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Bacterial Communities Associated with Production Facilities of Two Newly Drilled Thermogenic Natural Gas Wells in the Barnett Shale (Texas, USA)

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Abstract We monitored the bacterial communities in the gas–water separator and water storage tank of two newly drilled natural gas wells in the Barnett Shale in north central Texas, using a 16S rRNA gene pyrosequencing approach over a period of 6 months. Overall, the communities were composed mainly of moderately halophilic and halotolerant members of the phyla Firmicutes and Proteobacteria (classes Beta-, Gamma-, and Epsilonproteobacteria) in both wells at all sampling times and locations. Many of the observed lineages were encountered in prior investigations of microbial communities from various fossil fluid formations and production facilities. In all of the samples, multiple H_2S-producing lineages were encountered; belonging to the sulfate- and sulfur-reducing class Deltaproteobacteria, order Clostridiales, and phylum *Synergistetes*, as well as the thiosulfate-reducing order Halanaerobiales. The bacterial communities from the separator and tank samples bore little resemblance to the bacterial communities in the drilling mud and hydraulic-fracture waters that were used to drill these wells, suggesting the in situ development of the unique bacterial communities in such well components was in response to the prevalent geochemical conditions present. Conversely, comparison of the bacterial communities on temporal and spatial scales suggested the establishment of a core microbial community in each sampled location. The results provide the first overview of bacterial dynamics and colonization patterns in newly drilled, thermogenic natural gas wells and highlights patterns of spatial and temporal variability observed in bacterial communities in natural gas production facilities.

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Introduction

It is well understood that the current consumption rate of crude oil is leading to an economic impasse, and the increased awareness of climate change has spurred interest in more abundant and cleaner alternative fuel sources [1]. Natural gas is viewed as a favorable alternative fuel because of its abundance and relatively low cost and because its combustion results in lower greenhouse gas emissions compared with other fossil fuels [2, 3]. Since 2000, the contribution of natural gas from geological shale to total US natural gas supplies has increased from 1 % to approximately 20 % and is expected to increase to nearly 50 % by 2025 [4, 5].

The Barnett Shale in north central Texas is one of the most important shale gas reservoirs in the United States and accounts for over a third of the total US shale gas production [5]. The gas in the Barnett Shale is completely thermogenic in origin [6]. The gas was formed when formation temperatures exceeded 175 °C, which caused cracking of the kerogen and petroleum that was present in the formation [6]. Due to these extreme conditions, no indigenous bacterial populations are present in the Barnett Shale [4]. Current temperatures of natural gas wells in the Barnett Shale have cooled to around 82 °C, which could theoretically support the growth of microorganisms [7]. However, repopulation of the shale by bacteria was likely prevented by the nanodarcy permeability and extremely small average pore throat size (typically <0.005 µm) of the shale [7, 8].

Although the gas from the Barnett Shale is abiotic in origin, there have been several reports of biogenic sulfide production and biocorrosion in various production facilities (e.g., gas water separator and water-storage tanks) at well sites located throughout the Barnett Shale [9]. There are several possible sources of bacterial populations that could account for such effects including: drilling mud utilized during the well drilling process, waters used during the hydraulic-fracturing processes, as well as secondary microbial development and colonization by above-surface, airborne microorganisms [10–12]. However, in spite of the increased importance of natural gas in the United States, only few studies have examined the microbial ecology of natural gas wells and their production components (gas–water separators and production–water storage tanks) [13]. Furthermore, all previous studies examined the microbial communities at a single time point and at one specific location, which only provides a snapshot of the microbial community and hence does not offer spatiotemporal insights on the stability and dynamics of microbial communities in natural gas production facilities.

In this study, the bacterial communities associated with production facilities at two newly drilled wells were investigated to better understand this little-studied, yet vital system. Natural gas emerges from the well as a gas–water mixture, and the water portion is referred to as production water. Samples of production water were collected at multiple time points over a period of 6 months from two locations at each well. The first location that was sampled was the gas–water separator (hereafter referred to as the separator), which separates the production water from natural gas after it emerges from the wellhead. The second location that was sampled was the production water storage tank, which is used to house production water after natural gas and water separation occurs (hereafter referred to as the tank). The goals of this work were: (1) to identify and document the origins and phylogenetic diversities of bacterial communities that developed in separators and production water storage tanks at these two newly drilled wells, (2) to compare the bacterial communities in the separator samples and production water tank samples (from both wells) to determine if the communities were highly similar to one another, (3) to compare the bacterial community from a single location at various time points to document temporal dynamics associated with natural gas production within a single location, and (4) to identify the presence, nature, and proportion of sulfidogenic lineages in the bacterial communities from these production facilities. We chose to monitor sulfidogenic lineages since previous studies have shown that sulfide is corrosive and can lead to reduced natural gas quality, increased refining costs, and corrosion of pipelines that are used to transport natural gas to refineries [49].

To date, very little is known about the origins of microorganisms in the production equipment from oil and natural gas wells [10]. We chose to monitor these two natural gas wells since they were the subjects of prior investigations in which the microbial communities in the drilling and fracturing fluids were characterized using pyrosequencing [11, 12]. This will allow us to address questions regarding the role of these processes in establishing microbial communities in natural gas production facilities. We also studied the microbial communities in this equipment from the time natural gas production started until several months after production began in order to better understand how the microbial communities in these ecosystems evolve over time and what factors influence the establishment of microbial communities in these ecosystems.

Materials and Methods

Description of Sampling Sites

Production water samples were collected from the separator and tank at two newly drilled natural gas wells located in the Barnett Shale. The two natural gas wells are geographically distinct and located approximately 60 miles from one another in Denton (SM well) and Johnson (AI well) counties in north central Texas. The two wells are similar in that both were drilled and hydro-fractured during the summer of 2009 and started actively producing natural gas in October 2009. Water samples from the gas–water separator, and the produced water storage tank were collected from October 2009 through March 2010. The separator and tank were located on the surface, approximately 20 m from the well-head. The separators were vertical units with an approximate diameter of 0.7 m and 2.5 to 3 m in height. The tanks had a total capacity of approximately 75 m^3. All samples were collected from sampling ports located at the bottom of separators and tanks with sterile Nalgene bottles that were filled to capacity, frozen on dry ice while in transit to the laboratory, and stored at −20 °C upon arrival at the laboratory. The temperature of the water (from the separator and tank) was near ambient environmental temperatures when collected (data not shown). Drilling mud and hydro-fracturing fluid (frac-water) were collected and described in detail elsewhere [11, 12].

Geochemistry of the Production Waters

The geochemical properties of the produced water from the tanks and separators were measured approximately 24 h after returning to the laboratory. Samples were not filtered prior to geochemical analysis. Total dissolved solids (TDS) and pH were measured using the ExStik® II pH/conductivity meter (Extech Instruments Corp., Waltham, MA). Salinity was measured with the VWR Portable Refractometer (VWR International, LLC, West Chester, PA). Alkalinity, ferrous

iron, total iron, sulfate, nitrate, and nitrite were all measured using Hach test kits (Hach Co., Loveland, CO). Samples for sulfide analysis were sampled separately and fixed using zinc acetate immediately on-site to precipitate soluble and most insoluble sulfides (e.g., FeS) as zinc sulfide, and sulfide was subsequently measured using the methylene blue assay [14].

DNA Extraction, 16S rRNA Gene Amplification, and Pyrosequencing

Five hundred milliliters of each water sample was centrifuged for 45 min at 10,000 RPM at 4 °C. The cell pellets were suspended in 200 μL of sterile 1× TE buffer, and DNA was extracted with FastDNA® Spin kit for soil (QBiogene, Carlsbad, CA). The 16S rRNA gene from the production water DNA was amplified using bacterial-specific, barcoded primers with FLX platform adaptors. The forward primer was modified so that it contained the 454 Roche adapter A (GCCTCCCTCGCGCCATCAG) followed by an 8 bp barcode sequence, a two-base linker sequence (CA), and the conserved bacterial primer 338 F [15, 16]. A unique 8 bp barcode sequence was used for each sample (data not shown). The reverse primer was modified so that it contained the 454 Roche adapter B (GCCTTGCCAGCCCGCTCAG) followed by a 2 bp linker (TC) and the conserved bacterial primer 518R [16]. Polymerase chain reaction (PCR) was conducted in a 50-μL volume containing (final concentration) 0.15–0.2 ng/μL template DNA, 1× goTaq PCR buffer (Promega, Madison, WI), 2.5 mM MgSO4, 0.2 mM dNTPs mixture, 0.4 mM each of both the forward and reverse primers, and 2.5 U of goTaq Flexi DNA polymerase (Promega). PCR amplification was conducted using the following cycling conditions: an initial denaturation step for 5 min at 95 °C, 30 cycles of denaturation at 95 °C for 45 s, annealing at 54 °C for 45 s, and elongation at 72 °C for 1.5 min, followed by a final elongation step at 72 °C for 15 min. Positive PCR products were pooled and then purified using a Purelink™ PCR purification kit (Invitrogen, Carlsbad, CA). DNA was sequenced at the Environmental Genomics Core Facility (EnGenCore) at the University of South Carolina, Columbia, SC, using FLX technology.

Sequence Processing

The software package, mothur, was used for processing the obtained pyrosequencing reads [17]. The raw sequences were screened, and low-quality sequences were removed based on: quality (>25 quality score threshold), minimum nucleotide length (>80 bp), maximum homopolymers (>8 bases), and sequences with ambiguous bases (*N*). In addition, sequences without an exact match to the primer sequence were also removed. The remaining high-quality sequences were aligned in mothur using a furthest neighbor algorithm and a Greengenes database template as previously described [18]. An uncorrected pair-wise distance matrix was created from the alignment with mothur, and the distance matrix was then used to assign the sequences into operational taxonomic units (OTUs), using a 97 % sequence similarity cutoff from the furthest neighbor. The OTUs were then classified using the Greengenes classifier program [18, 19]. Chao and ACE species richness indices and Good's coverage were also calculated using mothur [20–22].

Spatial and Temporal Comparisons of Bacterial Populations in Tanks and Separator Samples

We used multiple pair-wise diversity estimates to compare community membership and structure between all the datasets examined in this study. Shared OTUs between all possible pairs of samples were identified by creating a joint distance matrix of all sequences within all datasets in mothur and using this matrix to generate a shared OTUs file. The file was used to conduct pair-wise comparisons between every possible pair of samples using both qualitative similarity indices (those that use presence/absence data, e.g., Sørensen index), as well as quantitative indices (those that take OTUs abundance or relative abundance into consideration, e.g., abundance-based Sørensen index) [23]. Non-metric multidimensional scaling (NMDS) plots for communities using Sørensen similarity indices were created using the NMDS algorithm in mothur.

Three different groups of pair-wise comparisons were conducted. First, we compared the bacterial communities obtained from both tanks and separators at all time points to the bacterial communities in drilling mud formulations used during the well drilling process at each site and to the bacterial communities in water obtained during the hydraulic fracturing process at both well locations [11, 12]. These comparisons were conducted to identify the potential contribution of microorganisms that were introduced during the drilling and hydraulic-fracturing process on the microbial communities that developed in above-ground facilities post-production. Second, temporal comparisons of sequences obtained from a specific location (e.g., AI tank in October vs AI tank in November) were conducted to quantify changes within a specific location over time. Finally, spatial comparisons of sequences obtained at the same sampling time (e.g., AI tank vs AI separator samples in October 2009, or AI tank vs SM tank in October 2009) were conducted to quantify the relatedness of bacterial communities at two distinct locations at the same time within each well.

Nucleotides Sequence Accession Number

Sequences generated in this study have been deposited in GenBank short read archive (SRA) under the accession number SRA050195.1

Results

Sampling and Geochemical Characterization

A total of 17 water samples were collected and analyzed. Six tank samples and four separator samples were collected from the AI well, and five tank samples and two separator samples were collected from the SM well. Results of geochemical analysis from all samples are shown in Table 1. All samples were characterized by a slightly acidic pH. The salinities in the separator and tank samples from the AI well were initially around 8 % but gradually increased over time. The SM tank samples had lower salinity levels compared with tank samples from the AI well. However, as was the case with the AI tank samples, the salinity values in SM tank samples also increased over time. No change in salinity was observed in the two SM separator samples. Nitrate and nitrite levels were below detection limits (50 mg/L for nitrate and 0.5 mg/L for nitrite) in all separators and tank samples examined. Elevated concentrations of total iron and ferrous iron were detected in all of the tank and separator samples. Elevated concentrations of sulfate were detected at every sampling event in the separator and tank samples from the SM well. However, sulfate was only detected sporadically in separator and tank samples from the AI well. Sulfide concentrations were undetectable (detection limit, 0.1 mg/L) in all samples. The slow flow of water from the separators and tanks combined with the introduction of outside air during the sampling process could have prevented long-term accumulation of sulfide in the sampled locations.

Pyrosequencing and Diversity Estimates

A total of 50,209 high-quality sequences were obtained in this study, with an average of ~2,900 sequences per sample, and an average length of 203±66 bp (Table 2). Coverage estimates (Table 2) suggested that the majority of the bacterial community has been encountered. Multiple diversity estimates were used to gauge diversity within and across sites. There was a general trend (with few exceptions) of a gradual decrease in Chao and ACE values over time in AI and SM tanks but not in separator samples (Table 2), suggesting that the prevalent conditions in the tanks are selecting for specific lineages adapted to growth in tanks. Samples from the more saline, sulfate-depleted AI tanks and separators were in general less diverse than the less saline SM tanks and separators (ACE, tanks $p=0.23$; separators $p=0.09$). Finally, samples within the more sedentary tanks were more diverse than the separator samples, except for SM separator samples, which were only collected twice (ACE, AI $p=0.12$; SM $p=0.22$).

Table 1 Geochemical analysis of the tanks and separators for the two studied wells

Sample[a,b]	Date sampled[c]	SO_4	Total iron	Ferrous iron	Salinity	TDS	Alkalinity	pH
AI tank	19 Oct 2009	0	115	8	8 %	10,160	280	6.38
	13 Nov 2009	0	110	14	10 %	12,220	380	6.28
	18 Dec 2009	0	105	55	11 %	10,430	340	6.32
	19 Jan 2010	0	120	50	11 %	8,890	360	6.27
	10 Feb 2010	0	95	22	11 %	ADL[d]	280	6.15
	12 Mar 2010	5	65	50	11 %	ADL	200	6.44
AI separator	19 Oct 2009	20	180	11	8 %	7,880	280	6.53
	13 Nov 2009	0	80	11	10 %	11,520	340	6.23
	10 Feb 2010	0	120	24	10 %	ADL	400	6.13
	12 Mar 2010	3	100	70	11 %	ADL	380	6.12
SM tank	07 Oct 2009	52	25	3	6 %	9,310	360	6.56
	23 Nov 2009	10	35	12	6 %	9,690	300	6.34
	11 Dec 2009	14	65	16	8 %	10,260	340	6.38
	22 Feb 2010	38	15	12	8 %	ADL	160	6.41
	31 Mar 2010	36	23	16	8 %	ADL	180	6.38
SM separator	11 Dec 2009	18	50	16	8 %	10,050	320	6.37
	22 Feb 2010	18	36	10	8 %	ADL	200	6.23

[a] With the exception of salinity (values in percent *w/v*) and pH, all values shown are in milligrams per liter

[b] Sample refers to the origin of each sample. Two wells were sampled (AI and SM) and two sites were sampled per well (storage tank and gas–water separator)

[c] The dates correspond to the month–year when the sample was collected. Sampling points range between 2 (SM separator) and 6 (AI tank)

[d] Above detection limit (ADL) in "[b]"

Table 2 Alpha diversity estimates for all samples studied

Sample	Date sampled	No. of sequences	OTUs	Chao	ACE	Coverage
AI tank	19 Oct 2009	1,273	183	619	740	92 %
	13 Nov 2009	3,779	287	615	906	96 %
	18 Dec 2009	887	126	243	439	92 %
	19 Jan 2010	2,082	227	473	734	94 %
	10 Feb 2010	1,730	197	411	699	94 %
	12 Mar 2010	4,496	252	417	581	98 %
AI separator	19 Oct 2009	1,193	128	335	433	94 %
	13 Nov 2009	2,595	248	382	395	96 %
	10 Feb 2010	5,466	310	562	734	97 %
	12 Mar 2010	7,399	163	334	465	99 %
SM tank	07 Oct 2009	1,154	270	580	1045	86 %
	23 Nov 2009	1,697	202	349	564	94 %
	11 Dec 2009	3,729	298	624	931	96 %
	22 Feb 2010	2,909	230	612	845	96 %
	31 Mar 2010	4,541	265	455	700	97 %
SM separator	11 Dec 2009	1,369	295	1,383	736	89 %
	22 Feb 2010	3,910	676	582	1737	91 %

Phylogenetic Analysis

The phylogenetic affiliation of all of the major microbial lineages that were identified in the 17 production water samples that were collected from the four sampling locations is summarized below. More detailed information regarding the phylogeny of all of the sequences that were obtained in this study, the relative abundance of the major lineages that were observed in production water samples from the SM and AI wells, and information regarding the closest Genbank matches and closest cultured relatives of the major lineages that were observed in production water samples from the SM and AI wells is presented as Electronic supplementary material (Tables 1–3).

AI Tank

Within the AI tank, Proteobacteria-affiliated sequences represented the majority of the sequences in all six samples from the AI tank (ranging between 76 % and 94 % of sequences in examined samples), followed by members of the phylum Firmicutes (ranging between 6 % and 23 % of all AI samples examined) (Fig. 1a). The absolute majority (95–99 % in examined samples) of the Proteobacteria sequences belonged to the Gammaproteobacteria order Alteromonadales and the Epsilonproteobacteria order Campylobacterales. The absolute majority of Firmicutes sequences (99 %) belonged to the orders Bacillales, Clostridiales, and Halanaerobiales. Although these five order-level lineages represented the majority of the community, the proportion of each of these lineages could change significantly within a sampling incident (Figs. 1, 2, and 3).

Alteromonadales sequences were nearly all (99 % of the Alteromonadales fraction in various datasets) affiliated with the genus *Marinobacter*. The closest cultured relative of these sequences was *Marinobacter hydrocarbonoclasticus* (96–98 % sequence similarity), which is an aerobic, halotolerant, and iron-oxidizing aliphatic hydrocarbon degrader that was first isolated from petroleum contaminated seawater [24].

Sequences affiliated with the Epsilonproteobacteria order Campylobacterales were most closely related (97–98 %) to members of the genus *Arcobacter*. The closest cultured relative of these sequences was *Arcobacter mytili* (98 % sequence similarity), which is a mesophilic, halotolerant, sulfide-oxidizer [25]. *Arcobacter* sequences have often been detected in other petroleum studies and are capable of microaerophilic chemolithoautotrophic growth by utilizing sulfide as an electron donor [26].

As mentioned above, the majority of the Firmicutes sequences identified in the AI tank belonged to the orders Bacillales, Clostridiales, and Halanaerobiales. The majority of sequences affiliated with the order Bacillales belonged to the family Bacillaceae, with members of the families Alicyclobacillaceae, Paenibacillaceae, Planococcaceae, and Staphylococcaceae, representing a small fraction of the Bacillales community (Fig. 3). Bacillaceae sequences mostly belonged to the genera *Bacillus* and *Geobacillus*. The sequences classified as members of the genus *Bacillus* were closely related (97 % sequence similarity) to *Bacillus*

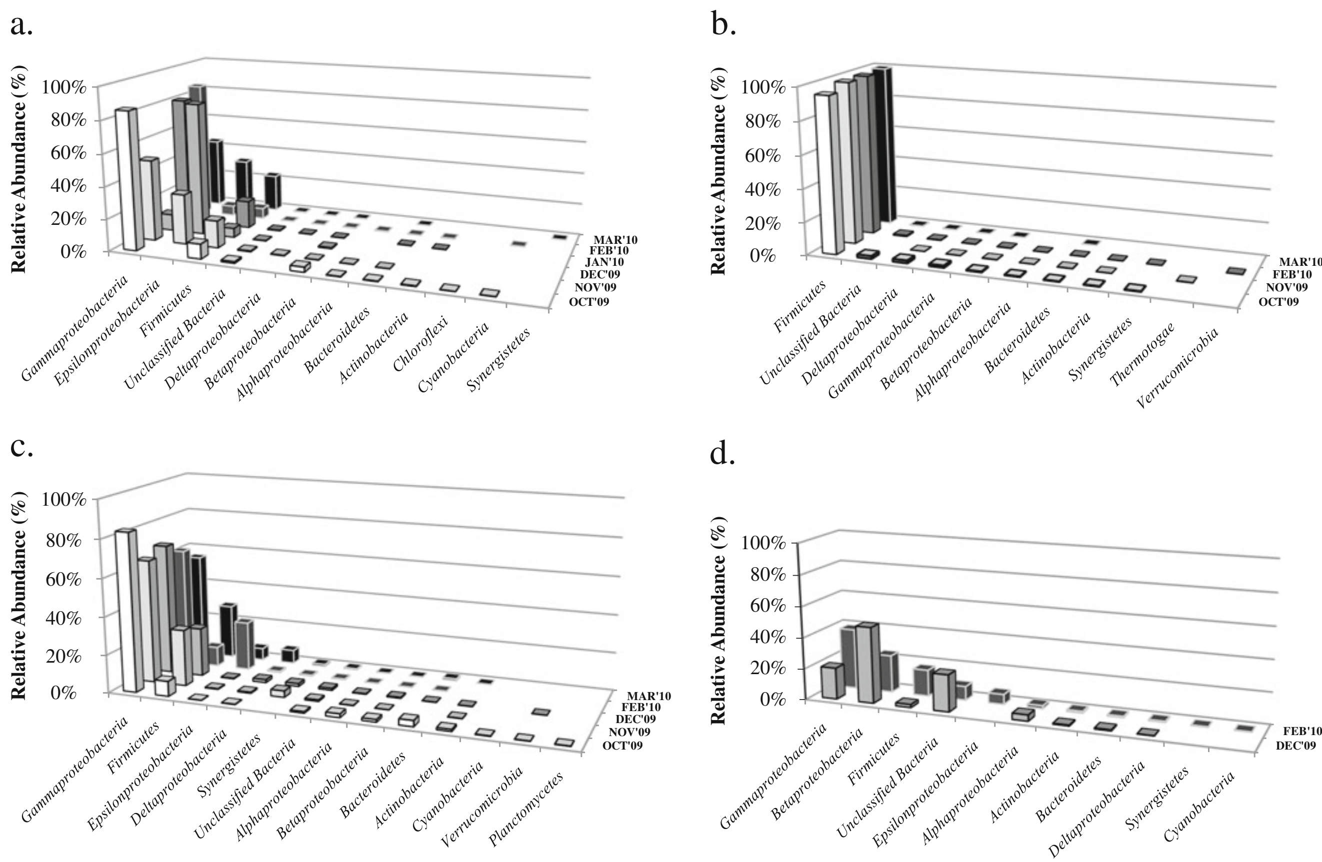

Figure 1 Phylum/class-level classifications of the bacterial 16S rRNA gene pyrosequences obtained from **a** AI tank, **b** AI separator, **c** SM tank, and **d** SM separator samples. The time of sampling is shown on the *Z*-axis for each sample

amyloliquefaciens, which is a halotolerant fermenter [27]. The sequences classified as members of the genus *Geobacillus* were closely related (97–98 % sequence similarity) to *Geobacillus thermoleovorans*, a thermophilic, hydrocarbon-degrading, facultative anaerobe that has frequently been observed in a variety of petroleum-impacted environments, hydrothermal vents, and hot springs [28, 29].

All the Clostridiales affiliated sequences belonged to the family Clostridiaceae, and most of these sequences were affiliated with the genus *Caminicella*. The closest cultured relative (96–98 % sequence similarity) to the *Caminicella*-related sequences was *Caminicella sporogenes*, which is a halophilic, thermophilic, anaerobic fermenter that is capable of reducing elemental sulfur, L-cystine, and thiosulfate to H_2S [30].

The Halanaerobiales-related sequences belonged to families Halanaerobiaceae and Halobacteroidaceae (Fig. 3a). All of the Halanaerobiaceae sequences were classified as members the genus *Halanaerobium*. Most of the *Halanaerobium* sequences were closely related (97–98 %) to *Halanaerobium congolense*, which is both a moderate halophile and strict anaerobe [32]. *H. congolense* oxidizes sugars and proteinaceous substrates and is the only species of *Halanaerobium* that can reduce both sulfur and thiosulfate [32]. *H. congolense* was isolated from an African oil field and has been detected in 16S rRNA gene surveys from a natural gas pipeline, an oil pipeline biofilm, a Brazilian oil reservoir, and a water–oil tank battery [31–35]. The sequences that were affiliated with the family Halobacteroidaceae were all classified as members of the genus *Orenia* and were closely related (94–98 % sequence similarity) to clones (GenBank accession no. DQ647102) from the high-temperature Troll oil field in the Norwegian sector of the North Sea [36]. *Orenia marismortui* (previously *Sporohalobacter marismortui*), which was isolated from the Dead Sea and is halophilic, moderately thermophilic, and a strict anaerobic fermenter, was the closest cultured relative (93–94 % sequence similarity) of these sequences [37, 38].

AI Separator

Richness estimates conducted at a putative species level ($OTU_{0.03}$ sequence divergence cutoff, Table 2) indicated that AI tank and AI separator have similar levels of diversity at the species-level. However, detailed phylogenetic analysis revealed that, at higher taxonomic levels (phyla, classes,

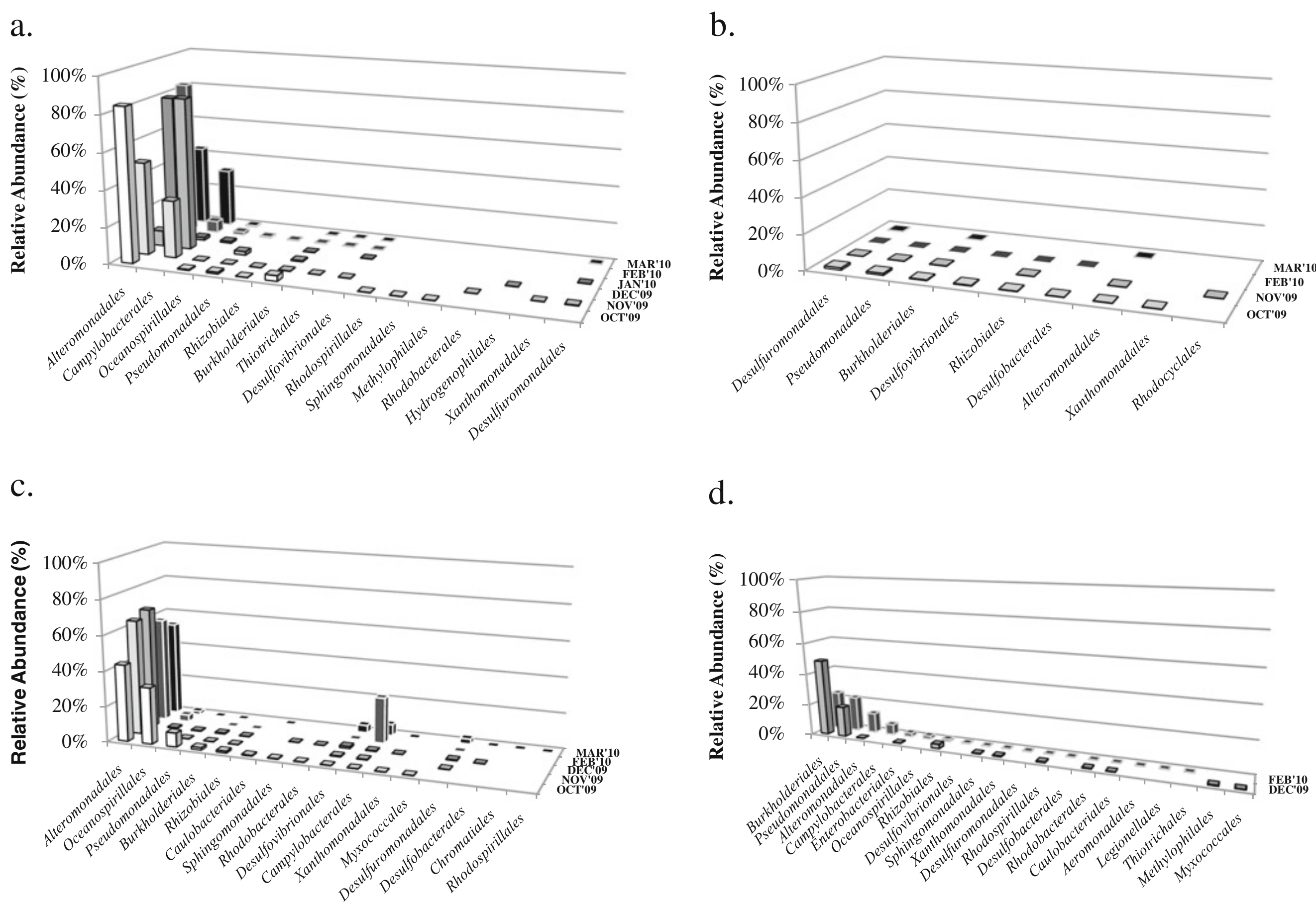

Figure 2 Order-level classifications of the Proteobacteria sequences obtained from **a** AI tank, **b** AI separator, **c** SM tank, and **d** SM separator samples. The time of sampling is shown on the *Z*-axis for each sample

and orders), AI separator samples were less diverse than the AI tank samples (Fig. 1a, b). Sequences affiliated with the phylum Firmicutes accounted for almost all (94–99 %) of the sequences in every AI separator sample that was collected (Fig. 1b). Various proportions of Clostridiales (1.4 % to 82 %) and Halanaerobiales (ranging between 16 % and 98 %) dominated these Firmicutes sequences. Most of the Clostridiales sequences were members of the genus *Caminicella*, while the Halanaerobiales sequences were most closely related to *H. congolense*.

SM Tank

The SM tank water samples had similar diversity indices to the AI tank samples, overall (Table 2). Furthermore, the SM tank samples exhibited some similarities in phylogenetic composition with the AI tank samples (Fig. 1c). The SM tank sample, like the AI tank sample, contained a core community that consisted primarily of sequences that were affiliated with the Gammaproteobacteria, Epsilonproteobacteria, and Firmicutes. The populations of Gammaproteobacteria in the SM tank samples were similar to those observed in the AI tank and consisted primarily of sequences, which were affiliated with the order Alteromonadales and closely related (96–97 % sequence similarity) to *M. hydrocarbonoclasticus*. In addition, a fraction (0–31.9 % of sequences in various time points) of the Gammaproteobacteria sequences in the SM tank samples were affiliated with the orders Oceanospirillales and Pseudomonadales, which were not detected in the AI tank samples (Fig. 2a, c). These Oceanospirillales sequences belonged to the genus *Chromohalobacter* and were most closely related (99–100 % sequence similarity) to *Chromohalobacter salexigens*, which is a strictly aerobic, mesophilic, halophilic microorganism [39]. The Pseudomonadales sequences were 97–100 % similar to *Pseudomonas stutzeri*, a thiosulfate-oxidizing, polycyclic aromatic hydrocarbon-degrader that has previously been detected in petroleum reservoirs in the US and Canada [26, 40–42]. The populations of Epsilonproteobacteria in the SM tank samples were very similar to those observed in the AI tank samples and consisted primarily of sequences that were affiliated with the order Campylobacterales and were closely related (96–97 % sequence similarity) to *A. mytili*.

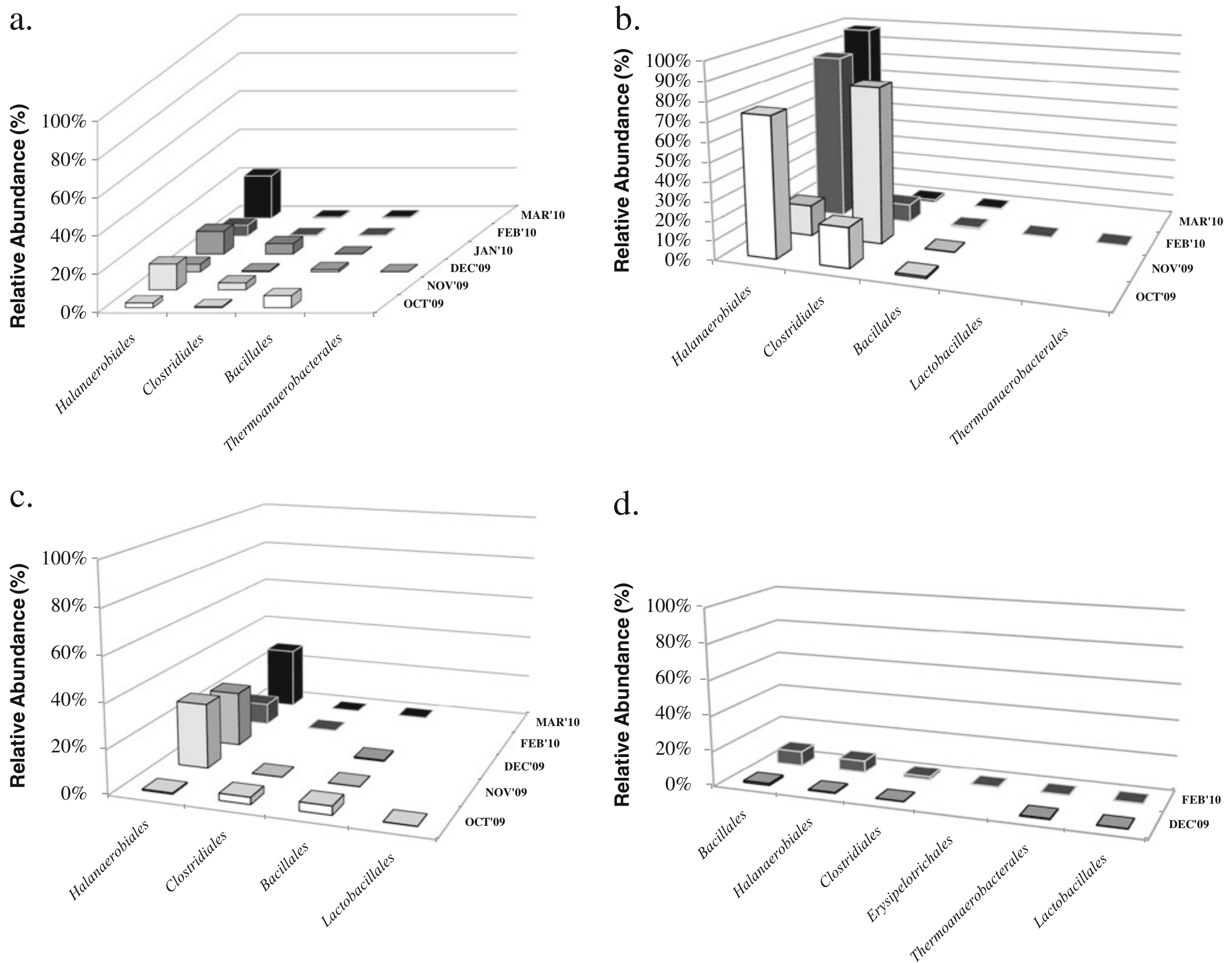

Figure 3 Order-level classifications of the Firmicutes sequences obtained from **a** AI tank, **b** AI separator, **c** SM tank, and **d** SM separator samples. The time of sampling is shown on the *Z*-axis for each sample

The Firmicutes sequences in the SM tank samples were also very similar to those observed in AI tank samples and consisted primarily of sequences that were affiliated with the orders Halanaerobiales, Bacillales, and Clostridiales. The majority of Firmicutes sequences in the SM tank were members of the order Halanaerobiales (Fig. 3c). Halanaerobiales sequences in SM tanks were closely related (98–99 % sequence similarity) to *H. congolense*, a facultative sulfur and thiosulfate-reducing, sulfide-producing bacterium. The Bacillales sequences were either related (94–96 % sequence similarity) to *B. amyloliquefaciens*, or to *Geobacillus pallidus* (97–98 % sequence similarity), a thermophile isolated from wastewater [43]. The majority of the Clostridiales sequences were related (94–96 % sequence similarity) to the genus *Caminicella*. The identification of sulfide-producing lineages within the Firmicutes population in SM tank is a reflection of the higher sulfate levels in SM tank compared with the AI tank. In addition to Gram-positive sulfate-reducing lineages, the SM tank samples also contained several groups of sequences that were affiliated with sulfide-producing bacteria from the class Deltaproteobacteria (0.5–7 % range at various sampling times) and the phylum Synergistetes (0–3.4 % range at various sampling times), which were detected in very low numbers (0–0.07 % of the community) in the AI tank samples. The sequences that were affiliated with sulfide-producing Deltaproteobacteria were classified as members of the orders Desulfobacterales, Desulfovibrionales, and Desulfuromonadales, which have been frequently detected in ecosystems that contain petroleum [26, 36, 44]. The Desulfobacterales classified sequences were related (92–93 % sequence similarity) to *Desulfobacter vibrioformis*. The Desulfovibrionales sequences were related (98 % sequence similarity) to *Desulfovibrio capillatus*, which was isolated from a crude oil storage tank [45]. The closest relatives (97–98 % sequence similarity) to the Desulfuromonadales classified sequences were clones

(GenBank accession no. FJ941600) from an oil–water separation tank [34]. Sequences affiliated with the phylum Synergistetes represented 0–3 % of the community in SM tanks. Synergistetes affiliated sequences were closely related (96–97 % sequence similarity) to *Dethiosulfovibrio acidaminovorans*, which is a sulfur and thiosulfate reducer [46].

SM Separator

The SM separator datasets were the most diverse out of all the sampled locations (Table 2 and Fig. 1). The majority of the sequences belonged to the Gammaproteobacteria (orders Pseudomonadales and Alteromonadales), Betaproteobacteria (order Burkholderiales), and Firmicutes (orders Bacillales, Clostridiales, and Halanaerobiales). The Pseudomonadales sequences belonged to the genus *Pseudomonas* (97–98 % sequence similarity), and the Alteromonadales sequences belonged to the genus *Marinobacter* (96–98 % sequence similarity). The Bacillales sequences were classified as members of the genus *Bacillus*. Other Bacillales sequences were affiliated with the genus *Planococcus*. The Clostridiales sequences were mostly related to the genus *Caminicella* (as described above). All of the Halanaerobiales sequences were 97–98 % similar to *H. congolense*. The Betaproteobacteria sequences were affiliated with the order Burkholderiales and were quite diverse at the genus level, but the majority of these sequences were related to the genera *Alcaligenes*, *Comamonas*, and *Ralstonia*.

Spatial and Temporal Comparisons of Bacterial Populations in Tanks and Separator Samples

The microbial community structure was compared between various tanks and separator samples (from this study) on temporal and spatial scales. The tank and separator communities were also compared with drilling mud and frac-water communities (previously characterized in both wells) to examine the role of drilling and hydraulic-fracturing processes in post-production microbial community establishment [11, 12]. The results indicate that the production (tank or separator) communities from both wells bear little resemblance to the mud or frac-water communities from these same wells, with average shared numbers ranging between 0.06 % and 3.15 % (Table 3, Fig. 4, Electronic supplementary material Table 4). This clearly indicates that the communities in the tank and separator samples are completely distinct from the communities introduced to the well during the drilling and hydraulic-fracturing processes. Spatial comparisons of the microbial communities in separator and tank samples from the same well at the same sampling time showed sequence similarities ranging between 25 % and 68 % (Table 4, Fig. 4).

Table 3 Percentage shared sequences between drilling mud and frac-water communities and the bacterial communities in the studied samples

Studied well[a]	Sample location	Communities compared	
		Frac-water	Mud
AI	Tank	2.55±0.015	0.07±0.001
	Separator	3.15±0.023	0.15±0.002
SM	Tank	0.12±0.003	0.1±0.001
	Separator	0.25±0.002	2.35±0.002

[a] Numbers are the averages ± standard deviations of all shared sequence percentages for all sampling points at each location (six sampling points for AI tank, four sampling points for AI separator, five sampling points for SM tank, and two sampling points for SM separator)

On the other hand, both spatial and temporal comparisons showed relatively high levels of similarities. Spatial similarities appear to be lowest at the early sampling stages, which correspond to the early stages of production within sampled wells, and increase with time (Table 4, Fig. 4). The highest level of similarities obtained were observed in temporal comparisons of microbial communities within the same locations, with percentage shared sequences values of up to 88 %, 91 %, and 94 % in AI tanks SM tank, and AI separator samples, respectively (Table 5, Fig. 4). Similarities between microbial communities on a temporal scale appear to be low at the early sampling stages, which corresponds to early stages of well production. Gradually, these percentages increase over time, suggesting the establishment of more stable microbial communities in those locations overtime.

Discussion

The predominance of specific lineages within the bacterial communities in AI and SM tanks and separators could best be understood by correlating the putative roles of these lineages to the observed geochemical conditions in such locations. The relatively high salinity within all samples (6–8 % in SM and 8–11 % in AI; Table 1) could explain the abundance of lineages that are exclusively halophilic (e.g., *Marinobacter, Halanaerobium, Chromohalobacter*, and *Caminicella*) in the bacterial communities from 14 out of 17 samples that were analyzed. Furthermore, sequences affiliated with lineages that contain multiple halophilic species (but are not exclusive to halophilic microorganisms, e.g., order Clostridiales) were also abundant members of the community in AI separator datasets as well.

Obligate halophilic and halotolerant members of the community were observed in lineages that contain a variety of metabolic and physiological capabilities ranging between

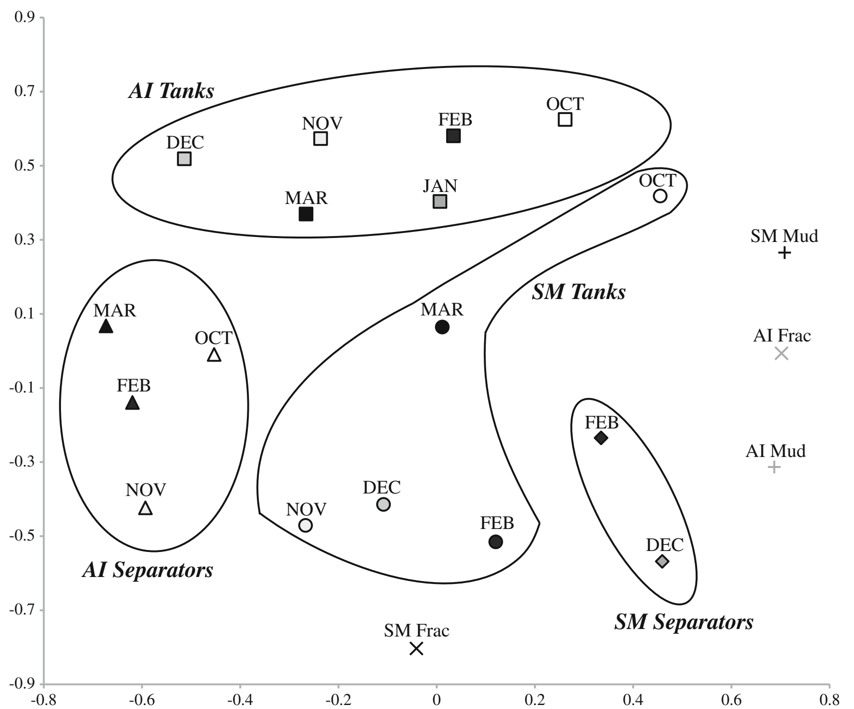

Figure 4 Non-metric multidimensional scaling plot of all samples studied (SM tanks (*closed squares*), SM separators (*closed stars*), AI tanks (*closed polygons*), AI separators (*closed circles*) compared with mud and frac-water (*open circles*) samples. Pair-wise abundance-based Sørensen indices were used to construct the plot

chemolithoautotrophs (*Arcobacter* sp.), aerobic heterotrophs (*Pseudomonas* sp.), anaerobic fermenters (*Halanaerobium* sp.), and anaerobic respiratory sulfate reducers (*Desulfovibrio* sp.), which is consistent with previous studies of the microbial communities in petroleum production facilities [26, 34, 47]. Lineages capable of growth at a wide range of redox potentials have also been observed in this study, including aerobes, facultative anaerobes, microaerophiles, and strict anaerobes (e.g., *Desulfovibrio* sp. and *Halanaerobium* sp.). Identification of such a wide, and seemingly contradictory, array of oxygen preference profiles in members of our community, often within a single sampling event, emphasizes the dynamic nature of sampled habitats. The flow of water from the well into the separators and then to the tanks is not constant, and the water often rests in the production equipment between the periods of flow. This may create temporary anaerobic conditions in these areas, especially in the separators. Also, since the water flow is not constant, the water at the bottom of storage tanks should be more anaerobic due to the lack of circulation and more aerobic at the top. Indeed, the coexistence of aerobic, microaerophilic,

Table 4 Effect of sampling time on the bacterial communities in the samples studied

Sampling location	Sampling times compared	Sørensen index[a]		% Shared sequences[b]
		Incidence-based	Abundance-based	
AI tank	Oct–Nov	0.33	0.62	53 %
	Nov–Dec	0.35	0.95	76 %
	Dec–Jan	0.27	0.90	61 %
	Jan–Feb	0.51	0.92	88 %
	Feb–Mar	0.45	0.88	85 %
SM tank	Oct–Nov	0.06	0.38	13 %
	Nov–Dec	0.48	0.94	91 %
	Dec–Feb	0.34	0.85	74 %
	Feb–Mar	0.42	0.91	74 %
AI separator	Oct–Nov	0.32	0.61	56 %
	Nov–Feb	0.40	0.92	85 %
	Feb–Mar	0.45	0.96	94 %
SM separator	Dec–Feb	0.29	0.63	52 %

[a]Numbers are pair-wise Sørensen similarity index between the bacterial communities of the samples shown in the first column at the two sampling times shown in the second column. Incidence-based Sørensen index uses only the presence and absence data while the abundance-based index takes the abundance into account

[b]Numbers are the percentages of shared sequences between the bacterial communities of the samples shown in the first column at the two sampling times shown in the second column

Table 5 Effect of sampling location on the bacterial communities in the samples studied

Sample	Sampling time[a]	Sørensen index		% Shared Sequences
		Incidence-based	Abundance-based	
AI	Oct	0.12	0.13	34 %
	Nov	0.15	0.25	28 %
	Feb	0.10	0.12	59 %
	Mar	0.27	0.35	68 %
SM	Dec	0.07	0.52	25 %
	Feb	0.22	0.33	49 %

The tank and separator communities are compared for each of the wells studied

[a] Sampling time refers to the sampling month at which the tank and separator communities of each well are compared

and anaerobic bacteria seems to be a hallmark of petroleum production facilities studies (e.g., oil–water separation tank samples taken from the Berkel oil field) [34].

Detailed phylogenetic analysis identified multiple members of the microbial communities within all samples that bear close resemblance to those observed in prior investigations of various fossil fluid production formations and facilities. Examples include sequences identified that are affiliated with genera *Pseudomonas*, *Arcobacter*, *Marinobacter*, *Geobacillus*, *Caminicella*, and *Halanaerobium*. These genera have previously been isolated or detected via 16S rRNA gene sequencing surveys from a high-temperature oil field of the San Joaquin Basin, oil formations (Troll, Dan, and Halfdan) of the North Sea, Pelican Lake oil field in Canada, a natural gas pipeline, and oil–water separator tanks in The Netherlands [26, 36, 44]. The general geochemical and environmental reasons rationalizing the selection of lineages with such metabolic capabilities and physiological characteristics in this study, as well as in prior fossil formation diversity studies are fairly well understood, e.g., elevated salinity, availability of SO_4^{2-}, sulfide, and elemental sulfur as described above. However, the reasons for the observed repeated selection of a relatively limited number of genera and species in petroleum formations and facilities, from the larger pool of hydrocarbon degraders, halophiles, and sulfur metabolizers is not yet fully understood.

Of special interest is the wide range of sulfur-metabolizing lineages within this dataset, which is of importance to the oil industry due to deleterious effects of sulfide [48, 49]. In general, sulfidogenic lineages could be divided into two main groups: obligate respiratory sulfate, sulfur, and thiosulfate reducers that utilize these compounds as a terminal electron acceptor during anaerobic respiration and microorganisms that are capable of sulfide production from sulfur and thiosulfate while growing fermentatively. Obligate sulfate-, sulfur-, and thiosulfate-reducing bacteria (members of the orders Desulfovibrionales and Desulfuromonadales) were more prevalent (range, 0.2–7 %) in SM tank samples, where sulfate levels were higher (10–52 mg/L, Table 1), and were present in much lower numbers in AI (0–1.6 % separator and tank) samples where sulfate is limited (Table 1). The numbers of sequences affiliated with sulfate-reducing bacteria do increase over time in the SM Tank samples (Fig. 1c), which implies an increase in the biocorrosive potential of the bacterial communities present in the production equipment. Sequences that were affiliated with the sulfur-reducing genus *Dethiosulfovibrio*, within the phylum Synergistetes, were also detected in all sampled locations (though not in all the sampling times, 8 out of the 17 samples) and were most abundant (3.4 %) in the SM tank in November (Fig. 1). Facultative sulfidogenic microorganisms (i.e., those capable of facultative sulfur and thiosulfate reduction to sulfide) were also identified in this study and were closely related to either *H. congolense* (order Halanaerobiales), which reduces thiosulfate and elemental sulfur, or sulfur- and sulfate-reducing microorganisms from the genus *Caminicella* (order Clostridiales) [32]. Sequences identified as close relatives of *H. congolense* were identified in most samples and represented the majority of the sequences in most of the AI separator samples (Fig. 3b). Sequences related to the genus *Caminicella* were detected at varying abundance (0–80 % of the total bacterial community) in many (13 out of 17) of the samples but were most abundant in SM separator in November (Fig. 3d). Thiosulfate is completely soluble, emits no odor, and therefore tends to be overlooked in the petroleum industry [10]. The large proportion of sequences affiliated with thiosulfate-reducing bacterial lineages (AI separator, Fig. 3b) indicates the possibility of deleterious, biocorrosive activity. Therefore, our results suggest that a consortium of both sulfate- and sulfur-reducing bacteria, in addition to facultative sulfidogenic bacteria, could contribute to the incidents of sulfidogenesis and corrosion that has been reported in separators and tanks from the Barnett Shale [9]. Multiple sources of sulfate and sulfur could be present in such locations, including barite and sulfonates that were present in drilling fluids [11]. A variety of sulfur-containing compounds are present in the shale (e.g., pyrite–FeS_2), which could be converted to sulfide by air exposure and subsequently oxidized under microaerophilic conditions by chemolithoautotrophic sulfide oxidizers (e.g., *Arcobacter* spp.) that were identified at various sampling points and locations (AI and SM tanks, Fig. 2a, c).

Finally, pair-wise Beta diversity comparisons revealed that the bacterial communities identified within the tanks and separators (at all time points) bore little resemblance to the communities of the drilling mud and frac-water (used to drill the wells). This indicates that microbial communities in these locations are not simply a carryover of microorganisms from the drilling mud and frac-water but are distinct communities that appear to develop in situ in response to the prevalent conditions at each sampling site. Several members of the

observed communities in the tanks and separators could have been minor components of the microbial communities in the drilling mud and frac-water that were ideally suited to propagate under specific prevalent conditions in the production equipment sampled. For example, Halanaerobiales sequences represented a small fraction of the total sequences in the SM and AI mud but accounted for the majority of sequences in the AI separators and AI/SM tanks [11]. Alternatively, soil, surface, and airborne microorganisms at these locations could provide an inoculum for the developing microbial community. Regardless of the origin of the microbial communities that developed at these locations, it appears that a core microbial community eventually developed in all of the production equipment that was sampled. Within specific locations, while the membership of the microbial communities remains similar, its community structure and relative proportions of various taxa appear to fluctuate over time. For example, the microbial communities in AI tank always contained Gammaproteobacteria and Firmicutes sequences (Fig. 1). However, the proportion of the orders (Alteromonadales, Oceanospirillales, Halanaerobiales, and Clostridiales) increases and decreases over time (Figs. 2–3). This shows the dynamic nature of the microbial communities in response to the sporadic influx of produced water from the formation.

In conclusion, we surveyed the bacterial communities in above-ground production facilities (gas water separators and tanks) at two newly drilled thermogenic natural gas wells in the Barnett Shale in north central Texas over a 6-month period. Analysis revealed that the bacterial communities from these locations: (1) reflect the geochemical properties of waters in the production facilities (salinity, availability of multiple electron acceptors, and availability of various sulfur-containing chemical species), (2) bear clear resemblance to communities identified in prior studies of the bacterial communities in production fluids from similar above-ground production facilities from other fossil fuel formations, (3) harbor multiple obligate and facultative sulfidogenic lineages, and (4) bear little resemblance to the microbial communities that were identified in the fluids that were utilized during the drilling and hydraulic fracturing processes, which suggests that these communities developed in situ post-production.

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