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The Effects of Sample Storage Conditions on the Microbial Community Composition in Hydraulic Fracturing Produced Water

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ABSTRACT

The petroleum industry has an increasing interest in understanding the microbial communities driving biofouling and biocorrosion in reservoirs, wells, and infrastructure. However, sampling of the relevant produced fluids from subsurface environments for microbiological analyses is often challenged by high liquid pressures, workplace regulations, operator liability concerns, and remote sampling locations. These challenges result in infrequent sampling opportunities and the need to store and preserve the collected samples for several days or weeks. Maintaining a representative microbial community structure from produced fluid samples throughout storage and handling is essential for accurate results of downstream microbial analyses. Currently, no sample handling or storage recommendations exist for microbiological analyses of produced fluid samples. We used 16S rRNA gene sequencing to monitor the changes in microbial communities in hypersaline produced water stored at room temperature or at 4 °C for up to 7 days. We also analyzed storage at −80 °C across a 3-week period. The results suggest ideal handling methods would include placing the collected sample on ice as soon as possible, but at least within 24 h, followed by shipping the samples on ice over 2–3 days, and finally, long-term storage in the −20 °C or −80 °C freezer.

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Fracturing fluids; hydraulic fracturing; microbial ecology; microbiology; produced water; sample storage

Introduction

Produced waters are often used to study microbial processes in subsurface environments, such as conventional and unconventional oil and gas fields, CO_2 storage reservoirs, coal seams, and other petroleum-related environments (Dahle et al. 2008; Gulliver, et al. 2014; Strąpoć, et al. 2008). Investigations into the microbial ecology related to hydraulic fracturing operations have particularly raised interest throughout the last few years. High-volume hydraulic fracturing operations typically produce hundreds of thousands to millions of gallons of produced water, hypersaline wastewater, throughout their completion and operation (Clark and Veil 2009; Gregory et al. 2011). 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, often resulting in a unique composition of anaerobic, halophilic, and extremophilic microorganisms of high scientific interest (Clark and Veil 2009; Gregory et al. 2011; Lipus et al. 2017; Struchtemeyer and Elshahed 2012).

Due to a growing interest in produced water biological activity, multiple studies have investigated the microbial ecology of produced water from these types of subsurface environments, in an effort to improve hydraulic fracturing operations, support produced water recycling, and understand mechanisms of microbial biocide resistance (Akob et al. 2015; Akyon et al. 2015; Cluff et al. 2014; Daly et al. 2016; Gregory et al. 2011; Kahrilas et al. 2015; Lipus et al. 2017; Mohan et al. 2014; Murali Mohan et al. 2013; Struchtemeyer et al. 2014; Struchtemeyer and Elshahed 2012; Vikram et al. 2014; et al. 2016; Wuchter et al. 2013). In addition, significant industrial effort is expended to minimize microbial activity during hydraulic fracturing operations, as microbial activity in produced water from hydraulic fracturing has the potential to cause corrosion, fouling, and sulfide release, resulting in production interruptions and environmental consequences (Daly et al. 2016; Gaspar et al. 2014; Gregory et al. 2011; Liang et al. 2016; Struchtemeyer and Elshahed 2012). Historically, microbial activity was determined through the 'bug-bottle' method, which has since been replaced with more accurate methods such as qPCR and 16S rRNA gene sequencing (Cluff et al. 2014;

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Lipus et al. 2017; Murali Mohan et al. 2013; Struchtemeyer and Elshahed 2012). Changes in the microbial composition of samples prior to these analyses may lead to inaccurate, non-representative data resulting in ineffective operational decisions, making appropriate sampling and storage guidelines indispensable. While we are specifically interested in produced water from hydraulic fracturing operations, the sampling of produced water from other operations and environments faces similar challenges.

The effects of storage conditions on microbial communities in other types of environmental samples have previously been investigated (Lee et al. 2007; Lin et al. 2010; Pepper et al. 2014; Petersen and Klug 1994; Rubin et al. 2013). Microorganisms in sediment cores were shown to remain active during storage at a temperature of 4 °C, resulting in altered microbial and geochemical patterns. Similar observations were made for bacteria inhabiting plant tissues, as storage at 4 °C resulted in a decrease of species richness (Mills et al. 2012). In contrast, Lauber et al. (2010) suggested different temperature conditions (between −80 °C and 20 °C) and storage times (up to 14 days) to only have minor effects on fecal, skin, and soil sample microbial communities. These studies provide some insights into the effects of storage on microbial communities in environmental samples. However, these guidelines are often applicable to environmental samples that do not have the same set of logistical challenges in the oil and gas industry, such as high liquid pressures, workplace regulations, operator liability concerns, and remote sampling locations. These methods also do not represent microbial communities in aqueous and hypersaline subsurface samples, that can be expected from produced fluids.

We believe fluid and produced water from hydraulic fracturing processes and other subsurface operations represent a unique environment (hypersaline, high metal, and organic concentrations), which can impact the microbial community structure during sample handling and storage. There are currently no guidelines on how to store or handle produced water samples for microbiological analyses. Produced water samples for microbiological analysis are typically obtained and handled onsite by well staff, due to workplace regulations and liability concerns, before being transferred to researchers for microbiological assessment, and thus are susceptible to inappropriate handling and storage practices. These circumstances, together with the unique chemical composition of hydraulic fracturing produced water, 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 focused on the effect of long term sample storage (>1 week) in the freezer, and the effect of short term storage (<1 week) at room temperature or in the fridge. The first is a commonly accepted method for environmental water samples, but the effects have not been evaluated for produced water. The latter can occur when samples need to be transported long distance (e.g. on ice) or the option to freeze the samples is not available. Produced water sample microbial communities were monitored for 7 days at two different storage temperatures (4 °C and room temperature) using 16S rRNA gene sequencing. Room temperature was utilized to represent conditions when samples could not be immediately placed on ice, while 4 °C was utilized to represent conditions expected in a shipping container full of ice. Furthermore, we evaluated the effect on the microbial ecology during storage at −80 °C for 3 weeks. −80 °C represents conditions expected in a shipping container full of dry ice, or long-term storage in the laboratory freezer. Sequences were taxonomically classified, and alpha- and beta-diversity were calculated to understand the changes in microbial ecology under varying storage times and temperatures. We specifically investigated the effects of storage condition and time on microbial communities in produced water from hydraulic fracturing operations, however, we believe findings from this study may also be applicable to produced waters from similar environments.

Materials and methods

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 (HP1 and HP2). A third sample was taken from a produced water hauling truck (FWT). For each type of produced water, 500 mL were filtered on-site and filters were immediately preserved in TRIZOL (Life Technologies, Carlsbad, CA) for analysis of the original sample ecology (referred to as 'Onsite' samples). Furthermore, 1–2 L of each produced water were sampled in sterile 1 L bottles. These fluids were intended for the storage experiments. All samples were stored on ice during transportation to the laboratory (less than 2 h). Upon arrival in the laboratory produced water samples were immediately frozen at −80 °C for 3 weeks prior to processing.

Temperature and conductivity were assessed on site for FWT and HP1 samples. Temperature and total dissolved solids (TDS) concentration for HP1 and FWT samples were also measured on-site. Sampling circumstances, timing, and access restrictions did not allow on-site temperature and TDS measurements for HP2 samples; however; the air temperature during sampling was ∼1 °C.

Sample processing

Produced water samples were thawed on ice. For each storage experiment condition, 250 ml of produced water sample was transferred to a sterile 1000 mL glass bottle. Each sample was processed in duplicate, for a total of two biological replicates per condition. Sub-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), ∼25 °C, in a closed box on the laboratory workbench.

Chemical analysis

TDS concentrations were determined using a Fisher Scientific Accumet AP75 Conductivity/TDS meter (Thermo Fisher Scientific, Pittsburgh, PA). The pH was measured using a Thermo Fisher Education pH meter (Thermo Fisher Scientific, Pittsburgh, PA). 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). 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. A detailed description of chemical methods can be found in the Supporting Information.

DNA extraction

A 15 mL sample from each treatment and each time point was collected and biomass was harvested through centrifugation at 10,000 rpm. Collected produced water biomass was then digested with 10 µl of 20 mg/mL lysozyme for 30 min at 37 °C followed by DNA extraction using TRIZOL (Life Technologies, Carlsbad, CA), according to manufacturer's instructions. DNA from on-site samples preserved in TRIZOL (Life Technologies, Carlsbad, CA) was also extracted according to the manufacturer's instructions. On-site samples were processed in duplicates. DNA extractions were performed under sterile conditions and control blanks were included.

PCR

DNA was amplified using 16S rRNA gene primers, targeting the V4 region, as described previously (Caporaso et al. 2012). Briefly, PCR samples underwent an initial denaturation step for 3 min at 96 °C. Samples were then run for 40 cycles under the following conditions: Denaturation occurred at 96 °C for 45 s, annealing at 50 °C for 60 s and elongation at 72 °C for 60 s. Final elongation was carried out at 72 °C for 10 min. Following amplification, 16S rRNA gene PCR products were purified using AMPure beads (Beckman Coulter, Pasadena, CA) and run on a 1% agarose gel for cleanup verification. Post clean up DNA concentrations were assessed using Qubit technology (Life Technologies, Carlsbad, CA). PCR amplifications were performed under sterile conditions and PCR controls were included.

Sequencing

Cleaned up 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 min at room temperature and further diluted to 10 pMol library with hybridization buffer HT1 according to the 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). MiSeq sequencing generated a single end read and barcode file.

Computational analyses

16S rRNA gene sequences were analyzed using QIIME version 1.7.0 (Caporaso et al. 2010). Sequences were quality trimmed at a quality score of 20 and demultiplexed. The number of sequences generated for each sample varied between 2506 and 21,147 with a median sequence length of 251.0 base pairs (Table S1). Operational Taxonomic Units (OTU) were picked using the pick_closed_reference_otus.py python script using UCLUST (Abdeljabbar et al. 2013) against the 2013 GreenGenes core set gg_97_otus.fasta reference database with a 97% sequence similarity (Brown et al. 2011). To remove bias introduced through varying number of sequences, 5000 sequences (1000 sequences for HP2 samples) successfully assigned to OTUs were randomly selected for each sample and used for alpha diversity analysis. Average microbial abundance data values were calculated based on OTU data from both replicates. Observed species, Chao1 and Shannon were calculated from OTU tables. Alpha diversity was estimated by the number of OTUs assigned per 1000 sequences for each sample. T-tests were used to assess statistical differences between OTU measurements across the sampling period. The data was prior determined to be normally distributed by plotting the sample data and comparing the histogram to a normal probability curve. Beta diversity was used to develop principal coordinate plots utilizing UniFrac distance metrics (Lozupone and Knight 2005). Weighted UniFrac distance matrixes were calculated and used to compare on-site samples with subsequent experimental samples (Day 0, 1, 2, 3, and 7). We used ANOSIM (analysis of similarity) to evaluate differences in community structure across the 3 week −80 °C storage period. Furthermore, we evaluated correlations between sample storage time and the relative abundance of the major observed taxa across the 7 day storage period. ANOSIM and Spearman calculations were performed using vegan in R (Team R 2014). Sequences for each sample were uploaded to MG-RAST and can be accessed under the accession numbers 4603074.3 (FWT), 4603075.3 (HP1), and 4603076.3 (HP2). Description for sequence FASTA headers can be found in the Supporting Information (Figure S1).

Results

Sampling, geochemical characterization, and sequencing statistics

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 samples (HP2) was taken in December 2013. Temperature and TDS concentration were found to be 23.8 °C and 11.60 mS for HP1 and 19.1 °C and 19.7 mS for

Table 1. Chemical composition of produced water samples used in the study.

Sample	Truck (FWT)	Pond (HP1)	Dec Pond (HP2)
Sampling date	Jun-14	Jun-14	Dec-13
pH	6.53	7.27	7.35
Concentration (mg/L)			
TDS (Total dissolved solids)	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

FWT. Chemical analysis results for all three samples were as expected for produced water samples, characterized by high TDS concentrations (Table 1) and in the range of previously reported data (Cluff et al. 2014; Murali Mohan et al. 2013; Struchtemeyer and Elshahed 2012). The highest TDS concentrations were measured for FWT samples at 52,500 mg/L. HP1 was found to have the lowest TDS concentration at 5300 mg/L. HP2 had a TDS concentration of 18,500 mg/L. DNA sequences generated for each sample varied between 2506 and 21,147 with a median sequence length of 251.0 base pairs (Table S1).

Taxonomy

The taxonomic distribution of all samples is shown in Figure 1, and taxonomic abundances for all samples are listed in detail in Tables S1–S3. We evaluated if sample handling (transport on ice from the sampling site) and storage of samples for 3 weeks at −80 °C affected the community's structure. Results suggested these practices to slightly alter the relative abundance of a few taxa, but to only minorly affect the overall community composition. Most notable changes in relative abundance between on-site and Day 0 samples were a 12% increase of *Campylobacterales* in FWT, a 5% decrease of *Rhodobacterales* in HP1, and a 10% increase of *Sphingomonadales* in HP1 (Figure 1). Statistical analysis using ANOSIM supported these observations for FWT samples ($R = 0.331$ and $P = .15$) and HP2 samples ($R = 0.333$, $P = .09$), but also suggested the change in community structure for HP1 samples to be more significant ($R = 0.968$, $P = .07$). These findings suggest that transport of produced water samples on ice and long term storage at −80 °C (a practice often used for environmental samples in many research efforts) can lead to minor (and sometimes significant) shifts in the relative abundances of individual taxa, but preserves the overall community structure.

In addition, we evaluated the effects of short term storage (up to seven7 days) at room temperature and at refrigerator conditions (4 °C). The microbial community structure of FWT samples remained constant during the first 3 days but changed at room temperature after 7 days (Figure 1A). Samples were initially dominated by bacteria of the order *Campylobacterales*, with a relative abundance of 78% at Day 0. A shift in the microbial community profile was detected under room temperature conditions after Day 3. The relative abundance of *Campylobacterales* decreased to 66% by Day 3 and to 2% by Day 7 (a decrease of 64% across a 4 day period), while the relative abundance of *Alteromonadales* increased to nearly 80%. No major changes were observed for the FWT samples stored at 4 °C, in which the community structure remained stable throughout the 7-day period (Figure 1A). For FWT room temperature samples we identified a negative correlation between storage time and *Campylobacterales* abundance ($R = -0.714$ and $P = .02$) and a positive correlation between storage time and *Alteromonadales* abundance ($R = 0.713$ and $P = .01$) using Spearman rank coefficient analysis (Table S4).

Storage results for the HP1 produced water samples demonstrated that communities that were stored at room temperature and 4 °C remained relatively stable over time, however a slight shift in community structure was observed for the RT samples (Figure 1B). Day 0 samples were dominated by the orders *Rhodobacterales* (31% relative abundance), *Sphingomonadales* (40%), *Oceanospirillales* (13%) and *Pseudomonadales* (6%). Bacterial community structure remained relatively constant for the first two days in both the RT and 4 °C samples. Through days 3 and 7, the fractions of *Sphingomonadales* (to 18% relative abundance) and *Oceanospirillales* (to 11%) bacteria decreased slightly, while bacteria of the order *Pseudomonadales* increased (up to 28% relative abundance) in the samples stored at room temperature. Only minor changes were observed in the samples stored at 4 °C up to 7 days. No significant correlations between the relative abundance of *Rhodobacterales*, *Pseudomonadales*, or *Oceanospirillales* and storage time were detected under either room temperature or 4 °C conditions. However; *Sphingomonadales* abundance was inversely correlated with storage time ($R = 0.911$, $P = .01$), supporting the observed decrease in *Sphingomonadales* throughout the experiment (Table S4).

The greatest effects of storage time and conditions on microbial communities in produced water were observed for the HP2 samples taken in December 2013. On-site sample community structure was found to be dominated by *Campylobacterales* (62% relative abundance), but to have higher abundances of *Bacteroidales*, *Desulfovibrionales*, and *Desulfuromonadales* than post freezing day 0 samples. Storage results suggested that the microbial community shifted quickly at room temperature while the shift was more gradual at 4 °C (Figure 1C). Day 0 samples were dominated by *Campylobacterales* (~75%). After 24 h at room temperature *Campylobacterales* relative abundance decreased to 17% relative abundance, while *Pseudomonadales* (45%) and *Alteromonadales* (13%) frequencies increased (Figure 1C). By Day 7 *Campylobacterales* relative abundance had decreased to 11%, while *Pseudomonadales* (up to 54%) and *Alteromonadales* (up to 40%) had become even more abundant (Figure 1C). In the samples stored at 4 °C, no changes in microbial community structure were detected within the first 3 days. Day 7 results revealed *Pseudomonadales* to be the dominant order (49% relative abundance), while

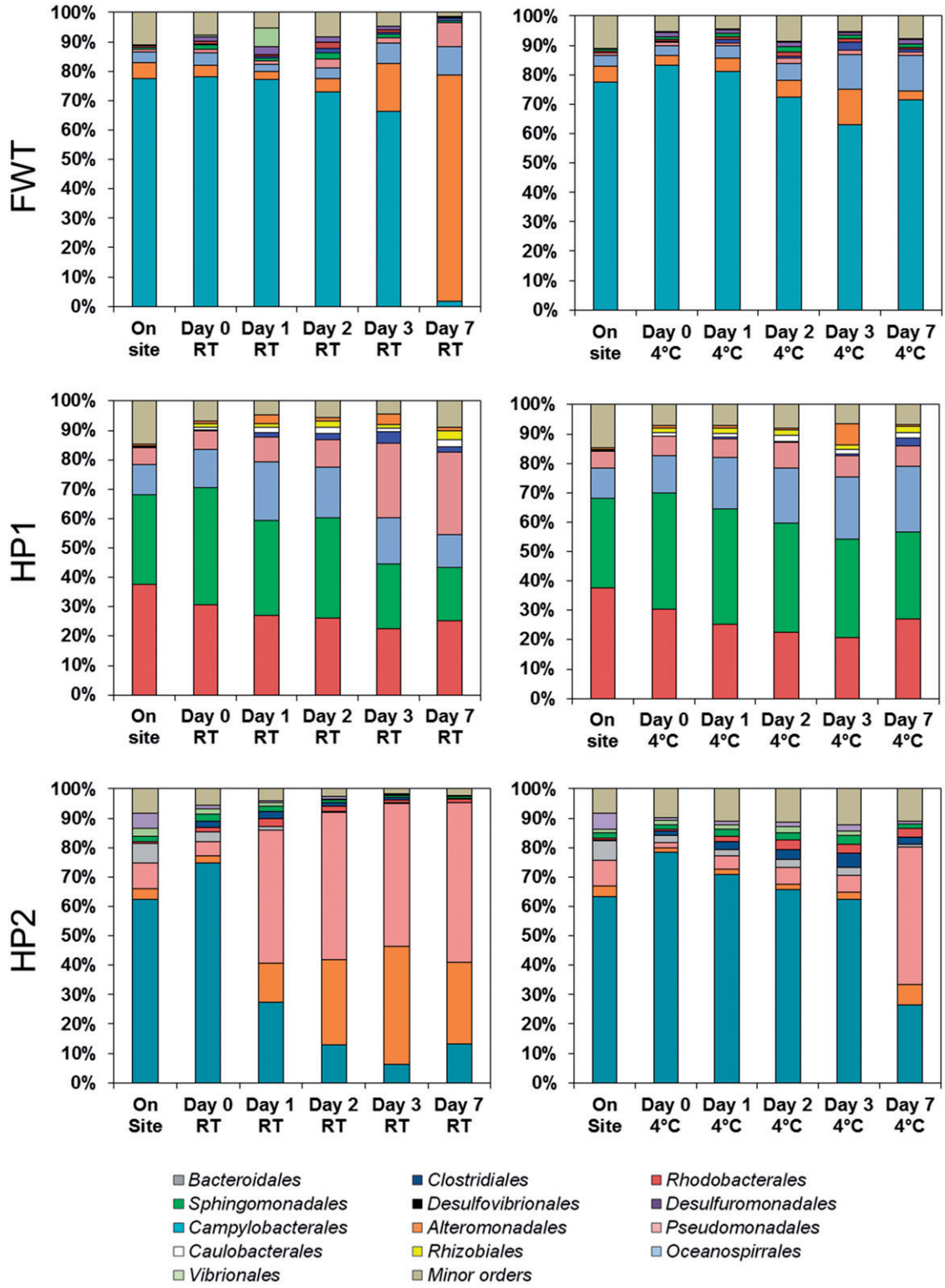

Figure 1. Microbial community structure in hydraulic fracturing produced water samples from a hauling truck (FWT), and two holding ponds (HP1 and HP2), preserved on-site and stored at room temperature or 4 °C over a time period of 7 days. On-site samples were stored at −80 °C for 3 weeks prior to storage experiment.

Campylobacterales relative abundance was found to have decreased to 28% (Figure 1C). For both room temperature and 4 °C conditions, our observations were supported by Spearman rank coefficient correlation analysis. We identified significant positive correlations between storage time and the relative abundance of *Pseudomonadales* ($R = 0.911$, $P < .01$) and *Alteromonadales* ($R = 0.788$, $P < .01$) under room temperature conditions. We also identified a negative correlation between storage time and relative *Campylobacterales* abundance under room temperature ($R = -0.881$, $P < 0.01$) and 4 °C conditions ($R = -0.812$, $P < .01$).

We also investigated to what extent the storage conditions, specifically temperature, resulted in the emergence of specific taxa. Genus level taxonomy analysis suggested the genera of *Marinobacter*, and *Shewanella* to increase in relative abundance in the room temperature samples (Figure S2A, Figure S2C). The genus *Arcobacter* was found to decrease in relative abundance under room temperature conditions in FWT and HP2 samples (Figure S2A, Figure S2C). We did not observe any effect of temperature of known thermophiles such as the genus *Thermoanaerobacter*; however relative abundances for this genus were generally low. Genus level taxonomy for all three samples and temperature conditions is summarized in Figure S2A–C.

Alpha diversity

Operational taxonomic units (OTUs) were utilized to determine the change in a number of microbial species of the samples. The number of OTUs assigned per 1000 sequences varied between samples (Table S5). The OTUs varied from as low as 56 OTUs (HP2 room temperature) to as high as 156 OTUs (HP2 4 °C). Results demonstrated the observed number of OTUs did not change under 4 °C conditions throughout the 7-day storage period but decreased under room temperature conditions throughout the 7-day storage period for FWT and HP2 samples (Table S5). Statistical analysis revealed the number of OTUs in FWT and HP2 room temperature samples to be significantly different at Day 7 compared to Day 0 (t-test, $P < .05$). No statistical differences in the number of OTUs were observed across the 7-day sampling period for HP1 room temperatures samples. The same observations were made for HP1 4 °C samples, HP2 4 °C samples, and FWT 4 °C samples.

The Chao1 and Shannon diversity measurements were used to assess species richness and evenness (Hill et al. 2003; Magurran 2004). Results for both approaches suggest diversity within samples to remain more stable at 4 °C than room temperature. Chao1 estimates richness by taking into account the abundance of each sequence belonging to a certain phylogeny in a sample and thus corrects for rare OTUs (Hill et al. 2003). Chao1 values were found to decrease under room temperature conditions over time, suggesting population richness declined (Table S5). Chao1 values were found to be significantly different in HP1 room temperature Day 7 samples when compared to on-site and Day 0 samples (*t*-test, $P < .05$). Chao1 values at 4 °C conditions suggested population richness to remain stable throughout storage, with the exception of the HP2 samples, for which population richness was found to increase within the first 2 days and then decrease until Day 7 (Table S5). The Shannon index was used to determine population evenness; microbial diversity and evenness were found to be the 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 (Table S5), suggesting microbial community diversity was affected throughout the storage period.

Beta diversity

Weighted UniFrac principal coordinate analyses (Figure 2) demonstrated that samples tend to cluster based on source (e.g. FWT, HP1, HP2), rather than by time or storage

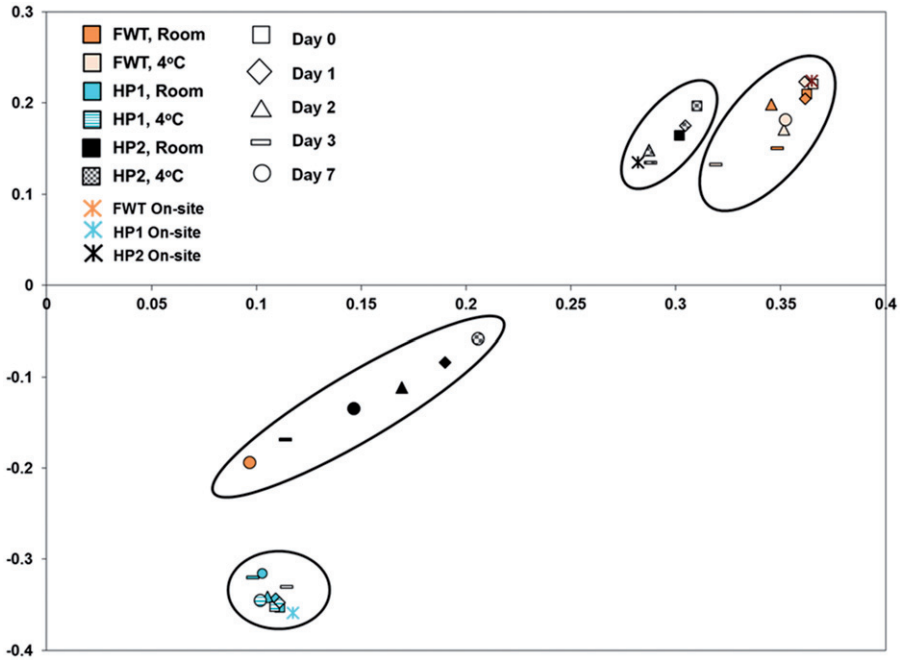

Figure 2. PCoA plot based on weighted UniFrac distances for microbial communities in three different types of hydraulic fracturing produced water preserved on-site and stored at two different storage conditions over a 7 day period. The three different colors represent the different produced water types, sampled from different locations. The different shapes represent the sampling time points, as depicted in the figure legend. Samples clustering closely together are very similar in community structure, while samples clustering apart from each other differ in community structure.

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 the other FWT samples and were found to group more closely with HP2 samples (Figure 2). Similarly, the HP2 room temperature Day 0 sample was found to be an outlier and cluster separately from the other HP2 room temperature samples (Figure 2). The HP2 4 °C Day 7 sample was found to cluster with the HP2 room temperature Day1, Day 2, Day 3, and Day 7 samples. Average weighted UniFrac distances were found to be the 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) (Figure S3). Weighted UniFrac distances relating HP2 samples stored at room temperature were greater than UniFrac distances obtained for samples under all other conditions (Figure S3). Although taxonomic and alpha diversity analyses demonstrate change according to the storage procedure, beta diversity analysis suggests the microbial communities in most samples to remain similar to the original samples across the storage period. Exceptions were the Day 7 FWT samples stored at room temperature, and the HP2 samples stored at room temperature for more than 24 h.

Discussion

Technical challenges with sample handling and transport of production water may lead to a considerable delay between sample collection and sample processes. Recommendations regarding the minimal handing requirements to maintain a relevant microbial ecology currently do not exist. To address this knowledge gap, we monitored the microbial ecology of produced water from three different sources (two water holding ponds and one truck sample), at two temperatures (room temperature and 4 °C) through a 7-day period (Days 0, 1, 2, 3, and 7). Furthermore, we evaluated how sample transport (on ice) and subsequent long-term storage (at −80 °C) may affect the microbial community structure in produced water.

We found that for all three produced water samples, microbial taxonomy observed in produced water samples stored at 4 °C on Day 3 was considered representative of the Day 0 community structure based upon relative abundances of major taxa and beta-diversity analyses. This suggests transport on ice over a three-day period should still result in a representative microbial community. 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 h after storage. These results were also confirmed through both alpha- and beta-diversity analyses. This suggests the placement of samples on ice as soon as possible to be ideal handling methods, but at least within 24 h. Our study also suggested produced water storage at −80 °C for 3 weeks can result in minor shifts in microbial community structure, however statistical analysis using ANOSIM suggested the change to be insignificant (all $P > .05$). In addition, our results also demonstrated that the unique chemical possession may play a role in how susceptible microbial communities in produced water are to changes. We observed significant changes in produced water samples, which were characterized by higher salinities (FWT, HP2), but did not observe these shifts in the produced water samples that were characterized by lower TDS concentrations. Thus, geochemistry factors should be taken into account when transporting and storing produced water samples.

We also specifically investigated if any potentially thermophilic taxa emerged under the tested conditions. Results suggested the genera *Marinobacter* and *Shewanella* to increase and *Arcobacter* to decrease under room temperature conditions. However, none of these organisms is considered specifically thermophilic and known to be selected by high-temperature environments; on the contrary, members of the genus *Marinobacter* have been described to survive in both very low as well as high-temperature environments (Gauthier et al. 1992; Kim et al. 2012). We also did not observe a disappearance of specifically thermophilic organisms throughout storage. The genus *Shewanella* typically grows at temperatures around 20 °C; however, it has also been shown to sustain growth under low-temperature conditions (Abboud et al. 2005). While certain *Arcobacter* species are known to live under colder conditions (Donachie et al. 2005), members of this genus are generally known to grow under a wide range of temperatures (Van Driessche and Houf 2008).

These findings are consistent with previous storage condition studies for environmental samples (Mills et al. 2012; Pepper et al. 2014; Rubin et al. 2013; Struchtemeyer et al. 2014). 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 (Mills et al. 2012). 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 (Lee et al. 2007; Stenberg et al. 1998). However, our results also show that guidelines proposed by Lauber et al. (2010), which suggest soil and human-associated samples stored at room temperature may be stored for up to 2 weeks at room temperature, cannot be applied to the unique produced water environment. Similar to our findings, Lauber et al. (2010) also highlight the impact environmental factors have on microbial community structure and their role in making samples more or less susceptible to changes in microbial composition.

This study extends the current state of knowledge on storage of environmental samples by investigating a saline environment and including an on-site sample. 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 (Cluff et al. 2014; Murali Mohan et al. 2013; Struchtemeyer et al. 2014). The microbial community structures observed in our study was similar (high relative abundance of halophilic taxa, such as *Marinobacter*, and member of the *Pseudomonadales* and *Campylobacterales*) to that previously observed in hydraulic

fracturing produced water (Cluff et al. 2014; Lipus et al. 2017; Struchtemeyer and Elshahed 2012), and overall characterized by halophilic aerobic and anaerobic microorganisms. This suggests that the microbial ecology of our samples was representative of that in other produced waters and that our findings are likely relevant to other studies evaluating microbial the community structures of hydraulic fracturing produced water. 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 various locations. Our results showed that storage of produced water at room temperature, and after a certain period of time at 4 °C, can degrade the original microbial community structure and suggests the implementation of a protocol for produced water handling that includes storage at 4 °C and processing within 3 days of sampling.

Nevertheless, it is important to point out that the changes in community structure observed in this study are specific to the evaluated samples. Produced water samples from different locations and environments are likely different and a similar experiment would lead to different shifts in the microbial community structure and potentially different outcomes. Results from this study do not necessarily imply that storage at the described conditions leads to significant changes in community structure, but rather that the risk for degraded samples and consequently inaccurate results is higher, should handling and storage under improper circumstance occur.

This study represents the first effort to evaluate the effects of different storage conditions and storage times on the microbial ecology of production waters from oil and gas environments. Our results suggest produced water storage and handling to be important for microbiological analyses. Storing samples at room temperature for 24 h or longer may lead to sample degradation, alter the taxonomic profile, and limit the validity of the downstream analysis. To maintain the original microbial community structure samples should ideally be preserved on-site. If on-site sample processing is not possible, our observations suggest storage of produced water samples intended for microbiological analysis at 4 °C for a short period of time (not more than 3 days) can maintain the original community structure. Long-term storage should occur at −20 °C or ideally, at −80 °C. Comparisons of samples preserved on-site and samples stored for 3 weeks at −80 °C suggested long term freezer storage (−80 °C) likely preserves the overall microbial taxonomy profiles, but can also result in small relative abundance shifts. While these results are based on analysis and observations made with produced waters from hydraulic fracturing operations, we believe this data to be also useful to scientists analyzing produced waters from closely related subsurface environments, such as petroleum reservoirs or coal beds.

Disclosure statement

This report/presentation was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference therein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed therein do not necessarily state or reflect those of the United States Government or any agency thereof.

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References

Abboud R, Popa R, Souza-Egipsy V, Giometti CS, Tollaksen S, Mosher JJ, Findlay RH, Nealson KH. 2005. Low-temperature growth of *Shewanella oneidensis* MR-1. Appl Environ Microbiol 71(2):811–816.

Abdeljabbar H, Cayol J-L, Hania WB, Boudabous A, Sadfi N, Fardeau M-L. 2013. *Halanaerobium sehlinense* sp. nov., an extremely halophilic, fermentative, strictly anaerobic bacterium from sediments of the hypersaline lake Sehline Sebkha. Int J Syst Evol Microbiol 63(6): 2069–2074.

Akob DM, Cozzarelli IM, Dunlap DS, Rowan EL, Lorah MM. 2015. Organic and inorganic composition and microbiology of produced waters from Pennsylvania Shale Gas Wells. Appl Geochem 60: 116–125.

Akyon B, Stachler E, Wei N, Bibby K. 2015. Microbial mats as a biological treatment approach for saline wastewaters: the case of produced water from hydraulic fracturing. Environ Sci Technol 49(10): 6172–6180.

Brown SD, Begemann MB, Mormile MR, Wall JD, Han CS, Goodwin LA, Pitluck S, Land ML, Hauser LJ, Elias DA, et al. 2011. Complete genome sequence of the haloalkaliphilic, hydrogen-producing bacterium Halanaerobium hydrogeniformans. J Bacteriol 193(14): 3682–3683.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI et al. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Meth 7(5):335–336.

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, et al. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. Isme J 6(8):1621–1624.

Clark CE, Veil JA. 2009. Produced Water Volumes and Management Practices in the United States. No. ANL/EVS/R-09-1. Argonne, IL: Argonne National Lab.(ANL). https://www.osti.gov/biblio/1007397

Cluff MA, Hartsock A, MacRae JD, Carter K, Mouser PJ. 2014. Temporal changes in microbial ecology and geochemistry in produced water from hydraulically fractured Marcellus shale gas wells. Environ Sci Technol 48(11):6508–6517.

Dahle H, Garshol F, Madsen M, Birkeland N-K. 2008. Microbial community structure analysis of produced water from a high-temperature North Sea oil-field. Antonie Van Leeuwenhoek 93(1–2):37–49.

Daly RA, Borton MA, Wilkins MJ, Hoyt DW, Kountz DJ, Wolfe RA, Welch SA, Marcus DN, Trexler RV, MacRae JD et al. 2016. Microbial metabolisms in a 2.5-km-deep ecosystem created by hydraulic fracturing in shales. Nature Microbiology 1:16146.

Donachie SP, Bowman JP, On SL, Alam M. 2005. Arcobacter halophilus sp. nov., the first obligate halophile in the genus *Arcobacter*. Int J Syst Evol Microbiol 55(3):1271–1277.

Gaspar J, Mathieu J, Yang Y, Tomson RC, Leyris J, Gregory KB, Alvarez PJ. 2014. Microbial dynamics and control in shale gas production. Environ Sci Technol Lett 1(12):465–473.

Gauthier MJ, Lafay B, Christen R, Fernandez L, Acquaviva M, Bonin P, Bertrand J-C. 1992. *Marinobacter hydrocarbonoclasticus* gen. nov., sp. Nov., a new, extremely halotolerant, hydrocarbon-degrading marine bacterium. Int J Syst Evol Microbiol 42:568–576.

Gregory KB, Vidic RD, Dzombak DA. 2011. Water management challenges associated with the production of shale gas by hydraulic fracturing. Elements 7(3):181–186.

Gulliver DM, Lowry GV, Gregory KB. 2014. CO_2 concentration and pH alters subsurface microbial ecology at reservoir temperature and pressure. RSC Adv 4(34):17443–17453.

Hill TC, Walsh KA, Harris JA, Moffett BF. 2003. Using ecological diversity measures with bacterial communities. FEMS Microbiol Ecol 43(1):1–11.

Kahrilas GA, Blotevogel J, Stewart P, Borch T. 2015. Biocides in hydraulic fracturing fluids: a critical review of their usage, mobility, degradation, and toxicity. Environ Sci Technol 49(1):16–32.

Kim SJ, Shin SC, Hong SG, Lee YM, Choi I-G, Park H. 2012. Genome sequence of a novel member of the genus *Psychrobacter* isolated from Antarctic soil. J Bacteriol 194(9):2403.

Lauber CL, Zhou N, Gordon JI, Knight R, Fierer N. 2010. Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples. FEMS Microbiol Lett 307(1):80–86.

Lee YB, Lorenz N, Dick LK, Dick RP. 2007. Cold storage and pretreatment incubation effects on soil microbial properties. Soil Sci Soc Am J 71(4):1299–1305.

Liang R, Davidova IA, Marks CR, Stamps BW, Harriman BH, Stevenson BS, Duncan KE, Suflita JM. 2016. Metabolic capability of a predominant Halanaerobium sp. in hydraulically fractured gas wells and its implication in pipeline corrosion. Front Microbiol 7:988.

Lin Y-S, Biddle JF, Lipp JS, Orcutt BN, Holler T, Teske A, Hinrichs K-U. 2010. Effect of storage conditions on archaeal and bacterial communities in subsurface marine sediments. Geomicrobiol J 27:261–272.

Lipus D, Vikram A, Ross D, Bain D, Gulliver D, Hammack R, Bibby K. 2017. Predominance and metabolic potential of *Halanaerobium* spp. in produced water from hydraulically fractured Marcellus shale wells. Appl Env Microbiol 83:e02659–e02616.

Lozupone C, Knight R. 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol 71(12):8228–8235.

Magurran AE. 2004. The commonness, and rarity, of species. In: Measuring Biological Diversity. Blackwell Science Ltd., Oxford, UK.

Mills HJ, Kiel Reese B, St Peter C. 2012. Characterization of microbial population shifts during sample storage. Front Microbiol 3:49.

Mohan AM, Bibby KJ, Lipus D, Hammack RW, Gregory KB. 2014. The functional potential of microbial communities in hydraulic fracturing source water and produced water from natural gas extraction characterized by metagenomic sequencing. PLoS One. 9(10):e107682.

Murali Mohan A, Hartsock A, Hammack RW, Vidic RD, Gregory KB. 2013. Microbial communities in flowback water impoundments from hydraulic fracturing for recovery of shale gas. FEMS Microbiol Ecol 86(3):567–580.

Pepper IL, Gerba CP, Maier RM. 2014. Environmental sample collection and processing. In: Environmental Microbiology (Third Edition), Elsevier, p137–155, Oxford, UK.

Petersen SO, Klug MJ. 1994. Effects of sieving, storage, and incubation temperature on the phospholipid fatty acid profile of a soil microbial community. Appl Environ Microbiol 60(7):2421–2430.

Rubin BE, Gibbons SM, Kennedy S, Hampton-Marcell J, Owens S, Gilbert JA. 2013. Investigating the impact of storage conditions on microbial community composition in soil samples. PLoS One 8(7): e70460.

Stenberg B, Johansson M, Pell M, Sjödahl-Svensson K, Stenström J, Torstensson L. 1998. Microbial biomass and activities in soil as affected by frozen and cold storage. Soil Biol Biochem 30(3):393–402.

Strąpoć D, Picardal FW, Turich C, Schaperdoth I, Macalady JL, Lipp JS, Lin Y-S, Ertefai TF, Schubotz F, Hinrichs K-U, et al. 2008. Methane-producing microbial community in a coal bed of the Illinois Basin. Appl Env Microbiol 74(8):2424–2432.

Struchtemeyer CG, Elshahed MS. 2012. Bacterial communities associated with hydraulic fracturing fluids in thermogenic natural gas wells in North Central Texas, USA. FEMS Microbiol Ecol 81(1):13–25.

Struchtemeyer Christopher, Youssef Noha, & Elshahed Mostafa. (2014). Protocols for Investigating the Microbiology of Drilling Fluids, Hydraulic Fracturing Fluids, and Formations in Unconventional Natural Gas Reservoirs. 10.1007/8623_2014_8.

Team R. 2014. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Van Driessche E, Houf K. 2008. Survival capacity in water of Arcobacter species under different temperature conditions. J Appl Microbiol 105(2):443–451.

Vikram A, Lipus D, Bibby K. 2014. Produced water exposure alters bacterial response to biocides. Environ Sci Technol 48(21):13001–13009.

Vikram A, Lipus D, Bibby K. 2016. Metatranscriptome analysis of active microbial communities in produced water samples from the Marcellus Shale. Microb Ecol 72(3):571–581.

Wuchter C, Banning E, Mincer TJ, Drenzek NJ, Coolen MJ. 2013. Microbial diversity and methanogenic activity of Antrim Shale formation waters from recently fractured wells. Front Microbiol 4:367.