs increased significantly when organic loads of 10 ppm of more were present (MICs of up to 400 ppm were observed for sodium hypochlorite). This is an important observation, as hydraulic fracturing fluids and produced water can be characterized by organic loads of up to several thousand ppm and could therefore contribute to increased biocide inefficacy.

In summary, this study also supports previous observations that the commonly used biocide glutaraldehyde has limited effectiveness in the treatment of undesirable microorganisms

during hydraulic fracturing operations and produced water storage. Biofilms were found to be more resistant to the evaluated antimicrobials. Findings from this study also suggest THPS and the biocide DDAC to be more efficient than glutaraldehyde. Both studies (Lauer et al. and Struchtemeyer et al.) did their testing based on cultures obtained from Barnett Shale produced water or on Barnett Shale produced waters. A third study evaluated the efficacy of glutaraldehyde, QAC, and THPS on a *Halanaerobium* produced water isolate, and was discussed previously [17]. QAC was found to be more efficient than glutaraldehyde and THPS. We believe no such studies are currently available for produced waters or produced water cultures from regions such as the Marcellus Shale or the Bakken Shale. As the Marcellus Shale and Bakken Shale have different physiochemical and operational characteristics (e.g. depth, temperature, and biocide application), and microbial community structures may be affected by these characteristics, it would be useful to explore biocide efficacy on produced water isolates from those regions in more detail.

Efficacy testing and current biocide application strategies suggest microbial resistance to exist in produced waters from hydraulic fracturing. Therefore, potential microbial resistance mechanisms are a research interest. This is particularly important for the commonly used biocide glutaraldehyde. While degradation and absorption of biocides (in, for example, biofilm matrixes) have been discussed, recent studies have suggested genetic responses play an important role in biocide resistance [31, 32].

Vikram et al. evaluated the transcriptomic response of *Pseudomonas fluorescens* and *Marinobacter* (a produced water isolate) to the biocide glutaraldehyde under hypersaline produced water conditions [32]. Results suggested the hypersaline environment leads to an enhanced tolerance, as the cellular osmotic stress response helped the organisms cope with

higher concentrations of biocide. Produced water exposure led to an upregulation of chaperones, the sec transclocon, and Tol-Pal system genes, which are involved in protein folding and stability and the maintenance of outer cell membrane integrity. Furthermore, the energy production systems were upregulated, suggesting that cells exposed to produced water have more energy to respond to stress. The induction of the NADH dehydrogenase in the electron transport chain suggests the potential for cellular glutaraldehyde oxidation. This response results in improved membrane stability, and production of enzymes able to inactivate glutaraldehyde. These mechanisms likely lead to an increased glutaraldehyde resistance in microorganisms. Furthermore, results suggest the hypersaline produced water environment to contribute to genetic resistance mechanisms to this biocide.

An additional resistance mechanism was proposed by Vikram et al. in a study evaluating *Pseudomonas* biofilm resistance to glutaraldehyde [31]. Upregulation of efflux pumps, lipid biosynthesis, and polyamine biosynthesis were proposed as active mechanisms cells can utilize in response to glutaraldehyde exposure. Efflux pumps have previously been suggested to play an important role in antimicrobial resistance as they allow active removal of foreign substances from the cell [48]. Based on this data it is possible that microorganisms living in produced waters may have the ability to protect themselves against biocide, through upregulated biofilm formation and the active removal of foreign compounds from the cell.

These examples show that microorganisms living in produced water have the potential for genetic responses to biocides, and that the hypersaline produced water environment may play an important role in cellular resistance mechanisms. Nevertheless, transcriptomic studies under produced water conditions have only been done for glutaraldehyde. It is likely that other biocides may induce additional cellular responses that have not been investigated at this point. Overall

observations from these studies do agree with previous biocide efficacy testing results, and further support the potential for glutaraldehyde resistance in produced water microbial populations.

2.7 CONCLUSIONS

Throughout this chapter we have evaluated the current understanding of microbial populations in produced waters from hydraulic fracturing and closely related environments, such as parts of the hydraulic fracturing infrastructure. Independent of location, produced waters were found to be dominated by halophilic microorganisms, of which several identified taxa are considered fermentative, sulfate or thiosulfate reducing, and biofilm forming. Microbial community structures were also found to evolve from aerobic dominated to anaerobic dominated with increasing production ages. Microbial populations in samples from storage tanks or ponds were also found to be more aerobic and diverse. Produced water from the Marcellus Shale is the best characterized from a microbial ecology standpoint. Both 16S rRNA and metagenomic sequencing efforts have shown microbial populations in Marcellus Shale produced water to have the potential for biocorrosion, biofouling, and souring events. Additional work confirming these trends across a larger number and diversity of samples will help to further enhance the current understanding of produced water microbial ecology. Several studies have also evaluated the Barnett Shale, identifying similar microbial community dynamics. Little data is available on other regions, especially the Bakken Shale, which has become one of the leading oil producing regions in the country and should be evaluated in more detail. The data that is available suggests

microbial populations very similar to those observed in the Marcellus and the Barnett Shale regions. The predominance of the genus *Halanaerobium* in later stage produced water (5-6 months post fracture) was observed in the majority of the studies, suggesting this taxon to play an important role in produced water microbial activity.

Finally, the current use and efficacy of biocides, and potential resistance mechanisms were investigated. The currently small study sample size suggests that more work in this field is necessary. Evaluating the available data revealed that the currently most used biocide glutaraldehyde has several disadvantages, such as the potential to self-polymerize, low efficacy, and potentially induces genetic resistance mechanisms in microbes often identified in produced water. Efflux, and osmotic stress induced membrane stabilization were identified as the main causes of genetic resistance. Effects of other biocides currently in use should be evaluated in more detail using the tools described in this review.

In conclusion, previous work has helped to identify the issue of microbial activity in produced water from hydraulic fracturing, provided novel insights into the ecology and biocide resistance of produced water microbial populations, and set the framework for additional investigations. Microbial communities were found to change throughout the flowback period, *Halanaerobium* was identified as an emerging microorganism of interest, and the potential for biocide resistance (especially to glutaraldehyde) was evaluated. However, continued research in this field is recommended. Additional efforts should further advance the current understanding of produced water microbial ecology by looking at a large range of more diverse samples. These research efforts will reveal similarities and differences to previous findings, study the produced water microbial ecology in previously little characterized oil and shale gas regions in more depth, and investigate genetic biocide resistance mechanisms in more detail.

3.0 THE INFLUENCE OF SAMPLE STORAGE CONDITIONS ON MICROBIAL COMMUNITY COMPOSITION OF HYDRAULIC FRACTURING PRODUCED WATER

Produced water sampling from high-volume hydraulic fracturing is challenged by high liquid pressures, workplace regulations, liability concerns on behalf of the operator, and remote sampling locations. Currently, no sample handling or storage recommendations exist for microbiological analyses of produced water. Maintaining a representative microbial community structure from hydraulic fracturing produced water samples throughout storage and handling is essential for accurate results of both culture-based and culture-independent microbial analyses. To provide sample handling and storage recommendations, we used 16S rRNA sequencing to monitor the changes in microbial communities in produced water stored at room temperature or 4^{o}C for seven days. Our results suggest that keeping samples at room temperature for more than 24 hours or at 4^{o}C for more than three days can lead to inaccurate representation of the original sample microbial ecology. In summary, our study highlights the importance of appropriate storage and handling practices when analyzing microbial community structures in saline environmental water samples and makes recommendations on how to best preserve the original sample ecology.

3.1 INTRODUCTION

High-volume hydraulic fracturing operations typically produces millions of gallons of wastewater throughout their completion and operation, generally referred to as produced water [5, 6]. Produced water is characterized by unique geochemical characteristics, particularly elevated salinity, dissolved metals, and the presence of organic compounds that are derived from both the fracturing fluid and subsurface formation [5, 6, 14, 36, 39]. Microbial activity in produced water from hydraulic fracturing has the potential to cause corrosion, fouling, and sulfide release, with potential negative production and environmental consequences [5, 10, 14, 49]. Additionally, microbial activity in the subsurface may result in sulfide production, or 'souring', of the gas stream [35, 36]. Due to a growing interest in produced water biological activity, multiple studies have investigated the microbial ecology of produced water from unconventional reservoirs in an effort to improve hydraulic fracturing operations, support produced water recycling, and understand mechanisms of microbial biocide resistance [5, 10, 13, 32, 35, 39, 41, 43, 45].

Significant industrial effort is expended to minimize microbial activity during hydraulic fracturing operations. The presence and abundance of acid or sulfide producing microbes in produced water is typically determined through the utilization of culture-based assays. Changes in the microbial composition of samples prior to analysis may lead to inaccurate, non-representative data resulting in ineffective operational decisions. There are currently no guidelines on how to treat, store, or handle produced water samples for microbiological analyses. Culture independent approaches, such as 16S rRNA sequencing, are also widely used tools for investigation of produced water microbial ecology [10, 13, 39]. Produced water samples for

sequencing are typically obtained on-site by well staff, due to workplace regulations and liability concerns, before being transferred to researchers for microbiological assessment. These circumstances, together with hydraulic fracturing produced water's unique chemical composition, call for defined sampling and storage procedures.

The goal of this study is to define specific handling and processing guidelines for produced water samples intended for microbiological analysis. We investigated the effects of storage condition and time on microbial communities in produced water. Produced water sample microbial communities were monitored for seven days at two different storage temperatures (4°C and room temperature) using 16S rRNA sequencing. Sequences were taxonomically classified, and alpha- and beta-diversity were calculated to understand the changes in microbial ecology under varying storage times and temperatures.

3.2 MATERIALS AND METHODS

3.2.1 Sampling

Samples used for time series experiments were obtained from two produced water holding ponds (impoundments used to store produced water) in Washington County, PA on December 9th 2013 (HP2) and June 13th 2014 (HP1). A third sample was taken from a produced water hauling truck on June 13th 2014 (FWT). All samples intended for storage experiment were collected in sterile 1L bottles and stored on ice during transportation to the laboratory (less than 2 hours). Temperature and total dissolved solids (TDS) for HP1 and FWT samples were measured

on-site and found to be 23.8°C and 11.60 mS for HP1 and 19.1°C and 19.7 mS for FWT. Sampling circumstances did not allow on-site temperature and TDS measurements for HP2 samples; however, the air temperature during sampling was approximately 1°C. Upon arrival in the laboratory produced water samples were immediately frozen at -80°C. For each produced water sample, 500 mL were also filtered on-site and filters were immediately preserved in TRIZOL (Life Technologies, Carlsbad, CA) for analysis of the original sample ecology.

3.2.2 Sample Processing

Samples were thawed at room temperature. Each sample (250 mL) was transferred to sterile 1000 mL bottles. Each sample was processed in duplicate. Samples for analysis (15 mL) were taken at the start of the experiment (Day 0), after 1 day, 2 days, 3 days, and 7 days. Samples were taken using a sterile 15 mL pipette and transferred into a 15 mL Falcon tube for further processing. During the experiment, sample bottles were stored at 4°C or room temperature (RT), approximately 25°C, in a closed box on the laboratory workbench.

3.2.3 Chemical analysis

pH was measured using a Thermo Fisher Education pH meter (Thermo Fisher Scientific, Pittsburgh, PA). TDS concentrations were determined using a Fisher Scientific Accumet AP75 Conductivity/TDS meter (Thermo Fisher Scientific, Pittsburgh, PA). If necessary, produced water samples were filtered through a 0.45 μm membrane filter to remove solids, which may

interfere with atomic absorption and ion chromatography analysis. Cation concentrations were measured using a Perkin Elmer Atomic Absorption Spectrometer 1100 (Perkin Elmer, Bridgeville, PA). Anion concentrations were determined using Thermo Scientific ICS-1100 Ion Chromatograph (Thermo Fisher Scientific, Waltham, MA). Serial dilutions for each sample were prepared (1:10, $1:10^2$, $1:10^3$) followed by absorbance measurements. Sample concentrations were determined using standard curves.

3.2.4 DNA Extraction

A 15 mL sample was aliquoted from each treatment and each time point and biomass was collected via centrifugation at 10000 rpm. Collected produced water biomass was then digested with 10µl of 20mg/mL lysozyme for 30 minutes at 37ºC followed by DNA extraction using a MoBio PowerSoil kit (Carlsbad, CA), according to manufacturer's instructions. DNA from on-site samples preserved in TRIZOL was extracted according to manufacturer's instructions.

3.2.5 DNA processing and sequencing

DNA was amplified using 16S rRNA primers as described previously [50]. PCR samples underwent an initial denaturation step for 3 minutes at 96ºC. Samples were then run for 40 cycles under the following conditions: denaturation occurred at 96ºC for 45 seconds, annealing at 50ºC for 60 seconds and elongation at 72ºC for 60 seconds. Final elongation was carried out at 72ºC for 10 minutes. Following amplification, 16S rRNA PCR products were purified using AMPure beads (Beckman Coulter, Pasadena, CA) and run on a 1% agarose gel for cleanup verification.

DNA concentrations were assessed using a Qubit (Life Technologies, Carlsbad, CA). Cleaned up PCR products were pooled and diluted to a concentration of 20nM. Diluted samples were then denatured using fresh 0.2 N sodium hydroxide for 5 minutes at room temperature and further diluted to 10 pMol library with hybridization buffer HT1 according to manufacturer's instructions (Illumina, San Diego, CA). The 10 pMol library was spiked with 5% of 12.5 pMol PhiX control and sequenced using a 300 cycle V2 Nano kit on an Illumina MiSeq sequencer (Illumina, San Diego, CA).

3.2.6 Computational analysis

16S rRNA sequences were analyzed using QIIME version 1.7.0 [51]. Sequences were quality trimmed at a quality score of 20 and demultiplexed. Operational Taxonomic Units (OTU) were picked using the pick_closed_reference_otus.py python script using UCLUST [52] against the 2013 GreenGenes core set gg_97_otus.fasta reference database with a 97% sequence similarity [53]. Average microbial abundance data values were calculated based on OTU data from both replicates. To remove bias introduced through varying number of sequences, 1000 sequences successfully assigned to OTUs were randomly selected for each sample and used for alpha diversity analysis. As part of this analysis observed species, Chao1, and Shannon were calculated from OTU tables. T-tests were used to assess statistical differences between number of OTUs measurements across the sampling period. Beta diversity was used to develop principal coordinate plots utilizing UniFrac distance metrics [54]. Weighted UniFrac distance matrices were calculated and used to compare baseline samples (Day 0) with subsequent experimental samples (Day 1, 2, 3, and 7). Sequences for each sample were uploaded to MG-RAST and are

available under the accession numbers 4603074.3 (FWT), 4603075.3 (HP1), and 4603076.3 (HP2).

3.3 RESULTS AND DISCUSSION

3.3.1 Sampling and geochemical characterization

Samples were collected from two different produced water holding ponds and from a produced water hauling truck. Produced water holding pond 1 (HP1) and truck (FWT) samples were taken in June 2014, produced water holding pond 2 sample (HP2) was taken in December 2013. Chemical analysis results for all three samples were as expected for produced water samples, characterized by high TDS concentrations (Table 3-1) and in the range of previously reported data [10, 13, 39]. The highest TDS concentrations were measured for FWT samples at 52,500 mg/L. HP1 was found to have the lowest TDS concentration at 5,300 mg/L. HP2 had a TDS concentration of 18,500 mg/L.

3.3.2 Changes in microbial community structure

The taxonomic distribution of all samples is shown in Figure 5-1, and taxonomic abundances for all samples are listed in Appendix A, Tables A1-A3. Generally, taxonomy results demonstrated that the microbial community changed slowly when stored at 4^{o}C, and that samples cannot be considered microbiologically stable when stored at room temperature. Freezing produced water samples slightly altered the taxonomic profile in two out of three samples.

The microbial community taxonomic structure of FWT samples remained constant during the first three days and changed at room temperature after seven days (Figure 3-1A). Samples were initially dominated by bacteria of the order *Campylobacterales*. A shift in the microbial community profile was detected under room temperature conditions after Day 3. The relative abundance of *Campylobacterales* decreased while the relative abundance of *Alteromonadales* increased to nearly 80%. No changes were observed for samples stored at 4^{o}C, in which the community structure remained stable throughout Day 7 (Figure 3-1A).

Table 3-1: Chemical composition of produced water samples used in this study.

Sample	**Truck (FWT)**	**Pond (HP1)**	**Pond (HP2)**
Sampling date	Jun-14	Jun-14	Dec-13
pH	6.53	7.27	7.35
		Concentration (ppm)	
TDS	52500.0	5300.0	18500.0
Calcium	6360.0	4850.0	1691.0
Sodium	18300.0	1720.0	5272.0
Barium	62.5	5.0	14.6
Strontium	727.0	39.2	1051.3
Iron	18.3	0.9	4.2
Magnesium	449.0	40.0	193.0
Manganese	2.0	BDL	0.3
Chloride	37600.0	3400.0	13867.0
Sulfate	3.7	71.5	66.5

Storage results for HP1 produced water samples demonstrated that communities that were stored at room temperature and 4^{o}C remained relatively stable over time (Figure 3-1B). Baseline samples were dominated by the orders *Rhodobacterales*, *Sphingomonadales*,

Oceanospirillales, and *Pseudomonadales*. Bacterial community structure remained relatively constant for the first two days in both the RT and 4ºC samples. Through Days 3 and 7, the fractions of *Sphingomonadales* and *Oceanospirillales* bacteria decreased slightly while bacteria of the order *Pseudomonadales* increased in samples stored at room temperature. Only minor changes (up to +/- 5% relative abundance) were observed in samples stored at 4ºC up to seven days.

The greatest effects of storage time and conditions on microbial communities in produced water were observed for HP2 samples taken December 2013. On-site sample community structure was found to be dominated by *Campylobacterales*, but to have higher relative abundances of *Bacteroidales*, *Desulfovibrionales*, and *Desulfuromonadales* than post freezing day 0 samples. Storage results suggested that the microbial community shifted rapidly at room temperature, while the shift was more gradual at 4ºC (Figure 3-1C). Baseline samples were dominated by *Campylobacterales* (~75%). After 24 hours at room temperature *Campylobacterales* relative abundance decreased, while *Pseudomonadales* and *Alteromonadales* relative abundances increased (Figure 3-1C). In samples stored at 4°C, no changes in microbial community structure were detected within the first three days. Day 7 results revealed *Pseudomonadales* to be the dominant order while relative *Campylobacterales* abundance was found to have decreased (Figure 3-1C).

3.3.3 Changes in microbial diversity within and between samples

The number of OTUs assigned per 1000 sequences varied between samples (Appendix A, Table A4). Results demonstrated the observed number of OTUs to remain relatively more stable

under 4°C conditions, but to decrease under room temperature conditions throughout the seven-day storage period for FWT and HP2 samples (Figure 3-1). Statistical analysis revealed number of OTUs in FWT and HP2 room temperature samples to be significant different at Day 7 compared to Day 0 (t-test, $P < 0.05$). No statistical differences in number of OTUs across the 7 day sampling period were identified for HP1 room temperatures and 4°C samples, HP2 4°C samples, and FWT 4°C samples. The highest number of OTUs was observed for HP2 4°C samples (as high as 156 OTUs) and lowest number of OTUs was observed for HP2 room temperature samples (as low as 56 OTUs).

The Chao1 and Shannon diversity measurements were used to assess species richness and evenness [55, 56]. Results for both approaches suggest diversity within samples to remain more stable at 4°C than room temperature. Chao1 estimates richness by correcting for rare OTUs; Chao1 values were found to decrease under room temperature conditions over time, suggesting population richness to decline (Appendix A, Table A4). Chao1 values were found to be statistically significantly different in HP1 room temperature Day 7 samples, when compared to on-site and Day 0 samples (t-test, $P < 0.05$). Chao1 values at 4°C conditions suggested population richness to remain stable throughout storage, with the exception of HP2 samples, for which population richness was found to increase within the first two days and then decrease until Day 7 (Appendix A, Table A4). The Shannon index was used to determine population evenness; microbial diversity and evenness were found to be greatest within the HP1 sample set and lowest within the FWT sample set. Evenness values increased slightly over time in FWT, HP1 and HP2 room temperature samples and HP1 4°C samples (Appendix A, Table A4), suggesting microbial community diversity to be altered throughout the storage period.

Weighted UniFrac principal coordinate analyses (Figure 3-2) demonstrated that samples tend to cluster based on source (e.g. FWT, HP1, HP2), rather than by time or storage condition. All on-site samples were found to cluster with the Day 0 sample. The FWT room temperature Day 7 samples clustered further away from other FWT samples and were found to group more closely with HP2 samples (Figure 3-2).

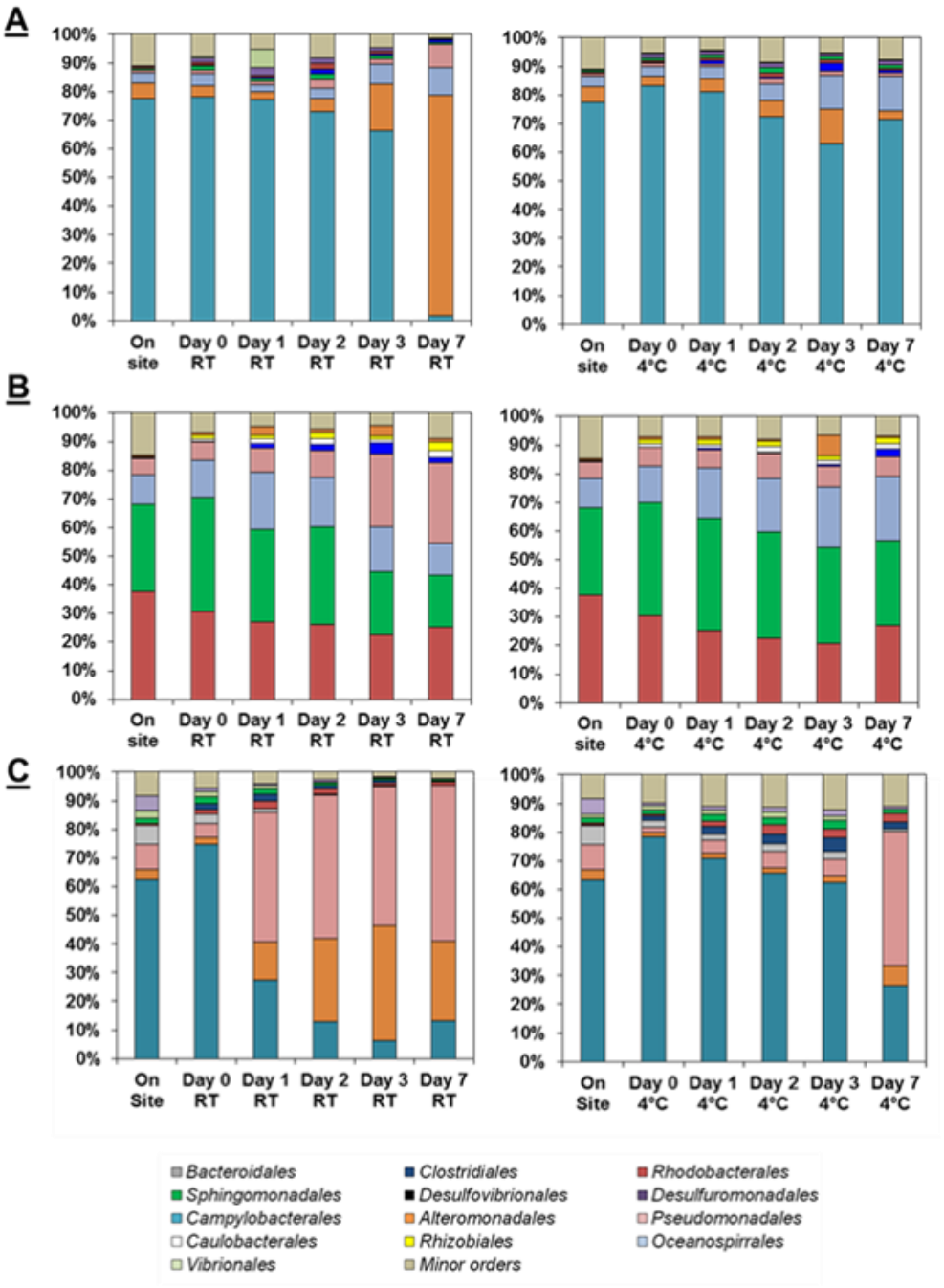

Figure 3-1: Microbial community structure in hydraulic fracturing produced water samples FWT (A), HP1 (B) and HP2 (C) samples preserved on-site and stored at room temperature or 4^{o}C over a time period of 7 days.

Similarly, the HP2 room temperature Day 7 sample was found to be an outlier and cluster separately from other HP2 room temperature samples (Figure 3-2). Average weighted UniFrac distances were found to be greatest for FWT room temperature Day 7 samples (0.62 +/- 0.01) and HP2 room temperature Day 2 (0.46 +/- 0.02), Day 3 (0.52 +/- 0.01) and Day 7 (0.48 +/- 0.05). Weighted UniFrac distances relating HP2 samples stored at room temperature were greater than UniFrac distances obtained for samples under all other conditions (Appendix A, Figure A2).

3.3.4 Implications and produced water storage recommendations

In all three produced water samples, microbial taxonomy observed in produced water samples stored at 4^{o}C on Day 3 was considered representative of the baseline on-site community structure based upon relative abundances of major taxa and beta-diversity analyses. Microbial community composition in produced water samples stored at room temperature was found to be more variable, with major taxonomic profile changes being observed as soon as 24 hours after storage. Results were also confirmed through both alpha- and beta-diversity analyses. Evaluation of species richness and evenness within each sample also supported the convention that microbial communities are more susceptible to changes at room temperature. These results suggest produced water storage and handling to be important for microbiological analyses. Storing samples at room temperature for 24 hours or longer can significantly alter the taxonomic profile and limit the validity of downstream analysis. To preserve the original community structure samples should ideally be preserved on-site. If on-site sample processing is not possible produced water samples intended for microbiological analysis can be stored at 4^{o}C for up to three days. Long term storage (between three days and up to several weeks) should occur at -

80°C. Comparisons of samples preserved on-site and samples stored at -80°C suggested to preserve the overall microbial taxonomy profiles, but to result in small abundance shifts.

These findings are consistent with previous storage condition studies for environmental samples [57, 58]. Changes in microbial community structure in sediment core samples were observed when stored long term at 4°C, confirming observations that microbes remain active at these storage conditions resulting in changes over extended periods of time [58]. Similarly, studies investigating storage conditions for soil samples intended for microbial analysis suggested -20°C or -80°C as best long term storage options and advised against storage at 4°C [59, 60].

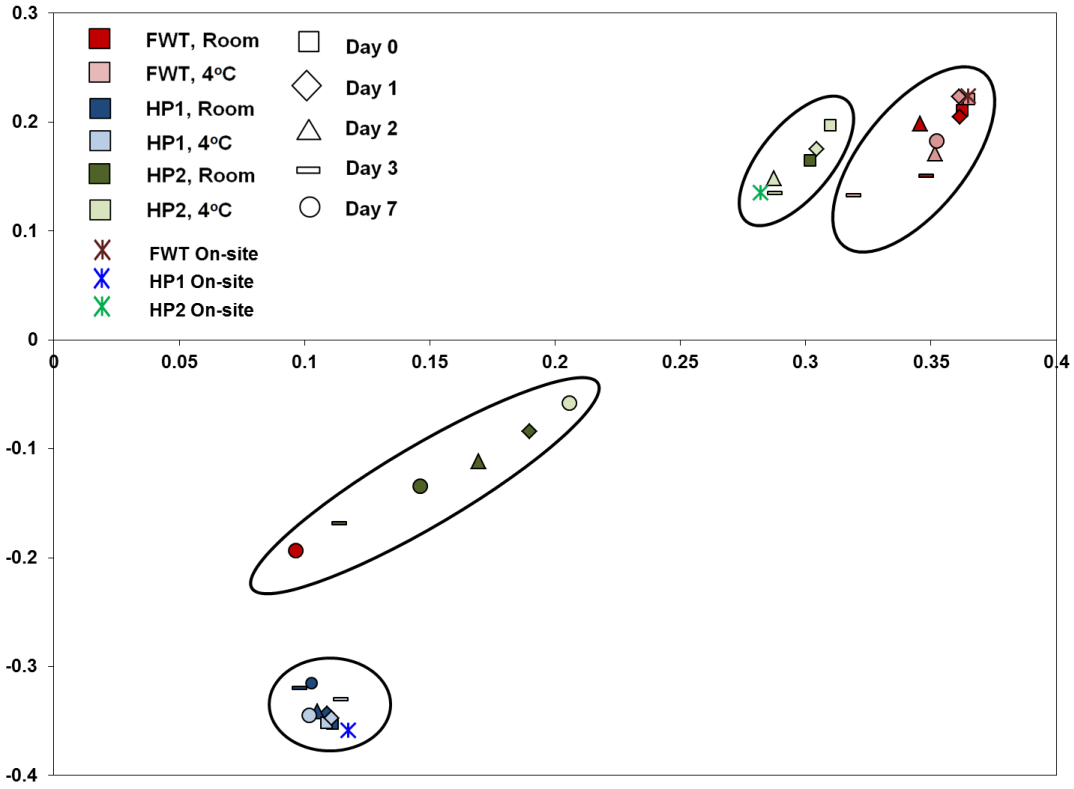

Figure 3-2: Weighted Unifrac plot for microbial communities in three different types of hydraulic fracturing produced water at two different storage conditions over seven days.

3.4 SUMMARY AND CONCLUSIONS

Changes in microbial community structure and composition during transport and storage of environmental samples may lead to results not representative of the original environment. Due to technical challenges with collection of produced water samples from hydraulic fracturing, there is the potential for a delay between sample collections and processing that may alter sample microbial ecology, and sample processing recommendations do not currently exist. To address this knowledge gap, we monitored the changes in microbial communities in produced water subjected to different storage conditions. Microbial ecology of produced water from three different sources (two water holding ponds and one truck sample), at two temperatures (room temperature and 4^{o}C) through a seven-day period (Days 0, 1, 2, 3, and 7) was analyzed to assess the change in microbial community structure. Results suggest hydraulic fracturing produced water microbial communities to remain stable for up to three days, when stored at 4^{0}C and to change within 24 hours when stored at room temperature. These findings extend the current state of knowledge on storage of environmental samples by investigating a saline environment and including true baseline samples. Furthermore, these results are particularly important as analysis of microbial communities in produced water is an emerging focus area and necessary to understand the role of microbes during unconventional oil and gas production [10, 13, 36, 43, 61]. Proper produced water handling strategies will be necessary when undergoing large scale studies that include samples from many wells, sampled at different time points and at different locations. These results strongly encourage implementation of a strict protocol for produced water handling that includes storage at 4^{o}C and processing within three days of sampling.

4.0 PREDOMINANCE AND METABOLIC POTENTIAL OF HALANAEROBIUM IN PRODUCED WATER FROM HYDRAULICALLY FRACTURED MARCELLUS SHALE WELLS

This work has been published as:

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Microbial activity in the produced water from hydraulically fractured oil and gas wells may potentially interfere with hydrocarbon production and cause damage to the well and surface infrastructure via corrosion, sulfide release, and fouling. This study surveyed the microbial abundance and community structure of produced water sampled from 42 Marcellus Shale wells in southwestern Pennsylvania (well age ranged from 150 to 1846 days) to better understand the microbial diversity of produced water. We sequenced the V4 region of the 16S rRNA gene to assess taxonomy and utilized qPCR to evaluate the microbial abundance across all 42 produced water samples. Bacteria of the order *Halanaerobiales* were found to be the most abundant organisms in the majority of the produced water samples, emphasizing their previously

suggested role in hydraulic fracturing related microbial activity. Statistical analyses identified correlations between well age and biocide formulation and the microbial community, in particular the relative abundance of *Halanaerobiales*. We further investigated the role of the order *Halanaerobiales* in produced water by reconstructing and annotating a *Halanaerobium* draft genome (named MDAL1), using metagenomic sequencing and metagenomic binning. The recovered draft genome was found to be closely related to the species *Halanaerobium congolense*, an oil-field isolate, and *Halanaerobium* sp. T82-1, also recovered from produced water. Reconstruction of metabolic pathways revealed *Halanaerobium* sp. MDAL1 to have the potential for acid production, thiosulfate reduction, and biofilm formation, suggesting it have the capability to contribute to corrosion, souring, and biofouling events in the hydraulic fracturing infrastructure.

4.1 INTRODUCTION

Oil and gas are now produced from previously unproductive (unconventional) hydrocarbon reservoirs due to the widespread use of horizontal drilling in conjunction with multi-stage, high-volume hydraulic fracturing. Hydraulic fracturing uses up to 25 million liters of water per well as the working fluid to fracture and increase the permeability of the hydrocarbon containing formation [9, 33]. Approximately 10-60% of the injected fluid returns to the surface after hydraulic fracturing as produced water and is characterized by total dissolved solids (TDS), with concentrations as high as 300,000 mg/L [5, 33]. Wells continue to generate produced water throughout their operational lifetime, generating up to 8000 liters of saline to hypersaline

wastewater per day [5]. Biological activity is generally undesirable during produced water holding prior to reuse or disposal, as well as during well operation [44, 49, 62]. Microorganisms in produced water may have the potential to produce acids and sulfides, leading to corrosion and gas souring [16, 37, 42, 44, 63, 64], and form biofilms, resulting in clogging and fouling events [13, 16, 39, 42, 64]. Microbial activity is controlled by biocide addition during the fracture process and produced water holding, and there is concern about the ecological impacts of biocides due to inadvertent release [45]. Understanding the microbial ecology of produced water is a critical component of controlling undesirable microbial activity.

Previous studies have investigated the microbial ecology of Marcellus Shale produced water, reporting a rapid transition of the microbial community in produced water from an aerobic surface water microbial community, to a fermentative, anaerobic community [10, 13, 16, 36, 37, 43, 64]. Notably, bacteria of the genus *Halanaerobium* (order *Halanaerobiales*) have been shown to be a predominant members of the produced water microbial community [10, 13, 16, 17, 36, 43, 64], representing a potential operational concern due to its fermentative [16, 53, 65, 66], thiosulfate reducing nature [16, 17, 67-69]. Previous taxonomic characterization of microbial communities in produced water from the Marcellus Shale is from a sum total of six wells, with a maximum of three per study, all less than 18 months following fracture [10, 13, 16, 36, 37, 43, 64, 70][37]. As there are greater than 15,000 unconventional wells in the Marcellus Shale region alone [71] and the produced water and well infrastructure are expected to be managed and maintained for 30 years or more, investigation of additional sites and well ages is necessary to confirm observed microbiological trends.

In addition, operational and geochemical factors may influence the microbial ecology of produced water, including the produced water salinity and the biocide composition used in the hydraulic fracturing fluid. Salinity has been suggested to be a major factor controlling the bacterial community composition and activity in a variety of aquatic environments [32, 72, 73]. Biocide composition likely affects the microbial ecology due to its wide variety of application approaches, as many different types and treatment combinations of biocides have been used during hydraulic fracturing with limited efficacy [39]. No study has yet investigated the influence of these factors on produced water microbial community structure.

The objective of this study was to analyze the microbial abundance and community structure in produced water sampled from a greater number of wells, well sites, and well ages than previously considered. An additional goal was to identify possible correlations between the produced water microbial community and biocide composition, well age, and salinity as measured by total dissolved solids (TDS). Furthermore, this study aimed to specifically evaluate the abundance and metabolic potential of the order *Halanaerobiales*, which previous studies suggested to be one of the most abundant produced water organisms. Ultimately, enhanced understanding of produced water microbial ecology will inform microbial monitoring and control efforts in produced water, leading to enhanced protection of well infrastructure.

4.2 MATERIALS AND METHODS

4.2.1 Samples

Sample parameters reported in this study were based on previously suggested guidelines for the investigation of wastewater from unconventional shale gas extraction [74]. All Marcellus Shale produced water samples were obtained during one sampling day in June 2014 from wells in Southwest Pennsylvania. Samples were directly taken from the gas-water separator, collected in sterile 200 mL bottles, kept on ice during transport, and stored at -80°C within 24 hours of sampling. The gas-water separator represented the closest available sampling port to the production well. Samples analyzed in this study represent 42 wells from 18 different well sites in the Marcellus Shale region in Southwest Pennsylvania from production ages of 150 to 1846 days (Appendix B, Table B1).

4.2.2 Chemical analysis

Prior to chemical analyses, produced water samples were filtered through a 0.45 μm membrane filter to remove solids which could interfere with mass spectrometry and ion chromatography analysis. Cation and trace element concentrations were determined in dilute sub-samples using inductively coupled plasma mass spectrometry (NeXION 300x ICP-MS). Chloride concentrations were assessed using Thermo Scientific ICS-1100 Ion Chromatograph (Thermo Fisher Scientific, Waltham, MA). Total dissolved solid (TDS) concentrations were determined based on measured cation and anion concentrations. Biocide utilization data for the

42 wells was obtained from Fracfocus [75]. Nine different biocide treatment combinations were used across the analyzed wells, with biocide treatment combinations one (19 wells) and biocide treatment combination two (13 wells) used in the majority of the wells (Appendix B, Table B2). Biocides were part of the fracturing fluid and applied during hydraulic fracturing.

4.2.3 DNA extraction, PCR, and sequencing

For each sample, 30-50 mL of produced water was centrifuged (15,900 x g) to collect biomass. This volume was used as it represents a volume range successfully applied by our and other research groups for DNA extraction from produced water [39, 43]. Collected biomass was then digested with 10 µl of 20 mg/mL lysozyme for 30 minutes at 37ºC followed by DNA extraction using MoBio PowerSoil kit (Carlsbad, CA) according to manufacturer's instructions. DNA from all samples was amplified using V4 region 16S rRNA gene primers as described previously [50, 51], Samples that could initially not be amplified were diluted 10 fold prior to amplification to limit inhibition. Negative controls were utilized for each PCR reaction. Following amplification, 16S rRNA gene PCR products were purified using AMPure beads (Beckman Coulter, Pasadena, CA), run on a 1% agarose gel for cleanup verification, and quantified using Qubit (Life Technologies, Carlsbad, CA). Purified PCR products were pooled and diluted to a concentration of 20 nM. Diluted samples were then denatured using fresh 0.2 N sodium hydroxide for 5 minutes at room temperature and further diluted to 10 pMol library with hybridization buffer HT1 according to manufacturer's instructions (Illumina, San Diego, CA). The 10 pMol library was spiked with 5% of 12.5 pMol PhiX control and sequenced using a 300 cycle V2 Nano kit on an Illumina MiSeq sequencer (Illumina, San Diego, CA).

4.2.4 qPCR

The bacterial load for 42 produced water samples was determined using SYBR Green based quantitative PCR (qPCR). Triplicate reactions were prepared for all samples each containing 1 µl DNA, 10 µl SYBR Green qPCR Mastermix (Bio-Rad, Hercules, CA), 8 µl ultrapure water, 0.5 µl reverse 16S rRNA primer, and 5 µl forward 16S rRNA primer, designed by Maeda et al [76]. Standard curves were generated using genomic DNA from *Pseudomonas fluorescens* (ATCC 13525). Reactions were run using a Bio-Rad qPCR thermocycler (Bio-Rad, Hercules, CA) using default settings. The number of 16S rRNA gene copies per mL was calculated as described previously [77]. The detection limit ranged between 141.7 and 426.4 16S rRNA gene copies per mL of sample.

4.2.5 16S rRNA gene data processing

16S rRNA sequences from all samples were analyzed using QIIME version 1.7.0 [51]. Sequences were trimmed at a quality score of 20 and demultiplexed. Operational Taxonomic Units (OTU) were picked using the pick_closed_reference_otus.py python script using UCLUST [78] against the 2014 GreenGenes core set gg_97_otus.fasta reference database [79]. Beta diversity was assessed by calculating weighted UniFrac distances [54]. Alpha diversity was assessed by determining the number of operational taxonomic units (OTUs), Chao1, and Shannon indices per 2000 sequences to remove bias introduced through varying number of sequences. For samples with less than 2000 sequences, the available sequences were used for alpha diversity estimation. DNA sequences were also annotated and deposited on MG-RAST and

can be accessed under the accession number 4696241.3. Description of fastq headers for sample sequences can be found in Appendix B, Figure B1.

4.2.6 Correlation analysis

Spearman rank coefficients correlating taxonomy and diversity measures with TDS, well age, and bacterial abundance were calculated using R [80]. Additionally, correlations were investigated using linear regression analysis. Differences in diversity and taxonomy by biocide treatment combination were assessed using two tailed t-tests. Microbial diversity between well sites was assessed by calculating weighted UniFrac distances and visualized using NMDS (nonmetric multidimensional scaling) ordination. Analysis of similarities (ANOSIM) based on Bray-Curtis and Euclidean distances was used to investigate statistical differences in community structure in R and Past [80, 81]. Furthermore, samples were clustered based on weighted UniFrac distances using UPGMA (Unweighted Pair Group Method with Arithmetic mean) analysis in QIIME [51].

4.2.7 Metagenome library preparation

DNA from one produced water sample (Site 13, Well 2), previously analyzed using 16S rRNA sequencing, was selected for metagenome sequencing. The metagenome sequencing library was processed using Nextera XT (Illumina, San Diego, CA) according to manufacturer's instructions. Briefly, 1 ng of input sample DNA was tagmented with Illumina primers containing sequencing adapters and barcodes in a 12 cycle PCR step. PCR products were cleaned up using

AMPure XP beads (Life Technologies, Carlsbad, CA). DNA libraries was normalized using Illumina bead technology, quantified using Qubit (Life Technologies, Carlsbad, CA), and diluted to a concentration of 20-40 pM. The DNA library was denatured by heating the sample at 96^{o}C for 2 minutes, cooled in an ice bath for 5 minutes, and sequenced using a 300 cycle V2 kit on an Illumina MiSeq sequencer (Illumina, San Diego, CA).

4.2.8 Quality control and assembly

Sequencing data was quality trimmed using CLC Genomics workbench version 8.5.1 (CLC Bio, Aarhus, Denmark). Reads with poor quality score <Q30 and length <100 nucleotides were discarded. Trimmed reads were assembled into contiguous sequences ('contigs') using metaSPAdes version 3.5.1 [82]. Ribosomal rRNA genes in assembled contigs were predicted using RNAmmer [83]. Phylogenetic analysis based on extracted 16S rRNA sequence was performed using CLC workbench version 8.5.1 (CLC Bio, Aarhus, Denmark).

4.2.9 Reference genome mappings

Halanaerobium reference genomes were downloaded from NCBI using CLC Genomic workbench version 8.5.1 (CLC Bio, Aarhus, Denmark). Trimmed sequencing data was then mapped against each reference genome using the 'map reads to reference' tool using default settings.

4.2.10 Binning, genome annotation, and functional gene mapping

Halanaerobium contigs were recovered from metagenomic data based on taxonomy using PhyloPythiaS [84] and tetranucleotide frequency, differential coverage, and marker genes using Maxbin [85]. The resulting *Halanaerobium* draft genome was assessed for completeness using CheckM [86, 87]. The generated *Halanaerobium* draft genome was uploaded to NCBI Genbank (accession number MIJU01000000.1) and RAST to be annotated using the SEED database [88, 89]. The draft genome can be accessed under the RAST accession number 6666666.207575. KEGG orthology terms [90] were assigned to contigs and exported using RAST. Data was then mapped against KEGG pathways using the KEGG mapper tool [90, 91]. Functional gene sequences of interest were downloaded from RAST and evaluated using BLASTx or mapped against selected references genomes (obtained from NCBI) using CLC workbench version 8.5.1 using default parameters.

4.3 RESULTS

4.3.1 Sample background and geochemistry

Produced water samples were analyzed from 42 hydraulically fractured, horizontal Marcellus Shale gas wells, representing 18 well sites, in southwestern Pennsylvania in June 2014. In this study, the terminology ‘well site’ refers to a single well pad. A well pad is a single site consisting of multiple wells tapping laterally into the same formation. None of the wells

sampled in this study had previously been remediated for fouling or souring issues. The production ages of analyzed wells ranged from 150 to 1846 days and TDS concentrations for the 42 samples ranged between 38,000 and 223,000 mg/L (Appendix B, Table B1). Sulfate concentrations were found to be below the detectable limit in 26 samples and low across the remaining samples, with maximum concentrations of 30 mg/L. Further inorganic ion composition data is shown in Table B3, Appendix B.

4.3.2 Microbial abundance

Microbial abundance for each sample is reported in Appendix B, Table B1. Microbial abundance in the produced water samples as determined by qPCR varied between $1.5 \times 10^5 – 2.1 \times 10^8$ 16S rRNA gene copies per mL of produced water, within the range of previously reported values for produced water from the Marcellus Shale [10, 14, 36, 39]. Correlation analysis based on Spearman rank coefficients and linear regression analysis suggested microbial abundance to be positively related with well age (Appendix B, Tables B1, B4, B5) and did not reveal any correlations between microbial abundance and TDS (Appendix B, Tables B1, B4, B5) and biocide treatment combination (Appendix B, Figure B2, t-test: all $P < 0.05$).

4.3.3 16S rRNA gene analysis and bacterial community structure determination

The Firmicutes phylum, specifically the orders *Halanaerobiales* and *Clostridiales*, was the dominant phyla across all produced water samples (Figure 4-1). *Halanaerobiales* were identified in all produced water samples and were the most dominant order in 40 of the 42

samples (Figure 4-1). For most *Halanaerobiales* sequences we were not able to achieve classification below the family level using the RDP classifier within QIME (Appendix B, Figure B3). Within *Halanaerobiales*, sequences were affiliated with the genus *Halanaerobium* (up to 8% of all sequences in a single sample), unassigned *Halobacteroidaceae* (up to 21%), or unassigned *Halanaerobiaceae* (up to 99%) (Appendix B, Figure B3). Annotation of 16S rRNA gene sequences using an alternative annotation strategy (MG-RAST) assigned the majority of unassigned *Halanaerobiaceae* reads to the genus *Halanaerobium*. *Clostridiales* were also observed in all produced water samples and were the second most abundant group behind *Halanaerobiales* (Figure 4-1). Abundant *Clostridiales* taxa included the families *Clostridiaceae* (up to 20% of all sequences in a single sample), *Acidaminobacteraceae* (up to 21%), and the *Lachnospiraceae* (up to 30%) (Appendix B, Figure B3). We were not able to classify *Clostridiales* sequences below the family level. Other abundant (>5%) orders included *Pseudomonadales* identified in 41 samples (up to 25%), *Bacteroidales* identified in 40 samples (up to 32%), *Campylobacterales* identified in 40 samples (up to 13%), *Bacillales* identified in 34 samples (up to 29%), *Oceanospirillales* identified in 32 samples (up to 13.9%), and *Desulfovibrionales* identified in 17 samples (up to 6%). Within the order *Pseudomonadales*, most sequences were affiliated with the genus *Pseudomonas* (up to 24% of all sequences in a single sample). Within the order *Campylobacterales*, most sequences were affiliated with the genus *Arcobacter* (up to 8%), and within the *Oceanospirillales* most sequences were assigned as unclassified *Halomonadaceae* (up to 10%) (Appendix B, Figure B3). Sequences identified as Archaea were detected in 17 samples as the orders *Methanosarcinales* (up to 0.5% of all sequences in a single sample) and *Methanomicrobiales* (up to 1.1%). All minor orders with abundances below 2% are summarized in Appendix B, Figure B4.

We used Spearman rank coefficients and ANOSIM to correlate the relative abundance of *Halanaerobiales* with operational parameters (Appendix B, Table B6). Results identified an inverse correlation between the relative abundance *Halanaerobiales* and well age ($P < 0.001$). *Halanaerobiales* relative abundances were also plotted against well age and TDS concentration for linear regression analysis, revealing no trends (Appendix B, Figure B5). We identified a correlation between biocide treatment combination and *Halanaerobiales* abundance (t-test: $P = 0.018$, ANOSIM: $P = 0.008$), as samples from wells treated with biocide treatment combination one were associated with higher relative abundance of *Halanaerobiales*. Biocide treatment combination one was 2,2-dibromo-3-nitrilopropionamide (DBNPA) based, biocide treatment combination two was tetrahydro-3,5-dimethyl-2H-1,3,5-thiadiazine-2-thione (Dazomet) based (Appendix B, Table B2). Both DBNPA and Dazomet are non-oxidizing biocides. Further mechanistic explanation for this observation will require additional analysis.

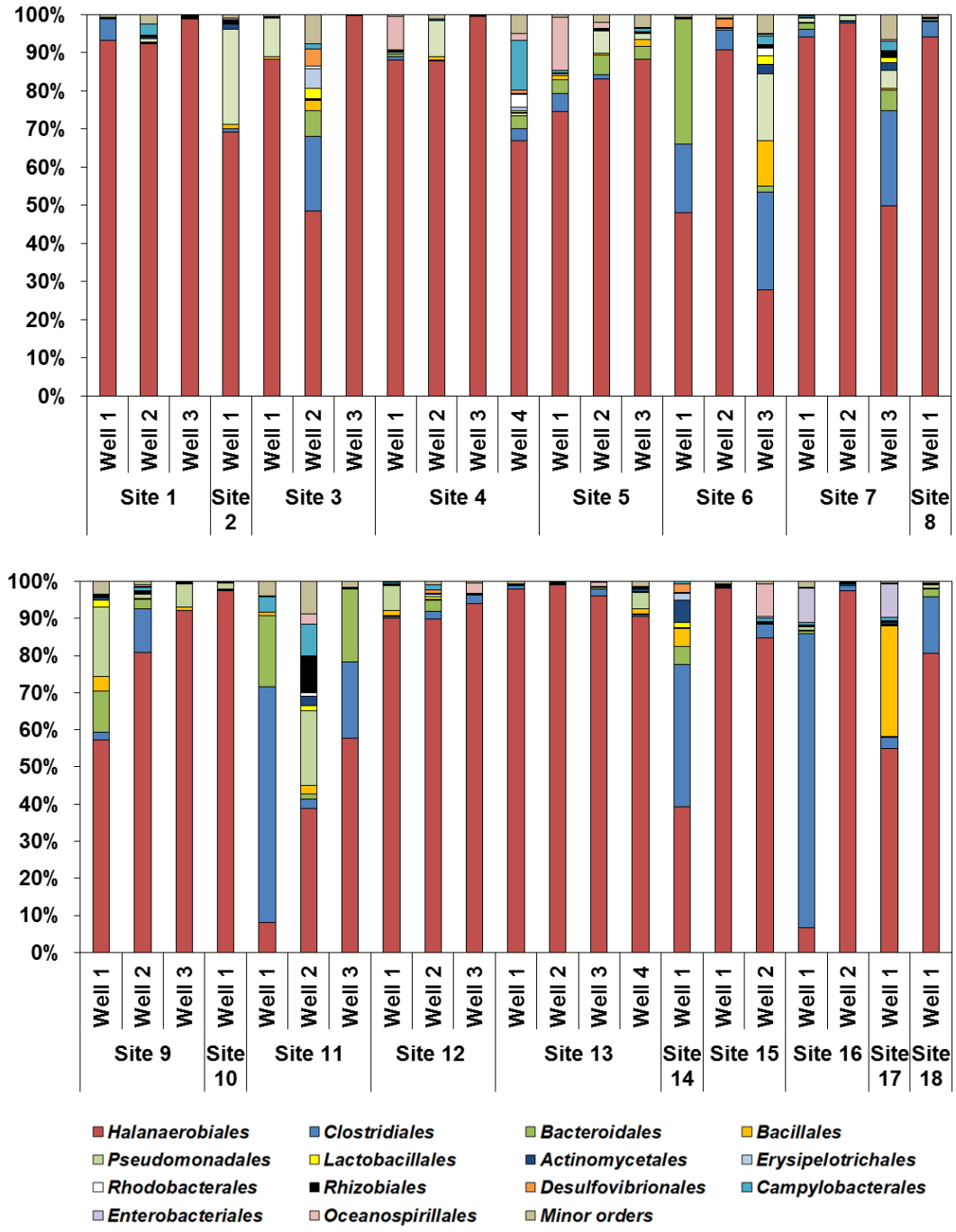

Figure 4-1: Relative abundance of microbial orders for 42 Marcellus Shale produced water samples analyzed using 16S rRNA gene sequencing in this study. Sites in this description refer to individual well pads. Orders that were less than 2% abundant in all samples were classified as minor orders and are summarized in Appendix B, Figure B4.

4.3.4 Alpha diversity

The produced water microbial community alpha diversity was calculated by the number of OTUs, Chao1 richness index (a measure of sample richness, i.e. number of OTUs), and Shannon index (a measure of both sample richness and the distribution of OTUs). Alpha diversity parameters were assessed per 2000 sequences to enable even cross-sample comparison. All sequences were used for the three samples with less than 2000 OTU assigned sequences available for analysis (Appendix B, Table B7). The number of observed OTUs ranged from 6 to 187; Chao1 richness analysis predicted richness values between 7 and 237; and the Shannon diversity ranged between 0.54 and 4.62 (Appendix B, Table B7). Correlation between alpha diversity parameters (number of OTUs, Chao1, and Shannon) and well age, TDS, and microbial abundance were assessed using Spearman rank coefficients and linear regression analysis. Results suggested a moderate correlation between Shannon diversity and well age, but did not reveal any other correlations (Appendix B, Figures B6 – B9). Alpha diversity parameters were also plotted against biocide treatment combinations, and no clustering by treatment combination was observed (Appendix B, Figure B10, t-test: all $P > 0.05$).

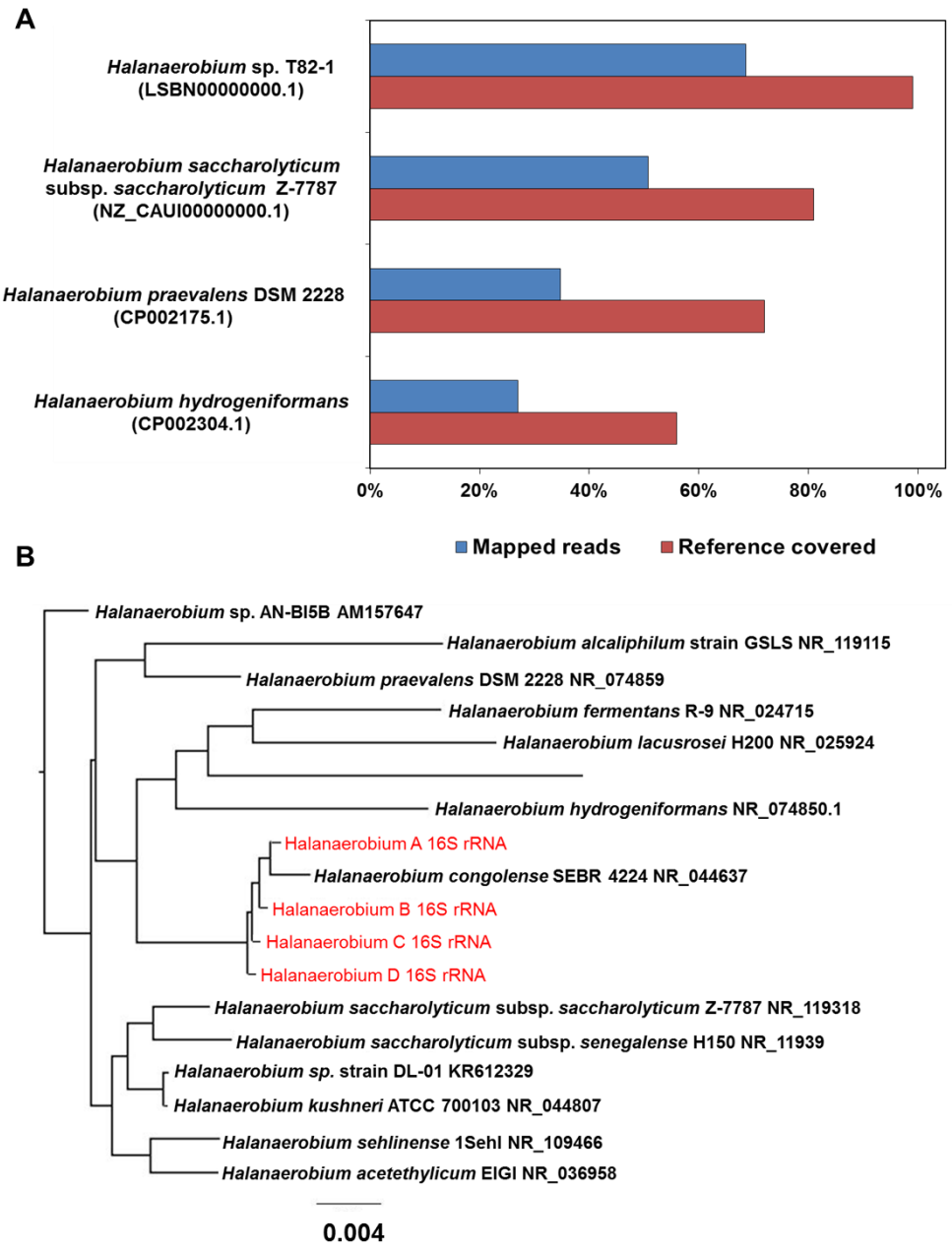

Figure 4-2: (A) Mapping results for metagenomic reads against other available *Halanaerobium* genomes and (B) unrooted phylogenetic tree showing the relationship between the 16S rRNA genes recovered from metagenomic contigs and the 16S rRNA genes of other *Halanaerobium* reference sequences downloaded from NCBI. Bar indicates 4 nucleotide substitutions per 1000 nucleotides.

4.3.5 Beta diversity

NMDS ordination analysis did not reveal any clustering by well site, or correlations between well sites and operational parameters (Appendix B, Figure B11). Weighted UniFrac distances were used to build an UPGMA tree and branches were labeled by TDS concentration, well age, and applied biocide composition (Appendix B, Figure B12). No clustering was observed for TDS concentration or well age (Appendix B, Figure B12). Analysis of similarity (ANOSIM) did not suggest any correlations for TDS concentration or well age (all $P > 0.05$). We observed clustering by biocide treatment combination (Appendix B, Figure B12, Table B2) and ANOSIM analysis revealed a difference in microbial community composition between samples treated with biocide treatment combinations one or two ($P > 0.004$).

4.3.6 Metagenome sequencing

Sample "Site 13, Well 2" was selected for shotgun metagenomic sequencing to further investigate the metabolic potential of the genus *Halanaerobium*, found to be abundant across all analyzed produced water samples. Based upon 16S rRNA gene data sample "Site 13, Well 2" was 99.1% *Halanaerobiales* and characterized by a low overall diversity (12 OTUs). Shotgun metagenome sequencing generated 6,089,871 trimmed, high-quality reads. Trimmed sequencing reads were then mapped against available *Halanaerobium* reference genome sequences (Figure 4-2A). The best mapping results were achieved for *Halanaerobium* sp. T82-1 (accession numbers LSBN00000000.1) [16], a draft genome recovered from produced water (68% mapped

reads and 99% coverage), and *Halananaerobium saccharolyticum* strain DSM 6643 (accession numbers NZ_CAUI00000000.1) (50% mapped reads and 81% coverage) (Figure 4-2A).

Sequencing reads were then assembled into 446 contigs using metaSPAdes. The minimum contig length was 5,000 bp, the maximum length was 96,535 bp, and the N50 was 15,595 bp. Taxonomy assignment with PhyloPythiaS using the 2013 generic model with default settings [84] identified 82% of contigs to belong to the genus *Halanaerobium*. 16S rRNA gene prediction identified four 16S rRNA genes, between 1023 bp and 1260 bp in length [83]. Extraction and phylogenetic analysis of 16S rRNA genes identified the extracted genes to be identical to each other (ANI 100%, across 1023 bp) and closely related to the 16S rRNA gene of the species *Halanaerobium congolense* (ANI 99%, across 1023bp) (Figure 4-2B). Phylogenetic analysis showed that the 16S rRNA genes did not cluster closely with the only other available produced water *Halanaerobium* isolate (*Halanaerobium* sp. DL-01) (ANI 98%, across 1023 bp) (Figure 4-2B) [17]. No 16S rRNA gene is currently available or could be identified for the recently published produced water draft genome *Halanaerobium* sp. T82-1 [16].

4.3.7 Metagenomic contig binning and annotation

Metagenomic binning resulted in one *Halanaerobium* draft genome, named *Halanerobiu*m sp. MDAL1, containing 129 contigs with a total size of 2,389,586 bp and a GC content of 34.2%, consistent with previously sequenced *Halanaerobium* genomes with sizes between 2.3 and 2.9 million bp and GC contents between 30.3% and 33.3% (Appendix B, Table B9) [53, 92, 93]. The *Halanaerobium* draft genome was the only phylogenetic bin obtained from the metagenomic library. Contigs that could not be binned were affiliated with the genus

Acetohalobium and *Clostridia* or could not be classified. More than half (53.9%) of original sample sequencing reads were successfully mapped back onto the binned contigs belonging to the *Halanerobium* sp. MDAL1 draft genome. The *Halanaerobium* sp. MDAL1 draft genome bin was found to be 83% complete using 898 *Halanaerobium* marker genes in CheckM [86, 87]. Annotation identified 2219 gene coding sequences (CDs) and 23 RNA sequences, representing 304 SEED subsystems for the recovered *Halanaerobium* draft genome. Phylogenomic analysis of *Halanaerobium* genomes and calculations of average nucleotide identities (ANI) and average amino acid identities (AAI) in comparison to other available *Halanaerobium* genomes suggested the recovered *Halanaerobium* sp. MDAL1 draft genome to be closely related to *Halanaerobium* sp. T82-1 (ANI = 98.48%, AAI = 93.82%, Table 4-1).

Of particular interest was the metabolic potential for fermentation pathways, sulfur metabolism, and biofilm formation, as acid production, sulfide production, and biofouling are undesirable and therefore of high interest to the hydraulic fracturing industry and broader oil and gas industry [9, 45]. In addition, we evaluated the presence of genes involved in stress response mechanisms, as these processes have previously been shown to enable increased resistance to biocides in produced water [32].

Genes associated with mixed acid fermentation were identified in the *Halanaerobium* sp. MDAL1 draft genome and include *ldh*, which encodes a lactate dehydrogenase responsible for the conversion of pyruvate to lactate, *ptaA*, encoding a phosphate acetyltransferase that converts pyruvate to acetate, and *adh* which encodes an alcohol dehydrogenase involved in the fermentation of simple sugars into ethanol (Appendix B, Table B10). Furthermore, a gene encoding pyruvate formate lyase Pfl involved in the transformation of pyruvate to hydrogen or carbon dioxide was identified (Appendix B, Table B10). BLASTx analysis confirmed the

presence of the described fermentation genes in *Halanaerobium* sp. T82-1 (99% identity). The discovery of these genes allowed reconstruction of putative metabolic pathways for the conversion of pyruvate into the fermentation products lactate, acetate, ethanol, hydrogen, and carbon dioxide, confirming *Halanaerobium's* potential to contribute to acid production in produced water.

Table 4-1: Average nucleotide (ANI) and amino acid identity (AAI) between recovered produced water *Halanaerobium* sp. MDAL1 draft genome and other available *Halanaerobium* genomes.

Name	Genbank Accession Number	ANI (%)	AAI (%)
Halanaerobium sp. MDAL1 draft genome	MIJU00000000.1	-	-
Halanaerobium sp. strain T82-1	LSBN00000000.1	98.48	93.82
Halanaerobium saccharolyticum subsp. *saccharolyticum* DSM 6643	NZ_CAUI00000000.1	84.62	82.04
Halanaerobium praevalens DSM 2228	CP002175.1	83.93	73.73
Halanaerobium hydrogeniformans	CP002304.1	82.65	67.59

The production of sulfides during hydraulic fracturing operations can lead to gas souring and is therefore a significant concern to hydraulic fracturing operations [44, 94]. While we did not identify any classical sulfate reduction genes (e.g. *dsrAB*, *aps*), our analysis revealed several genes involved in thiosulfate reduction, a process previously reported to contribute to sulfide production and observed across various anaerobic, halophilic and thermophilic bacteria taxa [69,

95]. Identified genes included an unclassified rhodanese-like gene, a thiosulfate sulfurtransferase rhodanese (*mpst*), an unclassified sulfurtransferase rhodanese gene, and the anaerobic sulfite reductase genes *asrA*, *asrB*, and *asrC* in the *Halanaerobium* sp. MDAL1 draft genome (Appendix B, Table B10). BLASTx analysis revealed the unclassified rhodanese-like gene to share 100% homology with the previously described thiosulfate reduction RdlA rhodanese proteins in *Halanaerobium* sp. T82-1, *H. congolense*, and *H. saccharolyticum* [16, 68, 95]. Furthermore, a Trk type sulfate permease and a putative ABC type sulfate-like transporter were identified, allowing the reconstruction of a putative thiosulfate reduction pathway (Appendix B, Table B10). Thiosulfate is transported into the cell by a sulfate ABC-type transporter, converted into adenyl sulfate by the Rdl rhodanese protein, and into sulfide by a sulfurtransferase and the AsrABC complex. The utilization of thiosulfate and production of hydrogen sulfide has recently been reported for a *Halanaerobium* isolate DL-01 from produced water [17]. In the same study researchers found *Halanaerobium* DL-01 to produce acetate when thiosulfate was used as an electron acceptor [17]. Our results, together with observations from previous studies, suggest that *Halanaerobium* identified in produced water have the ability to reduce thiosulfate and potentially produce sulfides and acetate. These characteristics have also been described for *Halanaerobium* species isolated from different environments [68, 92].

Biofilm formation during or after hydraulic fracturing can cause damage to the hydraulic fracturing infrastructure, and lead to clogging, interfering with hydraulic fracturing operations [14, 34]. Genes encoding for proteins suggested to be involved in biofilm formation processes were identified and include the sporulation two-component response regulator Spo0A, associated with surface attachment initiation, the glycosyl transferase group 2 family protein Glt2, and the diguanylate cyclase AdrA, which have both been associated with

exopolysaccharide production (Appendix B, Table B10) [96, 97]. Furthermore, we identified several flagella and motility genes, including *fliC*, *flhA*, *motA*, and *motB*, which have been suggested to be important for cellular attachment and initial stages of biofilm formation [98, 99].

Produced water contains elevated salinity levels and the presence of heavy metals, resulting in osmotic, oxidative, and periplasmic stress responses to protect the cell [100-102]. Multiple genes associated with salinity tolerance were identified in the *Halanaerobium* sp. MDAL1 draft genome (Appendix B, Table B10). The genome encodes for the Trk and Ktr transmembrane transporter complexes, including the potassium and sodium uptake proteins TrkA, TrkH, and KtrA [103-105]. The presence of the *trkH* and *trkA* genes supports previously reported characteristics for bacteria of the genus *Halanaerobium*, which have been shown to use a specific "salt in strategy" to counter osmotic stress through the uptake of potassium from the environment utilizing membrane proteins of the Trk and Ktr complexes [67, 102, 104]. In addition, the glycine and betaine ABC transport protein ProX, the L-proline glycine betaine ABC transport system permease protein ProW, and the high-affinity betaine transport system OpuA were identified (Appendix B, Table B10) [106, 107]. BLASTx analysis of *proW*, *proX*, and *opuU* gene sequences revealed close homology to *Halanaerobium* sp. T82-1 (>99% identity). Other identified genes with a potentially important role in microbial stress response under produced water conditions included *perR*, a redox-sensitive transcriptional regulator *sor*, encoding a superoxide reductase, and unidentified genes encoding rubredoxin, glutaredoxin, and rubrethyn. These genes are potentially involved in oxidative stress response pathways, which have been suggested to be triggered by a secondary response to osmotic stress as well as the high concentration of cations in produced water [108]. In addition, sequences encoding the periplasmic stress response gene *ompH* [109], the universal stress protein UspA, the heat shock

protein GrpE, Hsp20, and the heat shock chaperones GroES and GroEL were identified (Appendix B, Table B10). We also discovered gene sequences for the chemotaxis related proteins MotA and MotB, and the flagella assembly protein family Flg, Fli, and, Flh in *Halanaerobium* sp. MDAL1 draft genome (Appendix B, Table B10).

4.4 DISCUSSION

This study evaluated microbial communities in 42 produced water samples taken from wells of varying ages within the Southwest Pennsylvania region of the Marcellus Shale and investigated the metabolic potential of the dominant produced water genus *Halanaerobium*. The goal of this study was to improve the understanding of the microbial community associated with hydraulic fracturing operations by analyzing samples from the largest number and diversity of wells to date. Furthermore, the recovery and annotation of a metagenome-assembled *Halanaerobium* sp. MDAL1 draft genome bin provides new insights into the metabolic potential of microbial populations in produced water, specifically highlighting acid and sulfide production. Ultimately, improved understanding of produced water microbial ecology will enable the development of better produced water management strategies to minimize corrosion, produced water souring, and fouling events, and encourage produced water reuse.

4.4.1 *Halanaerobiales* dominates Marcellus Shale produced water

Microbial community structure analysis revealed bacteria of the phylum Firmicutes to be dominant across all produced water samples analyzed, constituting as much as 99% of the microbial community. Within the phylum Firmicutes, the majority of the sequences were associated with the orders *Halanaerobiales* (up to 99.8% total relative abundance) and *Clostridiales* (up to 79.2% total relative abundance). *Halanaerobiales* are fermentative, obligate anaerobic halophiles that have previously been shown to be abundant in produced water following the flowback period in the Marcellus [10, 13, 36, 67] and Antrim Shales [41]. Members of the order *Halanaerobiales* have been isolated from conventional oil wells [68] and have been identified in a variety of other ecosystems such as the Dead Sea, marine salterns, cyanobacterial mats, and hypersaline lakes [67, 110]. Phylogenetic analysis of recovered 16S rRNA genes suggested the recovered draft genome to be closely related to the genus *H. congolense* (25), which was isolated from an oil field and shares multiple functional characteristics identified in the *Halanaerobium* sp. MDAL1 draft genome.

Clostridiales sequences were identified in all but eight produced water samples, constituting more than 20% of the microbial community in six samples. Similar to *Halanaerobiales*, the order *Clostridiales* comprises fermentative, obligate anaerobes, some of which are spore forming [111]. Unlike *Halanaerobiales*, the order *Clostridiales* consists of many families with a diverse range of characteristics. The actetogenic families *Clostridiaceae, Lachnospiraceae,* and *Acidaminobacteraceae* were abundant in our samples. These families include putative sulfate reducing genera, such as *Desulfotomaculum*, which was identified [112].

Within the *Clostridiaceae*, the moderately halophilic, acid producing, biofilm forming genus *Clostridium* was also identified [113, 114].

We compared the most abundant orders identified with findings reported in previous produced water microbial ecology studies (Figure 4-3) [10, 13, 14, 36, 39, 43, 70]. Our data suggested *Halanaerobiales* to be the most dominant order across Marcellus Shale produced water samples. This observation supports previous studies, which have shown *Halanaerobiales* to exist at lower abundances in wells after a short operational lifetime and account for as much as 99% of the population in wells older than six months (Figure 4-3) [10, 13, 36]. Sequences affiliated with the *Clostridiales*, *Campylobacterales*, *Rhodobacterales*, *Bacillales*, *Pseudomonadales*, *Oceanospirillales*, and *Bacteroidales* were found to be abundant in this study and have also been identified in previous produced water studies (Figure 4-3) [10, 13, 16, 36, 43]. Comparison to previous studies showed that all orders identified in our study at greater than 2% abundances have previously been detected in produced water, with the exception of the order *Erysipelotrichales* which has not been identified in produced water from unconventional wells. *Erysipelotrichales* has been previously identified in petroleum reservoirs, formation waters, and subsurface hot springs [115, 116].

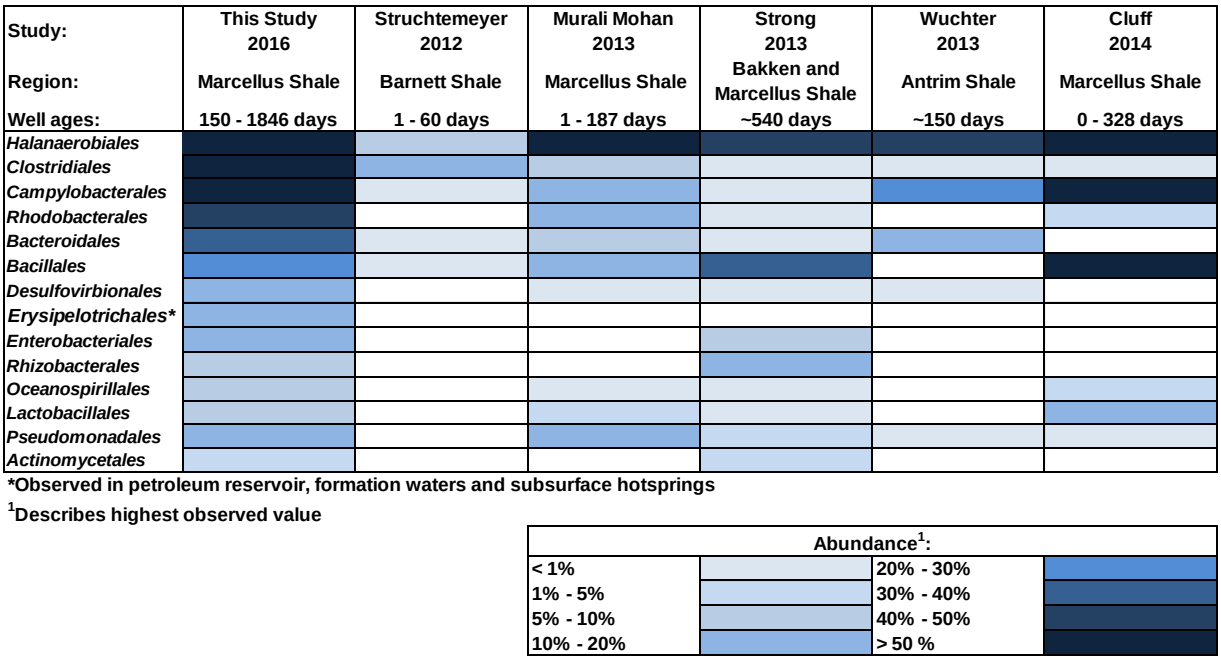

Figure 4-3: Abundance of identified Marcellus Shale produced water microbial orders identified in this study and in other produced water studies. Reported values represent the highest observed abundance in any single sample.

4.4.2 Influence of well age, salinity, and biocide treatment combination on produced water microbial communities

We evaluated the correlation between TDS, well age, and biocide application in the fracturing fluid on the microbial community composition across all samples, the occurrence of the bacterial order *Halanaerobiales*, the microbial diversity within each sample, and the microbial abundance to identify operational or geochemical factors that impact microbial community structure in produced water. Previous studies have suggested TDS and well age to influence microbial community composition in produced water [10, 13, 36], however we did not observe any correlations between TDS concentration and the microbial ecology in produced

water and only identified a moderate correlation between well age and the Shannon index, a measure of diversity within samples.

In this study we were particularly focused on the abundance of *Halanaerobiales*, as this taxon was shown to be predominant across all samples. We identified a moderate inverse correlation between *Halanaerobiales* relative abundance and well age, a result standing in contrast to previous data suggesting *Halanaerobiales* relative abundance to increase with well age; however, these studies only investigated a limited number of samples with well ages at the lower end of the time spectrum analyzed in this study [10, 13, 36]. We also identified a correlation between *Halanaerobiales* abundance and biocide treatment combination. A DBNPA based biocide treatment combination correlated with a higher *Halanaerobiales* relative abundance. The biocide treatment combinations also contained additives such as polyethylene glycol and sodium hydroxide, serving other functions in the hydraulic fracturing fluid [8, 117, 118]. Future research efforts are necessary to develop mechanistic explanations for these findings.

4.4.3 Functional potential of *Halanaerobium* from produced water

Microbial acid and sulfide production are of high interest to the oil and gas industry, due to microbial influenced corrosion, fouling, and gas souring [13, 37, 39, 44]. Taxonomic analysis of produced water allowed identification of multiple microbial taxa that are potentially involved in these processes, such as the orders *Halanaerobiales* and *Clostridiales*. Metagenomic investigation of *Halanaerobiales* acid production pathways identified putative metabolic fermentation pathways for lactate, acetate, ethanol, hydrogen, and carbon dioxide, consistent

with previous reports for *Halanaerobium* species isolated from diverse environments [52, 53, 67, 68, 92, 93, 119]. In particular, these findings agree with data recently reported for a similar produced water *Halanaerobium* draft genome (strain T82-1) with the capacity to yield ethanol, hydrogen, and acetate as fermentation products [16]. In addition, our analysis identified potential sulfate reducing bacteria. Specifically, sequences identified as the sulfide-producing order *Desulfovibrionales* were more abundant than previously suggested [10, 36, 43, 70]. Reconstruction of putative sulfide production pathways in the recovered *Halanaerobium* sp. MDAL1 draft genome revealed the metabolic potential for thiosulfate reduction via a rhodanese thiosulfate reductase (Rdl). This pathway, utilizing thiosulfate or elemental sulfur instead of sulfate, has been previously described for *H. congolense*, a *Halanaerobium* species isolated from an oil field, and for a *Halanaerobium* sp. T82-1 and *Halanaerobium* sp. DL-01 recently recovered from produced water [16, 64, 68, 95]. These results suggest that multiple metabolic pathways, some of which would not be detected by current sulfate-reducing bacteria tests, have the potential to contribute to microbial sulfide production in the produced water environment. Genetic evidence for thiosulfate reduction also suggests the need to evaluate thiosulfate concentrations in produced water in future research efforts to generate additional geochemical support for these processes.

Fouling incidents in hydraulic fracturing infrastructure are also commonly attributed to microbial activity [9, 33]. This study revealed several putative biofilm forming microbial taxa to exist in produced water, in particular the genera *Pseudomonas* and *Clostridium* have previously been suggested to be involved in biofilm formation [32, 120-122]. In addition, several genes involved in biofilm formation processes were identified in the *Halanaerobium* sp. MDAL1 draft genome, suggesting the biofilm formation potential of produced water *Halanaerobium*. Our data

therefore confirms recent work which reported genes for biofilm formation pathways to exist in draft genomes of *Halanaerobium* from fractured shale formations, despite their absence in other currently available *Halanaerobium* genomes [16]. In addition, these findings also confirm previous studies that have reported the presence of *Halanaerobium* in biofilms found in hydrocarbon environments [123].

Finally, draft genome analysis revealed the potential for diverse stress response mechanisms in produced water *Halanaerobium*. Produced water *Halanaerobium* populations overcome osmotic stress through the uptake of potassium (i.e. salt in strategy) and the utilization of osmoprotectants. In addition, we observed the genetic potential for motility, oxidative stress protection mechanisms characterized by rubredoxin, glutaredoxin, and superoxide reductase activity, and several heat shock and periplasmic stress associated genes, enabling *Halanaerobium* survival in the saline, heavy metal rich produced water environment. These findings are of particular interest as the stress response in microorganisms exposed to produced water has been shown to lead to enhanced biocide resistance and should be taken into consideration when evaluating biocide application strategies [32].

4.4.4 Study implications

An enhanced understanding of produced water microbial ecology is critical to limit corrosion, fouling, and souring issues, protect well infrastructure; minimize unnecessary biocide application; and encourage produced water recycling. This study represents the largest sampling and characterization of unconventional produced water microbial ecology to date. Recent studies have extensively analyzed unconventional produced water microbiology data based on samples

obtained from one to two Marcellus Shale well pads, producing valuable information limited by the small number of included sites [10, 13, 16, 36, 64]. The broader sampling effort in this produced water microbial ecology study allowed the confirmation of general trends observed during these previous temporal studies of a smaller number of wells, specifically the predominance of the putative biofilm-forming and fermentative *Halanaerobiales*. In addition, correlation analysis revealed statistically significant influence of fracturing fluid biocide composition on the produced water microbial community, and in particular *Halanaerobiales* abundance.

Finally, this study evaluated the metabolic potential of *Halanaerobium* in produced water by successfully recovering a *Halanaerobium* draft genome and comparing it to other available *Halanaerobium* genomes. Annotation revealed genetic potential for several fermentation pathways, thiosulfate reduction, biofilm formation, and a diverse stress response, suggesting *Halanaerobium* sp. MDAL1 contributes to acid and sulfide production in produced water. These genetic traits have also been previously observed in other *Halanaerobium* isolates and draft genomes, in particular the species *Halanaerobium* sp. T82-1, *H. congolense* and *Halanaerobium* sp. DL-01[16, 17, 68].

In conclusion, this study was able to confirm the dominance of halophilic, fermentative microorganisms, in particular the taxa *Halanaerobium*, across a wide range of produced water samples. Correlation analysis results suggest TDS concentration to have little influence on the microbial ecology in produced water and biocide treatment combination may affect the abundance of *Halanaerobiales* in produced water. This study was one of the first efforts to evaluate the metabolic potential of microorganisms associated with hydraulic fracturing

operations, supporting the role of *Halanaerobium* as a major contributor to microbial activity and source for corrosion, souring, and biofouling in the hydraulic fracturing infrastructure.

5.0 MICROBIAL COMMUNITIES IN BAKKEN REGION HYDRAULIC FRACTURING PRODUCED WATER

To be submitted for publication as:

Lipus, D., Roy, D., Khan, K., Ross, D., Vikram, A., Gulliver, D., Hammack, R., and Bibby, K. Microbial Communities in Bakken Region Hydraulic Fracturing Produced Water.

The Bakken Shale region (including both the Bakken and Three Forks formations) has become one of the United States' most important oil and gas producing regions. This study examines the microbiology of Bakken region produced water from 17 wells sampled over a six-month time frame. We also measured basic geochemical characteristics (TDS, DOC, and pH) across all evaluated samples. Produced water samples were characterized by high total dissolved solids (TDS) (220,000 mg/L – 350,000 mg/L) and low dissolved organic carbon (DOC) concentrations (41 mg/L – 132 mg/L). Microbial abundances varied between 10^1 – 10^4 16S rRNA gene copies/mL, approximately four orders of magnitude below those observed for produced waters from other unconventional resource regions. The most abundant bacterial orders found in produced water samples were *Bacillales*, *Halanaerobiales*, and *Pseudomonadales*, consistent with observations from other unconventional resource plays. Our observations suggest

temporal community structuring, as produced waters sampled early in our sampling period were dominated by *Halanaerobiales*, and produced waters sampled at the remaining winter sampling time points were characterized by high relative abundances of *Bacillales*. Data from this study extends the current available knowledge of the microbiology and chemistry associated with hydraulic fracturing produced water from the Bakken and Three Forks formations.

5.1 INTRODUCTION

Recent developments in unconventional resource exploration have motivated investigations into the microbial communities associated with produced water from hydraulic fracturing. The Bakken formation, together with the underlying Three Forks formation, represents one of the most important oil and gas reservoirs in the United States with proven reserves of 7.4 billion barrels of oil and 190 trillion m^3 of natural gas [124, 125] and is currently the third most productive oil region in the United States [125, 126]. Oil and gas production in the Bakken region generates an average of more than 11 million liters of produced water throughout a single well's lifetime [127]. Microbial activity during produced water holding is of particular concern, as microorganisms may cause sulfide release (souring), and corrode and foul infrastructure, resulting in increased operating costs and adverse environmental issues [5, 8, 10, 13, 16, 35, 44, 49]. As the downhole environment of the Bakken and Three Forks reservoirs is greater than 93^{o}C [44], there are likely limited indigenous microorganisms in the subsurface and that the majority of microorganisms in produced water originate in the well casing or surface infrastructure. Nonetheless, microbial processes are still a concern, as reports of biocorrosion,

biofouling, and biogenic sulfide production in production facilities in the Barnett Shale, which is believed to be free of microbial growth due to high subsurface temperatures, have been published previously [39, 128, 129].

Previous studies have investigated microbial communities and geochemistry throughout the life cycle of produced water associated with unconventional gas and oil extraction from other plays [10, 13, 16, 17, 35, 39, 41, 43, 49, 130]; however, little is known about the microbial communities in Bakken region produced waters. One previous study evaluated two produced water samples from western North Dakota that were dominated by the bacterial taxa *Halanaerobium*, *Marinobacterium*, and *Pseudomonas* [43]. Other studies evaluating Bakken Shale produced waters have only analyzed geochemical data. A 2010 study reported overall total dissolved solids (TDS) concentrations ranging between 150,000 and 219,000 mg/L, with sulfate concentrations as high as 1000 mg/L [131, 132], while a 2016 study found TDS values as high as 350,000 mg/L [133]. Additional sample analyses are necessary to confirm preliminary observed trends, and establish a better understanding of the microbial ecology in Bakken Shale produced waters.

The goal of this study was to better characterize the microbial ecology of Bakken region produced water from 17 unconventional hydraulic fracturing wells in the Bakken and Three Forks formations sampled across a six-month time frame. Additionally, TDS, DOC, and pH data was collected. Findings from this study will expand the current microbiological characterization available for Bakken region produced waters.

5.2 MATERIALS AND METHODS

5.2.1 Sampling

Produced water samples analyzed in this study were sampled from hydraulic fracturing wells in North Dakota actively producing oil from the Bakken formation or the underlying Three Forks formation. As production data from the Bakken and Three Forks formations is usually reported together and labeled "Bakken Shale Data", we refer to the sampled region as Bakken Shale or Bakken region throughout the chapter. Produced water samples were collected from the three-phase separators and final storage tanks of 17 different well sites (Appendix C, Table C1) on four dates: 10/8/2014, 11/5/2014, 1/14/2015, and 3/25/2015. The locations of the wells are shown in Figure 5-1. The samples were collected in 1 L sterile polypropylene bottles and transported on ice to the Environmental Engineering Laboratory at North Dakota State University. Approximately 100 mL of the sample were centrifuged immediately upon arrival and pellets were sent overnight on ice to the University of Pittsburgh for DNA extraction or stored at -80ºC until shipment. Sampling and biomass pelleting were performed by collaborators at North Dakota State University.

5.2.2 Chemical analysis

Dissolved organic carbon (DOC) was measured after diluting the samples with deionized distilled water using a Phoenix 8000 UV-persulfate total organic carbon analyzer (Teledyne, Ohio). The pH was measured using an automatic temperature corrected pH meter (Orion 230A,

Thermo Scientific, CO). Turbidity was determined using a 2100N Laboratory Turbidimeter and Formazin standards (Hach Company, Iowa). Alkalinity was assessed by the titrimetric method using a standard 0.02 N sulfuric acid titrant and interference checking was performed using American Public Health Association standard method 2005 [134]. TDS was measured using gravimetric method according to American Public Health Association standard method 2005 [134]. The sample was filtered through a 0.45 µm pore-size cellulose acetate membrane prior to TDS determination. Analyses were conducted at least in duplicates. Chemical analysis of produced waters was performed by collaborators at North Dakota State University.

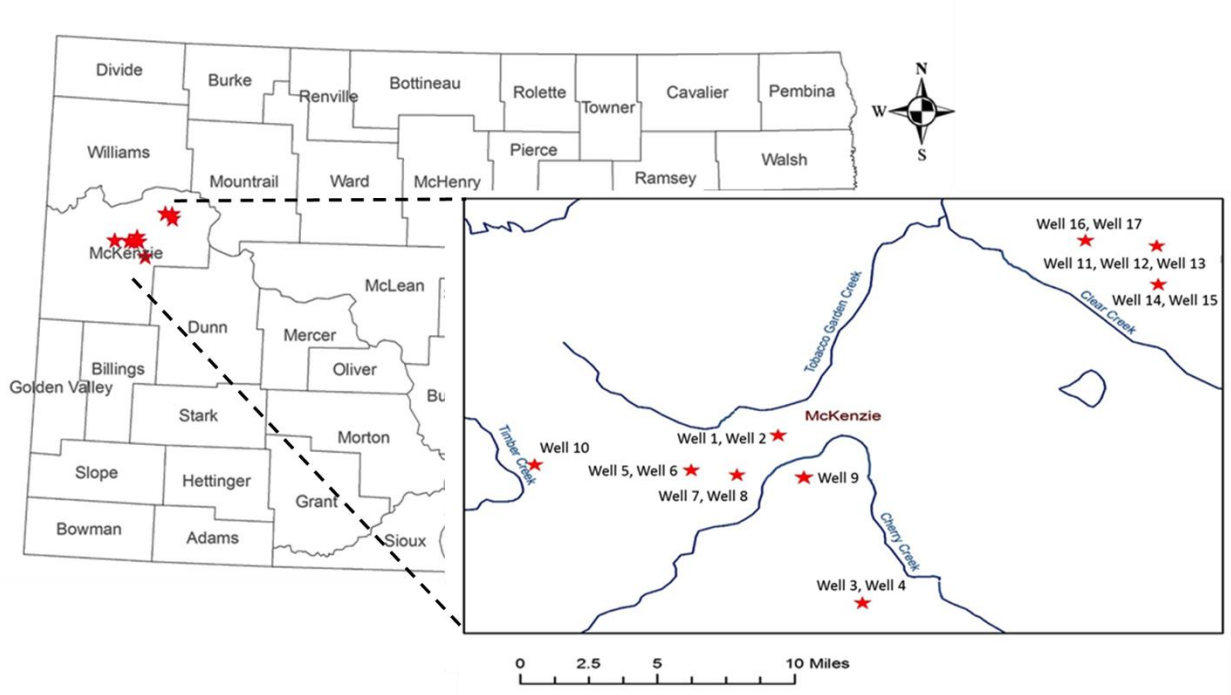

Figure 5-1: Sampling locations (red star) in North Dakota. Produced water samples from the separator and storage tank were collected from 17 different wells at nine different locations.

5.2.3 DNA extraction and sequencing

DNA extraction and sequencing was achieved as described previously [135]. Briefly, collected biomass was digested with 10 µl of 20 mg/mL lysozyme for 30 minutes at 37ºC followed by DNA extraction using a MoBio PowerSoil kit (Carlsbad, CA) according to the manufacturer's instructions. DNA from all samples was amplified using 16S rRNA primers as described previously [50, 136]. Samples initially not amplified were diluted 10 fold prior to amplification to limit inhibition. Negative controls were utilized for each PCR reaction and all negative controls were negative for contamination. Following amplification, 16S rRNA gene PCR products were purified using AMPure beads (Beckman Coulter, Pasadena, CA), run on a 1% agarose gel for cleanup verification, and quantified using Qubit (Life Technologies, Carlsbad, CA). Purified PCR products were pooled and diluted to a concentration of 20nM. Diluted samples were then denatured using fresh 0.2 N sodium hydroxide for 5 minutes at room temperature and further diluted to 10 pMol library with hybridization buffer HT1 according to manufacturer's instructions (Illumina, San Diego, CA). The 10 pMol library was spiked with 5% of 12.5 pMol PhiX control and sequenced using a 300 cycle V2 Nano kit on an Illumina MiSeq sequencer (Illumina, San Diego, CA).

5.2.4 qPCR

The microbial load for all produced water samples was determined using quantitative PCR (qPCR) using 16S rRNA gene primers designed by Maeda et al [76], as described previously [130]. Briefly, qPCR reactions were run in triplicate, each containing 1 µl DNA, 10 µl

SYBR Green qPCR Mastermix (BioRad, Hercules, CA), 8 µl ultrapure water, 0.5 µl reverse 16S rRNA gene primer, and 0.5 µl forward 16S rRNA gene primer. Standard curves were generated using genomic DNA from *Pseudomonas fluorescens* (ATCC 13525). Reactions were run using a BioRad qPCR thermocycler (BioRad, Hercules, CA) using default settings. The number of 16S rRNA gene copies/mL was calculated as described previously [135]. The theoretical detection limit was 10^1 16S rRNA gene copies/mL.

5.2.5 Data analysis

16S rRNA sequences from all samples were analyzed using QIIME version 1.7.0, as reported previously [51, 135, 136]. Sequences were quality trimmed (Q20) and demultiplexed in QIIME. Operational Taxonomic Units (OTU) were then picked using the pick_closed_reference_otus.py python script using UCLUST against the 2014 GreenGenes core set gg_97_otus.fasta reference database [78, 79]. Beta diversity was assessed by calculating weighted UniFrac distances [54]. Alpha diversity was assessed by determining the number of operational taxonomic units (OTUs), Chao1, and Shannon indices per 1000 sequences to remove bias introduced through varying number of sequences. For samples with less than 1000 sequences, the available number of sequences was used for alpha diversity estimation. Statistical differences in diversity, microbial load, and TDS concentration between separator and storage tank samples, Bakken and Three Forks formation samples, and between the four evaluated time points were assessed using two tailed t-tests. DNA sequences were deposited on MG-RAST [137] and can be accessed under the library accession number mgp17856. Sequence headers are described in Appendix C, Figure C1.

5.3 RESULTS

5.3.1 Sample characteristics and geochemistry

Hydraulic fracturing produced water samples were taken from 17 hydraulically fractured, horizontal, Bakken formation (9 wells) and Three Forks formation (8 wells) well sites (Figure 5-1). Overall, 64 separator samples and 57 holding tank samples were collected at four different time points over a six-month time frame (October 2014, November 2014, January 2015, and March 2015) (Appendix C, Table C1). Each well had its own produced water holding tank. The holding tank and separator from Well 6 were not sampled in November 2014, holding tanks from Wells 1, 2, 7, 8, 10, 11, 12, and 16 and separators from Wells 9 and 15 were not sampled in January 2015, and the holding tank and separator from Well 9 were not sampled in March 2015 due to logistical challenges. The production ages of the wells analyzed in this study ranged from 270 to 1241 days (Appendix C, Table C1). Total dissolved solids (TDS), dissolved organic carbon (DOC), pH, alkalinity, and turbidity concentrations were measured across all four time points (Table 5-1, Appendix C, Table C1). The overall total dissolved solids (TDS) concentrations ranged between 250,750 mg/L and 335,000 mg/L in the separator samples and between 259,750 mg/L and 330,500 mg/L in the storage tank samples (Table 5-1). No significant changes in TDS concentrations were observed between separator and storage tank samples and across the four sampling time points (t-test, all $P > 0.05$). No significant differences in TDS concentrations were identified between Bakken and Three Forks formation samples (t-test, all $P > 0.05$). Dissolved organic carbon (DOC) concentrations in Bakken Shale produced waters ranged between 41 and 80 mg/L in separator samples and 49 and 132 mg/L in storage tank

samples (Table 5-1), levels lower than those up to 400 mg/L, previously reported for early produced waters from the Marcellus Shale [11] and similar to levels of 19 mg/L - 46 mg/L, previously reported for later Marcellus and Devonian New Albany Shale produced waters [13, 138]. The pH varied between 5.0 and 7.0, in the range previously reported for Bakken Shale produced waters [131].

Table 5-1: Physiochemical characteristics of analyzed produced water samples. Total dissolved solids (TDS), dis-solved organic carbon (DOC), pH, alkalinity, and turbidity were measured across all four sampling time points. Ambient air temperatures were 8^{o}C to 10^{o}C on 10/7/2014 (October), 0^{o}C to – 2^{o}C on 11/1/2014 (November), -3^{o}C to -4^{o}C on 1/14/2015 (January), and 15^{o}C to 16^{o}C on 3/25/2015 (March).

	Separator			**Storage Tank**		
	Max	**Min**	**Avg**	**Max**	**Min**	**Avg**
TDS (mg/L)	340750	223000	301507	332250	196000	296803
DOC (mg/L)	225	19	70	132	14	65
pH	6.9	5.0	6.0	7.0	5.2	6.1
Alkalinity (mg/L)	900	300	550	900	250	525
Turbidity (NTU)	128.0	6.2	140.6	890.0	10.1	139.7

5.3.2 Microbial abundance

Microbial abundance in the collected produced water samples was determined by quantitative PCR. Eleven separator and fourteen storage tank samples could not be quantified due to low DNA yield. Microbial abundance in the remaining samples varied between $10^1 – 10^4$ 16S rRNA gene copies/mL (Figure 5-2). No statistically significant differences in microbial abundance were observed between separator and storage tank samples (t-test, $P > 0.05$), between sampling time points across all samples (t-test, all $P > 0.05$), or between Bakken and Three Forks formation samples (t-test, all $P > 0.05$).

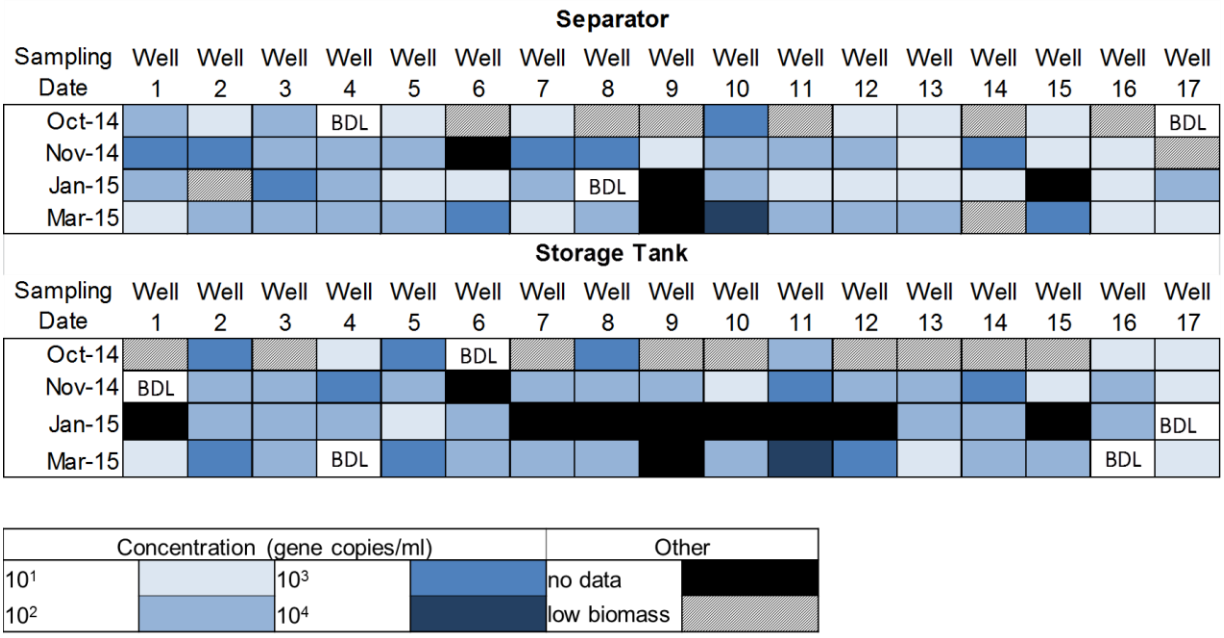

Figure 5-2: Microbial abundance as 16S rRNA gene copies per milliliter across the evaluated Bakken formation and Three Forks formation hydraulic fracturing produced water samples, as determined by qPCR. Well and time points for which no data is available were either not sampled or compromised during processing. DNA extraction from several samples failed due to low biomass. Samples labeled BDL were found to have DNA concentrations below the detectable limit.

5.3.3 Microbial community structure

The microbial community structure was determined in 44 separator samples and 38 storage tank samples using 16S rRNA sequencing. Samples from 20 separators and 19 storage tanks were not analyzed, as they failed DNA extraction due to low biomass, could not be PCR amplified, or yielded low sequence counts due to low biomass. Abundances for all major orders

are summarized in Appendix C, Figure C2. All minor orders (<2%) are summarized in Appendix C, Figure C3. Genus level taxonomy data for all samples is summarized in Appendix C, Figure C4. The anaerobic, fermentative Firmicutes orders *Bacillales* and *Halanaerobiales* and the Proteobacteria order *Pseudomonadales* were the most abundant taxa across all evaluated samples. Figure 5-3 shows taxonomy data for these three most abundant orders. *Bacillales* were identified in all 82 samples and were the most dominant order in 45 samples. *Bacillales* were particularly abundant in November, January, and March samples, accounting for as much as 99% of all sequences in a single sample. Within *Bacillales*, most sequences were unclassified *Bacillaceae* (up to 90% relative abundance), *Bacillus* (up to 17% relative abundance), or *Staphylococcus* (up to 14% relative abundance). These *Bacillales* taxa have previously been associated with acid production and spore formation [14, 39, 139-141]. The order *Pseudomonadales* was identified in all samples and most abundant in the November, January, and March samples, accounting for up to 69% relative abundance in a single sample (Figure 5.3; Appendix C, Figure C2). *Pseudomonadales* was the most dominant order in 13 samples. Within the order *Pseudomonadales*, the majority of sequences were *Pseudomonas* (up to 53% total relative abundance), *Psychrobacter* (up to 23% total relative abundance), or unclassified *Pseudomonadaceae* (up to 6% total relative abundance). *Psychrobacter* was particularly abundant in January and March produced water samples. *Halanaerobiales* were identified in all but three samples and were the most abundant order in all October separator and storage tank samples (up to 94% relative abundance) and 10 samples across the remaining three sampling time points. The majority of sequences within the order *Halanaerobiales* were unclassified *Halanaerobiaceae* (up to 48% total relative abundance), *Halanaerobium* (up to 19% total relative abundance), or *Haloanaerobacter* (up to 6% total relative abundance).

Other observed taxa identified at greater than 10% relative abundance included *Clostridiales*, *Bacteroidales*, *Lactobacillales*, *Oceanospirillales*, *Campylobacterales*, and *Actinomycetales* (Appendix C, Figure C2). *Bacteroidales, Lactobacillales, Oceanospirillales,* and *Actinomycetales* were only abundant (>10% relative abundance) in October samples. All of these taxa have been previously identified in produced water from hydraulic fracturing [10, 13, 14, 39, 135]. Within the *Clostridiales*, most sequences were affiliated with the genus *Clostridium* (up to 9% relative abundance). Within the *Oceanospirillales*, most sequences were associated with the genera *Halomonas* (up to 13% relative abundance) or *Marinobacterium* (up to 9% relative abundance). *Clostridium*, *Halomona*s, and *Marinobacterium* were all previously observed in produced water [10, 135, 142]. The majority of the order *Campylobacterales* was *Arcobacter*, and 11 samples were found to have relative *Arcobacter* abundances of 10% or greater. The majority of sequences within the order *Actinomycterales* were classified as the genus *Corynebacterium*, a taxa previously identified in hydrocarbon polluted environments [143]. This taxon was found to be particularly abundant in October samples.

We also investigated the presence of putative sulfate reducers. The genus *Desulfomicrobium* (order *Desulfovibrionales*) was identified in 12 samples, with a highest observed relative abundance of 1.6%. The genus *Desulfuromonas* (order *Desulfuromonadales*) was identified in 19 samples with a highest observed relative abundance of 1.8%.

5.3.4 Microbial diversity

Alpha diversity parameters (number of operational taxonomic units (OTUs), Chao1 diversity index, and Shannon diversity index) and beta diversity measurements (weighted

UniFrac distances) were calculated to investigate microbial diversity. The range of OTUs across all samples ranged between 44 and 347 OTUs (Appendix C, Table C3), the Chao1 index ranged between 34 and 185 and the Shannon diversity index ranged between 2.18 and 5.39 (Appendix C, Table C3). All three alpha diversity measurements were found to be within the range (OTUs = 9 – 381, Chao1 = 23 – 991, Shannon diversity = 0.02 – 7.17) of those previously reported for hydraulic fracturing produced waters [10, 14, 39]. No statistical differences in number of OTUs, Chao1 Index, and Shannon diversity measurements were identified between Bakken formation and Three Forks formation samples (t-test, all $P > 0.05$). PCoA analysis of weighted UniFrac distances revealed three clusters, each defined by high relative abundances of *Bacillales*, *Pseudomonadales*, or *Halanaerobiales* (Figure 5-4). The majority (71%) of October samples grouped in the *Halanaerobiales* cluster. PCoA analysis did not reveal any clustering by formation (Appendix C, Figure C5).

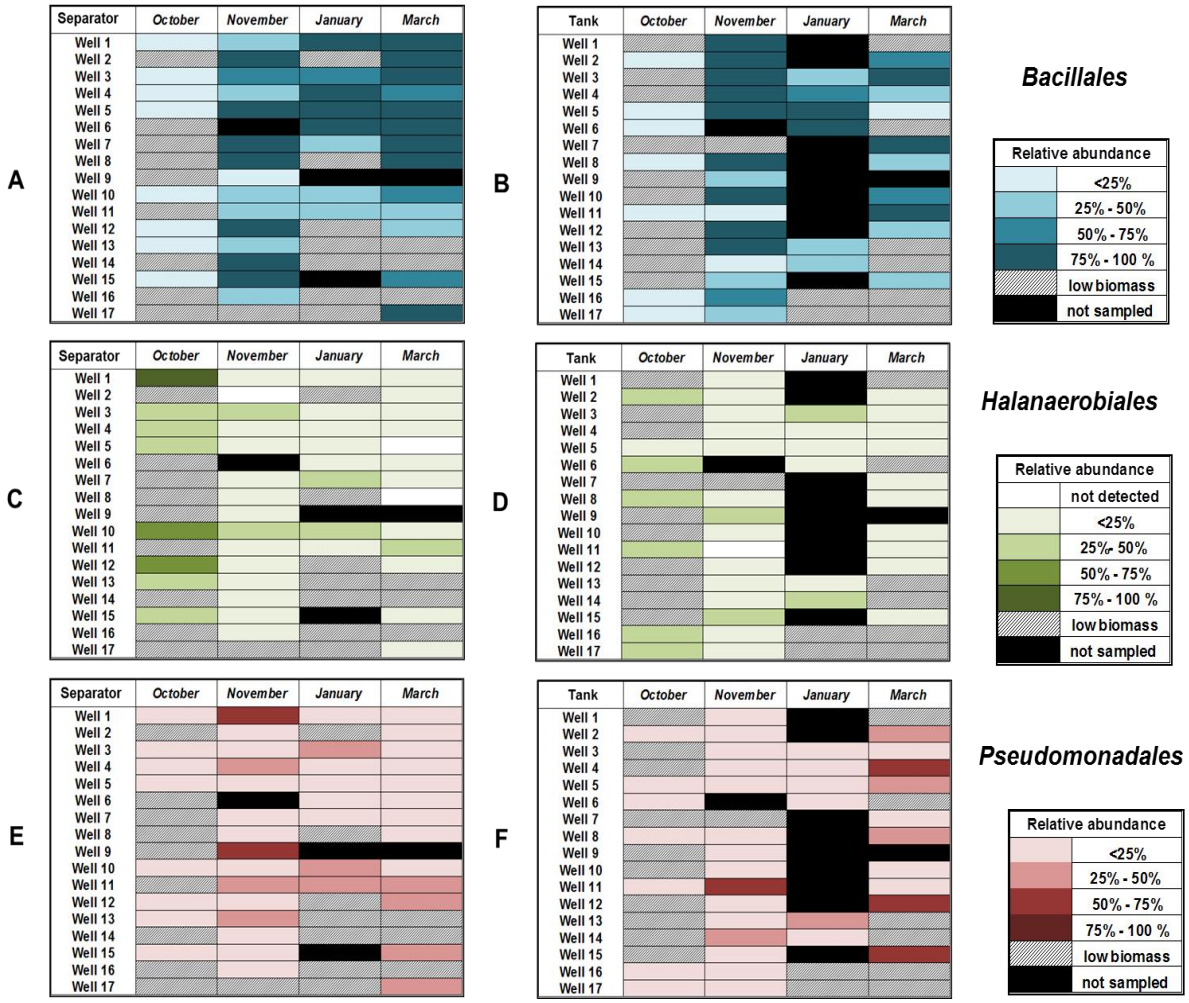

Figure 5-3: Heatmap of relative abundances of the three most dominant orders *Bacillales* (Panel A, B), *Halanaerobiales* (Panel C, D), and *Pseudomonadales* (Panel E, F) in separator (left) and storage tank (right) samples across all analyzed samples and time points. Samples labeled "low biomass" failed DNA extraction, could not be PCR amplified, or yielded low sequence counts.

5.4 DISCUSSION

Despite several recent studies evaluating hydraulic fracturing produced waters from oil and shale gas regions around the United States, little data is currently available on the microbiology of produced water generated in the Bakken region. The current study evaluated the microbial community structure and pH, total dissolved solids (TDS), dissolved organic carbon (DOC) concentrations in produced waters from 17 Bakken and Three Forks formation wells across a six month time frame.

5.4.1 Bakken and Three Forks formation produced water has low biomass compared to other shale regions

Microbial abundance was three to five orders of magnitude below microbial abundances reported for produced waters from other shale regions [10, 14, 39, 135, 144]. These observations agree with a recent Bakken Shale produced water study that reported low biomass [44]. High temperatures in the Bakken and Three Forks subsurface create an unfavorable environment for growth of the taxa identified in this study, suggesting that microorganisms identified were introduced to produced waters in the upper casing regions or the surface infrastructure [44]. Furthermore, microbial abundances were not found to vary temporally, and microbial abundances in the separator and storage tank were not found to be statistically different (all $P > 0.05$). These observations suggest that samples at either the tank or separator will produce comparable results.

5.4.2 Bakken and Three Forks formation produced water is dominated by the taxa *Bacillales, Halanaerobiales,* and *Pseudomonadales*

Analysis of the microbial community structure using 16S rRNA sequencing revealed both separator and storage tank produced water samples were dominated by the orders *Bacillales, Halanaerobiales*, and *Pseudomonadales*. A shift in community structure was observed from the October samples to the November samples. October samples had high relative abundances of *Halanaerobiales*, while November, January, and March samples had high relative abundances of *Bacillales* and *Pseudomonadales*. Ambient temperatures were approximately 10ºC at the time of sampling in October, 0ºC at the time of sampling in November, and -3ºC to -4ºC at the time of sampling in January. Ambient air temperatures had increased to 15ºC at the March sampling event; however, temperatures leading up to that day had been below 5ºC. Thus, the microbial population shift was observed in the 'winter' samples (Nov., Jan., and Mar.) with *Pseudomonas* and *Psychrobacter* becoming more prevalent (Figure 5-3, Appendix C, Table C4). The genus *Psychrobacter* is considered osmotolerant and psychrophilic, making it suitable for cold hypersaline environments [41, 145-148] and a likely candidate to outcompete other produced water microorganisms during colder months. These observations suggest microbial communities in hydraulic fracturing produced waters change temporally in response to environmental conditions, thus consideration of seasonal variation in microbial communities should be included in produced water management strategies.

Halanaerobiales, *Pseudomonadales*, and *Bacillales* have all previously been shown to be abundant and widespread in produced water [10, 13, 14, 39, 41, 43, 135]. The order *Halanaerobiales*, especially the genus *Halanaerobium*, is halophilic and previous isolates have

the metabolic potential to produce sulfides, produce acids, and form biofilms [17, 52, 67, 95]. Several *Halanaerobium* draft genomes have recently been recovered from hydraulic fracturing produced water [16, 135], and analysis of metabolic pathways further supports their role in acid and sulfide production in hydraulic fracturing systems [16, 17, 135]. The *Bacillales* were the most abundant taxa across the winter months (November, January, March). Members of *Bacillales*, including *Bacillus* and *Staphylococcus*, have both been previously identified in produced water and are considered anaerobic or facultative anaerobes, fermentative, and spore forming [139, 140]. Spore forming microorganisms have been linked to an increased biocide resistance [149]. *Pseudomonadales* were dominated by the genera *Pseudomonas* and *Psychrobacter*. *Pseudomonas* has been frequently detected in produced water and has been suggested to be involved in the oxidation of organic compounds and nitrate reduction in this environment [13, 14, 39, 150]. Recovery and annotation of a *Pseudomonas* draft genome via metagenome sequencing of one of the here analyzed samples (Well 10, March 2015, storage tank) revealed several genes involved in biofilm formation pathways [130]. Sequences affiliated with the genus *Psychrobacter* were identified in 45 samples. Six samples were characterized by relative *Psychrobacter* abundances of 10% or greater. We believe *Psychrobacter* has not been previously identified in produced water at this frequency and abundance. Both *Pseudomonas* and *Psychrobacter* contain biofilm forming species [121, 146]. The high relative abundance of *Arcobacter* across multiple samples was of particular interest. *Arcobacter* are autotrophic, halophilic, microaerophilic, nitrogen fixing, and sulfate oxidizing, and have been reported to survive and form biofilms at temperatures below 5^{o}C [13, 151-155]. The genus *Arcobacter* is often detected in hydrocarbon environments and has been suggested to be important for sulfur cycling in these systems [10, 13, 14, 39, 151]. *Psychrobacter* and *Arcobacter* relative abundances

were highest in samples with high relative abundances of *Halanaerobiales* and low relative abundances of *Bacillales*. *Psychrobacter* and *Arcobacter* have also been discovered together in sub-Antarctic seawater contaminated with hydrocarbons previously [156], supporting their ability to thrive in cold, hypersaline hydrocarbon contaminated environments.

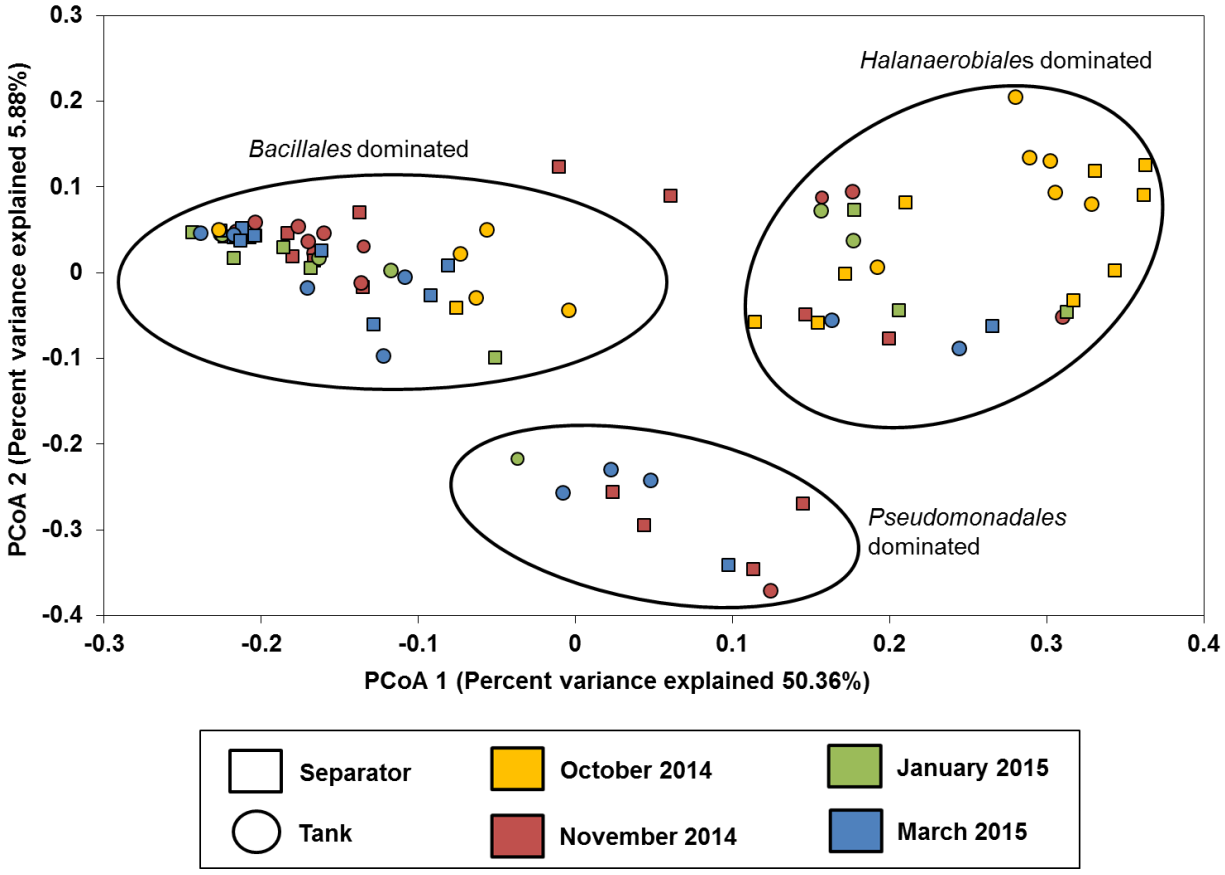

Figure 5-4: PCoA plot based on weighted UniFrac distances, showing all analyzed samples coded by sampling date (color) or sampling source (shape).

The presence of *Desulfomicrobium* and *Desulfomonas* suggests the potential for sulfide production through sulfate reduction; however the low relative abundance (<2%) agrees with

previous work suggesting classical sulfate reducers likely play a minor role in sulfide production in hydraulic fracturing produced water environments [13].

5.4.3 Bakken and Three Forks formation produced waters have a microbial community composition similar to produced waters from other oil shale gas regions, but have a distinct community structure

Comparison of these findings with results from previous hydraulic fracturing produced water studies suggests the Bakken Shale microbial community to be similar in composition and alpha diversity but unique in community structure (i.e. relative abundance of specific taxa) to produced water from other plays. This is exemplified by the high relative abundance of *Bacillales* and lower than expected relative abundances of *Halanaerobiales* in Bakken Shale produced water. In contrast, data from studies investigating Marcellus Shale and Barnett Shale produced water suggested this role to be reversed with *Halanaerobiales* as the more abundant taxa. All identified taxa have been previously observed in produced water, adding to a growing consensus on common produced water taxa. Despite increasing understanding of produced water microbiology, it is necessary to evaluate produced waters from different areas separately, as regional, seasonal, and operational factors structure the microbial community. Other operational factors, such as biocide composition, have also been shown to affect the microbial community structure [10, 13, 135].

5.4.4 Implications

Evaluating the biological characteristics of hydraulic fracturing produced water is necessary to inform produced water management. Unlike other oil and shale gas regions, such as the Marcellus Shale, little data is available in the literature about the microbiology of produced waters from the Bakken region. In this work we characterized the microbial ecology from the greatest number of Bakken Shale separator and storage tanks produced water samples to date.

One of the primary motivations for this study was to investigate the presence of microorganisms potentially involved in microbial acid and sulfide production and biofilm formation processes. Taxonomic analysis of produced water enabled identification of multiple microbial taxa putatively involved in corrosion, fouling, and gas souring, namely members of the orders *Halanaerobiales*, *Bacillales,* and *Clostridiales*. While the sulfidogenic taxa *Desulfomicrobium* and *Desulfomonas* were detected at low relative abundances, organisms belonging to genus *Halanaerobium* have been shown to produce sulfide through an alternative metabolic pathway, namely the reduction of sulfur or thiosulfates [17, 95, 135]. Finally, results demonstrated no community difference between separator or storage tank samples, but the community structure varied temporally, necessitating periodic sampling to capture microbial diversity.

Findings from this study are based on the analysis of produced water samples from a single region of the Bakken Shale region and are not directly transferrable to produced water from other oil and shale gas regions. In addition, the roles of identified taxa from produced water in biofilm formation, acid production, and sulfur metabolism were inferred from related isolates, which limits the ability to draw conclusions regarding the metabolic activity of microorganisms

based solely on sample taxonomic assignment. Further work to better understand the metabolic potential and activity of relevant isolates, particularly *Halanaerobiales*, *Bacillales*, and *Pseudomonadales* in the produced water environment is necessary to investigate how these organisms are involved with corrosion, sulfide production, and biofilm formation processes.

5.5 CONCLUSIONS

This study expands the current understanding of the microbial ecology in Bakken Shale region produced water. The microbial composition was found to be similar to that of produced waters from other regions with altered structure (i.e. relative abundances). Temporal changes in microbial community structure, presumably caused by environmental changes, were observed. Communities were dominated by taxa of potential operational significance, such as *Bacillus*, *Halanaerobium*, or *Pseudomona*s with implications for biocorrosion, biofouling, and sulfide production in the Bakken Shale hydraulic fracturing infrastructure. Results from this study also highlight the need to evaluate produced waters from different shale regions in greater detail to further advance the understanding of produced water chemistry and microbiology and improve produced water management.

6.0 PEROXIDE SCAVENGING AND MULTIDRUG EFFLUX HIGHLIGHT AN ACTIVE, GENETIC PSEUDOMONAS FLUORESCENS BIOFILM RESPONSE TO THE BROAD SPECTRUM ANTIMICROBIAL SODIUM HYPOCHLORITE

To be submitted for publication as:

Lipus, D., Bibby, K. (2017). Peroxide scavenging and multidrug efflux highlight an active *Pseudomonas fluorescens* biofilm response to the broad-spectrum antimicrobial sodium hypochlorite.

Sodium hypochlorite is one of most commonly used antimicrobial agents in industrial applications. Despite its popularity microbial resistance against sodium hypochlorite has been observed previously, and especially represents a challenge in biofilms. Here, we investigate the transcriptomic response of *Pseudomonas* biofilms, a taxon abundant in multiple industries, including water treatment, food, and oil and gas, to sub-lethal concentrations of sodium hypochlorite. RNA-seq results suggest *Pseudomonas* biofilm populations to use peroxide scavenging enzymes, oxidative stress repair, and multidrug efflux to protect themselves against reactive oxygen species produced by sodium hypochlorite exposure. Furthermore, genes involved in amino acid synthesis and energy metabolism were downregulated. Findings from this

work will help to improve the current understanding of genetic biocide resistance mechanisms and may help to optimize current biocide application strategies.

6.1 INTRODUCTION

Formation of biofilms is considered a major issue in many industries, including food and beverage processing, water and wastewater treatment, health care, and other sectors using industrial water systems [157-160]. Biofilms may cause food spoilage and food safety issues, corrosion of stainless steel, block membrane pores in filtration processes, lead to the release of unpleasant odors, and generally reduce production efficiency. Biofilms may also harbor pathogenic microorganisms responsible for a multitude of infections, and therefore represent a serious health risk [122, 161]. For example the two well known, pathogenic organisms *Pseudomonas aeruginosa* and *Clostridium difficile* are often discovered in biofilms [162]. In the hydraulic fracturing industry biofilm formation may lead to clogging of fractures [5, 45]. Furthermore, biofilms may damage the infrastructure through biofouling and corrosion [130, 135, 163]. *Pseudomonas fluorescens* populations have been suggested to contribute to corrosion in buried steel pipeline [164].

Biofilms usually comprise a population of microorganisms enclosed in extracellular polymeric substance consisting of lipids, proteins, and polysaccharides, attached to a surface. Biofilms are usually controlled through the use of biocides, a chemical or biological agent that disrupts and destroys microorganisms in biofilms. However, biofilms often prove to be resistant to commonly used biocides such as quaternary ammonium compounds, benzalkonium chloride,

chlorine, 2,2-dibromo-3-nitrilopropionamide (DBNPA), and sodium hypochlorite [165-167]. Different active, genetic responses in microbial biofilms have been observed previously. Biocides may have limited penetration into the exopolysaccharide layer and bind or adsorb to the outside matrix components of biofilms. For example, chlorine was shown to only penetrate 100 μm into a complex dairy biofilm [168]. In addition, biofilm communities may exhibit specific phenotypes, characterized by an upregulation of proteins involved in oxidative stress response, periplasmic stress, and cell envelope synthesis [167]. Several studies have shown genes encoding flagellar proteins are suppressed and genes encoding exopolysaccharide (EPS) production and general stress response proteins (e.g. RpoS) are induced upon surface attachment [165, 169, 170]. Biocide exposure may also trigger genetic factors. Efflux pumps and lipid biosynthesis were shown to contribute to biocide resistance in *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* when exposed to the biocide glutaraldehyde [32, 130]. Similarly, multidrug efflux pumps were upregulated in biofilms exposed to triclosan and cetylpyridinium chloride [171, 172]. Exposure to the quaternary ammonium compound benzethonium chloride (BZT) lead to the upregulation of efflux transporters and the peptidoglycan biosynthesis gene family *mur* [173].

Sodium hypochlorite (NaOCl) is a broad spectrum anti-microbial that is used in various domestic and industrial settings, including healthcare facilities, food and agriculture industries, and waste disposal industries [174]. Sodium hypochlorite plays one of the most important roles in water treatment, where it is used for disinfection [175]. Sodium hypochlorite has also been used to control microbial growth in the oil and gas industry [45, 47, 176]. Upon contact with water sodium hypochlorite forms hypochlorous acid, which divides into hydrochloric acid and produces reactive oxygen species (ROS) with strong antimicrobial properties. The resulting

oxidative action damages proteins, leads to strand breaks in DNA, and causes lipid and fatty acid degradation [177, 178]. Hypochlorous acid and hypochlorite ions cause amino acid degradation and hydrolysis. Furthermore, hypochlorous acid releases chlorine, which leads to the formation of chloramines that interfere with the cellular metabolism and enzymatic activity [177, 178]. Resistance to sodium hypochlorite and mechanistically similar antimicrobials has been previously reported for a variety of microorganisms [179-182], including *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Sodium hypochlorite exposure has been suggested to trigger stress by generating superoxide anions ($O_2^{\bullet-}$), through for example the oxidation of organic compounds, and hydroxyl radicals through a Fenton type reaction ($HOCl + Fe(II) \rightarrow {\cdot}OH + Cl^- + Fe(III)$) and a reaction between hypochlorous acid and superoxide anions ($HOCl + O_2^{\bullet-} \rightarrow OH + Cl^- + O_2$) [183, 184]. Correspondingly, sublethal exposure to sodium hypochlorite in *Xanthomonas campestris* lead to the transcription of peroxide scavenging enzymes within the OxyR and OhrR regulons [185]. Examples of primary scavengers in many bacteria are catalases (Kat), alkyl hydroperoxide reductases (Ahp), and glutathione peroxidases and reductases [186-188]. These enzymes work by actively reducing peroxides and hydroperoxides to their nontoxic forms [189, 190]. Monochloramine stress in *E. coli* induced oxidative stress, DNA repair, multidrug efflux, and cell wall repair [147]. Exposure of *Pseudomonas aeruginosa* to the biocides hypochlorite, peracetic acid, and hydrogen peroxide lead to the upregulation of DNA repair genes and protein secretion and downregulation of energy metabolism related genes [186]. These findings suggest sodium hypochlorite triggers a multifaceted genetic response and to specifically cause an induction of oxidative stress genes.

In this study we systematically analyzed the effect of sodium hypochlorite on biofilms formed by the model organism *Pseudomonas fluorescens*. Furthermore, we evaluated the genetic

response of *Pseudomonas fluorescens* to a sublethal exposure of sodium hypochlorite using RNA sequencing, with the goal to identify active, genetic response mechanisms that may contribute to biocide resistance. Exposure of *Pseudomonas fluorescens* to sodium hypochlorite was hypothesized to lead to an upregulated transcription of oxidative stress genes, and the induction of general stress response mechanisms such as DNA repair and multidrug efflux. Findings from this study will inform biocide resistance in microorganisms of industrial relevance and may help to improve industrial biocide application strategies.

6.2 MATERIALS AND METHODS

6.2.1 Bacterial strain maintenance and culturing

Pseudomonas fluorescens cultures (strain 13525) were obtained from American Type Culture Collection (ATCC) and maintained as laboratory glycerol stock. *Pseudomonas fluorescens* cultures were grown in Luria-Bertani (LB) broth at 25^{o}C.

6.2.2 Biofilm assays

Biofilm assays were performed using a modified version of a protocol previously described by Vikram et al. [31]. In this study, the term biofilm refers to surface attached cells growing in a 96-well or 6-well plate. Briefly, *Pseudomonas fluorescens* cultures were grown overnight, diluted 100 fold in LB-broth and 200 µl of inoculant was placed in each well of a 96-well plate. Plates were incubated at 25^{o}C for 48 hours. Post incubation, biofilms were washed

with phosphate-buffered saline (PBS). Washed biofilms were there then exposed to different concentrations of a sodium hypochlorite solution (NOCl) for 10 minutes, with 200 µl of treatment added to each well. Residual free chlorine concentration was assessed after the ten minute exposure for the 0.6 mg/L condition. Residual chlorine after ten minutes was found to be 0.41 mg/L (+/- 0.04 mg/L). Exposure experiments were performed in quadruplicate. After 10 minutes, the treatment was removed and residual chlorine was immediately quenched by adding 10% sodium thiosulfate. Biofilms were then washed again with PBS. Biofilm viability was assessed by adding 200 µl of 250 µg/mL 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution in PBS to each well and incubation for 1 h at 25°C. Viable biofilms produced formazan, that was dissolved in 200 µl of dimethyl sulfoxide (DMSO), as previously described [31, 191]. Biofilm viability was assessed by measuring absorbance at 570nm using a 96-well plate reader. The average absorbance and standard deviations (SD) from four replicates were calculated for each treatment group.

6.2.3 Biofilm growth and RNA extraction for RNA-seq

Overnight cultures of *Pseudomonas fluorescens* were diluted 100-fold and 2 mL of inoculant were placed in each well of a 6-well plate. Biofilms were grown for 48 hours at 25^{o}C. Post incubation biofilms were washed with PBS and exposed to a control solution (PBS) and 0.6 mg/L sodium hypochlorite solution for 10 minutes (six wells each). Post exposure the residual chlorine was immediately quenched with 10% sodium thiosulfate. Biofilms were then lysed in 1 mL TRIZOL (Life Technologies, Carlsbad, CA) and placed in fresh 1.5 mL tubes. RNA was immediately extracted using TRIZOL, according to the manufacturer's protocol. RNA from six

biological replicates on each plate was pooled. DNA contamination was removed using Turbo DNAse (Life Technologies, Carlsbad, CA), according to manufacturer's recommendations. Overall, four replicate experiments were performed.

6.2.4 RNA-seq library preparation and sequencing

Prior to library preparation ribosomal RNA was removed using Ribo-Zero rRNA removal kit (Illumina, San Diego, CA). Briefly, DNase-treated RNA samples were treated with RiboZero to remove rRNA, and purified using the RNeasy MiniElute Cleanup Kit (Qiagen, Valencia, Spain). RNA was prepared for sequencing using the ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicentre Biotechnologies, Madison, WI). 5 ng rRNA-depleted sample were mixed with the RNA fragmentation solution and cDNA synthesis primer provided in the ScriptSeq kit, and cDNA was synthesized using StarScript reverse transcriptase. Synthesized cDNA was purified using Agencourt AMPure beads (Beckman Coulter, Indianapolis, IN). Di-tagged cDNA was PCR amplified using provided failsafe primers with Illumina adapters and barcodes and sequenced on an Illumina Miseq sequencer.

6.2.5 Bioinformatics

Libraries were quality trimmed (<Q30, <35 nucleotides min length) using CLC Genomics workbench 10.0 (CLC bio, Aarhus, Denmark). Differential gene expression was analyzed as described previously [31], using the RNA-seq and small RNA analysis tools in CLC Genomics workbench 10.0 (CLC bio, Aarhus, Denmark). Reads mapping to *Pseudomonas*

ribosomal RNA (5S, 16S, 23S) and intergenic regions were removed manually. Furthermore, sequencing data was carefully screened for any sequences mapping against phiX (used as sequencing control) and other contaminants. Reads for each sample were then mapped against *Pseudomonas fluorescens* strain SBW25 reference genome (Genbank accession NC_012660) and reads per kilobase million (RPKM) were determined [192]. The data was then normalized by scaling [193], and differentially expressed genes were determined by comparing normalized gene reads, between control biofilm samples (PBS treated) and sodium hypochlorite treated biofilm samples, using Baggerly's test on proportions [194]. A minimum of 2-fold up or down regulation and a FDR corrected p-value of 0.01 were selected as criteria for differential expression. Genes found to be differentially expressed (either up or downregulated) in all or three of the four replicates were selected for annotation analysis. Furthermore, genes that were upregulated in two replicates and not downregulated in any other replicates were also selected for annotation analysis. Genes found to vary in differential regulation between replicates (e.g. upregulated in one replicate and downregulated in another) were not included. Selected genes were annotated using the online Database for Annotation, Visualization, and Integrated Discovery tool (DAVID) using default settings [195]. In addition, differentially expressed genes were annotated with the National Center of Biotechnology Information BLASTx tool using default settings [196].

6.3 RESULTS

6.3.1 Biofilm viability in response to sodium hypochlorite

Viability of 48-hour *Pseudomonas fluorescens* biofilms was evaluated across a range of ten concentrations between 0.1 mg/L and 20 mg/L sodium hypochlorite (Figure 6-1). Biofilm viability (measured as an equivalent of biofilm activity through formazan production) was found to be highest in control biofilms. A decrease in viability was observed for biofilms exposed to concentrations of 0.1 mg/L (17.7% reduction, compared to control) and 0.5 mg/L sodium hypochlorite (10.4%, compared to 0.1 mg/L). Sodium hypochlorite concentrations of 0.6 mg/L resulted in the largest percent decrease in viability (29.5%, compared to 0.5 mg/L). Further increase of sodium hypochlorite concentration resulted in a gradual decrease of biofilm viability (Figure 6-1). Therefore, 0.6 mg/L sodium hypochlorite was selected as the sub-inhibitory concentration for the purpose of this study.

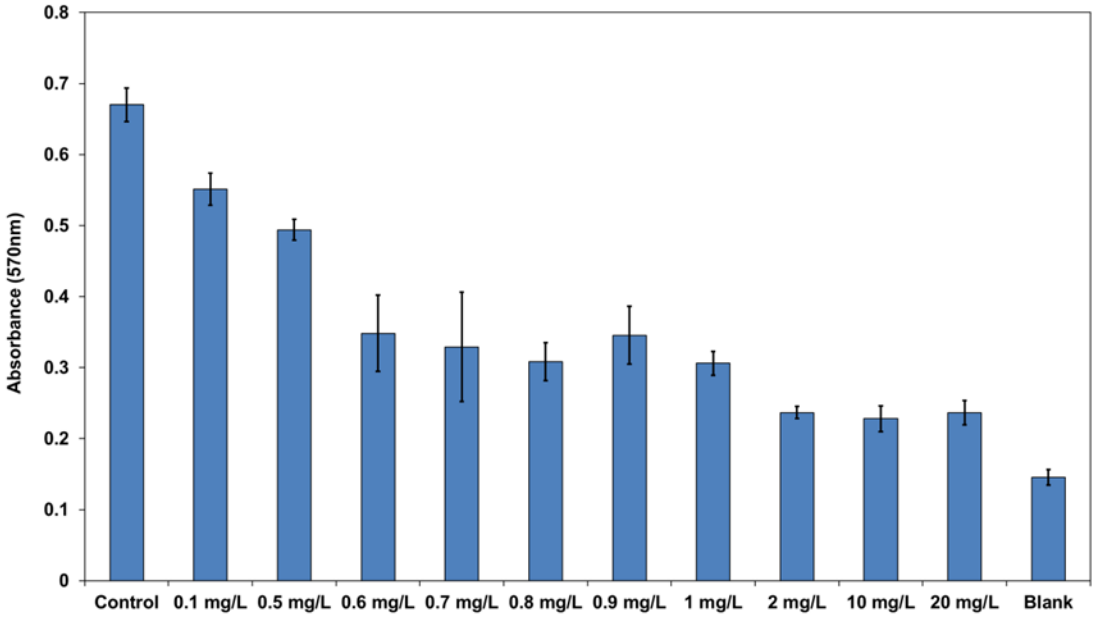

Figure 6-1: *Pseudomonas fluorescens* viability measured as absorbance at 570nm following a MTT assays. Biofilms were grown for 48-hours and treated with sodium hypochlorite concentrations between 0 mg/L and 20 mg/L for ten minutes.

6.3.2 Sequencing data analysis

The genetic response of *Pseudomonas fluorescens* biofilms was determined by evaluating 48-hour old biofilms exposed to 0.6 mg/L sodium hypochlorite using RNA-seq analysis. Four different experiments, each with six control and six experimental biofilms were performed. Prior to sequencing library preparation, for each experiment, extracted RNA from the six biofilms exposed to sodium hypochlorite (experimental group) was pooled and extracted RNA from the six biofilms exposed to PBS (control) was pooled. Sequencing analysis statistics are summarized in Table 6-1. Post screening, trimming, and ribosomal RNA removal 8,990,357 sequencing reads across all four replicates were used for RNA-seq analysis. Between 63% and 75% of all

sequencing reads were successfully mapped against *Pseudomonas fluorescens* strain SBW25 reference genome during RNA-seq analysis (Table 6-1).

Table 6-1: RNA-seq sequencing data for control and experimental samples for all four replicates.

		Reads generated	Reads post trimming, screening, and rRNA removal	RNA seq reads mapped (%)
Replicate 1	Control	3,509,088	1,543,767	75.80
	Experimental	5,905,900	1,247,471	68.12
Replicate 2	Control	3,995,686	854,427	70.34
	Experimental	4,439,474	910,474	66.35
Replicate 3	Control	4,786,779	405,874	70.09
	Experimental	4,230,106	1,074,702	63.30
Replicate 4	Control	4,315,809	858,848	69.87
	Experimental	3,965,471	2,094,794	67.34

6.3.3 RNA-seq results

The statistical analysis of RNA-seq data identified a total of 298 genes (4.9% of all genes in *Pseudomonas fluorescens* strain SBW25) that were statistically significantly upregulated (FDR corrected p-value < 0.01, at least 2-fold change) in at least one of the four replicates (Table 6-2). In addition, 285 genes (4.7% of genes) were found to be statistically significantly downregulated in at least one of the four replicates (Table 6-2). Genes that were up or downregulated in at least two out of the four replicates were selected for downstream analysis. Eight genes were found to be upregulated in all four replicates, four genes were found to be upregulated in three of the four replicates, and 31 genes were found to be upregulated in two of

the four replicates (Appendix D, Table D1). Two genes were found to be downregulated in all four replicates, eight genes were downregulated in three of the four replicate, and 18 genes were downregulated in two of the four replicates (Appendix D, Table D2).

Table 6-2: Differentially expressed genes across all four replicates (at least 2-fold change and $P < 0.01$).

	upregulated genes	**downregulated genes**
Replicate 1	95	107
Replicate 2	49	41
Replicate 3	88	46
Replicate 4	66	91

6.3.4 Identification of upregulated genes

Differentially regulated genes meeting above described criteria were selected for annotation using DAVID and BLASTx. All upregulated genes are listed in Appendix D, Table D1. As expected, multiple upregulated genes were affiliated with oxidative stress response mechanisms (Table 6-3). Oxidative stress response genes induced in all four replicates included PFLU_RS08440, PFLU_RS14570, PFLU_RS14575, and PFLU_RS25330. The highest upregulation across all genes was observed for PFLU_RS08440, with an average 30 fold increase in expression. PFLU_RS08440 encodes the organic hydroperoxide resistance protein Ohr. Ohr is produced in *Pseudomonas* and other bacteria in response to oxidative stress, and converts reactive oxygen species such as organic hydrogen peroxides (H_2O_2) to less toxic

metabolites [197, 198]. PFLU_RS14570 and PFLU_RS14575 encode the alkyl hydroperoxide reductase subunit C AhpC and subunit F AhpF (Table 6-3). The alkyl hydroperoxide reductases AhpC and AhpF are antioxidant enzymes that also control peroxide levels [183, 199]. PFLU_RS25330 shared homology with a thioredoxin-disulfide reductase, potentially trxB, which reduces thioredoxin and is also involved in the defense against oxidative stress (Table 6-3) [183, 200-202]. In addition, PFLU_RS25630, encoding the periplasmic sulfoxide reductase subunit YedY, and PFLU_RS26240, likely encoding the catalase KatA, were upregulated in two of four replicates (Table 6-3). Both of these genes have been associated with oxidative stress response in bacteria [186, 187, 203]. YedY is part of the MsrPQ (methionine sulfoxide reductase) system, and repairs oxidized periplasmic proteins [204]. YedY is attached to the sulfite transmembrane spanning sulfite oxidase subunit YedZ (PFLU_RS25625), forming the YedYZ reductase complex. YedZ was found to be differentially expressed in replicate one (2.33-fold) and in replicate two (2.74-fold, P = 0.01). The catalase KatA has been suggested to protect DNA from oxidative damage and is considered one of the key enzymes in the prevention of oxidative damage [205, 206].

Several genes affiliated with multidrug efflux, membrane transport, and membrane stability were also induced. These included a MexE family multidrug efflux RND transporter subunit (PFLU_RS14265). MexE was induced an average of 5.95-fold (Table 6-3). MexE is a membrane fusion protein as part of the MexEF complex, which plays an integral part in the detoxification of organic substances, and has been shown to be involved in antibiotic resistance [207, 208]. The compliment gene PFLU_RS14260, encoding MexE, did not meet the analysis criteria of differential expression used in this study. PFLU_RS14260 was found to be upregulated between 1.89-fold and 3.15-fold in three out of the four replicates (P-values 0.06 and

below). Furthermore, PFLU_RS07315, which shared homology with the drug resistance MFS transporter protein AraJ, was upregulated in two of four replicates. Moreover, PFLU_RS03250, an ortholog to the substrate-binding periplasmic chaperone and copper resistance protein CopZ, was upregulated in three of the four replicates. CopZ is a heavy metal binding protein and is induced by copper stress [209]. PFLU_RS08225, sharing homology to potassium-transporting ATPase subunit KdpA, and PFLU_RS13625 showing similarity to the organosulfonate utilization protein and transporter SsuF were also upregulated in two of the four replicates (Table 6-3). The Kdp complex contributes to the maintenance of intracellular K^+ concentrations through uptake of potassium and has been shown to be upregulated in response to acid stress [210]. SsuF is a molybdopterin-binding protein and part of the *ssu* locus regulating organosulfur metabolism [211]. PFLU_RS10540 shares homology with the membrane protein TerC. TerC has been suggested to play a role in the efflux of tellurium ions [212]. A hypothetical membrane protein (PFLU_RS02795) and a hypothetical protein of unknown function (PFLU_RS17150) were also upregulated in all four replicates (Table 6-3). PFLU_RS02795 was upregulated as high as 9.20-fold and PFLU_RS17150 was upregulated as high as 19.55-fold. Therefore, these genes potentially play an important, yet unidentified, role in oxidative stress response in *Pseudomonas fluorescens*.

Several genes involved in transcriptional regulation were induced in response to sodium hypochlorite (Table 6-3). PFLU_RS28745, encoding an AraC family transcriptional regulator, was upregulated in three replicates and induced as much as 21.00-fold. AraC type transcriptional regulators are widely distributed and can be involved in the regulation of stress response pathways [213]. We also observed the upregulation of PFLU_RS03275, a LysR family transcriptional regulator. PFLU_RS03275 was upregulated in two replicates. LysR family

regulators play an important role in activating oxidative stress-inducible genes [214]. One of the members of the LysR family is the redox-sensitive OxyR, responsible for inducing the Ahp complex [215]. PFLU_RS14210, encoding a TetR family transcriptional regulator, was induced in two replicates. TetR family transcriptional regulators control transcription of multidrug efflux pumps and oxidative stress and chemical stress response pathways [216]. PFLU_RS24860 is an ortholog of the transcriptional regulator IscR. PFLU_RS06855 encodes an ArsR family transcriptional regulator. Both were significantly upregulated in two replicates. Moreover, IscR (PFLU_RS24860) was also upregulated 1.95-fold in a third replicate. IscR activates the *suf* operon encoding Fe-S assembly proteins in response to oxidative stress [217]. ArsR is a transcriptional repressor and part of a metal ion resistance response pathway [218].

Finally, an ABC transporter permease sharing homology to a spermidine/putrescine abc transporter PotA or PotB, an isochorismatase, and an anti-sigma factor were also induced in two replicates (Table 6-3).

Table 6-3: Upregulated *Pseudomonas fluorescens* genes related to oxidative stress, efflux, transport, and transcription regulation.

Locus Tag	Gene	Description	Fold Change			
			Rep 1	Rep 2	Rep 3	Rep 4
Oxidative stress related genes						
PFLU_RS08440	*ohr*	organic hydroperoxide resistance protein	82.24	31.13	4.40	10.05
PFLU_RS14570	*ahpC*	alkyl hydroperoxide reductase subunit C	3.40	3.10	5.93	11.69
PFLU_RS14575	*ahpF*	alkyl hydroperoxide reductase subunit F	2.05	2.56	2.09	4.11
PFLU_RS25330	*trxB*	thioredoxin reductase	2.36	2.88	3.28	3.58
PFLU_RS25630	*yedY*	periplasmic sulfoxide reductase subunit Y	4.77	3.20		
PFLU_RS26240	*katA*	catalase		1.73*	2.15	3.89

Table 6-3 (continued)

Efflux, transport, and membrane related genes						
PFLU_RS14265	*mexE*	multidrug efflux RND transporter periplasmic subunit	4.97	8.77	3.40	6.65
PFLU_RS02795	-	membrane protein	5.41	6.14	2.52	9.20
PFLU_RS10540	*terC*	TerC like membrane protein	14.26	7.10		
PFLU_RS19180	*ssuF*	organosulfonate utilization protein, transporter	3.81	2.38		
PFLU_RS03250	*copZ*	substrate-binding periplasmic protein	2.43		3.41	3.80
PFLU_RS06620	-	*Pseudomonas* membrane protein	4.07	13.01		
PFLU_RS28760	*potAB*	ABC transporter permease potA or potB	59.60	18.43		
PFLU_RS08225	*kdpA*	potassium-transporting ATPase subunit KdpA			2.34	2.31
PFLU_RS07315	*araJ*	Arabinose efflux permease transporter		∞		2.4
Transcription regulation related genes						
PFLU_RS28745	*araC*	AraC family transcriptional regulator	12.76	15.34	2.63	1.89*
PFLU_RS27245	-	translocase, transcription regulator	12.8	4.76	4.2	1.83*
PFLU_RS14210	*tetR*	TetR family transcriptional regulator	3.60		2.93[#]	8.35
PFLU_RS03275	*lysR*	LysR family transcriptional regulator	2.57		3.37	
PFLU_RS06855	*arsR*	ArsR family transcriptional regulator			2.23	5.20
PFLU_RS24860	*iscR*	iron-sulfur cluster assembly transcription regulator		1.95*	2.07	2.67
PFLU_RS22250	-	anti-sigma factor	2.96	5.48		

*upregulated less than 2-fold
[#] P-value > 0.01

6.3.5 Identification of downregulated genes

RNA-seq analysis also identified multiple genes that were downregulated. All genes found to be downregulated are listed in Appendix D, Table D2. Two groups of genes were downregulated across multiple replicates. One group of downregulated genes were membrane

proteins involved in uptake of small molecules and secretion mechanisms (Table 6-4). Three repressed genes were affiliated with sulfur uptake. The *ssuB* gene, encoding an aliphatic sulfonate import ATP-binding protein, was downregulated in three replicates, and contributes to sulfonate accumulation, which can be used as a sulfur source in *Pseudomonas* [211]. Two sulfate ABC transporter genes were also downregulated. The sulfate ABC transporter permease subunit CysW (PFLU_RS00955) and the sulfate ABC transporter ATP-binding protein CysA (PFLU_RS00960) form a transporter unit and are involved in sulfate and thiosulfate uptake [219, 220]. In addition, PFLU_RS01135, likely representing the *pbpB* gene, PFLU_RS24285, encoding the α-ketoglutarate permease KgtP, and *actP* were downregulated (Table 6-4). The *pbpB* gene encodes the penicillin binding protein III (also called peptidoglycan synthase) and is involved in cell division and a target for β-lactam antibiotics [221]. The cation/acetate symporter ActP transports pyruvate or acetate across the plasma membrane and the α-ketoglutarate transporter KgtP is a sodium-dependent dicarboxylate transporter and responsible for the movement of α-ketoglutaric acid and other TCA cycle intermediates into the cell [222]. PFLU_RS04590, showing homology with the *aidA* gene and likely encoding an autotransporter type VI secretion protein, was also repressed in two replicates. PFLU_RS01245 is an ortholog to the α-ketoglutarate-dependent taurine dioxygenase TauD and was also downregulated in two replicates. In addition, PFLU_RS06425 was downregulated in two replicates and could be identified as a secretion related protein, homologous to the peptidoglycan-binding protein domain LysM, but could not be characterized further. The LysM family encompasses membrane proteins, lipoproteins, and cell wall proteins, and is important for secretion and peptidoglycan attachment [223]. We also observed the downregulation of PFLU_RS04960, likely encoding an unidentified amino acid ABC transporter substrate-binding protein, and of PFLU_RS10525,

sharing homology with a purine efflux pump PbuE. Previous studies have observed membrane proteins and transporters to be repressed in response to hydrogen peroxide, suggesting oxidative stress impacts the regulation of these types of proteins [224].

The second group of genes found to be downregulated included PFLU_RS10165 and PFLU_RS07415, encoding an acetylornithine deacetylase and an NAD(P)-dependent oxidoreductase. Furthermore, PFLU_RS21225, sharing similarity to a NADP-dependent 3-hydroxy acid dehydrogenase-like gene *ydfG*, PFLU_RS23355, an ortholog to an acetyl-coenzyme A synthetase like gene, and PFLU_RS03330, a gene homologous to the methylmalonate semialdehyde dehydrogenase MSDH, were also repressed (Table 6-4). These genes are all affiliated with amino acid synthesis and energy metabolism pathways. Acetylornithine deacetylases are broad specificity hydrolases. The acetylornithine deacetylase ArgE is involved in arginine biosynthesis in *Pseudomonas syringae* [225]. YdfG is a broad substrate specificity dehydrogenase and is part of amino acid synthesis, specifically pyrimidine, glycine, serine, and threonine metabolism, in *Escherichia coli* [226]. The acetyl-coenzyme A synthetase likely participates in acetate metabolisms and the methylmalonate semialdehyde dehydrogenase participates in the degradation of the amino acids valine, leucine, and isoleucine. Two other genes of interest were also downregulated: PFLU_RS28830 encodes an alkanesulfonate monooxygenase gene involved in sulfur metabolism, and PFLU_RS23670 is homologous to a CsbD, a stress response protein (PFLU_RS23670) (Appendix D, Table D2).

Table 6-4: Downregulated *Pseudomonas fluorescens* genes related to membrane transport, amino acid metabolism, and energy metabolism.

Locus Tag	Gene	Description	Fold Change			
			Rep 1	Rep 2	Rep 3	Rep 4
Membrane proteins and membrane transport						
ssuB	*ssuB*	aliphatic sulfonates import ATP-binding protein	-2.82		-2.34	-3.10
PFLU_RS01135	*pbpB*	amino acid ABC transporter, substrate-binding protein		-2.24	-2.05	-2.24
PFLU_RS08920	*yjcH*	membrane protein YjcH		-3.75	-4.00	-2.69
PFLU_RS10525	*pbuE*	MFS transporter, PbuE like		-6.51	-2.12	-2.58
PFLU_RS00955	*cysW*	sulfate ABC transporter permease subunit		-2.53	-3.81	-3.21
PFLU_RS00960	*cysA*	sulfate ABC transporter ATP-binding protein		-2.13	-2.77	-5.50
PFLU_RS24285	kgtP	alpha-ketoglutarate permease		-2.43	-1.97*	-2.24
actP	actP	cation/acetate symporter ActP		-1.73*	-2.85	-2.10
PFLU_RS24655	*ptsA*	PTS N-acetyl-D-glucosamine transporter	-2.15		-1.99*	-2.24
PFLU_RS04960	-	amino acid ABC transporter substrate-binding protein			-2.96	-2.03
PFLU_RS26395	-	lipoprotein/hydrolase	-2.09		-4.68	
Amino acid metabolism and energy metabolism						
PFLU_RS21225	*ydfG*	NADP-dependent 3-hydroxy acid dehydrogenase	-2.56		-5.17	-3.62
PFLU_RS10165	-	acetylornithine deacetylase			-18.00	-6.28
PFLU_RS07415	-	NAD(P)-dependent oxidoreductase			-3.84	-3.52
PFLU_RS23355	-	acetyl-coenzyme A synthetase			-3.87	-2.03
PFLU_RS03330	*msdh*	methylmalonate semialdehyde dehydrogenase			-2.29	-2.02

*downregulated less than 2-fold

6.4 DISCUSSION

The purpose of this work was to investigate active, genetic pathways in *Pseudomonas fluorescens* biofilms, which may contribute to genetic resistance, in response to the broad spectrum antimicrobial sodium hypochlorite. Previous studies have evaluated the response to oxidative agents, such as hydrogen peroxides and sodium hypochlorite, for planktonic populations, suggesting genes involved in oxidant defense systems and cell repair to be induced [182, 185, 224, 227]. As biofilms have been suggested to exhibit increased antimicrobial resistance in comparison to planktonic cells [31, 167], we expected a similar and additional stress response pathways to be upregulated.

To investigate the effect of sublethal oxidative stress, 48-hour *Pseudomonas fluorescens* biofilms were exposed to sodium hypochlorite. A sodium hypochlorite concentration of 0.6 mg/L resulted in the largest percent decrease in viability (29.5%), but did not completely inactivate the biofilms, and was used for transcriptome evaluation. This concentration was lower than sodium hypochlorite or hydrogen peroxide concentrations used in previous studies analyzing oxidative stress responses in bacteria [186, 187, 227]. However, exposure of up to 20 mg/L (the highest tested concentration) resulted in further decrease of biofilm viability.

6.4.1 Oxidative stress response is driven by oxidant defense system genes

As expected, many upregulated genes were involved in oxidative stress adaption and defense pathways. Oxidative stress response was highlighted by the organic hydroperoxide

resistance protein Ohr and the alkyl hydroperoxide reductase subunits AhpC and AhpF. Ohr is considered one of the key enzymes in microbial oxidative stress response and has been shown to be essential for optimal resistance against oxidative stress in *Pseudomonas aeruginosa* [197, 198]. Ohr is activated by the presence of peroxide or similar molecules, and functions as a hyperoxide reductase and metabolizes organic hyperoxides and inorganic H_2O_2 into less toxic metabolites [197, 198]. In addition to Ohr, the alkyl hydroperoxide reductase *ahp* complex is also one of the most important oxidative stress response pathways. AhpC and AhpF are peroxide scavenging enzymes. AhpC, is the component with peroxidase activity, while AhpF is the NADH or NADPH binding component [189, 228]. When responding to oxidative stress, AhpF uses NADH or NADPH as electron donor to activate AhpC. AhpC then reduces peroxides and hyperperoxide to their nontoxic alcohol forms [189, 228]. AhpC and AhpF are both activated by the OxyR transcriptional regulator, a positive regulator of hydrogen peroxide inducible genes, and global regulator of the oxidative stress response [185, 200, 215]. Significant OxyR upregulation was not detected in this study, but OxyR has been shown to be induced, together with AhpC and AhpF, in response to oxidative stress previously [186, 187, 227]. Moreover, we detected the upregulation of a LysR family regulator, which could not be classified more specifically. OxyR belongs to the LysR family, suggesting these proteins may have similar functions [215]. The catalase KatA (induced in two replicates) is also activated by OxyR and has been shown to respond to H_2O_2 and oxidative stress [229, 230]. The catalase KatA, together with KatB, is considered an important peroxidase and plays an important role in oxidative stress response in bacteria [215, 231]. The induction of a thioredoxin reductase gene was also expected, as thioredoxin reductases are regulated by OxyR in *Pseudomonas* [232]. Thioredoxin reductases are disulfide reductases and have been shown to maintain cytoplasmic proteins in a reduced state

in response to oxidative stress [232, 233]. The upregulated gene in this study likely encodes the thioredoxin reductase subunit TrxB, the flavoprotein component, which reduces other oxidized thioredoxin reductases [232, 233].

Finally, we observed the upregulation of additional genes involved in the repair of cellular components, which may be damaged through oxidative stress. Two of those genes are the periplasmic sulfoxide reductase subunit YedY, and the periplasmic sulfoxide reductase subunit YedZ (which was only upregulated in one replicate). Like thioredoxin reductases, these enzymes are likely involved in the repair of oxidative damage [204]. In particular, sulfoxide reductases repair methionine and other residues in the cytoplasm and in the bacterial cell envelope [204], thereby playing an important role in cell integrity during oxidative stress. Similarly, IscR is a transcriptional regulator that regulates the transcription of the *isc* gene cluster, which includes genes involved in the repair of iron sulfur clusters and triggers the production of oxidation resistance Suf proteins [227].

Sodium hypochlorite has been suggested to trigger a similar cellular response as hydrogen peroxide (H_2O_2), as its antimicrobial activity can generate superoxide and hypochlorite ions and hydroxyl radicals [183, 234, 235]. Here, we observed the upregulation of several oxidant defense system genes, known to be involved in peroxide scavenging and the neutralization of reactive oxygen species in response to hydrogen peroxide exposure. These enzymes may therefore also be able to neutralize hypochlorite and superoxide ions.

Based on these findings and the upregulation of several transcriptional regulators potentially involved in the control of oxidative stress response pathways (such as LysrR, AraC, TetR, and IscR) putative oxidative stress response mechanisms for *Pseudomonas fluorescens* biofilms exposed to sodium hypochlorite can be proposed (Figure 6-2). Sodium hypochlorite

generates hypochlorite ions and reactive oxygen species, which are directly neutralized by the hyperoxide reductase Ohr. In addition, the oxyR regulator activates the alkyl hydroperoxide reductase complex *ahp* (encoding AhpC and AhpF) and the catalase KatA. These proteins then begin neutralizing reactive oxygen species. Furthermore, OxyR triggers thioredoxin reductase activity, which together with sulfoxide reductases repairs cytoplasmic and cell envelope proteins that may have been damaged through oxidative stress.

6.4.2 Multidrug efflux potentially contributes to sodium hypochlorite resistance

Multidrug efflux pumps have been shown to contribute to antimicrobial resistance in response to multiple types of antibiotics and biocides in the genus *Pseudomonas* and other bacteria, and are considered important antimicrobial resistance mechanism [31, 236]. Furthermore, multidrug efflux pumps were also reported to be upregulated in response to oxidative stress in *Pseudomonas* [237]. Here, we observed the upregulation of the periplasmic subunit of a multidrug efflux RND transporter periplasmic subunit MexE, the cytoplasmic membrane protein MexF, and the induction of a MFS transporter with homology to the AraJ transporter protein. MexE is important for antibiotic resistance and has previously been associated with chloramphenicol and fluoroquinolone resistance in *Pseudomonas aeruginosa* [238]. Homologs of MexE and MexF were also induced in response to glutaraldehyde stress in *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* [31]. MexE and MexF are part of a *Pseudomonas* antibiotic efflux system MexEF-OprN. OprN is the third component in this system and the outer membrane channel-forming protein. Together these three proteins form a resistance-nodulation-cell division (RND) efflux pump. MexE plays a key role in this system, as

mutations in MexE lead to the loss of multidrug-resistance [238, 239]. A possible reason is that the MexE membrane fusion protein links the inner and outer membrane-associated efflux components MexF and OprN and is therefore essential [239]. However, it was notable that the third component of the system (OprN) was not identified in the group of upregulated genes.

In addition, a TetR transcriptional regulator was upregulated. The *tetR* gene is located slightly downstream of the MexE and is known to control multidrug efflux, in particular for tetracycline resistance [240].

MFS transporters are another large group of transport proteins, of which several are involved in multidrug efflux mechanisms [241, 242]. The MFS transporter induced in this study shares homology with AraJ. AraJ has been described as a secondary active export pump protein and proposed to function as a transporter for arabinose-containing oligosaccharides in *E. coli* [243]. AraJ has also been proposed to transport arabinose-containing antibiotics [243]. These observations suggest that AraJ mainly functions as a sugar import and export protein. It may have the ability to transport certain types of antibiotics.

The upregulation of the potassium transport protein KdpA and the copper resistance protein CopZ suggests that oxidative stress might also trigger indirect genetic responses. KdpA upregulation has been observed in response to osmotic stress [244], suggesting sodium hypochlorite exposure may have altered salt transport across the membrane. The CopZ chaperone serves in the intracellular routing of copper in *Enterococcus hirae* and has been identified in *Pseudomonas aeruginosa* copper stress response [245].

Finally, the *ssuF* gene has been suggested to be regulated by sulfate, and to be specifically expressed under sulfate starvation conditions in *Pseudomonas putida* [211].

Therefore, SsuF is likely induced through the absence of sulfate in the cell, as the presence of sodium hypochlorite interfering with sulfate transport across the cell membrane.

In summary, two genes associated with multidrug efflux were upregulated in response to sodium hypochlorite. MexE and MexF are components of a known *Pseudomonas* antibiotic efflux MexEF-OprN operon, but the third protein in the system (OprN) was not upregulated. The MexEF-OprN efflux system has previously been shown to be upregulated in response to oxidative and nitrosative stress suggesting it to respond to stressors other than antibiotics [246]. Although our data only suggested two of the MexEF-OprN operon components to be induced, we believe there is evidence for oxidative stress response through multidrug efflux in *Pseudomonas fluorescens* biofilms (Figure 6-2).

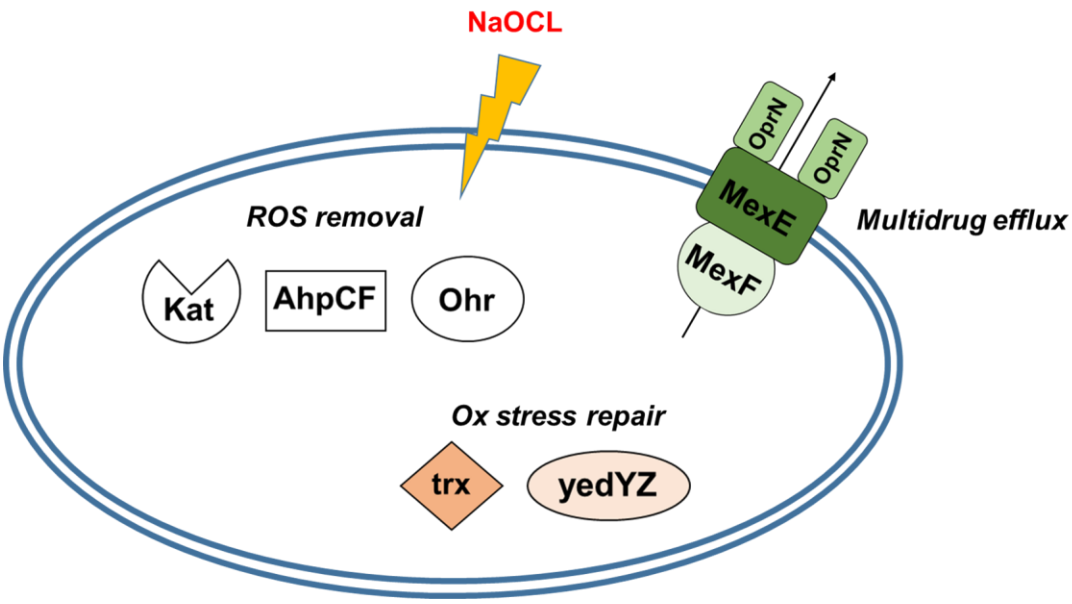

Figure 6-2: Schematic summarizing active, genetic response pathways against sodium hypochlorite in *Pseudomonas fluorescens* biofilms.

6.4.3 Sodium hypochlorite exposure downregulates genes encoding membrane transport proteins, amino acid synthesis proteins, and genes involved in energy pathways

Only a small group of genes was found to be downregulated in in two or more replicates. Overall, it was difficult to assign these genes to functional groups and identify how they were affected by oxidative stress, as no obvious pattern could be observed. Several genes encoding membrane proteins involved in the transport of small molecules in and out of the cell, such as sulfate, were downregulated. Previous studies have made similar observations in response to hydrogen peroxide, suggesting that oxidative stress changes the regulation of these types of proteins [224]. Furthermore, genes encoding glucose, α-ketogluconate, gluconate, and fructose transport have been shown to be downregulated in response to oxidative stress [186, 187], supporting our observations. One possible explanation is that sodium hypochlorite interferes with transport proteins at the membrane surface. Stressed cells might also neglect non-essential active and facilitated transport through the cell membrane, resulting in decreased transcription levels. The same applies to genes encoding proteins involved in amino acid synthesis and metabolism and energy metabolism related processes, which were also downregulated. Similarly, these types of genes have been observed to be repressed in response to oxidative stress previously [186, 187, 224]. Unexpectedly, we found a gene similar to the gene encoding the general stress response protein CsdD to be downregulated. CsbD's function is unknown, but it has is induced in response to various stresses, including oxidative and osmotic stress [247-249].

6.5 CONCLUSIONS AND IMPLICATIONS

In conclusion, results from this study predict *Pseudomonas fluorescens* biofilms to respond to oxidative stress induced by sodium hypochlorite through two mechanisms. One of them is active neutralization of oxidizing agents through Ohr and the Ahp complex. These mechanisms allow cells to neutralize and scavenge reactive oxygen species, such as peroxides. These mechanisms are also well studied and have been described previously for several bacteria as active oxidative stress response pathways. The other is active removal through multidrug efflux of the MexEF-OprN pump system. This mechanism may be evaluated for its importance in oxidative stress response in more detail, for example through the use of efflux pump inhibitors, proteomics, or knockout studies.

Findings from this study have implications for industries using sodium hypochlorite as disinfectant and biocide. Results suggest that *Pseudomonas* biofilms utilize active mechanisms, induced through the presence of reactive oxygen species, to protect themselves against oxidative stress. This is a possible explanation why sodium hypochlorite has shown poor efficacy when tested against microbial populations isolated from hydraulic fracturing produced water [47] and against bacteria observed in the food industry [250, 251]. Finally, observations from this study show that an active microbial response can play an important role when assessing the efficacy and usefulness of an antimicrobial agent. Thus, data from this and similar studies, supporting the presence of active, genetic pathways, likely contributing to biocide resistance, should be taken into consideration in the development of improved and more specific biocide application strategies.

7.0 SUMMARY, CONCULSIONS, AND FURTUE WORK

7.1 SUMMARY

Management of microbial activity in produced waters from hydraulic fracturing is critical to control corrosion, fouling and souring issues, protect well infrastructure, minimize unnecessary biocide application, and encourage produced water recycling. During hydraulic fracturing microbial populations can become established downhole, in the separator, in water tanks, and production lines through the distribution of hydraulic fracturing fluids and hydraulic fracturing produced waters. Previous investigations offered first insights into the microbial ecology of hydraulic fracturing systems and identified taxa likely to contribute to acid and sulfide production. Nevertheless, additional work on produced water microbial populations was necessary to confirm previous results, explore poorly characterized unconventional resource plays, and identify metabolic pathways of interest. Thus, the objective of the research presented in this dissertation was to: (1) review and summarize the current understanding of the microbial ecology, biocide efficacy, and genetic resistance in hydraulic fracturing produced waters; (2) investigate changes in microbial community structure during storage of hydraulic fracturing produced water samples; (3) investigate the microbial ecology and the role of the genus *Halanaerobium* in Marcellus Shale produced water; (4) analyze the microbial ecology of Bakken

Shale produced waters; (5) and investigate the active, genetic mechanisms in response to the broad spectrum antimicrobial sodium hypochlorite.

A review of studies having evaluated microbial populations in produced waters from hydraulic fracturing revealed microbial communities to be similar in structure, independent of well location and shale region (Chapter 2). However, microbial community structure was found to change over time, as early flowback water was dominated by halophilic, aerobic taxa, such as *Marinobacter*, while produced waters from older wells were characterized by high abundances of anaerobic, halophilic taxa, specifically the genus *Halanaerobium*. Furthermore, the potential for additional work was identified.

In chapter 3, the effects of storage conditions on produced water samples intended for microbiological analysis were evaluated. Results demonstrated that storage at room temperature for more than 24 hours and at 4^{o}C for more than three days can lead to changes in microbial community structure. Based on these findings, storage recommendations for produced water samples, which are being used in microbiological studies, were developed. To preserve the original community structure, samples should be kept refrigerated for short-term storage and in the freezer (ideally at -80^{o}C) for long-term storage.

Analysis of 42 Marcellus Shale produced water samples confirmed the predominance of the genus *Halanaerobium* (Chapter 4). Furthermore, correlations between the microbial community structure and biocide treatment combinations used in the fracturing fluid were identified, showing that operational factors need to be taken into consideration when evaluating the microbial ecology of hydraulic fracturing produced water. Analysis of the metabolic potential of *Halanaerobium*, through the assembly of a draft genome, revealed fermentation pathways and

thiosulfate reduction pathways, suggesting this taxa contributes to acid and sulfide production in hydraulic fracturing produced water systems.

Chapter 4 presented findings on the microbiological analysis of Bakken Shale produced waters from 18 different wells sites sampled between October 2014 and March 2015. Using quantitative PCR and 16S rRNA sequencing, the microbial abundance and microbial community structure were analyzed. Low biomass was detected across all analyzed samples. This data supported previous observations and was attributed to high subsurface temperatures in Bakken and Three Forks formations. Microbial community structure analysis identified the same taxa previously observed in produced water from other oil and gas regions; however, relative abundances were different. Investigations also revealed the microbial community structure to change across the sampling period, as ambient air temperatures dropped below 0^{o}C. Thus, findings from this chapter suggest the microbial ecology in produced water can differ from one unconventional oil and gas region to another. Results also show that seasonal changes in temperature can affect the microbial community structure, an observation that needs to be considered for produced water management.

Finally, the exposure of *Pseudomonas fluorescens* biofilms to the broad spectrum antimicrobial and biocide sodium hypochlorite revealed induction of active, genetic pathways involved in peroxide scavenging, oxidative stress repair, and multidrug efflux (Chapter 5). A concentration of 0.6 mg/L sodium hypochlorite was found have a sublethal effect on 48-hour old *Pseudomonas fluorescens* biofilms and resulted in the upregulation of genes encoding the organic hydroperoxide resistance protein Ohr, the alkyl hydroperoxide reductases subunits AhpC and AhpF, and two components of a multidrug efflux pump system. Thus, results suggested active removal of reactive oxygen species through peroxidases, and removal through efflux

systems to be the primary oxidative stress response mechanisms in *Pseudomonas fluorescens* biofilms. Findings from this analysis may contribute to the optimization of current biocide applications and disinfection strategies.

7.2 CONCLUSIONS

The overall conclusions from this work are:

1. Microorganisms are prevalent in produced waters from hydraulic fracturing across the United States; however, microbial abundances vary regionally and likely depend on the physicochemical characteristics of each play. Moreover, the hypersaline produced water environment drives the microbial ecology, resulting in similar community structures across shale oil and gas regions. It needs to be noted that the produced water community structure changes during production, as different microbial compositions have been observed throughout the lifetime of a well. Moreover, seasonal and operational changes can also impact the microbial ecology in hydraulic fracturing produced water and may lead to shifts within the community structure.
2. The genus *Halanaerobium* is one of the most abundant taxa in hydraulic fracturing produced water. This was supported by its dominance across produced water samples from different shale oil and gas regions and its metabolic potential for acid and sulfide production. However, findings presented in chapter 4 suggest

Halanaerobium can be outcompeted by other taxa under certain environmental conditions.

3. Observations from hydraulic fracturing produced water ecology studies may help to develop improved produced water management strategies. For example, microbial activity and microbial community dynamics should be monitored through regular testing, with the goal to identify changes caused by seasonal and operational factors.
4. Active, genetic response mechanisms, such as active reactive oxygen scavenging and multidrug efflux, likely contribute to biocide resistance against sodium hypochlorite in *Pseudomonas fluorescens* biofilms. These findings are potentially important for disinfection strategies in industrial settings, including the oil and gas industry, as sodium hypochlorite is a commonly used biocide and resistant microbial populations can interfere with industrial processes through, for example, biocorrosion.

7.3 FUTURE WORK

Findings from this research advance the current understanding of the microbial ecology in produced water, revealing novel insights into microbial community dynamics, the metabolic capabilities of *Halanaerobium*, and the active microbial response against biocides, in produced water. Nevertheless, future research is recommended to further evaluate and confirm observations made in the here presented work. Efforts should target the identification of active

microbial pathways in produced water through the use of metatranscriptomics. This method allows the establishment of active microbial communities and active metabolic pathways in produced water and can help to confirm and complement metagenomic data presented here and in previous studies. We believe one such study, which evaluated active microbial mechanisms in holding pond and hauling truck produced water samples, currently exists [38]; however additional analyses using this approach, specifically targeting *Halanaerobium* dominated samples, are recommended.

Investigation of Bakken Shale produced water revealed high abundances of *Bacillales* and *Pseudomonadales* in the majority of the evaluated samples. These findings were made using 16S rRNA sequencing and should be further confirmed through metagenomic and metatranscriptomic analyses, with the goal to characterize *Bacillales* and *Pseudomonadales* populations in Bakken Shale produced water in more detail and identify their metabolic capabilities.

Finally, research efforts should be aimed at developing an efficient biocide treatment designed to control the genus *Halanaerobium*. As this taxon has repeatedly been suggested to significantly contribute to microbial activity in hydraulic fracturing produced water, minimizing its presence is desirable. Very little data is currently available on the efficacy of biocides against the genus *Halanaerobium*, all of it coming from a single study [17]. Thus, exposure of *Halanaerobium* isolates to different biocide combinations may help to identify treatments useful for the effective control of *Halanaerobium* populations in produced water. In addition, the active, genetic response of *Halanaerobium* against commonly used biocides should be evaluated to identify mechanisms that may contribute to *Halanaerobium* biocide resistance. These efforts

should ideally be conducted under produced water conditions (hypersaline environment), as produced water has previously been suggested to contribute to microbial biocide resistance [32].

Altogether, additional research efforts will further enhance the current understanding of produced water microbial activity and represent, combined with the already available data, the next step in the effort to develop specific control and management strategies. Furthermore, they will help to provide biocide application strategies to improve biocide efficacy and eliminate microbial populations contributing to corrosion and souring.

APPENDIX A

CHAPTER 3 - SUPPLEMENTAL INFORMATION

Table A1: Abundance fractions for microbial orders in FWT samples stored at either room temperature (RT) or at 4°C.

Order	On-site	Day 0 RT	Day 0 4°C	Day 1 RT	Day 1 4°C	Day 2 RT	Day 2 4°C	Day 3 RT	Day 3 4°C	Day 7 RT	Day 7 4°C
Halanaerobiales	0.001	0.002	0.003	0.005	0.011	0.016	0.007	0.006	0.026	0.011	0.007
Rhodobacterales	0.005	0.010	0.007	0.007	0.009	0.021	0.015	0.008	0.013	0.002	0.008
Sphingomonadales	0.007	0.015	0.010	0.010	0.011	0.021	0.016	0.011	0.012	0.004	0.011
Burkholderiales	0.001	0.017	0.014	0.028	0.013	0.018	0.016	0.013	0.009	0.003	0.016
Campylobacterales	0.763	0.782	0.832	0.771	0.807	0.731	0.722	0.662	0.629	0.017	0.713
Alteromonadales	0.054	0.038	0.035	0.028	0.045	0.044	0.060	0.165	0.122	0.771	0.031
Oceanospirillales	0.034	0.042	0.032	0.024	0.042	0.037	0.056	0.070	0.119	0.096	0.122
Pseudomonadales	0.010	0.013	0.012	0.011	0.009	0.028	0.018	0.017	0.015	0.082	0.012
Vibrionales	0.001	0.006	0.003	0.063	0.004	0.001	0.004	0.001	0.003	0.000	0.003
Minor Orders	0.110	0.076	0.054	0.052	0.043	0.083	0.086	0.047	0.054	0.013	0.078

Table A2: Abundance fractions for microbial orders in HP1 samples stored at either room temperature (RT) or at 4ºC.

Order	On-site	Day 0 RT	Day 0 4°C	Day 1 RT	Day 1 4°C	Day 2 RT	Day 2 4°C	Day 3 RT	Day 3 4°C	Day 7 RT	Day 7 4°C
Halanaerobiales	0.003	0.002	0.002	0.014	0.006	0.020	0.004	0.039	0.008	0.018	0.026
Caulobacterales	0.002	0.009	0.011	0.017	0.014	0.021	0.018	0.013	0.016	0.023	0.020
Rhizobiales	0.000	0.013	0.015	0.012	0.017	0.020	0.019	0.010	0.013	0.031	0.021
Rhodobacterales	0.356	0.306	0.303	0.271	0.253	0.261	0.226	0.227	0.206	0.253	0.270
Sphingomonadales	0.290	0.400	0.397	0.322	0.393	0.341	0.370	0.219	0.337	0.180	0.298
Alteromonadales	0.006	0.007	0.008	0.032	0.009	0.014	0.008	0.037	0.073	0.013	0.005
Oceanospirillales	0.098	0.129	0.126	0.200	0.173	0.172	0.189	0.156	0.210	0.113	0.223
Pseudomonadales	0.053	0.065	0.066	0.085	0.064	0.096	0.087	0.254	0.072	0.281	0.070
Minor Orders	0.140	0.069	0.072	0.047	0.072	0.055	0.079	0.045	0.065	0.089	0.068

Table A3: Abundance fractions for microbial orders in HP2 samples stored at either room temperature (RT) or at 4ºC.

Taxon	On-site	Day 0 RT	Day 0 4°C	Day 1 RT	Day 1 4°C	Day 2 RT	Day 2 4°C	Day 3 RT	Day 3 4°C	Day 7 RT	Day 7 4°C
Bacteroidales	0.064	0.033	0.025	0.013	0.024	0.005	0.028	0.002	0.029	0.002	0.011
Clostridiales	0.001	0.021	0.015	0.023	0.028	0.011	0.036	0.008	0.048	0.002	0.026
Rhodobacterales	0.000	0.016	0.007	0.028	0.020	0.018	0.033	0.010	0.034	0.011	0.030
Sphingomonadales	0.000	0.024	0.015	0.017	0.027	0.009	0.027	0.006	0.030	0.006	0.014
Desulfovibrionales	0.053	0.014	0.009	0.008	0.014	0.008	0.016	0.003	0.021	0.002	0.012
Desulfuromonadales	0.026	0.016	0.013	0.011	0.017	0.005	0.016	0.004	0.021	0.002	0.017
Campylobacterales	0.624	0.748	0.843	0.274	0.753	0.128	0.680	0.061	0.651	0.132	0.276
Alteromonadales	0.037	0.024	0.017	0.133	0.020	0.290	0.021	0.403	0.022	0.279	0.075
Pseudomonadales	0.087	0.049	0.022	0.453	0.047	0.501	0.059	0.487	0.062	0.542	0.488
Minor orders:	0.100	0.086	0.078	0.076	0.086	0.069	0.088	0.063	0.098	0.060	0.085

Table A4: Sequence abundances and alpha diversity measurements for each sample

	# of sequences°			OTUs*°			Chao1*°			Shannon*°			Evenness*°		
Sample:	**FWT**	**HP1**	**HP2**	**FWT**	**HP1**	**HP2**	**FWT**	**HP1**	**HP2**	**FWT**	**HP1**	**HP2**	**FWT**	**HP1**	**HP2**
On-site	9444	11401	2506	95	105	133	127.5	195.4	263.8	4.07	1.93	3.65	0.89	0.41	0.75
Day 0, RT	19306	14983	9862	112	102	110	309.8	192.3	180.6	2.16	4.07	2.64	0.46	0.88	0.56
Day 1, RT	10576	12277	8166	107	128	113	332.5	252.5	223.7	2.09	4.44	3.50	0.45	0.92	0.74
Day 2, RT	8286	12699	9400	145	131	87	326.5	271.6	201.8	2.63	4.49	3.10	0.53	0.92	0.69
Day 3, RT	14119	12263	12206	102	117	63	274.9	232.2	188.1	2.63	4.69	2.92	0.57	0.99	0.70
Day 7, RT	21147	7704	9597	62	128	56	150.6	227.9	115.8	3.31	4.98	2.94	0.80	1.03	0.73
Day 0, 4°C	15134	14139	12177	106	113	81	260.2	241.5	178.2	2.01	4.09	1.84	0.43	0.87	0.42
Day 1, 4°C	12078	17142	8924	111	125	109	282.1	252.8	215.2	2.28	4.18	2.51	0.48	0.87	0.53
Day 2. 4°C	13641	14630	10239	147	124	156	312.3	244.1	317.6	2.87	4.44	3.29	0.57	0.92	0.65
Day 3, 4°C	12130	12682	9921	110	123	149	263.2	316.8	320.9	2.86	4.59	3.54	0.61	0.95	0.71
Day 7, 4°C	18847	14566	8803	66	116	135	222.1	246.0	282.5	2.62	4.52	3.70	0.62	0.95	0.75

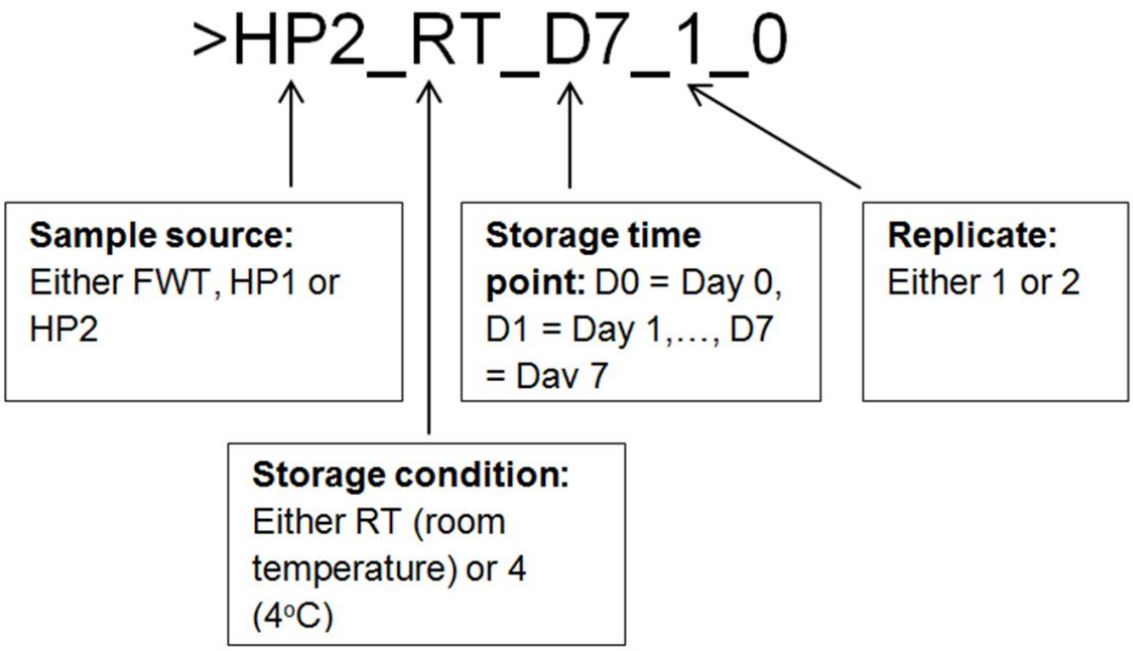

Figure A1: Explanation of FASTA headers for 16S sequences deposited on MG-RAST. Sequences were deposited by sample Source (FWT, HP1, HP2). Header of each sequence in each file indicates sample source, storage condition, storage time point and replicate, as shown above.

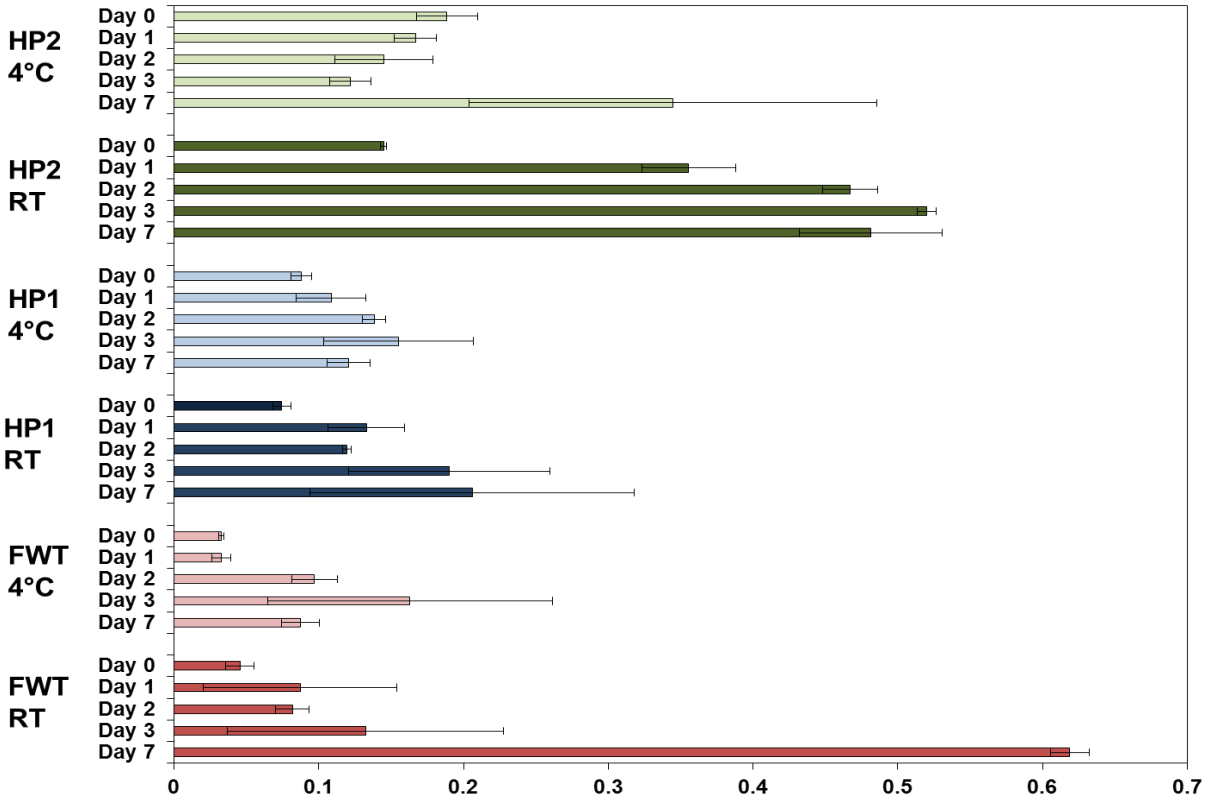

Figure A2: Average weighted UniFrac distances (+/- SDEV) between on-site samples and

APPENDIX B

CHAPTER 3 - SUPPLEMENTAL INFORMATION

Table B1: Characteristics of samples used in this study. All samples were taken in June 2014

Sample ID	Volume (ml)	Well age (Days)	TDS (mg/L)	Microbial abundance (16S rRNA gene copies per ml)	Sequences
Site 1, Well 1	200	785	71840	2.20E+05	14932
Site 1, Well 2	200	826	127834	1.88E+06	6811
Site 1, Well 3	200	790	135515	5.49E+06	13613
Site 2, Well 1	200	888	181386	4.53E+05	3406
Site 3, Well 1	200	525	115152	7.85E+05	2739
Site 3, Well 2	200	525	223034	2.76E+06	6122
Site 3, Well 3	200	257	166615	1.42E+06	12960
Site 4, Well 1	200	182	144284	1.38E+05	10388
Site 4, Well 2	200	182	163392	5.12E+06	3570
Site 4, Well 3	200	182	116772	1.57E+06	11886
Site 4, Well 4	200	182	182853	3.12E+06	5944
Site 5, Well 1	200	1075	38749	2.13E+06	2832
Site 5, Well 2	200	1324	130180	2.14E+06	17656
Site 5, Well 3	200	1324	75650	1.98E+06	7123
Site 6, Well 1	200	1812	151534	1.01E+07	8689
Site 6, Well 2	200	1114	134414	7.80E+05	10322
Site 6, Well 3	200	1114	82062	4.73E+05	2147
Site 7, Well 1	200l	1022	78900	2.38E+07	3987
Site 7, Well 2	200	1022	97436	2.31E+07	3943
Site 7, Well 3	200	1022	185814	4.66E+05	4242
Site 8, Well 1	200	1231	115730	4.77E+06	5381
Site 9, Well 1	200	1196	69902	5.14E+06	3117
Site 9, Well 2	200	1196	50383	1.50E+06	7894
Site 9, Well 3	200	150	127418	2.81E+06	3856
Site 10, Well 1	200	960	122015	1.02E+06	3153
Site 11, Well 1	200	1447	46461	5.88E+07	8662
Site 11. Well 2	200	1447	207357	1.00E+08	2058
Site 11, Well 3	200	1447	142000	1.06E+07	8419
Site 12, Well 1	200	718	191556	1.35E+06	2065
Site 12, Well 2	200	718	137232	1.42E+05	12630
Site 12, Well 3	200	694	76226	2.71E+06	10104
Site 13, Well 1	200	405	187352	2.14E+08	22292
Site 13, Well 2	200	405	196459	1.45E+05	19296
Site 13, Well 3	200	405	152629	1.73E+07	15481
Site 13, Well 4	200	405	90131	3.33E+06	2451
Site 14, Well 1	200	1423	157714	1.06E+07	5817
Site 15, Well 1	200	562	160723	2.24E+05	4291
Site 15, Well 2	200	562	179771	5.39E+05	7564
Site 16, Well 1	200	1788	152948	6.76E+06	8317
Site 16, Well 2	200	926	119739	1.67E+06	8018
Site 17, Well 1	200	669	160183	9.40E+06	10102

Table B1 (continued)

Site 18, Well 1	200	1847	117844	2.07E+07	6151

Table B2: Composition of biocide treatments applied to sampled wells during the fracturing process. Information obtained from fracfocus.

	Constituents	# of wells	# of well sites
Treatment 1	Polyethylene Glycol 2,2-dibromo-3-nitrilopropionamide Dibromoacetonitrile	19	8
Treatment 2	Sodium Hydroxide Tetrahydro-3,5-dimethyl-2H-1,3,5-thiadiazine-2-thione	13	7
Treatment 3	Methanol Tributyl Tetradecyl Phosphonium Chloride	2	2
Treatment 4	Hydrogen Chloride Sodium Hydroxide Sodium Hypochlorite Sodium Chlorite 2,2-dibromo-3-nitrilopropionamide Dibromoacetonitrile	1	1
Treatment 5	Polyethylene glycol mixture 2,2-dibromo-3-nitrolopropionamide	2	1
Treatment 6	Sodium Hydroxide Tetrahydro-3,5-dimethyl-2H-1,3,5-thiadiazine-2-thione Hydrogen Chloride Sodium Hypochlorite	2	1
Treatment 7	Sodium Hydroxide Tetrahydro-3,5-dimethyl-2H-1,3,5-thiadiazine-2-thione Alkyl Dimethyl Benzyl Ammonium Chloride Glutaraldehyde Ethanol Quaternary Ammonium Chloride	1	1
Treatment 8	Methanol Tributyl Tetradecyl Phosphonium Chloride Alkyl Dimethyl Benzyl Ammonium Ethanol Gluteraldehyde Quaternary Ammonium Chloride	1	1
Treatment 9	2,2-dibromo-3-nitrilopropionamide	1	1

Table B3: Composition of TDS for produced water samples analyzed in this study.

Sample	Sodium (mg/L)	Calcium (mg/L)	Barium (mg/L)	Chloride (mg/L)	Other Ions (mg/L)	Total (mg/L)
Site 1, Well 1	15490.0	7152.3	1983.1	44946.7	2267.9	71840.0
Site 1, Well 2	26309.0	11527.0	2829.8	83566.5	3601.8	127834.1
Site 1, Well 3	27238.3	16163.3	4034.3	83098.4	4980.3	135514.6
Site 2, Well 1	66029.7	27102.0	7094.5	72968.6	8191.0	181385.8
Site 3, Well 1	34631.3	12516.5	3095.7	61217.4	3690.9	115151.8
Site 3, Well 2	82867.0	30176.4	7087.2	93893.5	9009.7	223033.8
Site 3, Well 3	41366.1	15128.8	3870.9	101759.3	4489.6	166614.6
Site 4, Well 1	39394.2	14216.0	3528.4	82892.7	4252.4	144283.7
Site 4, Well 2	41540.1	14892.4	3788.5	98759.1	4411.7	163391.8
Site 4, Well 3	1242.6	11050.7	2608.6	98326.4	3544.1	116772.4
Site 4, Well 4	56362.4	19909.7	5113.7	95517.7	5949.1	182852.6
Site 5, Well 1	15618.0	6434.2	1556.5	13093.4	2046.5	38748.7
Site 5, Well 2	34664.1	13893.4	3589.6	73785.7	4250.3	130180.0
Site 5, Well 3	22390.9	8860.6	2112.3	39627.2	2658.8	75649.7
Site 6, Well 1	54561.2	22078.0	5343.4	62714.0	6837.9	151534.4
Site 6, Well 2	37058.4	13998.2	3288.2	76105.0	3964.7	134414.4
Site 6, Well 3	16375.0	6964.9	1800.3	54834.0	2087.8	82061.9
Site 7, Well 1	20880.8	7410.1	1425.6	46585.1	2597.9	78899.6
Site 7, Well 2	25178.3	9954.3	2617.0	56805.7	2880.5	97435.8
Site 7, Well 3	66050.8	27765.6	6754.1	76707.9	8535.5	185813.9
Site 8, Well 1	25364.2	11156.2	2900.3	72814.0	3495.6	115730.3
Site 9, Well 1	23523.2	9545.9	1921.3	31775.6	3135.8	69901.7
Site 9, Well 2	12629.8	3811.2	603.9	31846.5	1491.2	50382.7
Site 9, Well 3	31424.7	12346.0	2722.5	77154.7	3770.4	127418.3
Site 10, Well 1	33021.4	12194.1	2940.1	70303.1	3556.1	122014.9
Site 11, Well 1	18935.6	6493.8	1804.7	17455.4	1771.8	46461.4
Site 11. Well 2	58857.9	763.7	65.1	147436.3	234.1	207357.0
Site 11, Well 3	37760.0	15150.0	6191.0	64543.9	17429.3	142000.0
Site 12, Well 1	54967.0	22243.8	5616.0	101879.8	6849.6	191556.4
Site 12, Well 2	39779.0	16103.4	4261.9	71941.6	5145.9	137231.8
Site 12, Well 3	23825.5	8057.1	873.1	40322.3	3147.8	76225.8
Site 13, Well 1	14933.8	2750.0	6056.6	92113.2	71498.6	187352.3
Site 13, Well 2	61032.5	21886.3	2352.9	102248.7	8938.4	196458.7
Site 13, Well 3	34250.4	13790.4	3289.4	97021.3	4277.4	152628.9
Site 14, Well 1	43039.1	15808.9	4544.3	89318.7	5002.7	157713.7
Site 14, Well 2	31039.7	12014.1	3082.3	40641.5	3353.4	90130.9
Site 15, Well 1	37625.2	13925.3	3384.7	101603.2	4184.8	160723.2
Site 15, Well 2	48533.0	17908.7	4307.3	103567.9	5454.3	179771.1
Site 16, Well 1	102715.6	31668.3	2120.1	1745.1	14698.6	152947.6
Site 16, Well 2	31346.1	13906.1	3761.2	66778.1	3947.1	119738.6
Site 17, Well 1	46480.4	17413.6	4661.9	86332.6	5294.2	160182.8
Site 18, Well 1	32958.1	12470.2	2858.0	65572.3	3985.3	117843.9

Table B4: Spearman coefficients for the correlation of operational parameters TDS concentration and well age, number of OTUs, and the bacterial load (as 16S rRNA gene copies per mL). Spearman rank coefficients can be between -1 and +1. Values between 0 and 1 describe a correlation. Values between 0 and -1 described an inverse correlation. Correlations can be very weak (0.00 – 0.19), weak (0.20 – 0.39), moderate (0.40 - 0.59), strong (0.60 – 0.79) or very strong (0.80 – 1.00).

	TDS	**Well Age**	**Bacterial Load**	**OTUs**
TDS	1	-0.446	-0.166	0.106
Well Age	-0.446	1	0.340	0.204
Bacterial Load	-0.166	0.340	1	-0.004
OTUs	0.106	0.204	-0.004	1

Table B5: P-values for the correlation of operational parameters TDS concentration, and well age, number of OTUs, and the bacterial load (as 16S rRNA copies per mL).

	TDS	**Well Age**	**Bacterial Load**	**OTUs**
TDS		<0.01	0.30	0.50
Well Age	<0.01		0.03	0.20
Bacterial Load	0.30	0.03		0.98
OTUs	0.50	0.20	0.98	

Table B6: Spearman coefficients for the correlation of bacterial abundances (97% OTU table) with the operational parameters TDS concentration, well age, the bacterial load (as 16S rRNA copies) and the number of OTUs.

	Bacterial Load	**TDS**	**Well age**	**OTUs**
Halanaerobiales	-0.208	0.045	-0.519*	-0.812*
Pseudomonadales	-0.055	-0.020	0.147	0.457
Bacteroidales	0.306*	-0.213	0.573*	0.550*
Bacillales	0.170	-0.133	0.269	0.610*
Actinomycetales	0.072	0.089	0.139	0.547*
Lactobacillales	0.023	0.279	-0.000	0.684*
Clostridiales	0.262	-0.166	0.601*	0.511*
Erysipelotrichales	0.169	0.318	-0.251	0.433
Rhodobacterales	-0.169	0.156	0.0274	0.429
Rhizobiales	-0.076	0.147	0.098	0.632*
Desulfovibrionales	-0.170	0.112	0.015	0.498
Campylobacterales	-0.070	0.075	0.186	0.718*
Enterobacteriales	-0.102	0.031	0.187	0.445
Oceanospirales	-0.115	-0.009	0.127	0.263
Minor orders	0.0234	0.109	0.354	0.654*

*Denotes $P < 0.05$

Table B7: Alpha diversity results for all analyzed samples.

Sample	Observed OTUs	Chao1 Index	Shannon Index
Site 1, Well 1	25	38	1.24
Site 1, Well 2	54	60	1.29
Site 1, Well 3	19	30	0.78
Site 2, Well 1	77	129	2.28
Site 3, Well 1	25	33	1.64
Site 3, Well 2	187	252	4.62
Site 3, Well 3	6	7	0.51
Site 4, Well 1	42	75	1.70
Site 4, Well 2	48	75	1.63
Site 4, Well 3	12	17	0.54
Site 4, Well 4	147	237	2.76
Site 5, Well 1	39	41	2.51
Site 5, Well 2	39	70	2.13
Site 5, Well 3	42	66	2.23
Site 6, Well 1	26	30	2.43
Site 6, Well 2	20	24	1.75
Site 6, Well 3*	96	131	4.38
Site 7, Well 1	21	30	0.86
Site 7, Well 2	19	24	1.06
Site 7, Well 3	84	113	3.59
Site 8, Well 1	25	34	1.13
Site 9, Well 1	120	199	3.21
Site 9, Well 2	55	88	1.78
Site 9, Well 3	35	61	1.53
Site 10, Well 1	22	28	0.88
Site 11, Well 1	50	76	3.79
Site 11. Well 2	92	114	4.18
Site 11, Well 3	28	83	2.75
Site 12, Well 1*	43	89	1.38
Site 12, Well 2	101	178	1.74
Site 12, Well 3	38	65	1.16
Site 13, Well 1	19	41	0.66
Site 13, Well 2	12	33	0.73
Site 13, Well 3	22	40	0.78
Site 14, Well 1	151	233	4.34
Site 14, Well 2*	54	108	0.99
Site 15, Well 1	22	127	0.98
Site 15, Well 2	51	76	1.86
Site 16, Well 1	83	162	3.98
Site 16, Well 2	20	29	0.55
Site 17, Well 1	66	103	2.75
Site 18, Well 1	28	51	1.65

*** Samples for which less than 2000 OTU assigned sequence were available for alpha diversity analysis**

Table B8: Spearman coefficients for the correlation of alpha diversity (Number of OTUs, Shannon index and Chao1 index) with the operational parameters biocide treatment combination, TDS concentration, and well age and the bacterial load (as 16S rRNA copies per mL).* indicates $P < 0.05$.

	TDS	Well Age	Bacterial load
OTUs	0.106	0.204	-0.004
Chao1 Index	0.225	0.101	-0.021
Shannon Index	-0.058	0.494*	0.114

Table B9: Genome characteristics of Marcellus Shale draft genome *Halanaerobium* and four published, available *Halanaerobium* genomes.

Genome	Paper	Accession number	DNA Source	Genome size	Number of contigs	Coding sequences*	GC content	CheckM Complete-ness	CheckM Contamin-ation
Halanaerobium sp. MDAL1	This paper	MIJU00000000.1	Metagenome	2,389,586 bp	129	2219	34.20%	83.30%	9.60%
Halanaerobium sp.T82-1	Daly et al, 2016	LSBN00000000.1	Metagenome	2,765,245 bp	393	2825	33.30%	85.30%	28.90%
Halanaerobium saccharolyticum subsp. Saccharolyticum	Kivisito et al, 2013	NZ_CAUI00000000.1	Metagenome	2,873,865 bp	24	2645	32.30%	90.60%	16.20%
Halanaerobium praevalens	Ivanova et al, 2011	CP002175.1	Isolate	2,309,262 bp	1	2129	30.30%	98.20%	0.50%
Halanaerobium hydrogeniformans	Brown et al, 2011	CP002304.1	Isolate	2,613,116 bp	1	2474	33.10%	96.70%	1.80%

Table B10: Proteins of interest for hydraulic fracturing industry identified in *Halanaerobium* sp. Strain MDAL1

Fermentation		
Protein	**EC number**	**Putative function**
Lactose dehydrogenase **Ldh**	EC 1.1.1.27	Pyruvate to lactate
Phosphate acetyltransferase **Pta**	EC 2.3.1.8	Pyruvate to acetate
Alcohol dehydrogenase **Adh**	EC 1.1.1.1	Pyruvate to ethanol
Pyruvate formate lyase **Pfl**	EC 2.3.1.54	Pyruvate to hydrogen and carbon dioxide
Thiosulfate reduction		
Protein	**EC number**	**Putative function**
Thiosulfate sulfurtransferase rhodanese **Mpst**	EC 2.8.1.1	Thiosulfate to adenyl sulfate
Anaerobic sulfite reductases **AsrA**	N/A	
Anaerobic sulfite reductases **AsrB**	N/A	Sulfite to sulfide
Anaerobic sulfite reductases **AsrC**	EC 1.8.1.-	
Rhodanese-like gene **RdlA**	N/A	Adenyl sulfate to sulfite
Trk type sulfate permease	N/A	Thiosulfate uptake
ABC type sulfate like transporter	N/A	Thiosulfate uptake
Biofilm formation		
Protein	**EC number**	**Putative function**
Sporulation two-component response regulator **Spo0A**	N/A	Surface attachment initiation
Glycosyl transferase group 2 family protein gene **Glt2**	N/A	Capsular-polysaccharide synthesis
Diguanylate cyclase gene **AdrA**	N/A	Cellulose biosynthesis induction
Stress response		
Protein	**EC number**	**Putative function**
Potassium transporter **TrkA**	N/A	Hyperosmotic potassium uptake
Potassium transporter **TrkH**	N/A	Hyperosmotic potassium uptake
Potassium uptake protein **KtrB**	N/A	Potassium transport
Glycine and betaine ABC transport protein **ProX**	TC 3.A.1.12.1	Glycine and betaine uptake
L-proline glycine betaine ABC transport system permease protein **ProW**	TC 3.A.1.12.1	Glycine and betaine uptake
High-affinity betaine transport system **OpuA**	EC 3.6.3.32	Glycine and betaine uptake
Sensitive transcriptional regulator **PerR**	N/A	Oxidative stress response
Superoxide reductase **Sor**	EC 1.15.1.2	Oxidative stress response

Table B10 (continued)

Rubredoxin	N/A	Electron transfer, oxidative stress response
Outer membrane porin gene **OmpH**	N/A	Periplasmic stress response
Universal stress protein **UspA**	N/A	Environmental stress response, gene expression regulation, starvation, high temperature stress
The heat shock protein **GrpE**	N/A	Hyperosmotic and heat shock response, preventing the aggregation of stress-denatured proteins
Heat shock chaperones **GroES**	N/A	Protein folding
Heat shock chaperones **GroEL**	N/A	Protein folding
Chemotaxis protein **MotA**	N/A	Chemotaxis
Chemotaxis protein **MotB**	N/A	Chemotaxis
Flagella assembly protein family **Flg**	N/A	Flagella assembly and motility
Flagella assembly protein family **Fli**	N/A	Flagella assembly and motility
Flagella assembly protein family **Flh**	N/A	Flagella assembly and motility
Flagellin **FliC**	N/A	Flagella assembly

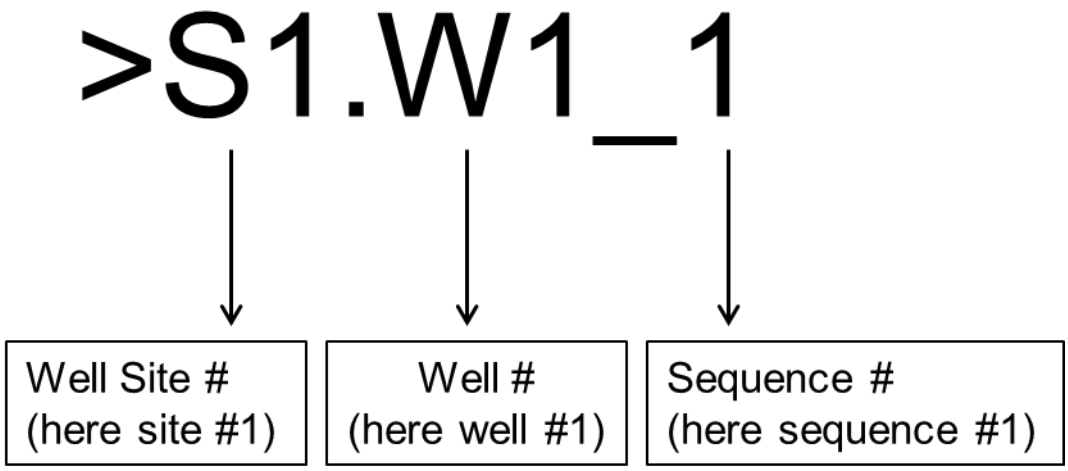

Figure B1: Sequence header descriptor for 16S rRNA sequences deposited in MG-RAST. Sequences were uploaded in FASTQ format and can be accesses under the accession number 4696241.3. This header represents an example.

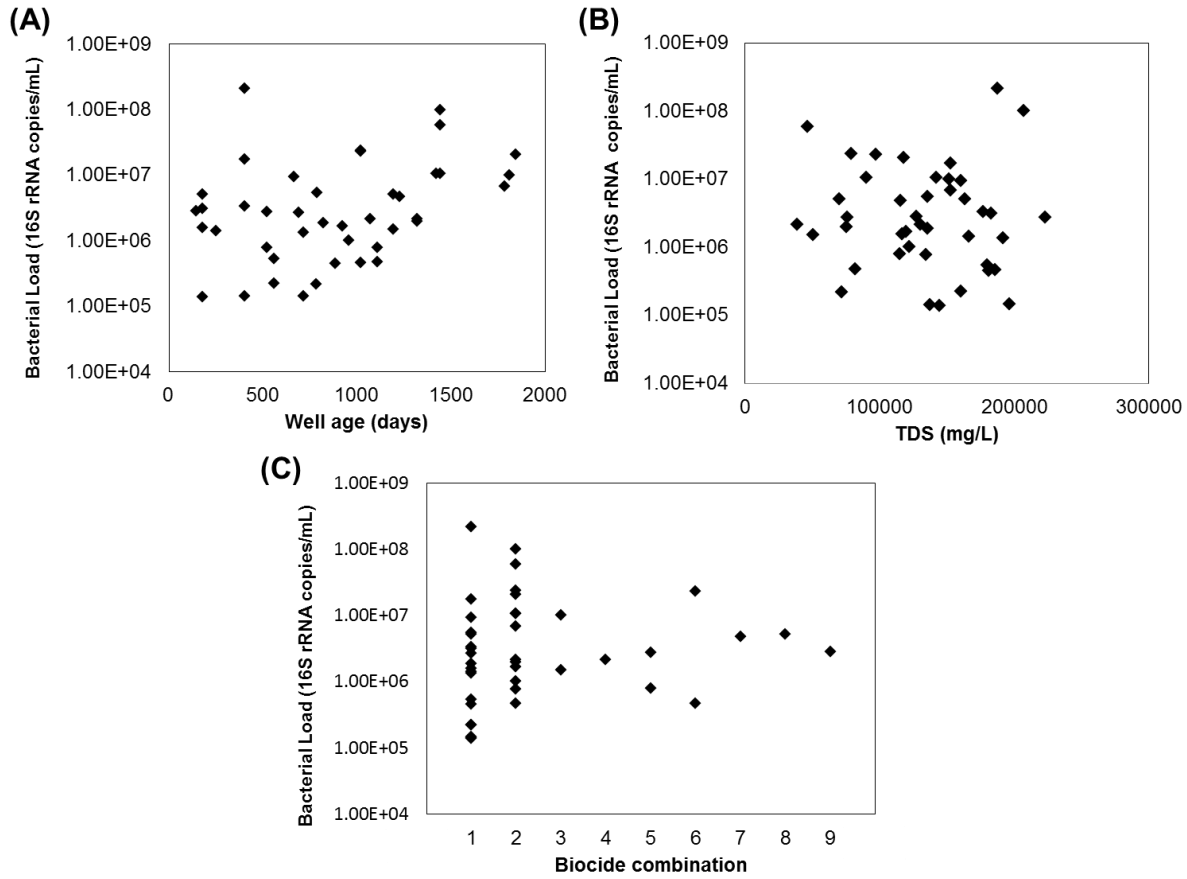

Figure B2: Well age (A), total dissolved solids (TDS) (B) and biocide treatment combinations (C) versus bacterial load (16S rRNA copies per mL) in hydraulic fracturing produced water samples. R^2 was found to be 0.12 for well age, and 0.01 for TDS. Biocide treatment combinations were determined from data reported on fracfocus.org (Table B2).

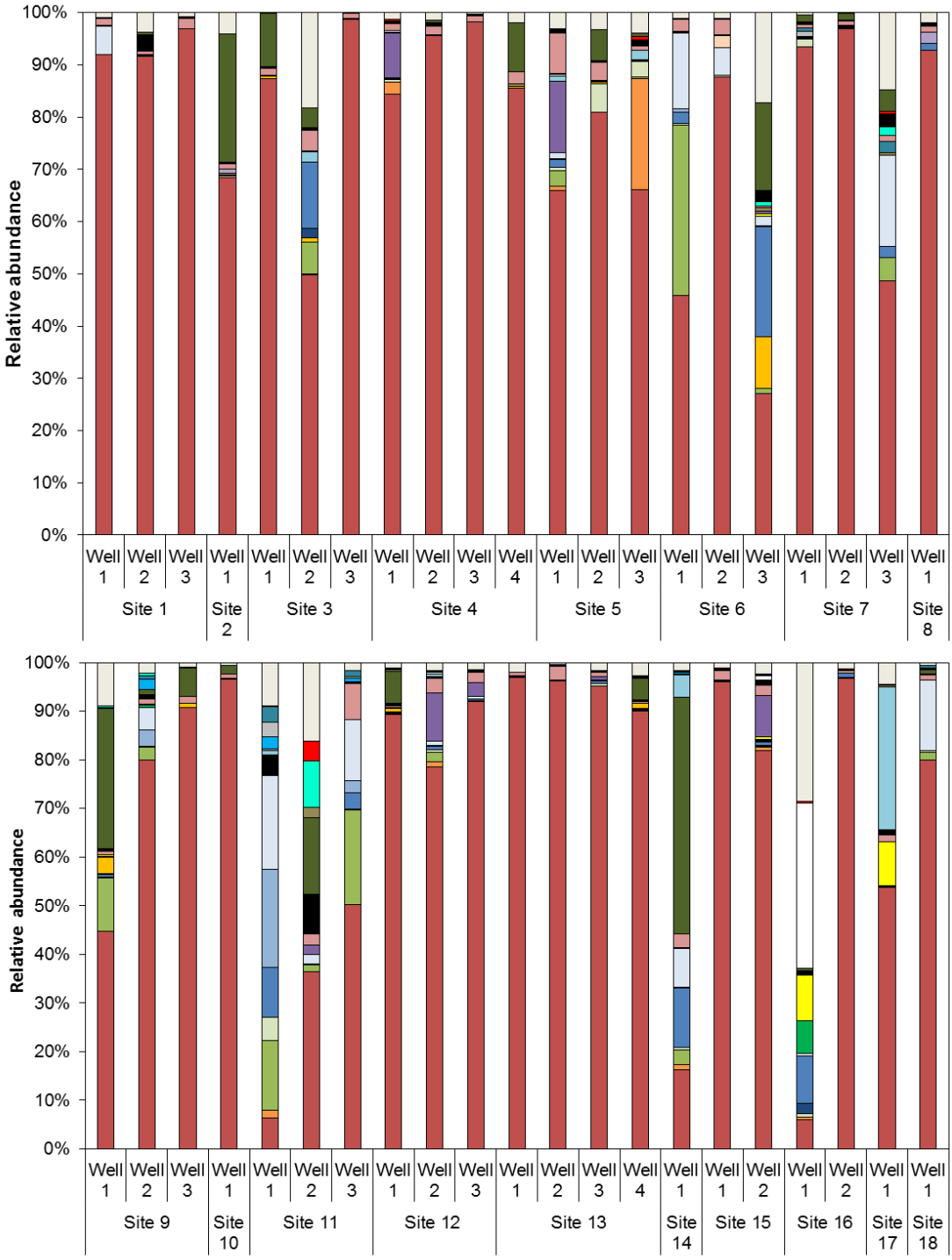

Figure B3: Genus level taxonomy based on 16S rRNA gene sequencing across all analyzed produced water samples. If sequenced could not be classified down to the genus level lowest identified phylogenetic level was listed.

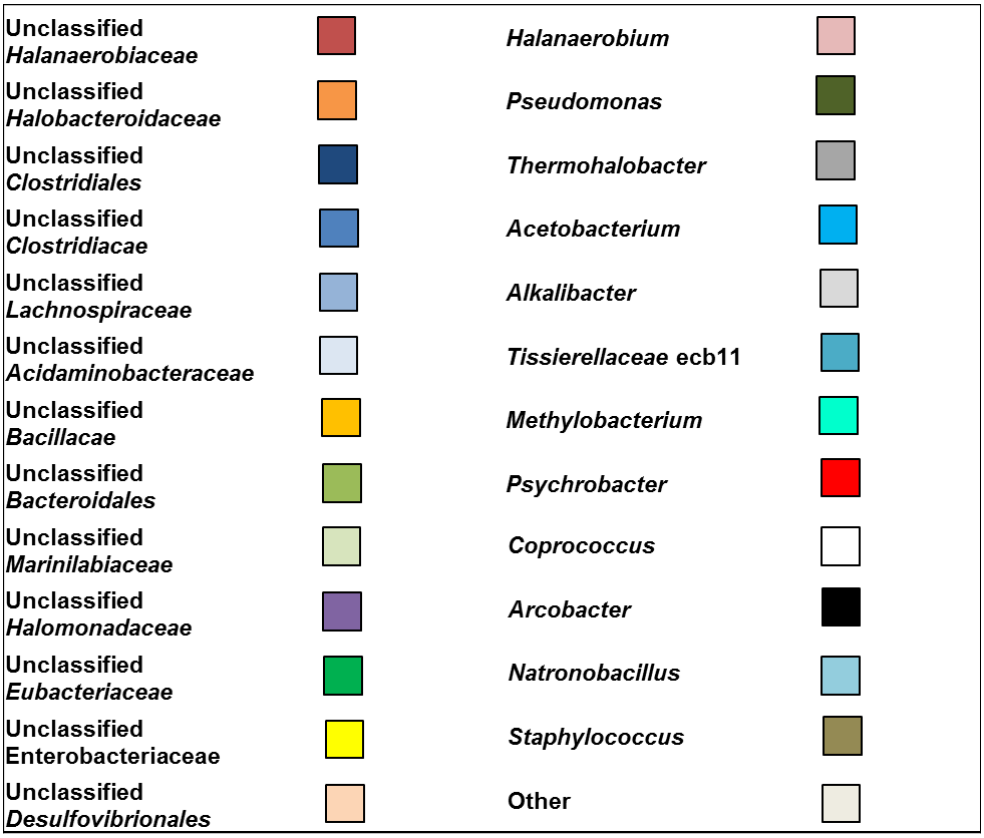

Figure B3 (continued)

Site	Site 1			Site 2	Site 3			Site 4				Site 5			Site 6			Site 7			Site 8	Site 9			Site 10	Site 11			Site 12			Site 13				Site 14	Site 15		Site 16		Site 17	Site 18
Well	Well 1	Well 2	Well 3	Well 1	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	Well 4	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	Well 1	Well 1	Well 2	Well 3	Well 1	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	Well 4	Well 1	Well 1	Well 2	Well 1	Well 2	Well 1	Well 1
Methanomicrobiales	0.20%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.07%	0.36%	0.39%	0.00%	0.05%	0.00%	0.00%	0.00%	1.13%	0.00%	0.00%	0.00%	0.00%	0.00%	0.76%	0.00%	0.74%	0.00%	0.03%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.10%
Methanosarcinales	0.07%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.82%	0.04%	0.02%	0.33%	0.00%	0.00%	0.03%	0.19%	0.00%	0.00%	0.01%	0.00%	0.00%	0.09%	0.00%	0.32%	0.00%	0.01%	0.01%	0.49%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.02%	0.03%
Acidobacteria-6 CCU21	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidobacteria-6 iii1-15	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidobacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidobacteria Sva0725	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidobacteria RB41	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidimicrobiales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.59%	0.00%	0.00%	0.42%	0.00%	0.00%	0.01%	0.00%	0.11%	0.00%	0.15%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Bifidobacteriales	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.24%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Coriobacteriales	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.24%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Gaiellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Solirubrobacterales	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.16%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Cytophagales	0.00%	0.00%	0.00%	0.10%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.10%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%
Flavobacteriales	0.00%	0.03%	0.01%	0.24%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.16%	0.00%	0.00%	0.01%	0.00%	0.00%	0.21%	0.00%	0.00%	0.17%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.10%	0.00%	0.00%	0.03%	0.03%	0.00%	0.01%	0.00%	0.10%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%
Sphingobacteriales	0.00%	0.00%	0.00%	0.10%	0.00%	0.10%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.10%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Saprospirales	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.26%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Anaerolineales	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Chloroflexi	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Cyanobacteria MLE1-12	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.29%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Stramenopiles	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%
Streptophyta	0.00%	0.19%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.19%	0.00%	0.00%	0.18%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.20%	0.02%	0.00%	0.00%
Oscillatoriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Pseudanabaenales	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Deferribacterales	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.10%	0.00%	0.01%	0.00%	0.00%	0.00%	0.03%	0.08%	0.00%	0.00%	0.00%
Gemellales	0.00%	0.00%	0.00%	0.14%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.51%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.21%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%
Thermoanaerobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.12%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Fusobacteriales	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.24%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Gemmatimonadales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.39%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Nitrospirales	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.13%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.08%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Planctomycetales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Alphaproteobacteria BD7-3	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Caulobacterales	0.00%	0.06%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.12%	0.00%	0.00%	0.08%	0.00%	0.01%	0.62%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.04%	0.04%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%
Kiloniellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%
Rhodospirillales	0.00%	0.21%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.10%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.07%	0.02%	0.24%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%
Rickettsiales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%
Sphingomonadales	0.03%	0.28%	0.05%	0.20%	0.00%	0.00%	0.00%	0.06%	0.00%	0.03%	1.02%	0.00%	0.03%	0.17%	0.06%	0.11%	0.41%	0.03%	0.00%	0.35%	0.07%	0.00%	0.22%	0.00%	0.00%	0.14%	1.94%	0.02%	0.00%	0.03%	0.03%	0.01%	0.07%	0.08%	0.00%	0.07%	0.61%	0.26%	0.05%	0.06%	0.26%	0.00%
Unclassified Betaproteobacteria	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.13%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.31%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Burkholderiales	0.03%	0.50%	0.03%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.02%	0.37%	0.00%	0.00%	0.28%	0.00%	0.01%	0.00%	0.00%	0.00%	1.23%	0.30%	0.00%	0.25%	0.00%	0.00%	0.05%	1.51%	0.01%	0.00%	0.00%	0.00%	0.02%	0.01%	0.01%	0.00%	0.00%	0.05%	0.00%	0.20%	0.09%	0.02%	0.00%
Gallionellales	0.00%	0.10%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.14%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%
Hydrogenophilales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%
Methylophilales	0.00%	0.09%	0.00%	0.00%	0.00%	0.48%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.62%	0.00%	0.00%	0.05%	0.00%	0.21%	0.01%	0.00%	0.00%	0.00%	1.17%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.02%	0.00%
Neisseriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.10%	0.00%	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Rhodocyclales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.10%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Deltaproteobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Bdellovibrionales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Desulfarculales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.17%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Desulfobacterales	0.11%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.26%	0.00%	0.00%	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.95%	0.00%	0.00%	0.00%	0.25%	0.25%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.07%
Desulfuromonadales	0.08%	0.09%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.07%	0.00%	0.00%	0.55%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.55%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.07%	0.00%	0.03%	0.05%	0.00%	0.00%	0.00%
Deltaproteobacteria GMD14H09	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.17%
Myxococcales	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Spirobacillales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.15%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unlcassified Gammaproteobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.31%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidithiobacillales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	1.04%	0.00%	0.08%	0.03%	0.00%	0.00%	0.00%	1.46%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Aeromonadales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Alteromonadales	0.00%	0.56%	0.02%	0.10%	0.00%	0.00%	0.00%	0.13%	0.03%	0.00%	0.45%	0.28%	0.06%	0.24%	0.09%	0.02%	0.52%	0.00%	0.00%	0.45%	0.11%	0.25%	0.19%	0.00%	0.00%	0.05%	0.34%	0.05%	0.06%	0.01%	0.00%	0.01%	0.11%	0.01%	0.00%	0.00%	0.07%	0.24%	0.50%	0.05%	0.08%	0.00%
Chromatiales	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Gammaproteobacteria HTCC2188	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Pasteurellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.15%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.10%	0.00%	0.00%	0.00%	0.01%	0.00%	0.02%	0.00%
Vibrionales	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.07%	0.06%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.90%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.53%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%
Xanthomonadales	0.00%	0.00%	0.00%	0.00%	0.00%	0.14%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.10%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.15%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.17%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Sphaerochaetales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.25%	0.00%	0.14%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Spirochaetales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Brachyspirales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Synergistales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.34%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
CW040	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
I025	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.10%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%
Unclassified Mollicutes	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acholeplasmatales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Anaeroplasmatales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Mollicutes RF39	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Thermotogales	0.04%	0.09%	0.21%	0.03%	0.00%	0.00%	0.02%	0.09%	0.00%	0.02%	0.00%	0.00%	0.67%	1.92%	0.00%	0.13%	0.00%	0.00%	0.00%	0.09%	0.07%	0.00%	0.00%	0.18%	0.00%	0.02%	0.05%	0.24%	0.00%	0.02%	0.02%	0.00%	0.01%	0.19%	0.05%	0.00%	0.00%	0.00%	0.42%	0.00%	0.00%	0.00%
Verrucomicrobiales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chthoniobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%

Figure B4: Abundances for minor orders (<2%) across all analyzed samples

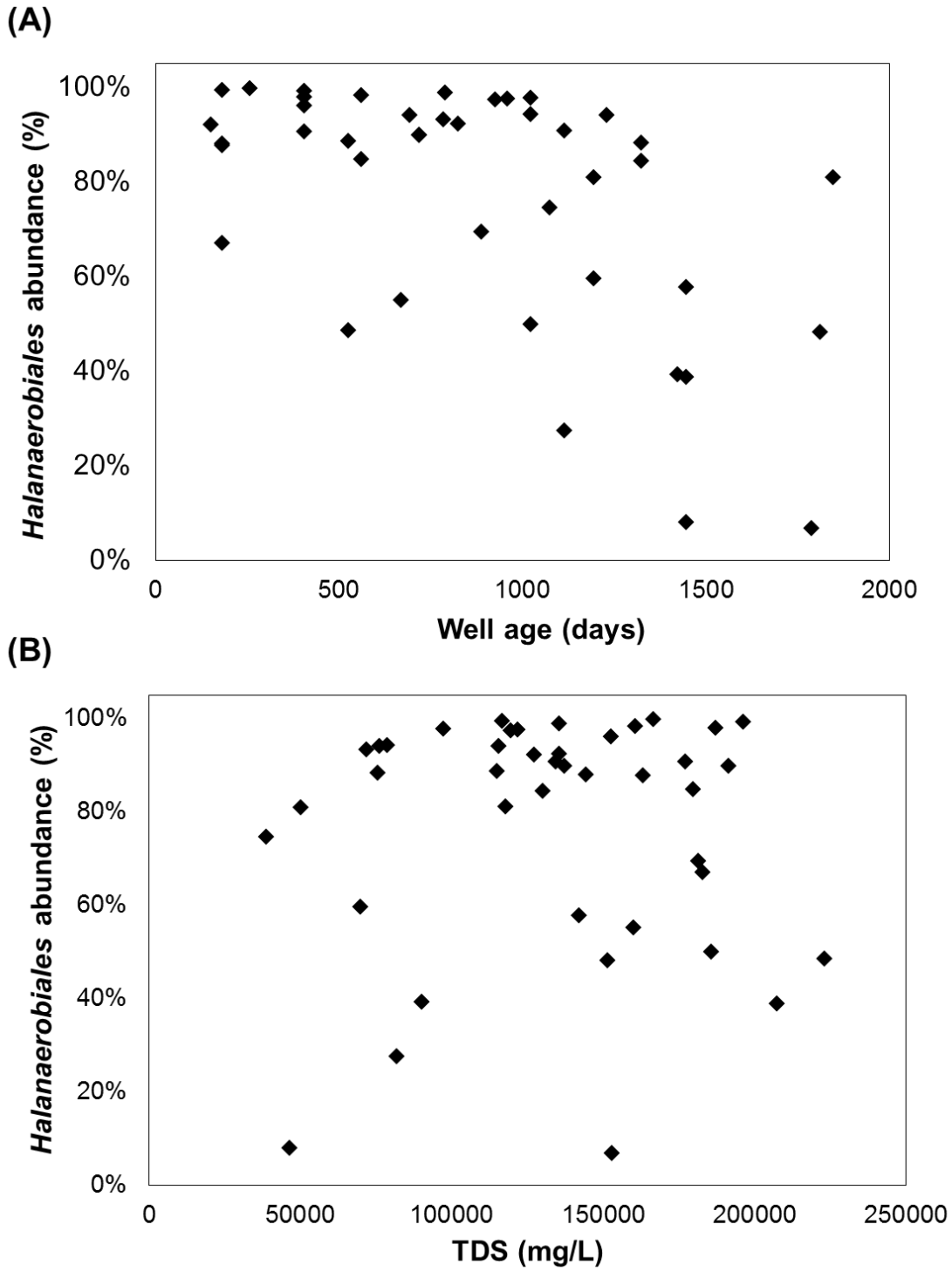

Figure B5: *Halanaerobiales* abundance versus well age (A) and TDS concentration (mg/L) (B). R^2 was found to be 0.30 for well age and < 0.01 for TDS concentration.

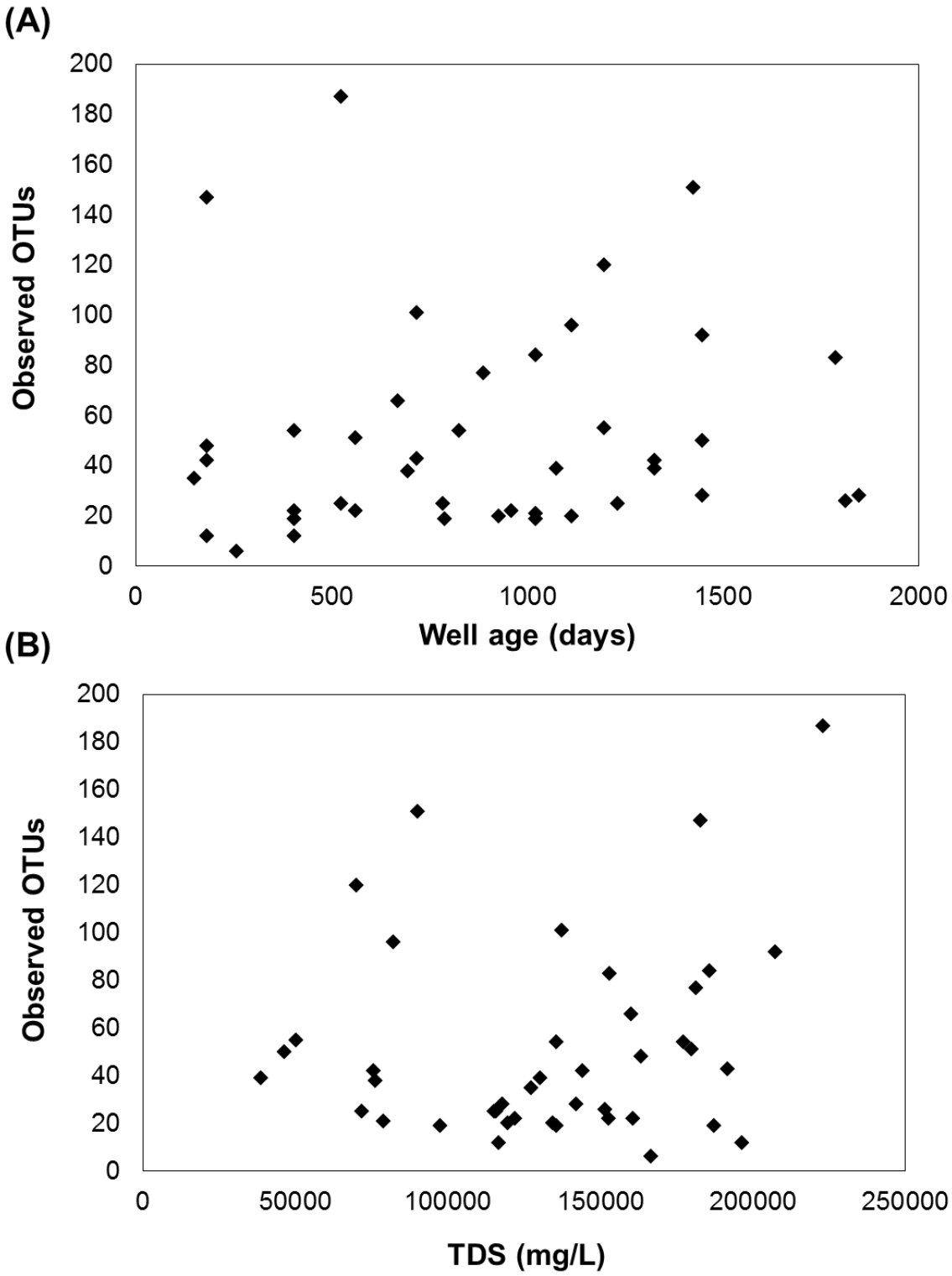

Figure B6: Well age (A) and TDS (B) versus number of observed OTUs (per 2000 sequences). R^2 was found to be 0.01 for well age and 0.03 for TDS.

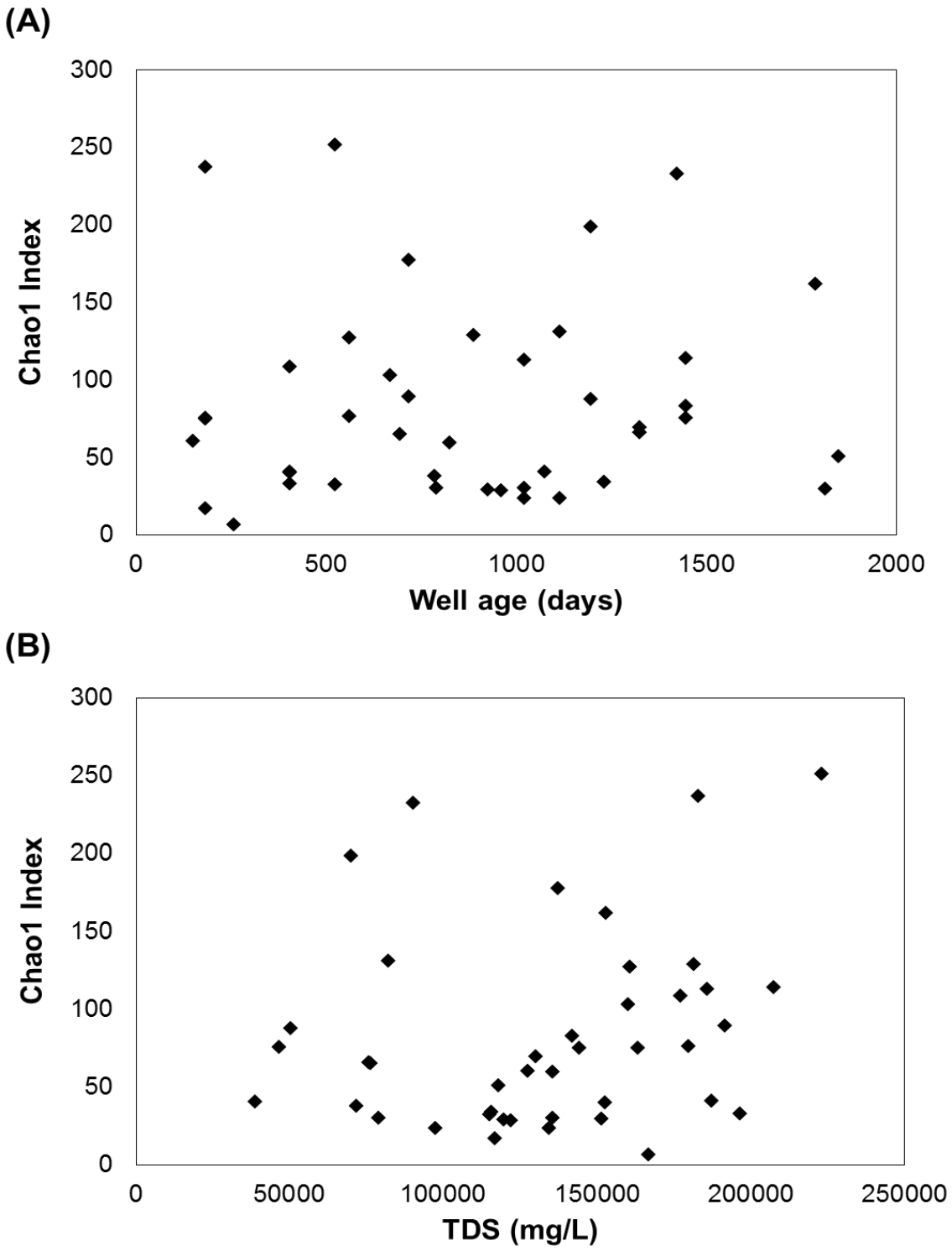

Figure B7: Well age (A) and TDS (B) versues Chao1 diversity index (per 2000 sequences). R^2 was found to be <0.01 for well age and 0.05 for TDS.

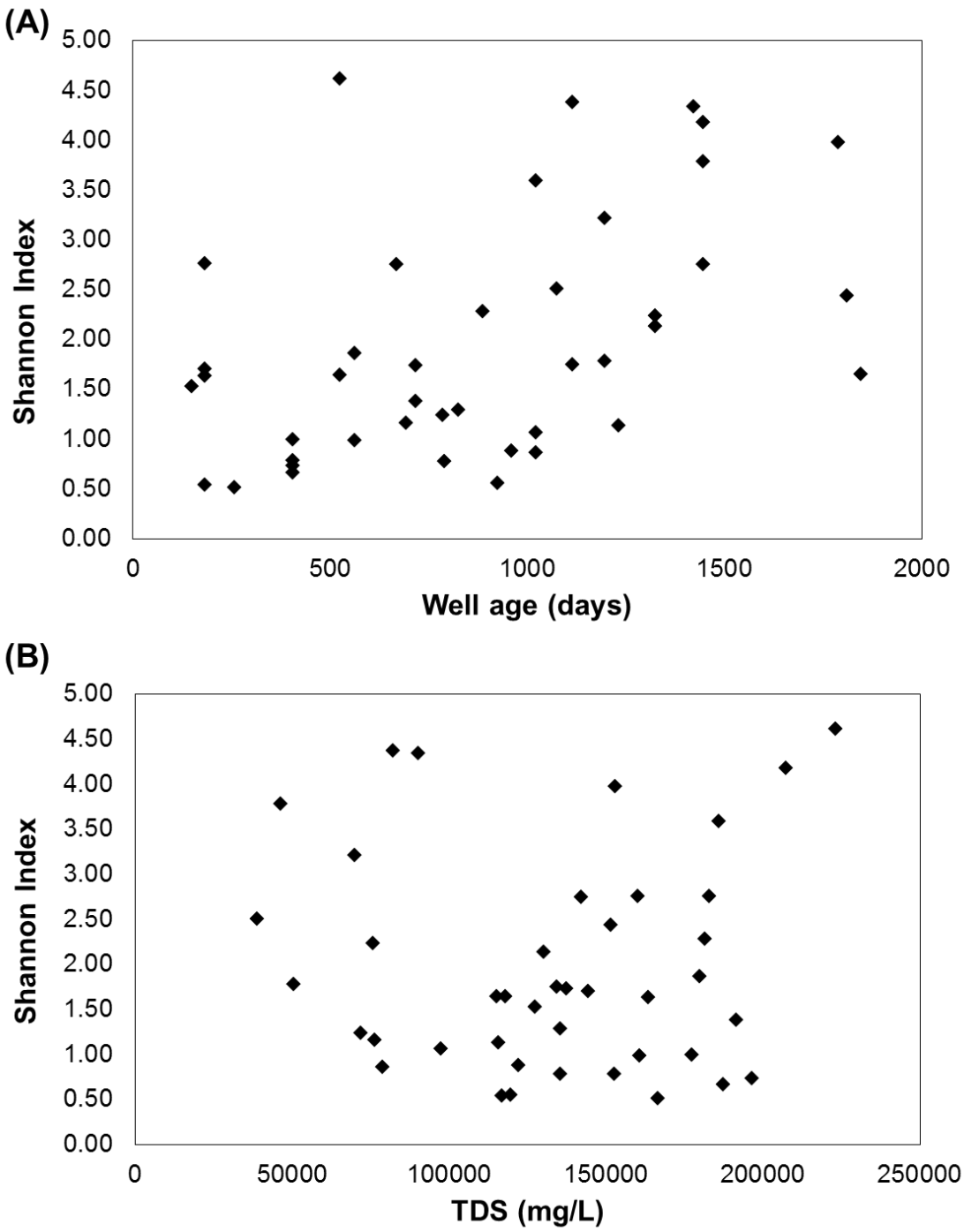

Figure B8: Well age (A) and TDS (B) versus Shannon diversity index (per 2000 sequences). R^2 was found to be 0.22 for well age and <0.01 for TDS.

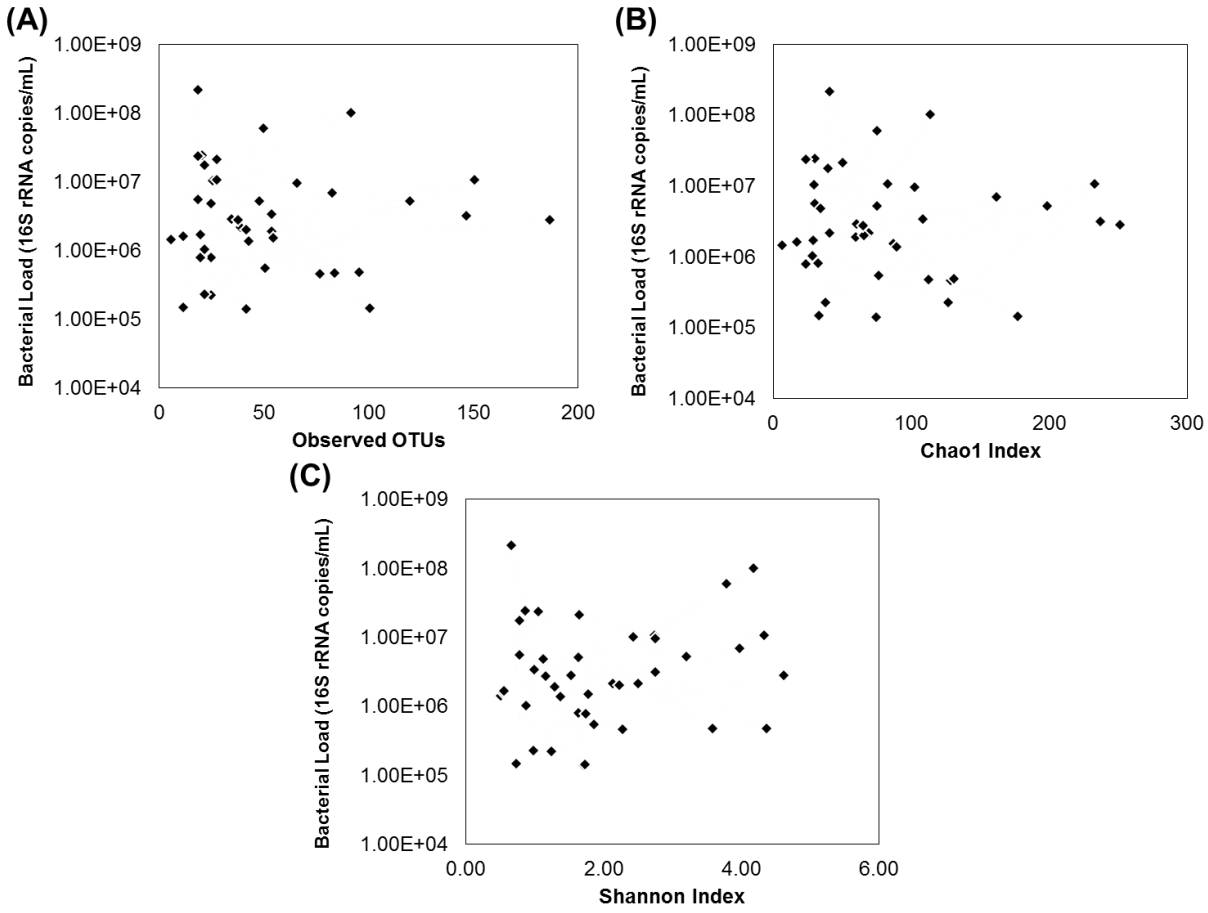

Figure B9: Number of observed OTUs (A), Chao1 diversity index (B) and Shannon diversity index (C) versus bacterial load (16S rRNA gene copies per mL) in hydraulic fracturing produced water samples. R^2 was found to be <0.01 for observed OTUs, <0.01 for Shannon diversity index, and <0.01 for Chao1 diversity index.

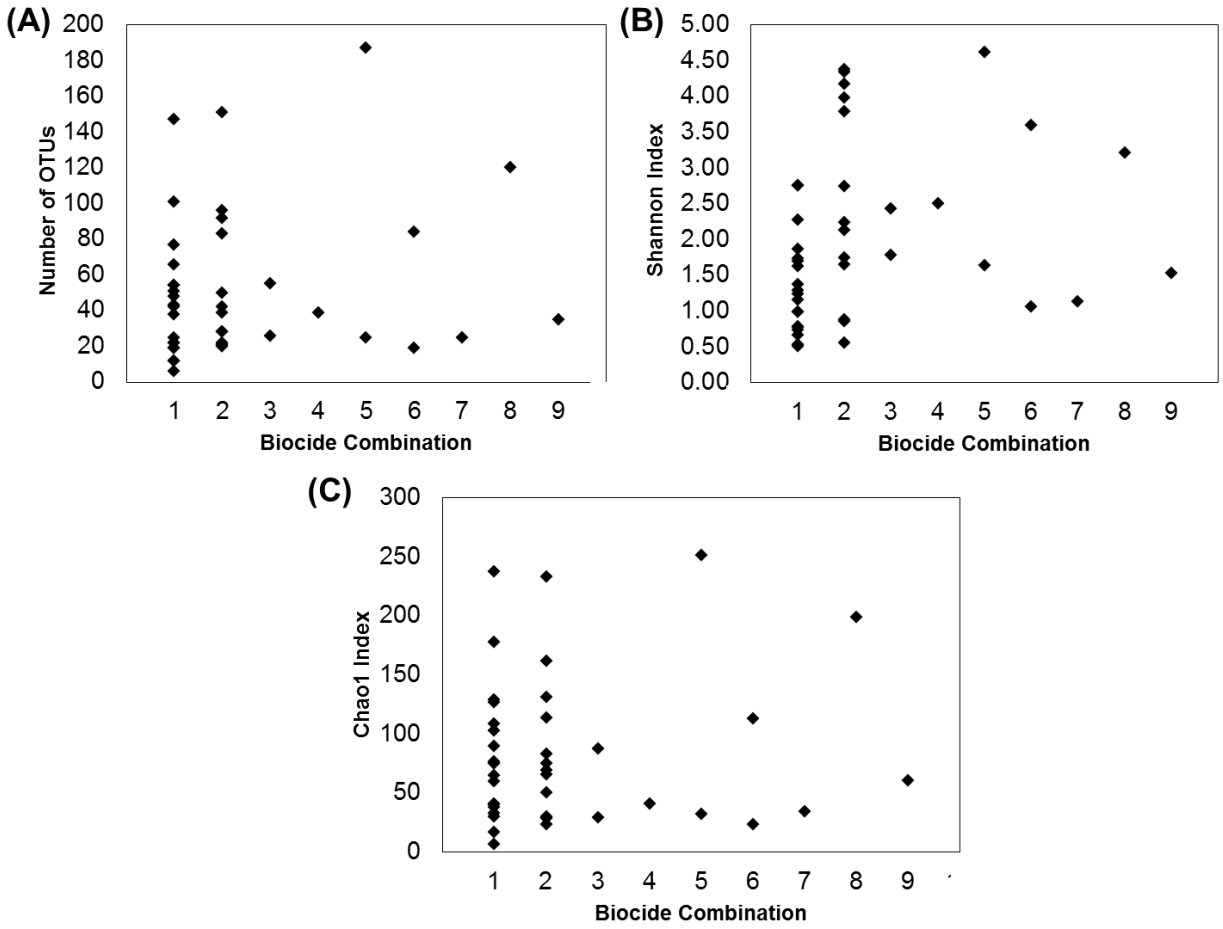

Figure B10: Number of observed OTUs (A), Shannon diversity index (B), and Chao1 diversity index (C) versus biocide treatment combination.

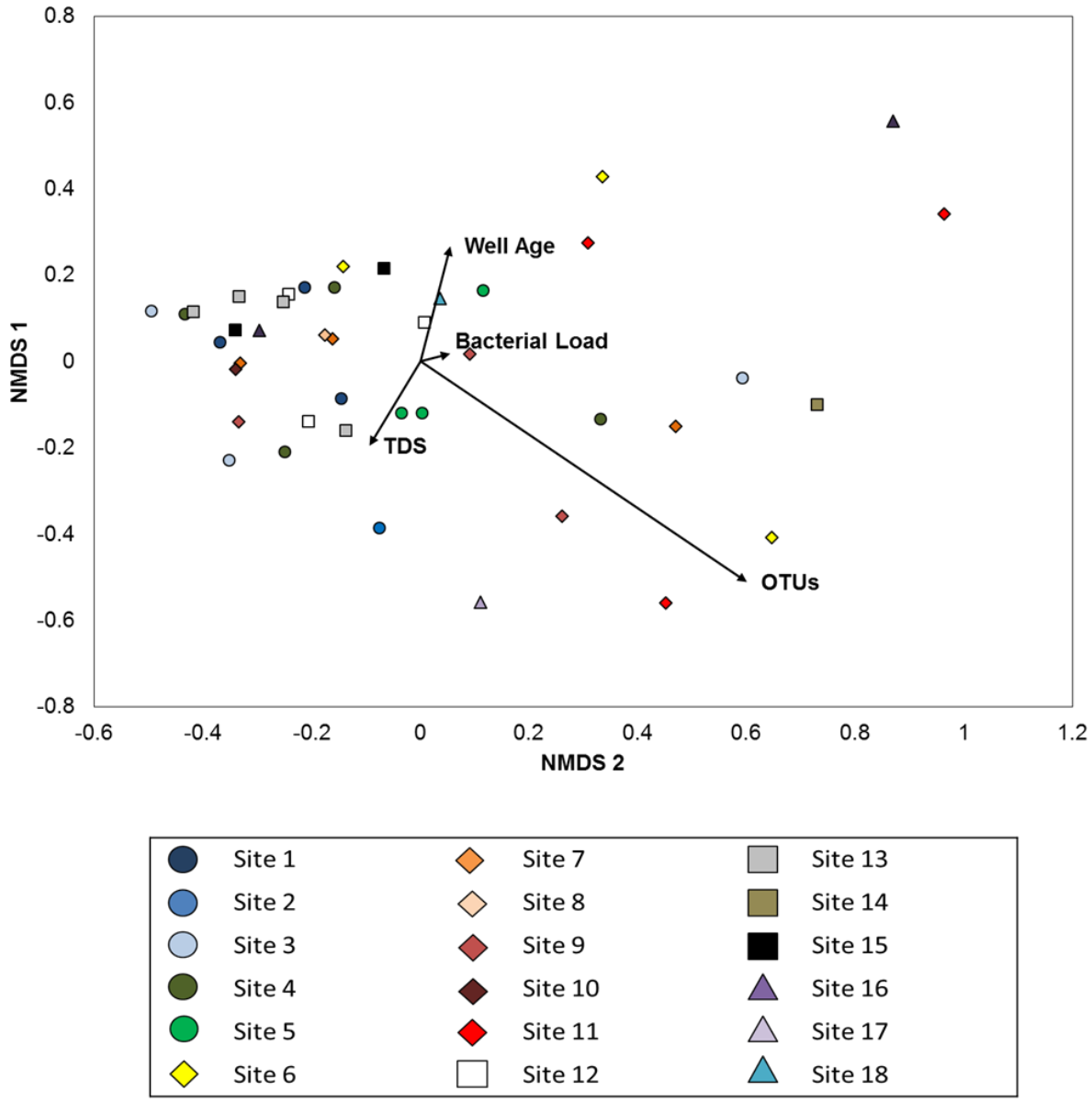

Figure B11: Nonmetric multidimensional scaling (NMDS) ordination plot showing the relationship between environmental variables and sites (based on weighted UniFrac distances).

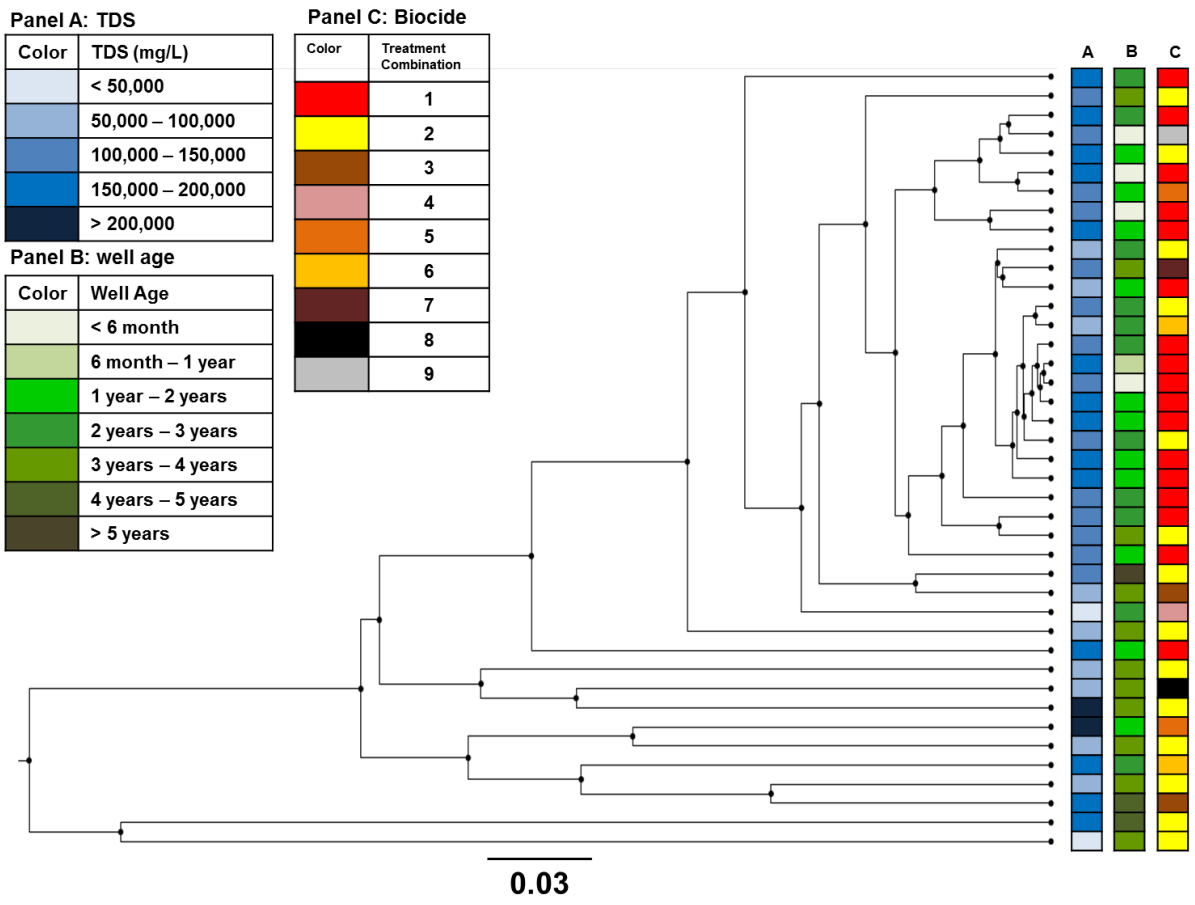

Figure B12: UPGMA tree based on weighted UniFrac distances**,** correlating operational parameters (Panels A-C) with analyzed samples. Sample were divided into five groups increasing by total TDS concentration (Panel A), seven groups increasing by well age (Panel B), and nine groups representing the nine biocide treatment combinations (Table B2). Each tree branch represents one sample, operational parameters are colorcoded based on TDS concentration, well age, or bioicde treatment combination. Bar distance represents weighted UniFrac distance.

APPENDIX C

CHAPTER 5 - SUPPLEMENTAL INFORMATION

Table C1: Well site characteristics. Formation abbreviations: BKN = Bakken formation, TF1 = First bench of Three Forks formation, TF2 = Second bench of Three Forks formation.

Well #	Well age at first sampling point	Formation
1	9 months	BKN
2	9 months	TF1
3	6 months	BKN
4	6 months	TF1
5	36 months	BKN
6	36 months	TF1
7	6 months	BKN
8	6 months	BKN
9	54-60 months*	TF1
10	18 months	BKN
11	12 months	BKN
12	12 months	TF1
13	12 months	TF2
14	12 months	BKN
15	12 months	TF1
16	10 months	BKN
17	10 months	TF1

*best available information at time of sampling

Table C2: Total dissolved solids (TDS) concentrations across all four sampling time point. For samples labeled N/A no data was obtained.

	Separator				Tank			
	October (10/7/2014)	**November 11/1/2014**	**January 1/14/2015**	**March 3/25/2015**	**October (10/7/2014)**	**November 11/1/2014**	**January 1/14/2015**	**March 3/25/2015**
Well 1	324250	325000	311000	245250	328750	323250	N/A	293250
Well 2	335000	340750	322500	285500	326250	332250	N/A	311750
Well 3	326500	N/A	312500	326250	292750	N/A	309500	298750
Well 4	321250	330750	320500	322750	313500	324000	286500	308000
Well 5	N/A	326500	317500	323750	317250	N/A	300750	321500
Well 6	N/A	N/A	326750	330000	330500	N/A	307750	275500
Well 7	307000	328000	305000	329750	310250	302250	N/A	298750
Well 8	311500	320250	319250	302750	321500	308750	N/A	303000
Well 9	250750	281000	N/A	N/A	N/A	281500	N/A	N/A
Well 10	307000	295250	293000	300750	293500	299250	N/A	301250
Well 11	311500	246250	258750	223000	259750	278000	N/A	196000
Well 12	274750	287500	284250	298500	269250	295250	N/A	288250
Well 13	315750	318000	279750	285000	305250	N/A	280000	285000
Well 15	283250	N/A	297750	265750	276000	270250	300250	278000
Well 16	277250	294250	N/A	285500	288750	285000	N/A	288250
Well 17	294750	305500	293500	301750	291500	297500	276500	305250
Well 18	324750	306500	315500	304000	308500	N/A	303250	308250

Table C3: Alpha Diversity results, *Samples for which less than 1000 OTU assigned sequence were available for alpha diversity analysis.

SEPARATOR					
Sampling Time	**Sample ID**	**Number of sequences**	**Number of OTUs**	**Chao1 Index**	**Shannon Index**
October	Well 1	1922	81	21	1.10
	Well 3	2020	169	106	4.62
	Well 4	2969	213	115	3.89
	Well 5	2367	249	98	5.59
	Well 10	1413	129	51	3.85
	Well 12	2236	186	103	4.34
	Well 13	3228	139	238	5.00
	Well 15	1622	158	96	4.81
November	Well 1	8469	107	181	3.33
	Well 2	3240	49	108	3.02
	Well 3	5496	64	122	3.86
	Well 4	4707	97	172	3.83
	Well 5	4323	78	107	4.02
	Well 7	2565	55	98	3.30
	Well 8	1862	76	113	3.67
	Well 9	1656	93	158	2.79
	Well 10	8531	112	190	4.22
	Well 11	9519	153	319	5.32
	Well 12	509*	63	44	3.75
	Well 13	6503	115	166	4.39
	Well 14	526*	60	143	3.50
	Well 15	7442	63	107	3.45
	Well 16	3113	162	334	5.37
March	Well 1	1366	31	53	2.86
	Well 3	678*	99	55	4.21
	Well 4	2670	99	195	3.95
	Well 5	3990	60	85	3.49
	Well 6	2690	58	86	3.45
	Well 7	2696	128	333	4.10
	Well 10	8385	150	316	4.98
	Well 11	10192	168	330	5.40
May	Well 1	7252	55	78	2.99
	Well 2	6495	70	123	2.88
	Well 3	3291	52	77	3.21
	Well 4	6198	83	110	4.27

Table C3 (continued)

Well 5	6019	57	73	3.13
Well 6	2105	59	217	3.38
Well 7	10209	58	71	3.28
Well 8	786*	37	31	3.06
Well 10	3953	94	145	4.34
Well 11	5721	175	348	5.28
Well 12	1215	87	181	2.90
Well 15	386*	169	45	3.68
Well 17	3282	60	105	3.44

Table C3 (continued): Alpha diversity results, *Samples for which less than 1000 OTU assigned sequence were available for alpha diversity analysis.

STORAGE TANK					
Sampling Time	**Sample ID**	**Number of sequences**	**Number of OTUs**	**Chao1 Index**	**Shannon Index**
January	Well 2	1874	168	90	5.10
	Well 5	1698	139	104	5.29
	Well 6	1567	105	76	4.50
	Well 8	1677	144	77	5.00
	Well 11	2469	147	78	4.75
	Well 16	332*	10	12	3.02
	Well 17	1327	87	53	4.61
November	Well 1	2970	73	130	2.77
	Well 2	8161	46	130	2.97
	Well 3	391*	48	30	3.46
	Well 4	6653	85	48	4.12
	Well 5	4837	53	44	3.86
	Well 8	7290	72	107	3.65
	Well 9	2774	103	148	4.39
	Well 10	2735	89	122	4.04
	Well 11	1348	65	121	2.19
	Well 12	7868	112	215	3.69
	Well 13	1886	67	112	3.68
	Well 14	2497	158	323	4.99
	Well 16	4725	104	161	4.25
	Well 17	389*	43	39	3.94
January	Well 3	2145	109	171	4.09
	Well 4	2912	27	23	3.03
	Well 5	6619	57	93	3.00
	Well 6	1735	78	122	3.77
	Well 13	1207	83	134	4.03
	Well 14	3597	88	174	4.04
March	Well 2	6108	173	49	2.26
	Well 3	1862	34	54	2.80
	Well 4	5947	89	138	3.51
	Well 5	6872	162	291	5.34
	Well 7	6573	61	93	3.20
	Well 8	7352	157	313	5.24
	Well 10	6846	100	147	4.20
	Well 11	5613	64	117	3.59
	Well 12	4882	54	87	2.57
	Well 15	1551	103	173	3.79

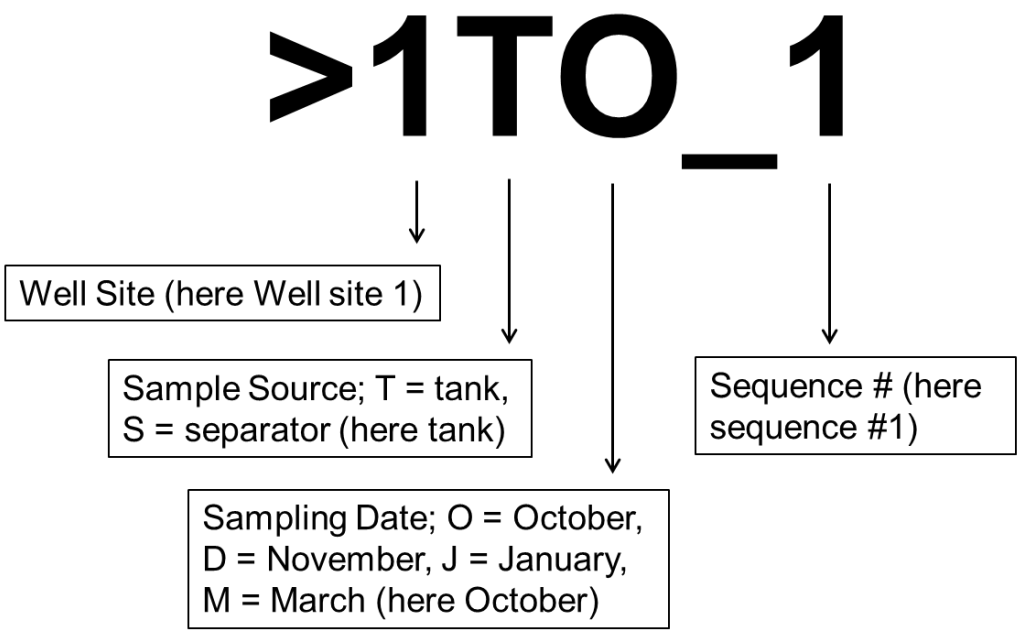

Figure C1: Sequence header descriptor for 16S rRNA sequences deposited in MG-RAST. Sequences were uploaded in FASTQ format and can be accesses under the accession number . This header represents an example.

Taxa	October 2014													
	Separator									Tank				
	Well 1	Well 3	Well 4	Well 5	Well 10	Well 12	Well 13	Well 15	Well 2	Well 5	Well 6	Well 8	Well 11	Well 17
Actinomycetales	0.33%	6.65%	6.94%	7.80%	6.64%	6.31%	6.90%	2.92%	20.12%	20.08%	8.27%	12.78%	13.50%	29.65%
Bacteroidales	0.33%	5.26%	9.29%	10.09%	16.61%	6.70%	9.03%	8.48%	5.41%	8.43%	5.66%	3.13%	4.18%	6.98%
Flavobacteriales	0.00%	0.46%	2.48%	2.75%	0.33%	1.16%	0.41%	0.58%	1.20%	0.40%	0.58%	1.70%	0.00%	0.58%
Bacillales	0.99%	0.46%	1.12%	0.92%	2.33%	2.84%	0.57%	1.75%	1.50%	2.61%	3.05%	3.13%	4.18%	2.91%
Lactobacillales	0.00%	2.63%	1.36%	1.38%	1.66%	0.52%	0.82%	1.46%	9.01%	15.46%	10.16%	10.23%	12.22%	5.81%
Halanaerobiales	93.70%	22.72%	32.47%	23.85%	50.50%	53.35%	25.04%	44.74%	25.23%	19.08%	32.22%	27.84%	36.33%	36.05%
Clostridiales	0.88%	5.10%	4.34%	3.67%	2.99%	2.71%	5.75%	7.02%	1.50%	2.21%	12.05%	7.67%	8.04%	6.40%
Fusobacteriales	0.00%	0.00%	0.37%	2.75%	0.33%	0.00%	0.00%	1.75%	0.00%	0.00%	0.00%	0.28%	0.00%	0.00%
Caulobacterales	0.00%	0.00%	0.00%	0.46%	0.00%	0.13%	0.00%	0.29%	0.00%	0.20%	0.00%	0.28%	0.96%	0.00%
Rhizobiales	0.11%	0.00%	0.62%	0.92%	3.99%	0.52%	0.08%	1.17%	0.90%	0.00%	0.00%	0.28%	2.25%	0.00%
Rhodobacterales	0.00%	2.16%	2.97%	2.29%	0.00%	0.64%	1.72%	3.22%	3.60%	5.22%	2.18%	0.57%	0.32%	0.58%
Rickettsiales	0.00%	0.00%	0.00%	0.46%	0.00%	0.00%	0.00%	0.29%	0.30%	0.00%	0.00%	2.27%	0.00%	0.58%
Sphingomonadales	0.00%	1.85%	3.47%	2.75%	0.00%	1.80%	0.66%	2.92%	2.70%	3.41%	2.32%	2.84%	3.86%	0.58%
Neisseriales	0.00%	0.00%	0.00%	0.46%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.64%	0.00%
Desulfovibrionales	0.00%	0.46%	0.25%	0.00%	0.00%	0.26%	0.82%	0.00%	0.00%	0.40%	2.47%	0.00%	0.00%	0.58%
Desulfuromonadales	0.00%	0.31%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Syntrophobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Campylobacterales	2.10%	6.03%	20.32%	9.63%	0.66%	2.32%	11.49%	6.14%	3.90%	11.45%	4.50%	8.81%	0.96%	2.91%
Alteromonadales	0.11%	0.93%	0.87%	0.92%	0.33%	2.71%	3.86%	0.88%	0.30%	0.40%	0.15%	0.85%	0.96%	0.00%
Oceanospirillales	0.33%	36.94%	5.20%	0.92%	0.66%	7.60%	28.24%	5.26%	4.80%	1.00%	6.97%	5.97%	0.96%	1.74%
Pseudomonadales	0.66%	6.80%	5.20%	13.30%	11.63%	9.15%	1.07%	7.89%	16.82%	6.22%	4.35%	5.40%	6.43%	0.58%
Vibrionales	0.00%	0.00%	1.24%	0.92%	0.00%	0.39%	2.63%	0.58%	0.30%	0.00%	0.00%	1.99%	0.64%	0.00%
Xanthomonadales	0.00%	0.00%	0.25%	0.92%	0.00%	0.00%	0.00%	0.29%	0.30%	0.00%	0.58%	0.00%	0.00%	2.33%
Minor	0.44%	1.24%	1.24%	12.84%	1.33%	0.90%	0.90%	2.34%	2.10%	3.41%	4.50%	3.98%	3.54%	1.74%

Taxa	November 2014																													
	Separator															Tank														
	Well 1	Well 2	Well 3	Well 4	Well 5	Well 7	Well 8	Well 9	Well 10	Well 11	Well 12	Well 13	Well 14	Well 15	Well 16	Well 1	Well 2	Well 3	Well 4	Well 5	Well 8	Well 9	Well 10	Well 11	Well 12	Well 13	Well 14	Well 16	Well 17
Actinomycetales	0.96%	1.32%	1.12%	3.02%	4.79%	0.60%	0.85%	2.10%	2.14%	0.67%	3.31%	3.66%	1.39%	0.68%	1.01%	2.56%	1.07%	2.46%	1.51%	5.21%	0.67%	2.08%	3.14%	1.35%	3.15%	0.77%	0.56%	1.53%	4.62%
Bacteroidales	0.23%	0.03%	1.68%	0.16%	0.04%	0.09%	0.52%	0.21%	0.36%	1.70%	0.55%	1.19%	0.00%	0.05%	1.33%	0.28%	0.08%	1.48%	0.02%	0.10%	0.20%	1.95%	0.22%	0.25%	0.61%	0.77%	3.71%	1.37%	0.00%
Flavobacteriales	0.00%	0.00%	0.00%	0.16%	0.00%	0.00%	0.00%	0.00%	0.13%	0.30%	0.00%	0.04%	0.00%	0.00%	0.32%	0.02%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.19%	0.37%	0.00%	0.00%
Bacillales	24.30%	96.01%	55.21%	29.58%	77.11%	92.15%	87.32%	8.27%	48.95%	18.65%	80.66%	15.52%	75.64%	87.60%	27.46%	94.22%	96.57%	76.35%	93.73%	90.16%	84.61%	26.17%	82.24%	3.37%	78.32%	86.59%	1.20%	23.25%	72.69%
Lactobacillales	0.75%	0.20%	0.39%	0.74%	1.88%	0.05%	0.59%	0.84%	1.11%	0.02%	0.55%	0.69%	0.93%	0.05%	0.19%	0.88%	0.07%	0.00%	0.18%	1.53%	0.23%	0.32%	0.85%	0.25%	1.14%	0.06%	0.09%	1.62%	0.00%
Halanaerobiales	0.62%	0.04%	32.60%	0.85%	5.50%	2.23%	2.88%	0.14%	26.71%	15.04%	1.38%	2.75%	0.46%	0.97%	15.16%	0.08%	0.01%	5.42%	0.04%	0.36%	2.37%	44.06%	2.54%	0.00%	6.41%	4.09%	18.63%	46.40%	2.10%
Clostridiales	8.86%	0.16%	2.40%	13.60%	1.35%	0.70%	0.59%	14.23%	1.78%	5.08%	3.59%	11.25%	2.32%	1.52%	6.47%	0.33%	0.99%	3.45%	1.09%	0.44%	0.83%	5.85%	3.59%	14.76%	0.68%	2.23%	8.71%	3.90%	4.20%
Fusobacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.72%	0.07%	0.13%	0.85%	0.00%	0.00%	0.00%	0.00%	0.63%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.03%	0.42%	0.08%	0.94%	0.00%	1.39%	0.03%	0.00%
Caulobacterales	0.00%	0.01%	0.00%	0.16%	0.00%	0.05%	0.00%	0.00%	0.13%	0.46%	0.00%	0.02%	0.46%	0.00%	0.25%	0.02%	0.00%	0.00%	0.04%	0.02%	0.00%	0.26%	0.02%	0.00%	0.11%	0.51%	0.65%	0.00%	0.00%
Rhizobiales	0.13%	0.01%	0.05%	0.32%	0.18%	0.23%	0.59%	0.56%	0.83%	1.17%	0.00%	1.56%	0.00%	0.06%	1.33%	0.08%	0.00%	0.00%	0.22%	0.17%	0.02%	0.73%	0.18%	0.00%	0.75%	0.00%	1.30%	0.03%	0.00%
Rhodobacterales	0.04%	0.10%	0.00%	0.16%	0.11%	0.00%	0.46%	0.14%	0.94%	2.97%	0.00%	0.33%	0.00%	0.00%	2.35%	0.11%	0.00%	0.00%	0.02%	0.00%	0.42%	0.19%	0.00%	0.17%	0.11%	0.00%	3.24%	3.08%	0.00%
Rickettsiales	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.24%	0.23%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Sphingomonadales	0.08%	0.22%	0.15%	0.08%	1.45%	0.00%	0.07%	0.00%	0.98%	1.61%	0.00%	0.46%	1.62%	0.23%	1.40%	0.00%	0.00%	0.00%	0.42%	0.31%	0.02%	1.05%	0.25%	0.00%	0.09%	0.13%	1.67%	0.64%	0.84%
Neisseriales	0.08%	0.00%	0.00%	0.29%	0.50%	0.00%	0.00%	0.07%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.11%	0.49%	0.04%	0.15%	0.00%	0.00%	0.00%	0.08%	0.02%	0.00%	0.00%	0.00%	0.00%
Desulfovibrionales	0.02%	0.00%	0.00%	0.19%	0.00%	0.00%	0.00%	0.00%	0.28%	1.38%	0.83%	0.37%	0.00%	0.00%	0.63%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	1.39%	0.15%	0.00%
Desulfuromonadales	0.00%	0.00%	0.00%	0.16%	0.00%	0.09%	0.00%	0.00%	0.17%	1.63%	0.00%	0.00%	0.00%	0.00%	0.76%	0.00%	0.00%	0.00%	0.02%	0.00%	0.35%	0.00%	0.34%	0.00%	0.00%	0.13%	1.76%	0.00%	0.00%
Syntrophobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Campylobacterales	1.12%	0.12%	0.12%	1.59%	3.23%	0.84%	0.78%	1.61%	7.53%	14.95%	1.10%	7.24%	0.93%	0.77%	14.65%	0.07%	0.26%	0.00%	0.05%	0.00%	0.60%	7.51%	2.13%	1.10%	0.02%	0.45%	22.43%	7.81%	0.00%
Alteromonadales	1.42%	0.15%	0.12%	1.40%	0.35%	0.19%	0.13%	1.40%	0.60%	2.23%	0.00%	2.25%	0.23%	0.24%	2.16%	0.00%	0.00%	0.00%	0.02%	0.00%	0.08%	0.22%	0.05%	1.43%	0.00%	0.13%	1.76%	0.46%	0.00%
Oceanospirillales	1.14%	0.03%	4.35%	1.83%	0.00%	0.05%	0.52%	2.03%	0.81%	4.37%	0.00%	4.05%	2.55%	0.87%	3.42%	0.00%	0.01%	0.00%	0.26%	0.10%	0.25%	1.57%	1.12%	1.77%	0.44%	0.32%	3.89%	2.01%	0.00%
Pseudomonadales	58.97%	1.27%	1.58%	44.92%	1.67%	2.42%	2.81%	67.55%	5.69%	25.09%	7.73%	47.14%	11.83%	6.81%	19.59%	1.09%	0.64%	9.36%	2.15%	0.99%	7.84%	5.97%	1.53%	74.37%	3.28%	3.58%	24.93%	6.04%	14.71%
Vibrionales	0.26%	0.00%	0.00%	0.05%	0.07%	0.00%	0.13%	0.28%	0.02%	0.28%	0.00%	0.00%	0.93%	0.05%	0.00%	0.07%	0.00%	0.00%	0.05%	0.15%	0.00%	0.06%	0.00%	0.25%	0.15%	0.00%	0.28%	0.27%	0.00%
Xanthomonadales	0.10%	0.00%	0.00%	0.00%	0.00%	0.14%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.01%	0.00%	0.02%	0.00%	0.68%	0.32%	0.00%	0.00%	2.84%	0.00%	0.00%	0.00%	0.00%
Minor	0.92%	0.32%	0.22%	0.77%	1.77%	0.19%	1.05%	0.49%	0.68%	1.49%	0.28%	1.21%	0.46%	0.08%	0.89%	0.18%	0.16%	0.99%	0.09%	0.31%	0.83%	1.60%	1.37%	0.76%	0.89%	0.06%	2.04%	1.40%	0.84%

Figure C2: Abundances for all major orders (>2%)

Taxa	January 2015													
	Separator								Tank					
	Well 1	Well 3	Well 4	Well 5	Well 6	Well 7	Well 10	Well 11	Well 3	Well 4	Well 5	Well 6	Well 13	Well 14
Actinomycetales	0.23%	6.81%	2.39%	0.92%	2.90%	2.50%	0.39%	0.92%	1.95%	3.39%	1.26%	4.11%	0.40%	1.73%
Bacteroidales	0.00%	1.43%	0.27%	0.07%	0.63%	1.83%	2.30%	1.98%	1.52%	0.33%	0.00%	0.00%	0.00%	0.56%
Flavobacteriales	0.00%	0.00%	0.14%	0.00%	0.00%	0.49%	0.42%	1.10%	0.36%	0.00%	0.00%	0.15%	0.00%	0.04%
Bacillales	98.91%	53.76%	82.85%	88.34%	87.49%	22.41%	1.69%	19.07%	21.23%	71.68%	96.04%	81.38%	44.01%	26.90%
Lactobacillales	0.00%	1.43%	0.45%	0.85%	1.29%	1.34%	0.17%	0.77%	1.37%	1.18%	0.52%	0.51%	0.60%	0.00%
Halanaerobiales	0.08%	0.36%	0.23%	0.36%	0.03%	47.07%	23.02%	17.76%	46.35%	3.48%	0.04%	0.81%	0.20%	43.10%
Clostridiales	0.63%	2.87%	1.89%	6.18%	0.90%	2.32%	6.19%	5.85%	4.19%	2.49%	0.43%	2.27%	4.33%	1.98%
Fusobacteriales	0.00%	0.00%	0.09%	0.00%	0.00%	0.00%	1.33%	0.67%	0.00%	0.05%	0.00%	0.07%	0.00%	0.00%
Caulobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.43%	0.39%	0.29%	0.00%	0.19%	0.00%	0.00%	0.00%	0.08%
Rhizobiales	0.00%	2.15%	0.00%	0.07%	0.02%	0.18%	0.78%	0.94%	0.00%	0.19%	0.05%	0.22%	0.00%	0.20%
Rhodobacterales	0.00%	0.00%	0.09%	0.00%	0.00%	0.55%	3.77%	2.57%	0.29%	0.24%	0.02%	0.00%	2.22%	2.50%
Rickettsiales	0.00%	0.36%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Sphingomonadales	0.00%	0.00%	0.27%	0.43%	0.00%	0.73%	1.61%	1.65%	0.36%	1.22%	0.20%	0.29%	0.00%	2.14%
Neisseriales	0.08%	0.00%	0.18%	0.07%	0.05%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.07%	0.00%	0.00%
Desulfovibrionales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	2.11%	1.20%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Desulfuromonadales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.97%	1.08%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Syntrophobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Campylobacterales	0.00%	0.00%	0.27%	0.07%	0.00%	5.86%	19.20%	15.30%	3.90%	0.28%	0.13%	0.37%	1.11%	9.76%
Alteromonadales	0.00%	0.72%	0.32%	0.00%	0.00%	0.18%	2.55%	2.20%	0.36%	0.19%	0.00%	0.00%	0.30%	0.00%
Oceanospirillales	0.00%	0.36%	0.32%	0.07%	0.00%	3.17%	5.19%	4.28%	3.25%	0.38%	0.22%	0.07%	1.51%	0.85%
Pseudomonadales	0.08%	29.03%	8.64%	2.42%	6.15%	8.49%	26.10%	20.78%	13.36%	12.46%	0.86%	8.87%	45.12%	7.70%
Vibrionales	0.00%	0.00%	0.36%	0.00%	0.00%	0.37%	0.25%	0.08%	0.51%	0.00%	0.04%	0.29%	0.00%	0.00%
Xanthomonadales	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.02%	0.00%	0.24%	0.00%	0.00%	0.00%	0.00%
Minor	0.00%	0.72%	1.26%	0.14%	0.52%	2.08%	1.58%	1.51%	1.01%	1.98%	0.20%	0.51%	0.20%	2.46%

Taxa	March 2015																					
	Separator												Tank									
	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Well 7	Well 8	Well 10	Well 11	Well 12	Well 15	Well 17	Well 2	Well 3	Well 4	Well 5	Well 7	Well 8	Well 10	Well 11	Well 12	Well 16
Actinomycetales	1.31%	1.90%	2.26%	9.60%	1.15%	1.76%	2.84%	0.00%	2.58%	1.20%	0.85%	2.63%	0.40%	0.93%	0.41%	3.06%	2.69%	1.60%	0.72%	1.91%	0.77%	0.73%	5.26%
Bacteroidales	0.20%	0.05%	0.00%	0.43%	0.04%	0.29%	0.08%	0.83%	0.47%	1.70%	0.19%	0.00%	0.06%	0.04%	0.06%	0.36%	2.11%	0.06%	1.17%	0.76%	0.34%	0.00%	0.59%
Flavobacteriales	0.03%	0.00%	0.37%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.25%	0.00%	0.00%	0.00%	0.00%	0.00%	0.44%	0.26%	0.19%	0.42%	0.80%	0.06%	0.03%	0.07%
Bacillales	92.99%	92.19%	95.58%	64.71%	94.00%	94.26%	92.69%	94.20%	67.52%	9.37%	11.23%	70.30%	83.80%	68.62%	98.55%	34.06%	11.26%	94.55%	25.16%	71.60%	82.26%	35.64%	27.95%
Lactobacillales	0.43%	0.25%	0.10%	0.43%	0.42%	0.12%	1.46%	0.00%	0.34%	0.12%	0.47%	1.50%	0.12%	0.43%	0.06%	0.31%	0.36%	0.32%	0.50%	0.71%	0.24%	0.29%	1.56%
Halanaerobiales	0.23%	1.65%	0.10%	4.16%	0.00%	0.23%	0.54%	0.00%	2.65%	20.53%	1.23%	0.75%	3.59%	0.26%	0.06%	1.20%	16.13%	0.11%	16.30%	3.86%	0.36%	0.34%	0.89%
Clostridiales	0.36%	0.99%	0.66%	1.18%	0.57%	0.47%	0.82%	0.00%	3.89%	4.52%	15.28%	1.13%	1.78%	0.67%	0.17%	1.84%	4.64%	1.10%	5.13%	3.90%	2.02%	0.16%	7.04%
Fusobacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.58%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.88%	0.00%	0.28%	0.02%	0.00%	0.00%	0.15%
Caulobacterales	0.03%	0.00%	0.00%	0.00%	0.14%	0.06%	0.01%	0.00%	0.03%	0.54%	0.00%	0.00%	0.01%	0.00%	0.00%	0.29%	0.68%	0.02%	0.59%	0.00%	0.02%	0.00%	0.00%
Rhizobiales	0.03%	0.00%	0.00%	0.00%	0.18%	0.12%	0.02%	0.28%	0.29%	1.12%	0.09%	0.75%	0.00%	0.87%	0.00%	0.00%	1.04%	0.06%	0.95%	0.16%	0.02%	0.57%	0.00%
Rhodobacterales	0.02%	0.00%	0.00%	0.18%	0.00%	0.00%	0.00%	0.00%	0.00%	3.53%	0.00%	0.00%	0.16%	0.04%	0.00%	0.00%	3.54%	0.00%	2.34%	0.31%	0.04%	0.00%	0.07%
Rickettsiales	0.00%	0.00%	0.07%	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Sphingomonadales	0.58%	0.14%	0.03%	0.00%	0.44%	0.00%	0.10%	0.00%	0.41%	1.04%	0.00%	0.00%	0.40%	0.00%	0.00%	0.00%	1.20%	0.06%	1.34%	0.05%	0.00%	0.00%	0.22%
Neisseriales	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.72%	0.04%	0.28%	0.00%	0.00%	0.02%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.02%	0.00%	0.22%
Desulfovibrionales	0.00%	0.00%	0.00%	0.47%	0.00%	0.00%	0.00%	0.00%	0.22%	1.58%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.79%	0.00%	1.03%	0.00%	0.00%	0.00%	0.00%
Desulfuromonadales	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	1.20%	0.00%	0.00%	0.03%	0.00%	0.00%	0.13%	0.81%	0.00%	1.00%	0.00%	0.00%	0.00%	0.00%
Syntrophobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Campylobacterales	0.02%	0.00%	0.00%	0.55%	0.04%	0.00%	0.01%	0.69%	3.76%	18.58%	1.79%	0.00%	0.75%	0.14%	0.00%	1.42%	16.20%	0.04%	12.18%	1.64%	0.44%	0.26%	1.11%
Alteromonadales	0.07%	0.00%	0.00%	0.92%	0.00%	0.00%	0.01%	0.00%	0.39%	2.12%	0.94%	0.75%	0.21%	0.10%	0.00%	0.87%	1.69%	0.00%	1.42%	0.33%	0.42%	0.00%	1.26%
Oceanospirillales	0.05%	0.00%	0.00%	0.55%	0.12%	0.23%	0.34%	0.00%	0.60%	4.27%	1.60%	0.38%	0.94%	0.30%	0.00%	1.11%	3.12%	0.10%	3.90%	0.91%	0.30%	0.42%	1.41%
Pseudomonadales	3.17%	2.21%	0.70%	15.14%	1.84%	2.23%	0.69%	4.01%	13.73%	25.13%	65.38%	21.80%	6.86%	27.08%	0.64%	54.34%	29.67%	1.56%	23.68%	11.69%	12.45%	60.43%	50.85%
Vibrionales	0.05%	0.11%	0.00%	0.04%	0.14%	0.00%	0.31%	0.00%	0.92%	0.12%	0.19%	0.00%	0.03%	0.10%	0.00%	0.07%	0.06%	0.00%	0.17%	0.13%	0.06%	0.55%	0.15%
Xanthomonadales	0.03%	0.11%	0.00%	0.04%	0.10%	0.06%	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.09%	0.06%	0.00%	0.00%	0.00%	0.02%	0.03%	0.38%	0.00%	0.00%	0.15%
Minor	0.40%	0.38%	0.13%	1.49%	0.81%	0.18%	0.07%	0.00%	0.50%	2.36%	0.47%	0.00%	0.72%	0.32%	0.06%	0.51%	1.75%	0.23%	1.70%	0.84%	0.20%	0.60%	1.04%

Figure C2 (continued): Abundances for all major orders (>2%)

Taxon	October 2014													
	Separator								Storage Tank					
	Well 1	Well 3	Well 4	Well 5	Well 10	Well 12	Well 13	Well 15	Well 2	Well 5	Well 6	Well 8	Well 11	Well 14
Methanomicrobiales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.30%	0.00%	0.00%	0.00%	0.00%	0.00%
Methanosarcinales	0.00%	0.00%	0.00%	0.46%	0.00%	0.13%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidobacteria iii1-15	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidobacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidobacteria B110	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chloracidobacteria RB41	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.28%	0.00%	0.00%
Acidimicrobiales	0.00%	0.00%	0.00%	1.38%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Bifidobacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Coriobacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Gaiellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.26%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Solirubrobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.29%	0.00%	0.00%	0.15%	0.00%	0.00%	0.00%
Armatimonadales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Bacteroidetes	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Cytophagales	0.00%	0.00%	0.00%	0.46%	0.00%	0.00%	0.00%	0.29%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Sphingobacteriales	0.00%	0.00%	0.12%	0.92%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.64%	0.00%
Rhodothermale	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Saprospirales	0.00%	0.00%	0.00%	1.83%	0.00%	0.39%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Anaerolineales	0.00%	0.31%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chloroflexi AKYG885	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Cyanobacteria MLE1-12	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Cyanobacteria YS2	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Stramenopiles	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Streptophyta	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.58%
Nostocales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chroococcales	0.00%	0.15%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Oscillatoriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.30%	0.00%	0.00%	0.00%	0.00%	0.00%
Pseudanabaenales	0.00%	0.00%	0.12%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Synechococcales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Deferribacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Gemellales	0.11%	0.15%	0.25%	0.00%	0.33%	0.13%	0.00%	0.29%	0.00%	1.81%	0.15%	0.28%	0.00%	0.00%
Thermoanaerobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Erysipelotrichales	0.00%	0.00%	0.00%	0.46%	0.00%	0.00%	0.00%	0.29%	0.60%	0.20%	0.15%	0.00%	0.00%	0.58%
Victivallales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Nitrospirales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Phycisphaerae MSBL9	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Phycisphaerales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Gemmatales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Pirellulale	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Planctomycetales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Alphaproteobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Alphaproteobacteria BD7-3	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Kiloniellales	0.11%	0.00%	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Rhodospirillales	0.00%	0.00%	0.37%	0.46%	0.00%	0.00%	0.49%	0.00%	0.30%	0.20%	0.00%	0.00%	0.00%	0.00%
Unclassified Betaproteobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.64%	0.00%
Burkholderiales	0.11%	0.15%	0.00%	0.46%	0.33%	0.00%	0.08%	0.29%	0.00%	0.00%	0.87%	0.28%	0.64%	0.00%
Hydrogenophilales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.73%	0.00%	0.00%	0.00%
Betaproteobacteria MND1	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Methylophilales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.32%	0.00%
Procabacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Rhodocyclales	0.00%	0.00%	0.12%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.15%	1.42%	0.00%	0.00%
Bdellovibrionales	0.00%	0.00%	0.00%	0.92%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Desulfobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Myxococcales	0.00%	0.00%	0.00%	0.46%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.15%	0.00%	0.00%	0.00%
Entotheonellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Gammaproteobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.42%	0.00%	0.00%
Gammaproteobacteria 34P16	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidithiobacillales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Aeromonadales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chromatiales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Enterobacteriales	0.00%	0.00%	0.12%	0.46%	0.33%	0.00%	0.00%	0.58%	0.00%	1.20%	0.29%	0.00%	1.29%	0.00%
Legionellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Methylococcales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Pasteurellales	0.00%	0.00%	0.00%	0.46%	0.33%	0.00%	0.00%	0.29%	0.00%	0.00%	0.44%	0.00%	0.00%	0.00%
Thiotrichales	0.00%	0.00%	0.00%	0.46%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Marinicellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Sphaerochaetales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Spirochaetales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Leptospirales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Synergistales	0.00%	0.00%	0.00%	0.92%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	1.45%	0.00%	0.00%	0.00%
Unclassified Mollicutes	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acholeplasmatales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Anaeroplasmatales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Mollicutes RF39	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Thermotogales	0.00%	0.00%	0.00%	0.46%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Verrucomicrobia	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Verrucomicrobiales	0.11%	0.31%	0.12%	1.38%	0.00%	0.00%	0.00%	0.00%	0.60%	0.00%	0.00%	0.28%	0.00%	0.58%
Pedosphaerales	0.00%	0.00%	0.00%	0.46%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chthoniobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Bacteria	0.00%	0.15%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%

Figure C3: Abundances for all minor orders (<2%), October samples.

Taxon	November 2014																												
	Separator															Tank													
	Well 1	Well 2	Well 3	Well 4	Well 5	Well 7	Well 8	Well 9	Well 10	Well 11	Well 12	Well 13	Well 14	Well 15	Well 16	Well 1	Well 2	Well 3	Well 4	Well 5	Well 8	Well 9	Well 10	Well 11	Well 12	Well 13	Well 14	Well 16	Well 17
Methanomicrobiales	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Methanosarcinales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%
Acidobacteria iii1-15	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%
Acidobacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidobacteria B110	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chloracidobacteria RB41	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidimicrobiales	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Bifidobacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Coriobacteriales	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.33%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Gaiellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Solirubrobacterales	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Armatimonadales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Bacteroidetes	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Cytophagales	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.99%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.37%	0.00%	0.00%
Sphingobacteriales	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%
Rhodothermale	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Saprospirales	0.02%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.40%	0.00%
Anaerolineales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chloroflexi AKYG885	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Cyanobacteria MLE1-12	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.15%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Cyanobacteria YS2	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Stramenopiles	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Streptophyta	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.28%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.02%	0.00%	0.00%	0.06%	0.00%	0.06%	0.00%
Nostocales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chroococcales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Oscillatoriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Pseudanabaenales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Synechococcales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Deferribacterales	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Gemellales	0.03%	0.01%	0.07%	0.03%	1.70%	0.00%	0.00%	0.07%	0.00%	0.02%	0.28%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.10%	0.10%	0.03%	0.20%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Thermoanaerobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Erysipelotrichales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.13%	0.00%	0.06%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.10%	0.00%	0.00%	0.00%	0.19%	0.09%	0.00%
Victivallales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Nitrospirales	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.33%	0.07%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.70%	0.17%	0.00%	0.00%	0.00%	0.00%	0.00%
Phycisphaerae MSBL9	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Phycisphaerales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Gemmatales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Pirellulale	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Planctomycetales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Alphaproteobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Alphaproteobacteria BD7-3	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Kiloniellales	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.11%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Rhodospirillales	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.06%	0.13%	0.00%	0.00%	0.00%	0.00%	0.37%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.46%	0.00%
Unclassified Betaproteobacteria	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.14%	0.00%	0.00%	0.00%	0.00%	0.51%	0.09%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Burkholderiales	0.01%	0.00%	0.00%	0.08%	0.00%	0.00%	0.07%	0.00%	0.02%	0.16%	0.00%	0.07%	0.00%	0.02%	0.25%	0.03%	0.00%	0.00%	0.00%	0.05%	0.10%	0.03%	0.00%	0.00%	0.64%	0.00%	0.19%	0.03%	0.00%
Hydrogenophilales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.32%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Betaproteobacteria MND1	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Methylophilales	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.13%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Procabacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Rhodocyclales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.23%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.42%	0.00%	0.00%	0.05%	0.00%	0.09%	0.00%	0.00%
Bdellovibrionales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%
Desulfobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.25%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%	0.09%	0.00%	0.00%
Myxococcales	0.00%	0.17%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Entotheonellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Gammaproteobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.13%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%
Gammaproteobacteria 34P16	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidithiobacillales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Aeromonadales	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%
Chromatiales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.18%	0.00%	0.00%	0.00%	0.00%	0.19%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%
Enterobacteriales	0.58%	0.00%	0.02%	0.56%	0.04%	0.09%	0.07%	0.35%	0.13%	0.37%	0.00%	0.85%	0.23%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.15%	0.16%	0.18%	0.51%	0.02%	0.00%	0.28%	0.37%	0.00%
Legionellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Methylococcales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Pasteurellales	0.10%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.02%	0.00%	0.07%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Thiotrichales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Marinicellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Sphaerochaetales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Spirochaetales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Leptospirales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Synergistales	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.02%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.28%	0.00%	0.00%
Unclassified Mollicutes	0.05%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acholeplasmatales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Anaeroplasmatales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Mollicutes RF39	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Thermotogales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Verrucomicrobia	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Verrucomicrobiales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Pedosphaerales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chthoniobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Bacteria	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%

Figure C3 (continued): Abundances for all minor orders (<2%), November samples.

Taxon	January 2015													
	Separator								Tank					
	Well 1	Well 3	Well 4	Well 5	Well 6	Well 7	Well 10	Well 11	Well 3	Well 4	Well 5	Well 6	Well 13	Well 14
Methanomicrobiales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Methanosarcinales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidobacteria iii1-15	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidobacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%
Acidobacteria B110	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chloracidobacteria RB41	0.00%	0.00%	0.09%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.14%	0.00%	0.00%	0.00%	0.00%
Acidimicrobiales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.85%	0.00%	0.00%	0.10%	0.00%
Bifidobacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%
Coriobacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%
Gaiellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Solirubrobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%	0.00%	0.00%
Armatimonadales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.38%	0.00%	0.00%	0.00%	0.00%
Unclassified Bacteroidetes	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Cytophagales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Sphingobacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.14%	0.00%	0.00%	0.00%	0.00%
Rhodothermale	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Saprospirales	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Anaerolineales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chloroflexi AKYG885	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Cyanobacteria MLE1-12	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Cyanobacteria YS2	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Stramenopiles	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Streptophyta	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Nostocales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chroococcales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Oscillatoriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Pseudanabaenales	0.00%	0.00%	0.00%	0.00%	0.00%	0.24%	0.06%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Synechococcales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.19%	0.16%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Deferribacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Gemellales	0.00%	0.00%	0.09%	0.14%	0.00%	0.43%	0.00%	0.02%	0.22%	0.00%	0.07%	0.29%	0.00%	0.36%
Thermoanaerobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Erysipelotrichales	0.00%	0.00%	0.00%	0.00%	0.00%	0.12%	0.00%	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.32%
Victivallales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Nitrospirales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.04%	0.00%	0.00%	0.12%
Phycisphaerae MSBL9	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Phycisphaerales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Gemmatales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%
Pirellulale	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Planctomycetales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Alphaproteobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Alphaproteobacteria BD7-3	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Kiloniellales	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Rhodospirillales	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.03%	0.10%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Betaproteobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Burkholderiales	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.11%	0.00%	0.00%	0.00%	0.00%	0.07%	0.00%	0.00%
Hydrogenophilales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.48%
Betaproteobacteria MND1	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Methylophilales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.29%	0.00%	0.00%	0.00%	0.00%	0.00%
Procabacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Rhodocyclales	0.00%	0.00%	0.00%	0.00%	0.50%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Bdellovibrionales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.08%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Desulfobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.19%	0.16%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Myxococcales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.10%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Entotheonellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Gammaproteobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Gammaproteobacteria 34P16	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidithiobacillales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Aeromonadales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chromatiales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Enterobacteriales	0.00%	0.36%	0.77%	0.00%	0.02%	0.49%	0.22%	0.33%	0.36%	0.05%	0.00%	0.07%	0.10%	0.40%
Legionellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Methylococcales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Pasteurellales	0.00%	0.36%	0.05%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.14%	0.05%	0.07%	0.00%	0.20%
Thiotrichales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.12%
Marinicellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Sphaerochaetales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Spirochaetales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Leptospirales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Synergistales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.36%	0.18%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Mollicutes	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acholeplasmatales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Anaeroplasmatales	0.00%	0.00%	0.00%	0.00%	0.00%	0.55%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Mollicutes RF39	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Thermotogales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Verrucomicrobia	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Verrucomicrobiales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Pedosphaerales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chthoniobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.40%
Unclassified Bacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%

Figure C3 (continued): Abundances for all minor orders (<2%), January samples.

Taxon	March 2015																							
	Separator													Tank										
	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Well 7	Well 8	Well 10	Well 11	Well 12	Well 15	Well 17	Well 2	Well 3	Well 4	Well 5	Well 7	Well 8	Well 10	Well 11	Well 12	Well 16
Methanomicrobiales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Methanosarcinales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidobacteria iii1-15	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidobacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidobacteria B110	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%
Chloracidobacteria RB41	0.00%	0.00%	0.00%	0.00%	0.14%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.22%
Acidimicrobiales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Bifidobacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Coriobacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.07%
Gaiellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Solirubrobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Armatimonadales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Bacteroidetes	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.16%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Cytophagales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.11%	0.02%	0.00%	0.00%	0.00%
Sphingobacteriales	0.00%	0.00%	0.03%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Rhodothermale	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Saprospirales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Anaerolineales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chloroflexi AKYG885	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Cyanobacteria MLE1-12	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Cyanobacteria YS2	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%
Stramenopiles	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Streptophyta	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%
Nostocales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.34%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chroococcales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%
Oscillatoriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Pseudanabaenales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Synechococcales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.17%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.16%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Deferribacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%
Gemellales	0.00%	0.00%	0.00%	0.49%	0.32%	0.12%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.07%	0.00%	0.00%	0.00%	0.04%	0.06%	0.16%	0.22%
Thermoanaerobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Erysipelotrichales	0.12%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.10%	0.00%	0.14%	0.00%	0.00%	0.00%	0.00%
Victivallales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Nitrospirales	0.00%	0.13%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Phycisphaerae MSBL9	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Phycisphaerales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.11%	0.00%	0.00%	0.00%	0.00%
Gemmatales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Pirellulale	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Planctomycetales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Alphaproteobacteria	0.13%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%
Alphaproteobacteria BD7-3	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%
Kiloniellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Rhodospirillales	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.17%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.16%	0.00%	0.08%	0.02%	0.00%	0.00%	0.00%
Unclassified Betaproteobacteria	0.00%	0.00%	0.00%	0.08%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.06%	0.00%	0.00%	0.03%	0.00%
Burkholderiales	0.03%	0.00%	0.00%	0.02%	0.02%	0.06%	0.01%	0.00%	0.00%	0.17%	0.00%	0.00%	0.01%	0.10%	0.00%	0.00%	0.06%	0.02%	0.14%	0.11%	0.00%	0.00%	0.00%
Hydrogenophilales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Betaproteobacteria MND1	0.00%	0.00%	0.00%	0.00%	0.12%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Methylophilales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Procabacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Rhodocyclales	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.12%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Bdellovibrionales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Desulfobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.21%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.23%	0.00%	0.22%	0.00%	0.00%	0.00%	0.00%
Myxococcales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Entotheonellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%
Unclassified Gammaproteobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Gammaproteobacteria 34P16	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.17%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acidithiobacillales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Aeromonadales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chromatiales	0.00%	0.00%	0.10%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.03%	0.00%	0.11%	0.00%	0.00%	0.00%	0.00%
Enterobacteriales	0.02%	0.07%	0.00%	0.88%	0.00%	0.00%	0.00%	0.00%	0.45%	0.83%	0.38%	0.00%	0.07%	0.06%	0.00%	0.24%	0.29%	0.02%	0.20%	0.29%	0.06%	0.34%	0.30%
Legionellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%
Methylococcales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%
Pasteurellales	0.00%	0.13%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.19%	0.06%	0.06%	0.02%	0.00%	0.00%	0.22%
Thiotrichales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%
Marinicellales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Sphaerochaetales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.13%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Spirochaetales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Leptospirales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%
Synergistales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.17%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.22%	0.16%	0.04%	0.00%	0.00%
Unclassified Mollicutes	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%	0.00%	0.00%	0.00%	0.10%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Acholeplasmatales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Anaeroplasmatales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Mollicutes RF39	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Thermotogales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%
Unclassified Verrucomicrobia	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Verrucomicrobiales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%
Pedosphaerales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Chthoniobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Bacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.02%	0.00%	0.00%

Figure C3 (continued): Abundances for all minor orders (<2%), March samples.

Taxon	October 2014														
	Separator								Storage Tank						
	Well 1	Well 3	Well 4	Well 5	Well 10	Well 12	Well 13	Well 15	Well 2	Well 5	Well 6	Well 8	Well 11	Well 16	Well 17
Corynebacterium	3.13%	5.41%	6.09%	5.07%	3.85%	0.00%	5.44%	1.47%	15.06%	15.29%	0.00%	8.55%	0.00%	8.00%	25.73%
Unclassified Micrococcaceae	0.00%	0.15%	0.00%	0.00%	7.69%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Micrococcus	3.13%	0.31%	0.50%	0.46%	0.00%	0.00%	0.25%	0.29%	2.11%	2.41%	0.00%	2.56%	0.00%	0.00%	1.75%
Rothia	0.00%	0.00%	0.00%	0.46%	3.85%	0.00%	0.16%	0.29%	0.90%	0.20%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Bacteroidales	0.00%	1.55%	1.49%	2.76%	0.00%	0.00%	3.62%	0.88%	0.90%	0.60%	0.00%	1.99%	0.00%	0.00%	0.00%
Bacteroides	0.00%	0.31%	0.12%	0.00%	0.00%	0.00%	0.08%	0.59%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Marinilabiaceae	0.00%	0.00%	2.49%	0.00%	0.00%	2.13%	2.88%	0.59%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Salegentibacter	0.00%	0.31%	0.62%	1.38%	0.00%	2.13%	0.08%	0.29%	0.90%	0.20%	0.00%	0.28%	0.00%	0.00%	0.00%
Unclassified Bacillales	6.25%	0.00%	0.00%	0.00%	3.85%	4.26%	0.00%	0.00%	0.00%	0.00%	5.45%	0.00%	4.76%	20.00%	0.00%
Unclassified Bacillaceae	21.88%	0.00%	0.12%	0.00%	0.00%	12.77%	0.00%	0.00%	0.00%	0.00%	16.36%	0.00%	33.33%	4.00%	0.00%
Bacillus	0.00%	0.00%	0.00%	0.00%	7.69%	4.26%	0.00%	0.29%	0.30%	0.00%	1.82%	0.00%	0.00%	0.00%	0.00%
Lysinibacillus	0.00%	0.00%	0.00%	0.00%	0.00%	2.13%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Staphylococcus	0.00%	0.31%	0.12%	0.92%	0.00%	0.00%	0.49%	1.47%	0.90%	2.01%	0.00%	2.28%	14.29%	0.00%	2.92%
Lactobacillus	0.00%	0.31%	0.12%	0.00%	0.00%	0.00%	0.08%	0.00%	0.60%	0.80%	0.00%	0.28%	9.52%	0.00%	2.92%
Lactococcus	0.00%	0.77%	0.75%	0.46%	0.00%	0.00%	0.16%	0.59%	7.23%	12.88%	1.82%	9.40%	0.00%	0.00%	0.00%
Streptococcus	0.00%	1.55%	0.37%	0.92%	0.00%	4.26%	0.41%	0.88%	1.20%	1.61%	0.00%	0.57%	0.00%	0.00%	2.92%
Unclassified Clostridiales	0.00%	0.31%	0.62%	0.46%	0.00%	0.00%	0.08%	0.88%	0.00%	0.00%	0.00%	0.57%	0.00%	0.00%	0.58%
Clostridiisalibacter	0.00%	0.00%	1.49%	0.00%	0.00%	0.00%	2.14%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Clostridium	0.00%	0.00%	0.00%	0.00%	0.00%	2.13%	0.00%	0.00%	0.30%	0.00%	0.00%	0.00%	9.52%	0.00%	0.00%
Unclassified Lachnospiraceae	0.00%	0.00%	0.12%	0.00%	0.00%	0.00%	0.25%	0.00%	0.00%	0.40%	0.00%	0.00%	0.00%	0.00%	1.17%
Unclassified Peptostreptococcaceae	0.00%	0.93%	0.25%	0.00%	0.00%	0.00%	0.00%	1.47%	0.00%	0.00%	1.82%	0.00%	0.00%	0.00%	0.58%
Sporomusa	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.25%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Peptoniphilus	0.00%	1.24%	0.00%	0.46%	0.00%	0.00%	0.00%	0.88%	0.30%	0.40%	1.82%	4.84%	0.00%	0.00%	0.00%
Unclassified Halanaerobiaceae	18.75%	21.79%	30.60%	23.50%	30.77%	10.64%	24.14%	44.12%	24.10%	18.51%	16.36%	26.50%	4.76%	36.00%	33.92%
Halanaerobium	6.25%	0.62%	1.49%	0.00%	19.23%	0.00%	0.74%	0.29%	0.60%	0.40%	3.64%	1.14%	4.76%	0.00%	0.58%
Halanaerobacter	0.00%	0.00%	0.00%	0.00%	0.00%	6.38%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Propionigenium	0.00%	0.00%	0.00%	2.76%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Thalassospira	3.13%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Hyphomonas	0.00%	0.00%	0.12%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.82%	0.00%	0.00%	0.00%	0.58%
Unclassified Rhodobacteraceae	0.00%	1.70%	2.11%	2.30%	0.00%	0.00%	1.48%	2.94%	3.61%	4.63%	5.45%	0.57%	0.00%	0.00%	0.00%
Loktanella	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.29%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Sphingomonadales	0.00%	0.77%	2.49%	2.76%	0.00%	0.00%	0.33%	2.35%	2.71%	3.42%	0.00%	2.85%	0.00%	0.00%	0.00%
Unclassified Erythrobacteraceae	0.00%	0.62%	0.00%	0.00%	0.00%	2.13%	0.00%	0.00%	0.00%	0.00%	1.82%	0.00%	0.00%	0.00%	0.00%
Erythrobacter	0.00%	0.00%	0.00%	0.00%	0.00%	6.38%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Methylophilales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	4.76%	0.00%	0.00%
Unclassified Neisseriaceae	0.00%	0.00%	0.00%	0.46%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Neisseria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	4.76%	0.00%	0.00%
Unclassified Rhodocyclaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.82%	1.42%	0.00%	0.00%	0.00%
Desulfomicrobium	0.00%	0.31%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Desulfuromonadaceae	0.00%	0.00%	0.12%	0.00%	0.00%	0.00%	0.66%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.58%
Desulfuromonas	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Arcobacter	15.63%	5.87%	20.40%	9.68%	0.00%	2.13%	11.12%	6.18%	3.92%	11.07%	1.82%	8.83%	4.76%	0.00%	2.92%
Campylobacter	0.00%	0.15%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.40%	3.64%	0.00%	0.00%	0.00%	0.00%
Marinobacter	0.00%	0.31%	0.50%	0.46%	3.85%	6.38%	0.99%	0.59%	0.30%	0.00%	1.82%	0.85%	0.00%	0.00%	0.00%
Idiomarina	0.00%	0.62%	0.37%	0.46%	0.00%	2.13%	2.80%	0.00%	0.00%	0.40%	0.00%	0.00%	0.00%	0.00%	0.00%
Shewanella	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Alcanivorax	0.00%	0.00%	0.37%	0.00%	0.00%	2.13%	2.31%	0.29%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Halomonadaceae	3.13%	35.24%	1.49%	0.46%	3.85%	2.13%	0.91%	0.88%	0.60%	0.60%	1.82%	0.85%	0.00%	0.00%	1.75%
Halomonas	0.00%	0.77%	1.62%	0.00%	0.00%	0.00%	13.10%	1.76%	1.20%	0.00%	1.82%	3.99%	0.00%	0.00%	0.00%
Marinobacterium	6.25%	0.00%	1.00%	0.46%	0.00%	4.26%	9.47%	1.47%	2.11%	0.00%	1.82%	1.14%	0.00%	0.00%	0.00%
Oleibacter	0.00%	0.77%	0.75%	0.00%	0.00%	0.00%	0.08%	0.59%	0.60%	0.40%	7.27%	0.00%	0.00%	0.00%	0.00%
Unclassified Moraxellaceae	3.13%	0.77%	0.87%	0.46%	0.00%	0.00%	0.16%	1.18%	0.30%	1.01%	0.00%	0.00%	0.00%	0.00%	0.00%
Acinetobacter	3.13%	0.62%	0.50%	1.84%	7.69%	8.51%	0.08%	0.59%	0.00%	0.40%	5.45%	0.28%	0.00%	4.00%	0.00%
Enydrobacter	0.00%	3.40%	1.37%	7.83%	0.00%	0.00%	0.41%	1.18%	13.25%	0.80%	0.00%	0.28%	0.00%	0.00%	0.58%
Psychrobacter	0.00%	0.62%	1.00%	0.92%	3.85%	2.13%	0.00%	2.35%	0.00%	1.21%	3.64%	0.00%	0.00%	8.00%	0.00%
Unclassified Pseudomonadaceae	0.00%	0.00%	0.00%	0.46%	0.00%	2.13%	0.00%	0.88%	0.00%	0.40%	0.00%	0.00%	0.00%	0.00%	0.00%
Pseudomonas	6.25%	1.39%	1.49%	1.84%	3.85%	2.13%	0.41%	1.76%	3.31%	2.41%	0.00%	4.84%	0.00%	12.00%	0.00%
Unclassified Xanthomonadaceae	0.00%	0.00%	0.25%	0.92%	0.00%	0.00%	0.00%	0.29%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	2.34%
Stenotrophomonas	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.30%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Minor genera	0.00%	7.42%	11.07%	23.50%	0.00%	6.38%	12.77%	13.24%	9.04%	9.26%	10.91%	14.25%	4.76%	8.00%	12.28%

Figure C4: Genus level taxonomy based on 16S rRNA sequencing, October samples.

Taxon	November 2014																												
	Separator																Tank												
	Well 1	Well 2	Well 3	Well 4	Well 5	Well 7	Well 8	Well 9	Well 10	Well 11	Well 12	Well 13	Well 14	Well 15	Well 16	Well 1	Well 2	Well 3	Well 4	Well 5	Well 8	Well 9	Well 10	Well 11	Well 12	Well 13	Well 14	Well 16	Well 17
Corynebacterium	0.46%	0.84%	0.70%	2.62%	3.87%	0.37%	0.33%	1.12%	0.70%	0.33%	2.76%	2.38%	0.95%	0.47%	0.57%	1.86%	0.93%	1.48%	1.01%	4.40%	0.38%	0.00%	1.92%	0.93%	2.27%	0.51%	0.37%	0.85%	2.94%
Unclassified Micrococcaceae	0.05%	0.02%	0.00%	0.03%	0.07%	0.00%	0.00%	0.00%	0.31%	0.03%	0.00%	0.09%	0.00%	0.03%	0.00%	0.05%	0.04%	0.00%	0.06%	0.22%	0.00%	0.04%	0.17%	0.00%	0.06%	0.00%	0.00%	0.07%	0.00%
Micrococcus	0.10%	0.09%	0.07%	0.08%	0.47%	0.00%	0.26%	0.21%	1.20%	0.08%	0.55%	0.59%	0.24%	0.03%	0.19%	0.28%	0.06%	0.00%	0.19%	0.47%	0.07%	0.27%	0.47%	0.00%	0.25%	0.19%	0.00%	0.11%	0.42%
Rothia	0.02%	0.06%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.02%	0.03%	0.00%	0.00%	0.11%	0.00%	0.15%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Bacteroidales	0.13%	0.00%	1.34%	0.13%	0.00%	0.09%	0.20%	0.14%	0.00%	1.28%	0.00%	0.04%	0.00%	0.00%	0.70%	0.00%	0.00%	0.99%	0.00%	0.00%	0.05%	0.57%	0.00%	0.25%	0.00%	0.64%	2.41%	0.11%	0.00%
Bacteroides	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.21%	0.00%	0.00%	0.00%	0.00%	0.25%	0.00%	0.00%	0.00%	0.00%	0.00%	0.12%	0.45%	0.00%	0.00%	0.00%	0.00%	0.28%	0.70%	0.00%
Unclassified Marinilabiaceae	0.00%	0.00%	0.22%	0.00%	0.00%	0.00%	0.00%	0.07%	0.00%	0.05%	0.00%	0.00%	0.00%	0.05%	0.19%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%
Salegentibacter	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Bacillales	7.23%	27.32%	1.07%	0.85%	21.02%	1.86%	1.96%	0.63%	7.88%	4.20%	1.66%	4.07%	20.57%	24.83%	0.63%	26.96%	26.69%	0.00%	27.27%	24.46%	24.17%	5.00%	23.51%	0.00%	22.43%	1.85%	0.00%	3.14%	1.26%
Unclassified Bacillaceae	14.23%	61.45%	53.22%	26.77%	47.99%	89.03%	84.38%	4.77%	11.55%	9.00%	75.97%	8.69%	50.83%	55.98%	26.32%	60.10%	63.10%	75.37%	59.88%	57.93%	53.86%	11.97%	49.39%	1.26%	49.28%	82.95%	0.93%	8.45%	68.91%
Bacillus	1.74%	5.34%	0.19%	0.74%	4.49%	0.79%	0.20%	1.75%	38.30%	0.92%	0.55%	1.61%	3.31%	4.69%	0.13%	5.08%	4.77%	0.49%	5.63%	5.25%	4.42%	1.21%	4.43%	1.52%	4.23%	0.83%	0.00%	0.48%	0.42%
Lysinibacillus	0.32%	0.17%	0.00%	0.26%	0.18%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.29%	0.00%	0.48%	0.00%	0.18%	0.14%	0.00%	0.19%	0.14%	0.34%	0.00%	0.60%	0.08%	0.21%	0.19%	0.00%	0.26%	0.00%
Staphylococcus	1.14%	0.62%	0.17%	0.69%	2.34%	0.09%	0.72%	0.84%	0.78%	0.23%	1.66%	0.26%	0.95%	0.43%	0.19%	0.69%	0.58%	0.00%	0.70%	1.62%	0.75%	0.72%	2.64%	0.42%	0.90%	0.51%	0.28%	0.52%	1.68%
Lactobacillus	0.16%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.14%	0.00%	0.00%	0.00%	0.00%	0.24%	0.00%	0.13%	0.28%	0.00%	0.00%	0.00%	0.19%	0.02%	0.23%	0.04%	0.08%	0.00%	0.00%	0.00%	0.07%	0.00%
Lactococcus	0.15%	0.00%	0.19%	0.19%	0.04%	0.00%	0.13%	0.00%	0.06%	0.03%	0.00%	0.09%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.04%	0.60%	0.07%	0.04%	0.00%	0.00%	0.33%	0.00%	0.09%	1.33%	0.00%
Streptococcus	0.17%	0.20%	0.17%	0.37%	1.82%	0.05%	0.46%	0.35%	1.29%	0.00%	0.00%	0.04%	0.47%	0.03%	0.06%	0.55%	0.06%	0.00%	0.14%	0.74%	0.09%	0.04%	0.82%	0.08%	0.84%	0.06%	0.00%	0.30%	0.00%
Unclassified Clostridiales	0.00%	0.00%	0.05%	0.08%	0.00%	0.23%	0.00%	0.14%	0.00%	0.10%	0.00%	0.00%	0.00%	0.00%	0.13%	0.00%	0.14%	0.00%	0.00%	0.00%	0.05%	0.34%	0.09%	0.00%	0.03%	0.00%	0.28%	1.07%	0.00%
Clostridiisalibacter	0.13%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.21%	0.00%	0.00%	0.55%	0.29%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	1.34%	0.00%	0.00%	0.00%
Clostridium	1.21%	0.00%	0.00%	0.61%	0.00%	0.00%	0.00%	1.68%	0.00%	2.02%	2.49%	1.50%	1.18%	0.40%	0.00%	0.00%	0.00%	1.48%	0.00%	0.00%	0.00%	1.25%	0.06%	2.02%	0.00%	0.00%	0.19%	0.04%	0.84%
Unclassified Lachnospiraceae	0.00%	0.00%	0.00%	0.05%	0.00%	0.05%	0.07%	0.07%	0.00%	0.87%	0.00%	0.00%	0.00%	0.00%	0.57%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.27%	0.00%	0.00%	0.00%	0.38%	0.56%	0.15%	0.00%
Unclassified Peptostreptococcaceae	6.66%	0.09%	0.49%	12.06%	0.00%	0.19%	0.00%	11.84%	0.00%	0.41%	0.28%	9.44%	0.24%	0.75%	3.23%	0.00%	0.00%	0.00%	0.60%	0.00%	0.00%	0.19%	2.57%	12.65%	0.00%	0.00%	4.82%	0.00%	1.68%
Sporomusa	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.71%	0.00%	0.00%	0.00%	0.00%	0.00%	0.26%	0.00%
Peptoniphilus	0.00%	0.03%	0.19%	0.56%	0.69%	0.00%	0.26%	0.00%	0.00%	0.05%	0.28%	0.00%	0.47%	0.00%	0.19%	0.23%	0.10%	0.99%	0.00%	0.14%	0.00%	0.00%	0.19%	0.00%	0.06%	0.00%	0.00%	0.00%	0.84%
Unclassified Halanaerobiaceae	0.51%	0.02%	28.90%	0.66%	5.26%	1.77%	2.48%	0.14%	26.47%	11.40%	0.83%	2.03%	0.24%	0.98%	14.33%	0.05%	0.01%	5.42%	0.04%	0.03%	2.19%	41.76%	1.55%	0.00%	0.09%	3.96%	17.42%	47.31%	2.10%
Halanaerobium	0.14%	0.03%	3.40%	0.19%	0.36%	0.05%	0.13%	0.00%	3.27%	5.00%	0.28%	0.77%	0.24%	0.00%	0.82%	0.03%	0.00%	0.00%	0.00%	0.00%	0.24%	9.44%	0.84%	0.00%	0.00%	0.13%	1.20%	7.71%	0.00%
Halanaerobacter	0.00%	0.00%	0.00%	0.00%	0.04%	0.42%	0.26%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.24%	0.00%	6.50%	0.00%	0.00%	0.00%	0.00%
Propionigenium	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.72%	0.00%	0.00%	0.79%	0.00%	0.00%	0.00%	0.00%	0.57%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	1.20%	0.04%	0.00%
Thalassospira	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.11%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Hyphomonas	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.85%	0.00%
Unclassified Rhodobacteraceae	0.03%	0.00%	0.00%	0.05%	0.04%	0.00%	0.13%	0.07%	0.00%	1.79%	0.00%	0.02%	0.00%	0.00%	0.63%	0.07%	0.00%	0.00%	0.00%	0.00%	0.38%	0.11%	0.00%	0.00%	0.02%	0.00%	0.65%	1.73%	0.00%
Loktanella	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Sphingomonadales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.21%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.15%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Erythrobacteraceae	0.00%	0.03%	0.02%	0.00%	0.91%	0.00%	0.00%	0.00%	0.00%	0.62%	0.00%	0.00%	0.71%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.87%	0.00%	0.00%	0.00%	0.00%	0.00%	0.15%	0.00%
Erythrobacter	0.00%	0.00%	0.00%	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.44%	0.00%
Unclassified Methylophilales	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.13%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Neisseriaceae	0.04%	0.00%	0.00%	0.00%	0.15%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.06%	0.49%	0.02%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Neisseria	0.05%	0.00%	0.00%	0.29%	0.36%	0.00%	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.02%	0.11%	0.00%	0.00%	0.00%	0.08%	0.02%	0.00%	0.00%	0.00%	0.00%
Unclassified Rhodocyclaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.24%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Desulfomicrobium	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	1.44%	0.83%	0.26%	0.00%	0.00%	0.44%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.83%	0.00%	0.00%
Unclassified Desulfuromonadaceae	0.00%	0.00%	0.00%	0.11%	0.00%	0.00%	0.00%	0.00%	0.00%	0.15%	0.00%	0.00%	0.00%	0.00%	0.19%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.46%	0.00%	0.00%
Desulfuromonas	0.00%	0.00%	0.00%	0.13%	0.00%	0.09%	0.00%	0.00%	0.00%	1.13%	0.00%	0.00%	0.00%	0.00%	0.51%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.11%	0.00%	0.00%
Arcobacter	1.08%	0.12%	0.12%	1.59%	3.03%	0.84%	0.78%	1.61%	4.19%	15.07%	1.10%	7.22%	0.95%	0.75%	14.01%	0.05%	0.27%	0.00%	0.02%	0.00%	0.58%	8.68%	1.86%	1.10%	0.02%	0.45%	21.41%	8.93%	0.00%
Campylobacter	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Marinobacter	0.39%	0.12%	0.12%	0.69%	0.00%	0.00%	0.00%	0.42%	0.00%	0.90%	0.00%	0.51%	0.00%	0.08%	0.76%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.28%	0.55%	0.00%
Idiomarina	1.01%	0.00%	0.00%	0.58%	0.22%	0.05%	0.00%	0.98%	0.00%	0.13%	0.00%	1.54%	0.24%	0.00%	0.25%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.27%	0.00%	1.35%	0.00%	0.13%	0.09%	0.00%	0.00%
Shewanella	0.01%	0.00%	0.00%	0.13%	0.18%	0.14%	0.13%	0.00%	0.00%	1.05%	0.00%	0.20%	0.00%	0.13%	0.95%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.06%	0.00%	0.00%	0.00%	1.39%	0.00%	0.00%
Alcanivorax	0.15%	0.00%	0.00%	0.21%	0.00%	0.00%	0.00%	0.28%	0.00%	0.00%	0.00%	0.44%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Halomonadaceae	0.48%	0.03%	4.25%	1.03%	0.00%	0.05%	0.52%	0.49%	0.00%	2.31%	0.00%	2.09%	2.13%	0.30%	2.66%	0.00%	0.00%	0.00%	0.12%	0.00%	0.12%	1.02%	0.20%	0.42%	0.36%	0.32%	3.52%	1.25%	0.00%
Halomonas	0.27%	0.00%	0.10%	0.42%	0.00%	0.00%	0.00%	0.77%	0.00%	1.26%	0.00%	1.14%	0.24%	0.32%	0.00%	0.00%	0.01%	0.00%	0.12%	0.03%	0.09%	0.72%	0.19%	1.35%	0.09%	0.00%	0.09%	0.92%	0.00%
Marinobacterium	0.15%	0.00%	0.00%	0.16%	0.00%	0.00%	0.00%	0.42%	0.00%	0.74%	0.00%	0.13%	0.00%	0.02%	0.19%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.50%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%
Oleibacter	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	0.18%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.15%	0.00%
Unclassified Moraxellaceae	0.01%	0.00%	0.02%	0.19%	0.00%	0.37%	0.59%	0.07%	0.03%	2.67%	0.00%	1.43%	0.00%	0.27%	2.47%	0.00%	0.00%	0.00%	0.00%	0.03%	0.68%	0.00%	0.02%	0.08%	0.28%	0.19%	3.52%	0.15%	0.00%
Acinetobacter	0.04%	0.00%	0.02%	0.03%	0.07%	0.00%	0.00%	0.07%	0.00%	1.08%	0.55%	0.15%	0.00%	0.00%	1.14%	0.03%	0.03%	0.49%	0.00%	0.16%	0.03%	0.00%	0.00%	0.00%	0.21%	0.26%	0.65%	0.00%	1.26%
Erythrobacter	0.52%	0.00%	0.07%	0.48%	0.00%	0.79%	1.11%	0.00%	0.00%	0.00%	0.00%	0.51%	0.00%	0.00%	9.19%	0.00%	0.00%	0.00%	0.23%	0.44%	0.00%	0.00%	0.11%	0.00%	0.13%	1.72%	16.03%	1.00%	0.00%
Psychrobacter	0.01%	0.00%	0.05%	0.05%	0.15%	0.00%	0.00%	0.00%	0.03%	16.91%	0.00%	1.10%	0.00%	1.70%	0.00%	0.00%	0.01%	0.00%	0.08%	0.00%	1.35%	0.34%	0.71%	0.00%	0.11%	0.19%	0.09%	3.06%	0.00%
Unclassified Pseudomonadaceae	5.18%	0.21%	0.00%	0.08%	0.51%	0.05%	0.00%	0.00%	0.62%	0.87%	0.00%	4.91%	0.95%	0.75%	0.19%	0.18%	0.06%	0.00%	0.04%	0.05%	0.63%	1.67%	0.22%	0.08%	0.62%	0.13%	0.09%	1.66%	1.68%
Pseudomonas	53.07%	1.01%	1.36%	44.10%	0.99%	1.21%	1.05%	67.41%	1.12%	4.82%	7.18%	39.38%	11.11%	4.26%	6.47%	0.90%	0.49%	8.87%	1.01%	0.16%	5.19%	3.49%	0.50%	74.20%	2.11%	1.09%	4.54%	0.52%	11.76%
Unclassified Xanthomonadaceae	0.05%	0.00%	0.00%	0.00%	0.00%	0.14%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.43%	0.00%	0.00%	0.00%	1.25%	0.00%	0.00%	0.00%	0.00%
Stenotrophomonas	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.27%	0.00%	0.00%	0.00%	1.67%	0.00%	0.00%	0.00%	0.00%
Minor genera	2.86%	2.21%	3.47%	2.54%	4.60%	1.30%	3.14%	3.22%	2.07%	9.58%	2.49%	6.54%	3.55%	1.98%	10.59%	2.37%	2.33%	3.94%	2.55%	2.67%	3.25%	7.01%	6.12%	1.85%	5.63%	1.98%	14.92%	5.68%	4.20%

Figure C4 (continued): Genus level taxonomy based on 16S rRNA sequencing, November samples.

Taxon	January 2015													
	Separator								Tank					
	Well 1	Well 3	Well 4	Well 5	Well 6	Well 7	Well 10	Well 11	Well 3	Well 4	Well 5	Well 6	Well 13	Well 14
Corynebacterium	0.00%	5.73%	1.76%	0.97%	2.44%	1.19%	0.37%	0.50%	1.36%	1.11%	0.61%	3.23%	0.21%	1.39%
Unclassified Micrococcaceae	0.00%	0.00%	0.00%	0.19%	0.02%	0.35%	0.03%	0.03%	0.00%	0.21%	0.02%	0.00%	0.00%	0.06%
Micrococcus	0.00%	0.36%	0.23%	0.00%	0.25%	0.91%	0.00%	0.05%	0.09%	0.74%	0.15%	0.37%	0.10%	0.06%
Rothia	0.00%	0.00%	0.14%	0.00%	0.00%	0.00%	0.00%	0.08%	0.09%	0.05%	0.06%	0.07%	0.00%	0.17%
Unclassified Bacteroidales	0.00%	0.00%	0.00%	0.00%	0.00%	0.28%	1.29%	0.97%	1.45%	0.00%	0.00%	0.00%	0.00%	0.00%
Bacteroides	0.00%	0.00%	0.00%	0.00%	0.00%	0.14%	0.29%	0.21%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Marinilabiaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.14%	0.11%	0.13%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Salegentibacter	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Bacillales	2.03%	0.36%	1.62%	26.90%	25.53%	4.88%	0.60%	5.57%	5.98%	19.14%	27.41%	2.57%	12.63%	8.03%
Unclassified Bacillaceae	96.25%	48.75%	78.80%	58.67%	55.37%	12.76%	0.92%	12.27%	10.42%	45.74%	61.17%	77.13%	26.18%	15.30%
Bacillus	0.23%	0.00%	1.49%	6.43%	4.42%	1.05%	0.06%	1.21%	2.08%	5.92%	5.16%	0.51%	2.87%	1.15%
Lysinibacillus	0.00%	0.00%	0.00%	0.19%	0.29%	0.56%	0.00%	0.11%	0.09%	0.21%	0.30%	0.00%	0.21%	0.35%
Staphylococcus	0.00%	2.87%	0.59%	0.97%	0.81%	1.88%	0.03%	0.29%	0.45%	3.44%	0.94%	0.73%	1.33%	1.79%
Lactobacillus	0.00%	0.36%	0.00%	0.58%	0.00%	0.28%	0.00%	0.00%	0.36%	0.05%	0.02%	0.00%	0.00%	0.00%
Lactococcus	0.00%	0.36%	0.00%	0.00%	0.29%	0.07%	0.00%	0.32%	0.18%	0.00%	0.22%	0.00%	0.00%	0.00%
Streptococcus	0.00%	0.72%	0.45%	1.36%	0.99%	0.91%	0.17%	0.08%	0.72%	1.16%	0.30%	0.51%	0.00%	0.00%
Unclassified Clostridiales	0.31%	0.00%	0.32%	1.17%	0.34%	0.21%	0.14%	0.13%	0.18%	0.00%	0.00%	0.00%	0.00%	0.00%
Clostridiisalibacter	0.00%	0.00%	0.00%	0.00%	0.00%	0.14%	0.00%	0.00%	0.00%	0.00%	0.00%	0.07%	0.00%	0.00%
Clostridium	0.08%	1.08%	0.14%	0.00%	0.02%	0.00%	2.29%	2.39%	0.00%	0.11%	0.00%	0.07%	0.31%	0.00%
Unclassified Lachnospiraceae	0.08%	0.00%	0.05%	0.00%	0.36%	0.70%	1.06%	0.58%	1.27%	0.05%	0.04%	0.00%	0.00%	0.00%
Unclassified Peptostreptococcaceae	0.00%	1.08%	0.68%	0.00%	0.00%	0.00%	0.72%	0.53%	0.00%	0.00%	0.04%	0.59%	2.98%	0.52%
Sporomusa	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Peptoniphilus	0.16%	0.36%	0.36%	0.19%	0.00%	0.00%	0.00%	0.03%	0.00%	0.21%	0.20%	0.37%	0.00%	0.00%
Unclassified Halanaerobiaceae	0.08%	0.36%	0.23%	0.39%	0.02%	47.63%	16.36%	13.06%	48.19%	3.60%	0.04%	0.81%	0.00%	39.38%
Halanaerobium	0.00%	0.00%	0.00%	0.00%	0.02%	5.72%	7.42%	5.86%	9.87%	0.32%	0.00%	0.00%	0.21%	6.64%
Halanaerobacter	0.00%	0.00%	0.00%	0.00%	0.00%	0.28%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%
Propionigenium	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.15%	0.55%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%
Thalassospira	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Hyphomonas	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.26%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Rhodobacteraceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.21%	1.95%	1.05%	0.27%	0.05%	0.00%	0.00%	1.13%	1.04%
Loktanella	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Sphingomonadales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.23%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Erythrobacteraceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.70%	0.37%	0.76%	0.09%	0.85%	0.00%	0.07%	0.00%	0.40%
Erythrobacter	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Methylophilales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.36%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Neisseriaceae	0.08%	0.00%	0.00%	0.19%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.07%	0.00%	0.00%
Neisseria	0.00%	0.00%	0.18%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Rhodocyclaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Desulfomicrobium	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.60%	0.68%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Desulfuromonadaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Desulfuromonas	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.60%	0.53%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Arcobacter	0.00%	0.00%	0.27%	0.00%	0.00%	6.56%	18.10%	14.48%	4.26%	0.21%	0.11%	0.37%	1.13%	8.66%
Campylobacter	0.00%	0.00%	0.00%	0.00%	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Marinobacter	0.00%	0.36%	0.00%	0.00%	0.00%	0.14%	0.72%	0.68%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Idiomarina	0.00%	0.36%	0.32%	0.00%	0.00%	0.00%	0.03%	0.03%	0.00%	0.00%	0.00%	0.00%	0.31%	0.00%
Shewanella	0.00%	0.00%	0.00%	0.00%	0.00%	0.07%	1.66%	1.21%	0.09%	0.21%	0.00%	0.00%	0.00%	0.00%
Alcanivorax	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.11%	0.00%	0.00%	0.00%
Unclassified Halomonadaceae	0.00%	0.36%	0.27%	0.00%	0.00%	1.60%	2.69%	2.26%	2.72%	0.26%	0.07%	0.07%	0.00%	0.69%
Halomonas	0.00%	0.00%	0.05%	0.19%	0.00%	0.91%	1.58%	1.00%	1.00%	0.11%	0.02%	0.00%	0.00%	0.12%
Marinobacterium	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.72%	0.53%	0.09%	0.00%	0.00%	0.00%	0.00%	0.00%
Oleibacter	0.00%	0.00%	0.00%	0.00%	0.00%	0.49%	0.06%	0.05%	0.00%	0.00%	0.00%	0.00%	1.54%	0.00%
Unclassified Moraxellaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	2.72%	1.60%	0.18%	0.26%	0.04%	0.07%	0.00%	0.00%
Acinetobacter	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	1.29%	0.68%	0.00%	0.11%	0.09%	0.07%	0.00%	0.12%
Enydrobacter	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.29%	0.00%	0.00%
Psychrobacter	0.00%	2.51%	0.23%	0.19%	0.00%	0.21%	17.59%	13.79%	1.63%	0.00%	0.00%	0.07%	0.00%	0.00%
Unclassified Pseudomonadaceae	0.00%	0.36%	0.05%	0.00%	0.68%	0.14%	0.66%	1.08%	0.18%	1.16%	0.26%	0.00%	4.21%	1.04%
Pseudomonas	0.00%	26.16%	8.37%	0.00%	5.59%	1.53%	4.10%	3.84%	1.72%	7.46%	0.37%	8.36%	41.48%	6.29%
Unclassified Xanthomonadaceae	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%
Stenotrophomonas	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.21%	0.00%	0.00%	0.00%	0.00%
Minor genera	0.63%	7.53%	3.42%	1.36%	2.55%	7.32%	10.08%	10.64%	4.53%	6.93%	2.26%	3.52%	3.18%	6.76%

Figure C4 (continued): Genus level taxonomy based on 16S rRNA sequencing**,** January samples.

Taxon	March 2015																					
	Separator													Tank								
	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Well 7	Well 8	Well 10	Well 11	Well 12	Well 15	Well 17	Well 2	Well 3	Well 4	Well 5	Well 7	Well 8	Well 10	Well 11	Well 12	Well 15
Corynebacterium	0.85%	1.16%	1.30%	8.29%	0.89%	1.35%	2.24%	0.00%	1.93%	0.81%	0.28%	0.75%	0.29%	0.71%	0.23%	1.49%	0.38%	1.25%	0.43%	1.24%	0.46%	0.53%	3.56%
Unclassified Micrococcaceae	0.07%	0.11%	0.00%	0.15%	0.08%	0.00%	0.06%	0.00%	0.17%	0.04%	0.00%	0.00%	0.03%	0.00%	0.00%	0.13%	0.00%	0.07%	0.04%	0.06%	0.00%	0.00%	0.00%
Micrococcus	0.22%	0.02%	0.13%	0.75%	0.10%	0.18%	0.16%	0.00%	0.22%	0.09%	0.28%	0.38%	0.05%	0.05%	0.00%	0.66%	0.08%	0.14%	0.04%	0.15%	0.04%	0.00%	0.74%
Rothia	0.02%	0.06%	0.30%	0.41%	0.00%	0.00%	0.00%	0.00%	0.02%	0.13%	0.00%	0.38%	0.00%	0.05%	0.00%	0.00%	0.04%	0.00%	0.07%	0.04%	0.00%	0.00%	0.15%
Unclassified Bacteroidales	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	1.50%	0.19%	0.00%	0.00%	0.00%	0.00%	0.00%	1.51%	0.00%	0.78%	0.17%	0.00%	0.00%	0.00%
Bacteroides	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.09%	0.00%	0.00%	0.00%	0.00%	0.00%	0.23%	0.33%	0.00%	0.21%	0.00%	0.00%	0.00%	0.00%
Unclassified Marinilabiaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.14%	0.00%	0.04%	0.00%	0.00%
Salegentibacter	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Bacillales	26.47%	26.20%	2.49%	17.37%	28.00%	1.70%	25.92%	1.24%	20.30%	2.69%	0.09%	1.88%	24.36%	20.44%	1.56%	12.69%	0.38%	26.84%	2.21%	20.69%	1.92%	9.14%	0.37%
Unclassified Bacillaceae	59.42%	59.24%	91.66%	40.30%	58.66%	90.98%	59.10%	92.27%	49.17%	5.65%	8.96%	65.41%	53.21%	47.59%	96.12%	25.28%	0.67%	59.31%	5.56%	44.69%	78.97%	20.73%	25.13%
Bacillus	4.75%	4.76%	0.27%	3.65%	5.37%	0.47%	4.83%	0.14%	3.34%	0.47%	1.32%	2.63%	4.30%	4.44%	0.35%	3.64%	0.08%	5.18%	0.46%	3.92%	0.67%	4.00%	0.82%
Lysinibacillus	0.19%	0.15%	0.00%	1.11%	0.12%	0.00%	0.16%	0.00%	0.14%	0.00%	0.28%	0.00%	0.20%	0.52%	0.00%	0.07%	0.00%	0.16%	0.07%	0.15%	0.08%	0.53%	0.07%
Staphylococcus	1.24%	0.66%	0.96%	1.31%	0.68%	0.41%	1.52%	0.14%	1.79%	0.30%	0.38%	0.00%	0.56%	0.71%	0.00%	1.06%	0.13%	1.36%	0.36%	0.81%	0.12%	0.53%	1.33%
Lactobacillus	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.22%	0.00%	0.00%	0.09%	0.09%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.59%
Lactococcus	0.24%	0.07%	0.10%	0.00%	0.00%	0.12%	0.72%	0.00%	0.02%	0.00%	0.00%	0.00%	0.02%	0.12%	0.00%	0.00%	0.13%	0.18%	0.43%	0.02%	0.10%	0.14%	0.00%
Streptococcus	0.12%	0.18%	0.00%	0.54%	0.35%	0.00%	0.37%	0.00%	0.33%	0.00%	0.38%	0.75%	0.03%	0.24%	0.00%	0.13%	0.08%	0.21%	0.18%	0.32%	0.10%	0.00%	0.67%
Unclassified Clostridiales	0.00%	0.00%	0.40%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%	0.13%	0.09%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.25%	0.04%	0.04%	0.00%	0.22%
Clostridiisalibacter	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.38%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	0.15%
Clostridium	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.98%	1.88%	1.32%	0.00%	0.29%	0.00%	0.00%	0.70%	2.05%	0.00%	2.35%	0.41%	0.14%	0.00%	0.89%
Unclassified Lachnospiraceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.81%	0.00%	0.00%	0.00%	0.00%	0.06%	0.13%	1.09%	0.00%	1.21%	0.09%	0.00%	0.00%	0.00%
Unclassified Peptostreptococcaceae	0.00%	0.15%	0.00%	0.10%	0.02%	0.06%	0.01%	0.00%	1.43%	0.51%	13.02%	0.38%	1.31%	0.31%	0.00%	0.60%	0.71%	0.02%	0.64%	2.53%	1.63%	0.00%	5.19%
Sporomusa	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%
Peptoniphilus	0.07%	0.28%	0.00%	0.00%	0.23%	0.18%	0.15%	0.00%	0.31%	0.04%	0.19%	0.38%	0.00%	0.00%	0.06%	0.00%	0.13%	0.72%	0.00%	0.15%	0.00%	0.00%	0.37%
Unclassified Halanaerobiaceae	0.24%	1.40%	0.10%	2.47%	0.00%	0.23%	0.45%	0.00%	2.39%	14.80%	0.09%	0.75%	3.26%	0.24%	0.06%	0.96%	14.06%	0.02%	13.87%	3.19%	0.24%	0.36%	0.89%
Halanaerobium	0.00%	0.28%	0.00%	1.16%	0.00%	0.00%	0.06%	0.00%	0.74%	6.37%	0.00%	0.00%	0.41%	0.00%	0.00%	0.56%	6.61%	0.07%	6.92%	0.75%	0.00%	0.00%	0.00%
Halanaerobacter	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.13%	0.00%	0.02%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.12%	0.00%	0.00%
Propionigenium	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.38%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.84%	0.00%	0.25%	0.02%	0.00%	0.00%	0.00%
Thalassospira	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Hyphomonas	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.29%	0.00%	0.11%	0.00%	0.00%	0.00%	0.00%
Unclassified Rhodobacteraceae	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	2.05%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	2.38%	0.00%	1.28%	0.15%	0.00%	0.00%	0.00%
Loktanella	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%
Unclassified Sphingomonadales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Erythrobacteraceae	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.28%	0.17%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.54%	0.00%	0.53%	0.02%	0.00%	0.00%	0.00%
Erythrobacter	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.04%	0.00%	0.00%	0.00%
Unclassified Methylophilales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Neisseriaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.93%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.22%
Neisseria	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.95%	0.00%	0.28%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%
Unclassified Rhodocyclaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Desulfomicrobium	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.24%	1.15%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.17%	0.00%	1.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Desulfuromonadaceae	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.13%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%
Desulfuromonas	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.68%	0.00%	0.00%	0.00%	0.00%	0.00%	0.17%	0.54%	0.00%	0.68%	0.00%	0.00%	0.00%	0.00%
Arcobacter	0.02%	0.00%	0.00%	0.59%	0.04%	0.00%	0.01%	0.69%	3.17%	17.54%	1.79%	0.00%	0.75%	0.07%	0.00%	1.49%	18.74%	0.05%	14.09%	1.67%	0.38%	0.25%	1.04%
Campylobacter	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.07%
Marinobacter	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.22%	0.94%	0.38%	0.00%	0.00%	0.00%	0.00%	0.50%	0.54%	0.00%	0.64%	0.02%	0.08%	0.00%	0.15%
Idiomarina	0.00%	0.00%	0.00%	0.98%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.57%	0.75%	0.08%	0.02%	0.00%	0.13%	0.04%	0.00%	0.00%	0.08%	0.34%	0.00%	1.11%
Shewanella	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.02%	0.86%	0.00%	0.00%	0.14%	0.02%	0.00%	0.27%	1.00%	0.00%	1.14%	0.24%	0.00%	0.00%	0.00%
Alcanivorax	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.19%	0.00%	0.03%	0.09%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.14%	0.00%
Unclassified Halomonadaceae	0.05%	0.00%	0.00%	0.00%	0.06%	0.12%	0.23%	0.00%	0.02%	2.22%	0.19%	0.38%	0.36%	0.02%	0.00%	1.09%	1.92%	0.09%	2.50%	0.47%	0.18%	0.03%	0.52%
Halomonas	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.12%	0.00%	0.22%	1.28%	0.94%	0.00%	0.45%	0.17%	0.00%	0.20%	1.05%	0.00%	1.39%	0.28%	0.12%	0.11%	0.89%
Marinobacterium	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.26%	0.34%	0.09%	0.00%	0.00%	0.05%	0.00%	0.00%	0.59%	0.00%	0.93%	0.11%	0.00%	0.14%	0.00%
Oleibacter	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.17%	0.00%	0.00%	0.09%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Unclassified Moraxellaceae	0.00%	0.00%	0.07%	0.08%	0.02%	0.00%	0.00%	0.00%	0.00%	2.05%	0.19%	0.38%	0.03%	0.00%	0.00%	0.17%	2.59%	0.00%	2.64%	0.21%	0.04%	0.00%	0.00%
Acinetobacter	0.12%	0.00%	0.03%	0.08%	0.00%	0.06%	0.03%	0.00%	0.00%	1.03%	0.19%	0.38%	0.08%	0.00%	0.00%	0.13%	0.96%	0.02%	1.03%	0.34%	0.00%	0.00%	0.15%
Enydrobacter	1.71%	0.07%	0.23%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.75%	0.00%	0.00%	0.00%	0.00%	0.00%	0.13%	0.05%	0.00%	0.02%	0.00%	0.00%	0.00%
Psychrobacter	0.00%	0.00%	0.00%	7.93%	0.00%	0.23%	0.04%	0.00%	0.84%	15.23%	0.09%	0.00%	2.64%	0.00%	0.00%	2.55%	22.89%	0.14%	19.15%	2.36%	0.00%	0.00%	2.15%
Unclassified Pseudomonadaceae	0.07%	0.26%	0.00%	2.19%	0.12%	0.00%	0.18%	0.00%	0.46%	1.37%	0.19%	0.00%	0.44%	1.86%	0.00%	5.60%	0.67%	0.14%	1.50%	0.86%	0.02%	4.58%	0.07%
Pseudomonas	1.32%	1.85%	0.37%	5.69%	1.70%	1.93%	0.38%	4.01%	3.80%	5.60%	63.96%	21.05%	3.82%	19.29%	0.64%	35.65%	3.56%	1.09%	4.56%	8.14%	12.39%	56.13%	50.33%
Unclassified Xanthomonadaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%	0.06%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.11%	0.00%	0.00%	0.15%
Stenotrophomonas	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.28%	0.00%	0.00%	0.00%
Minor genera	2.73%	3.06%	1.59%	4.84%	3.38%	1.87%	2.87%	1.52%	5.27%	10.18%	1.70%	3.38%	2.60%	2.90%	0.93%	3.68%	10.92%	2.87%	10.27%	5.01%	1.64%	2.65%	2.00%

Figure C4 (continued): Genus level taxonomy based on 16S rRNA sequencing**,** March samples.

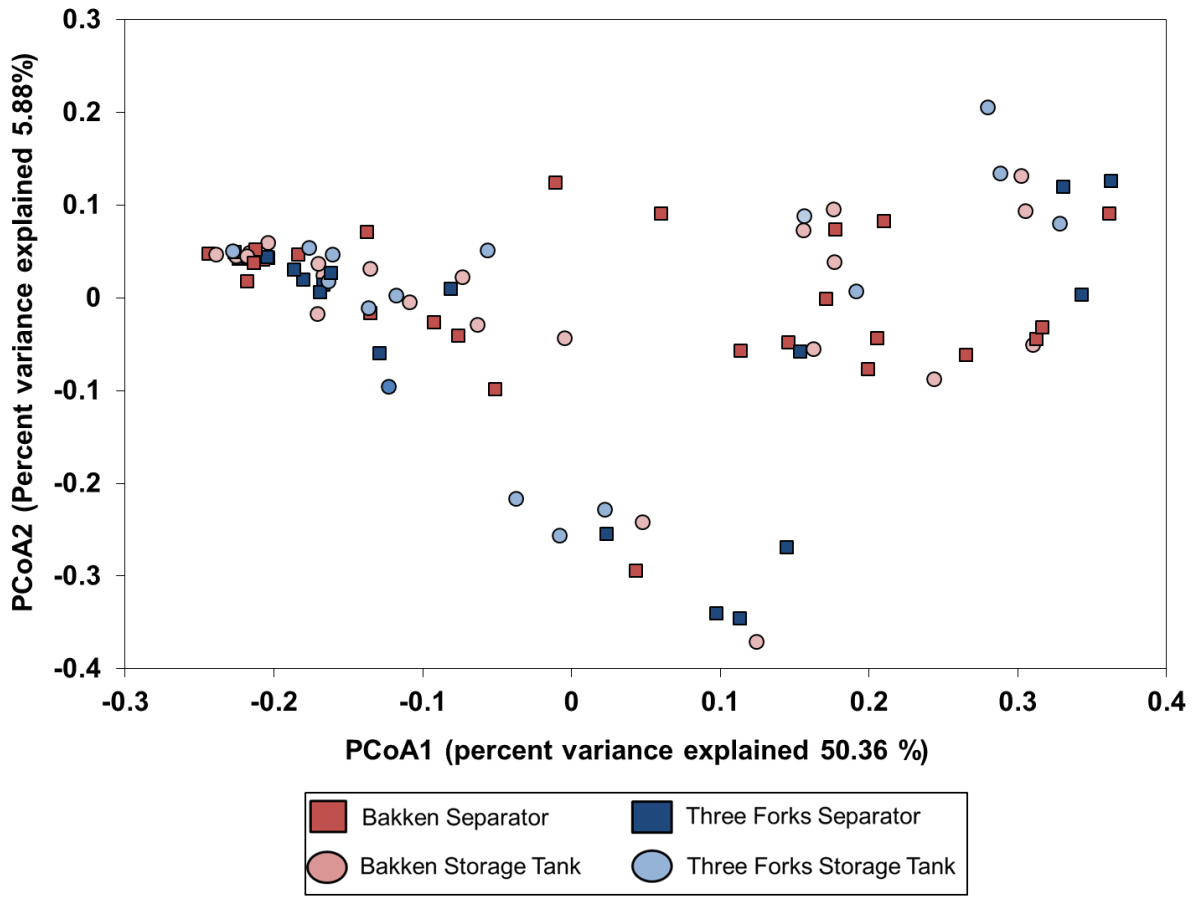

Figure C5: PCoA plot based on weighted UniFrac distances, showing all analyzed samples coded by formation (Bakken red, Three Forks blue) and source (Separator Square, Storage Tank round).

APPENDIX D

CHAPTER 6 - SUPPLEMENTAL INFORMATION

Table D1: *Pseudomonas fluorescens* genes found to be upregulated (at least 2-fold induced, with $P < 0.01$) in four, three, or two replicates.

Locus Tag	Gene	Annotation	Rep1	Rep2	Rep3	Rep4
			Fold upregulated			
		In all Replicates				
PFLU_RS02795	-	hypothetical membrane protein	5.41	6.14	2.52	9.20
PFLU_RS08440	*ohr*	organic hydroperoxide resistance protein	73.50	32.70	4.41	9.54
PFLU_RS14265	*mexE*	MexE family multidrug efflux RND transporter periplasmic adaptor	4.97	8.77	3.40	6.65
PFLU_RS14570	*ahpC*	alkyl hydroperoxide reductase subunit C	3.40	3.10	5.93	11.69
PFLU_RS14575	*ahpF*	alkyl hydroperoxide reductase subunit F	2.05	2.56	2.09	4.11
PFLU_RS17150	-	hypothetical protein	11.26	6.17	3.23	19.55
PFLU_RS25330	-	thioredoxin-disulfide reductase	2.36	2.88	3.28	3.58
PFLU_RS28745	*araC*	AraC family transcriptional regulator	13.57	21.00	2.53	2.04
		In 3 Replicates				
PFLU_RS03250	*copZ*	copper resistance protein copZ	2.43		3.41	3.80
PFLU_RS18105	-	hypothetical protein	13.49	6.68		2.48
PFLU_RS27245	-	translocase, transcription regulator	14.15	5.97	8.84	
PFLU_RS28155	-	thioesterase, DNA ligase B like	2.26		2.70	3.42
		In 2 Replicates				
PFLU_RS03275	*lysR*	LysR family transcriptional regulator	2.57		3.37	
PFLU_RS03890	tRNA	hypothetical protein, probably tRNA	15.47			2.47
PFLU_RS03900	*tRNA*	hypothetical protein, probably tRNA	13.97			2.41
PFLU_RS05735	-	hypothetical protein, leucyl-tRNA synthetase like	2.66	2.32		
PFLU_RS06620	-	Pseudomonas membrane protein	4.07	13.01		
PFLU_RS06855	*arsR*	ArsR family transcriptional regulator			2.17	5.19
PFLU_RS07315	*araJ*	MFS transporter, arabinose efflux permease like		∞		2.4
PFLU_RS08225	*kdpA*	potassium-transporting ATPase subunit KdpA			2.34	2.31
PFLU_RS08265	-	acyl-CoA thioesterase			4.23	3.18
PFLU_RS09260	*tRNA*	hypothetical protein, probably tRNA	2.77		∞	
PFLU_RS09270	*tRNA*	hypothetical protein, probably tRNA		∞		∞
PFLU_RS10265	-	lysine transporter LysE			3.50	4.58
PFLU_RS10505	-	hypothetical protein	2.33		2.60	
PFLU_RS10540	*terC*	TerC like membrane protein	14.26	7.10		
PFLU_RS13120	-	hypothetical protein	8.89	2.43	1.53*	
PFLU_RS13625	*ssuF*	organosulfonate utilization protein SsuF	3.81	2.38		
PFLU_RS14210	*tetR*	TetR family transcriptional regulator	2.97		2.75#	6.08
PFLU_RS17975	-	cysteine dioxygenase	3.31	10.45	1.98*	

Table D1 (continued)

PFLU_RS19180	-	hypothetical protein	7.99		7.80	
PFLU_RS20310	-	50S ribosomal protein	3.21	2.40	1.21*	
PFLU_RS20635	*tRNA*	hypothetical protein, probably tRNA	23.28	17.55		
PFLU_RS20645	-	hypothetical protein	∞	∞		
PFLU_RS21115	-	hypothetical protein	13.99		∞	
PFLU_RS21300	-	isochorismatase	2.20		3.18	
PFLU_RS22250	-	hypothetical protein, probably anti-sigma factor	2.91	5.57		
PFLU_RS24380	-	hypothetical protein	2.90		4.71	
PFLU_RS24860	*iscR*	Fe-S cluster assembly transcriptional regulator IscR		1.87*	2.02	2.36
PFLU_RS25630	*yedY*	sulfoxide reductase catalytic subunit YedY	4.77	3.20		
PFLU_RS27035	*catA*	catalase CatA		1.73	2.15	3.89
PFLU_RS28760	*potAB*	ABC transporter permease	59.60	18.43		
PFLU_RS29925	-	hypothetical protein			3.71	50.18

*upregulated less than 2-fold
[#] P-value > 0.01

Table D2: *Pseudomonas fluorescens* genes found to be downregulated (at least 2-fold induced, with $P < 0.01$) in four, three, or two replicates.

Locus Tag	Gene	Annotation	Rep1	Rep2	Rep3	Rep4
			Fold downregulated			
In all Replicates						
PFLU_RS05700	*bfd*	(2Fe-2S)-binding protein	-2.43	-3.66	-5.46	-2.18
PFLU_RS10595	-	hypothetical protein	-2.22	-2.80	-2.54	-3.61
In 3 Replicates						
ssuB	*ssuB*	aliphatic sulfonates import ATP-binding protein	-2.82		-2.34	-3.10
PFLU_RS08920	*yjcH*	membrane like membrane protein		-3.75	-4.00	-2.69
PFLU_RS00955	*cysW*	sulfate ABC transporter permease subunit CysW		-2.53	-3.81	-3.21
PFLU_RS00960	*cysA*	sulfate ABC transporter ATP-binding protein		-2.13	-2.77	-5.50
PFLU_RS10525	*pbuE*	MFS transporter, PbuE like		-6.51	-2.12	-2.58
PFLU_RS01135	*pbpB*	amino acid ABC transporter substrate-binding protein		-2.24	-2.05	-2.24
PFLU_RS24285	*kgtP*	alpha-ketoglutarate permease		-2.43	-1.97*	-2.24
PFLU_RS29235	-	uncharacterized DNA-binding protein	-1.70*	-2.31	-3.18	-2.01
In 2 Replicates						
actP	*actP*	Cation/acetate symporter ActP			-2.85	-2.10
PFLU_RS01185	-	N5,N10-methylene tetrahydromethanopterin reductase			-2.29	-2.02
PFLU_RS01245	*tauD*	alpha-ketoglutarate-dependent taurine dioxygenase		-2.73	-1.77*	-5.76
PFLU_RS03330	*msdh*	methylmalonate semialdehyde dehydrogenase	-1.19*		-2.92	-2.50
PFLU_RS04590	*aidA*	type V secretory pathway protein		-1.60*	-1.91*	-2.01
PFLU_RS04960	-	amino acid ABC transporter substrate-binding protein			-2.96	-2.03
PFLU_RS06425	*lysM*	peptidoglycan-binding protein		-1.72*	-2.08	-2.49
PFLU_RS06610	-	protein of unknown function			-2.81	-8.17
PFLU_RS07415	-	NAD(P)-dependent oxidoreductase			-3.84	-3.52
PFLU_RS10165	-	acetylornithine deacetylase			-18.00	-6.28
PFLU_RS19350	*arsR*	ArsR family transcriptional regulator	-2.25	-3.49		
PFLU_RS21225	*ydfG*	NADP-dependent 3-hydroxy acid dehydrogenase	-2.56		-5.17	-3.62

Table D2 (continued)

PFLU_RS23355	-	acetyl-coenzyme A synthetase			-3.87	-2.03
PFLU_RS23670	*csbD*	hypothetical protein, CsbD like			-2.56	-2.52
PFLU_RS24655	*ptsA*	PTS N-acetyl-D-glucosamine transporter, PtsA like	-2.15		-1.99*	-2.24
PFLU_RS24285	*kgtP*	ketoglutarate permease KgtP		-2.43	-1.97*	-2.24
PFLU_RS26395		lipoprotein/hydrolase	-2.00		-4.68	
PFLU_RS28830	*ssuD*	alkanesulfonate monooxygenase			-2.54	-4.13

*downregulated less than 2-fold
[#] P-value > 0.01

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