Microbial Metabolism and Community Dynamics in Hydraulic Fracturing Fluids Recovered From Deep Hydrocarbon-Rich Shale

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Hydraulic fracturing is a prominent method of natural gas production that uses injected, high-pressure fluids to fracture low permeability, hydrocarbon rich strata such as shale. Upon completion of a well, the fluid returns to the surface (produced water) and contains natural gas, subsurface constituents, and microorganisms (Barbot et al., 2013; Daly et al., 2016). While the microbial community of the produced fluids has been studied in multiple gas wells, the activity of these microorganisms and their relation to biogeochemical activity is not well understood. In this experiment, we supplemented produced fluid with ^{13}C-labeled carbon sources (glucose, acetate, bicarbonate, methanol, or methane), and ^{15}N-labeled ammonium chloride in order to isotopically trace microbial activity over multiple day in anoxic incubations. Nanoscale secondary ion mass spectrometry (NanoSIMS) was used to generate isotopic images of ^{13}C and ^{15}N incorporation in individual cells, while isotope ratio monitoring–gas chromatography–mass spectrometry (IRM–GC–MS) was used to measure $^{13}CO_2$, and $^{13}CH_4$ as metabolic byproducts. Glucose, acetate, and methanol were all assimilated by microorganisms under anoxic conditions. $^{13}CO_2$ production was only observed with glucose as a substrate indicating that catabolic activity was limited to this condition. The microbial communities observed at 0, 19, and 32 days of incubation did not vary between different carbon sources, were low in diversity, and composed primarily of the class *Clostridia*. The primary genera detected in the incubations, *Halanaerobium* and *Fusibacter*, are known to be adapted to harsh physical and chemical conditions consistent with those that occur in the hydrofracturing environment. This study provides evidence that microorganisms in produced fluid are revivable in laboratory incubations and retained the ability to metabolize added carbon and nitrogen substrates.

Keywords: NanoSIMS, hydraulic fracturing, microbial metabolism, microbial community, N and C assimilation

INTRODUCTION

Hydraulic fracturing (hydrofracturing, HF), injection of pressurized fluid into the subsurface to create new fractures and to extend natural fractures, is used to extract natural gas and oil from hydrocarbon-rich geological strata. These fracture networks greatly increase the amount of hydrocarbons that can be extracted (Arthur et al., 2008; Jenkins and Li, 2008; Kargbo et al., 2010). The injected fluids are composed primarily of water and a propping agent such as sand that holds the fractures open (Nicot and Scanlon, 2012). Chemical additives increase the efficacy of the process and include viscosity modifiers, chemical stabilizers, corrosion inhibitors, and biocides (Gregory et al., 2011; Nicot and Scanlon, 2012). After a well is completed, depressurization causes 10–70% of the hydrofracturing fluid to rapidly return to the surface while the remaining fluid stays in place or flows to the surface later in production (Olmstead et al., 2013). This expelled fluid from a hydrofractured well is called "produced fluids."

Produced fluids from hydrofractured wells are a concern because large volumes of the fluid are produced at the surface and in addition to the additives, they often contain high concentrations of salt, metals, organic compounds, radionuclides, and microorganisms due to the fluid-rock interactions at depth (Nicot and Scanlon, 2012; Barbot et al., 2013). Changes in chemical composition of produced fluids from the fluid originally injected for hydrofracturing occur due to shale dissolution, brine migration, and water-rock reactions (Blauch et al., 2009; Dresel and Rose, 2010; Warner et al., 2012). Biogeochemical activity may also change the chemical composition of produced fluids (Strong et al., 2013; Akob et al., 2015). Produced fluids must be treated for disposal or for recycling as they are important source of water for subsequent hydrofracturing fluid (Boschee, 2015); however, methods are often limited by resource availability, cost, and energy requirements (Gregory et al., 2011; Lutz et al., 2013; Rahm et al., 2013). Mitigating the compounds present in the produced fluids requires an accounting of their potential sources as well as an understanding of how they might be transformed in a hydrofractured well.

Microorganisms in the produced fluids can originate from the fractured geological formation or from anything at the surface that contacts the fluids used (e.g., water or sand used to produce hydrofracturing fluid, drilling equipment, drilling muds, well casing, gas separator) (Struchtemeyer et al., 2011; Mohan et al., 2013a,b). During hydrofracturing, the addition of chemicals such as biocides and the exposure to extreme conditions of the subsurface (high temperature, change in pH, high concentrations of salt and metals) leads to a microbial community that exhibits low diversity (Mohan et al., 2013a; Booker et al., 2017). Minor components of the community become dominant members of the produced fluids community (Mohan et al., 2013a; Cluff et al., 2014). Cluff et al. (2014) identified "indicator" genera in late stage produced fluids as being dominated by the bacterial *Halanaerobium*, unclassified *Halanaerobiaceae*, *Selenihalanaerobacter*, *Flexistipes*, and the archaeal *Methanohalophilus* and *Methanosarcinaceae*. These taxa are anaerobic halophiles that would appear to be adapted to hydrofracturing well conditions and may alter subsurface geochemistry and the legacy of the well (Cluff et al., 2014).

In order to know what long-term effects microorganisms could have on the produced fluid chemistry or in a hydrofractured well, it is important to know the fraction of these organisms that are alive in the fluids. Sulfate reducing, acid producing, and fermenting microorganisms have all been cultured from produced fluids which demonstrates their viability (Davis et al., 2012; Struchtemeyer et al., 2012; Liang et al., 2016; Booker et al., 2017); however, the ability to grow in culture may not indicate the physiological status of these cells (Rappé and Giovannoni, 2003; Puspita et al., 2012). In one metagenomic study, genes related to carbohydrate metabolism and stress response increased during the first 9 days of hydrofracturing suggesting that the microbial community responds to changes associated with hydrofracturing (Mohan et al., 2014). These investigations have clarified some aspects of the microbial ecology of the produced fluids but additional studies are needed to assess the degree to which microbes are alive in the produced fluids.

The aim of this research was to determine whether microbial communities in the produced fluids from hydrofractured systems retain the ability to assimilate carbon and nitrogen, the conditions under which such assimilation occurs, and the types of microbial communities associated with assimilation. We incubated a produced fluid for 60 days with ^{13}C-labeled glucose, acetate, methanol, bicarbonate, or methane, and ^{15}N-labeled ammonium chloride, all compounds that could be derived either from the subsurface or hydraulic fracturing fluid (Orberger et al., 2005; Horsfield et al., 2006; Environmental Protection Agency [EPA], 2012; Stringfellow et al., 2014). A nano-scale secondary ion mass spectrometry (NanoSIMS), a ultra-high spatial resolution isotope imaging instrument, was used to detect incorporation of stable isotopes in individual cells (Morono et al., 2011). Isotope ratio monitoring-gas chromatography-mass spectrometry, and high temperature catalytic oxidation were used to measure ^{13}C-labeled metabolic products. Community analysis was conducted to determine the microbial communities that developed due to the availability of different substrates.

MATERIALS AND METHODS

Sample Collection

Produced fluid samples were collected from the gas/liquid separator of a hydraulically fractured, horizontal well in Greene Co., Pennsylvania in May 2014. The well was drilled into the Marcellus shale which has a temperature that ranges from 35 to 51°C and an approximate pressure of 40 MPa at this location (USGS; Kargbo et al., 2010). The sample was collected in a sterile 4 L polypropylene bottle filled to capacity without headspace and shipped overnight to Oregon State University on ice.

Experiment Setup

Forty milliliter aliquots of the produced fluid were incubated in 50 mL serum bottles that had been acid washed and combusted at 400°C for 4 h. Killed controls were autoclaved prior to

allocation for 2 h at 121°C. Anoxic samples were sparged with nitrogen gas in an anaerobic chamber and capped with butyl rubber septa (Chemglass, CLS-4209-14). The absence of oxygen was confirmed by measuring oxygen saturation with a Micro TX3 – AOT Microsensor oxygen meter (PreSens, Regensburg, Germany). The supplemental carbon substrates were ^{13}C-labeled-glucose ($^{13}C_6$, 99%), -acetate (1,2-$^{13}C_2$, 99%), bicarbonate (^{13}C, 99%), methanol (^{13}C, 99%), and methane (^{13}C, 99%) and the nitrogen substrate was ^{15}N-labeled ammonium chloride (^{15}N, 99%) (Cambridge Isotope Laboratories), and added to final concentrations of 1 and 0.1 mM, respectively, excluding methane. For methane samples, 5 mL of gas was added with a syringe and tightly sealed with an aluminum cap afterward. No other supplemental nutrient was added. In total, 25 bottles (five substrates, four time points, one killed control) were prepared and shipped via express courier (48 h transit time) to the Kochi Institute for Core Sample Research, JAMSTEC in Kochi, Japan for downstream isotopic and genomic analysis. Once at Kochi, all samples were incubated at 25°C. Individual incubations with each supplemental carbon source were destructively sampled at 0, 19, 32, or 60 days for analyses.

Cell Counts

To prepare samples for cell counts, serum bottles were sonicated for 30 s and briefly vortexed to remove precipitates and cells from the sides of the bottles. Twenty milliliters were transferred to a Falcon tube and centrifuged. The sediment pellets were fixed with 4% paraformaldehyde in 1 × phosphate buffered saline (PBS) for 3 h at 4°C. The pellets were washed twice with 1 × PBS and then stored in a 1:1 solution of 1 × PBS: 100% ethanol at −20°C. Samples were prepared as described in Morono et al. (2013). Briefly, fixed sediment slurry was resuspended in 2.5% NaCl and treated with a detergent mix composed of 12.5 mM EDTA, 12.5 mM Na_3PO_4, 0.1% v/v Tween-80, 0.1% MeOH, final concentration. Cells were detached from the produced fluid precipitates by shaking at 500 rpm for 1 h (Shake Master, Bio Medical Science) and sonicating at 200 watts in 30 s intervals for 10 cycles (Bioruptor UCD-250, Cosmo Bio). Carbonate minerals were removed with hydrofluoric acid (1.1% v/v, final concentration) for 20 min and then samples were neutralized with 1.5 M Tris-base. The final solution was added to 2.5% NaCl and filtered through 0.22 μm black polycarbonate membranes (Isopore Membrane Filter GTBP, Merck). Cells were stained with a 1:40 dilution of SYBR-green I (Thermo): 1 × TE buffer. Five hundred microliters of 1:100 diluted microspheres were added as a reference for microscopic focusing and the resulting membrane was mounted with 3–10 μL of mounting solution (1:2 mixture of TE buffer and Vectashield [Vector Laboratories]). The cells were counted with an automated fluorescence microscope (Morono and Inagaki, 2010).

DNA Extraction and 16S rDNA Sequencing

To collect microbial community DNA, 10 mL aliquots of the produced fluids were collected after sonication and vortexing. The aliquots were centrifuged and the remaining pelleted solid was stored at −80°C. Genomic DNA was extracted using the hot alkaline method (Morono et al., 2014). In brief, pellets were warmed to 70°C in 12.5 mM EDTA for 10 min and cells were lysed at 70°C with lysis solution (1% SDS, and 1 N NaOH, final concentration) for 20 min. Supernatant was transferred to a tube with neutralization buffer (1 M N HCl, 0.3 M Tris-HCl). The remaining pellet was washed with prewarmed distilled water, centrifuged, and supernatant was transferred to the same sample tube. The extract was treated with equal volumes of phenol–chloroform–isoamyl alcohol (25:24:1) and chloroform–isoamyl alcohol (24:1) and then precipitated by adding a 1/10 volume of 3 M sodium acetate, and a 1:500 volume of polyacrylamide. The V4 region of the 16S rRNA gene was sequenced using Illumina MiSeq. The Illumina sequences were aligned to the SILVA database (release 123, Quast et al., 2013) using the program Mothur (version 1.37.0, Schloss et al., 2009). Chao richness, abundance-based coverage estimator (ACE), Jackknife, Shannon, and Simpson diversity estimators were calculated using Mothur.

The genera detected mainly constituted less than 1% of the community and were considered to be minor community. Genera that were abundant in PCR blanks or DNA negative controls and not abundant in the experimental samples (**Supplementary Table 1**) were deemed to be contaminants and were manually removed from the dataset. Some genera were also removed because they are often detected as contaminants during sequencing studies or because they are known to be associated with human contamination (**Supplementary Table 1**; Salter et al., 2014). Using this approach, approximately 5% of the sequences from all of the experimental samples were removed. However, the percent of sequences removed also varied from sample to sample (**Supplementary Table 2**). Two samples had notably high percentages of putative contaminants and fewer sequences as well.

Activity Detection Using NanoSIMS

Cells were separated from sediments using a multi-layer density gradient separation protocol (Morono et al., 2013). Samples were layered over a Nycodenz and sodium polytungstate multi-layer density solution. After centrifugation, the supernatant was removed and filtered through an Anodisc membrane (GE Healthcare) followed by staining with 1:40 dilution of SYBR Green I in 1 × TE buffer. Cells were resuspended by sonicating the membrane and the resulting cell suspension was subjected to fluorescence-activated cell sorting by using Moflo Cell Sorter (Beckman Coulter). Sorted cells were captured onto an indium-tin-oxide (ITO) coated black polycarbonate membrane.

Isotopic imaging analysis was conducted with a NanoSIMS 50L ion microprobe (AMETEK Co., Ltd., CAMECA BU) at the Kochi Institute for Core Sample Research, JAMSTEC. Samples on the ITO coated polycarbonate membrane were pre-sputtered at high beam currents (30–40 pA) for a few minutes to remove surface contamination and to obtain steady state of the secondary ion intensities before measurement. The secondary ions of $^{12}C^-$, $^{13}C^-$, $^{16}O^-$, $^{12}C^{14}N^-$, and $^{12}C^{15}N^-$ and $^{32}S^-$ were collected and measured in parallel at a mass resolution of ~9,000 that is sufficient to separate the $^{13}C^-$ from the $^{12}C^1H^-$ and $^{12}C^{15}N^-$

from the $^{13}C^{14}N^-$. Detailed isotopic images of the cells were obtained by rastering a 0.8–1.3 pA 16 keV Cs^+ primary ion beam ($\sim$100 nmφ) over an area of 25 × 25 μm field of view. Each image consisted of 256 × 256 pixels with a dwell time of 2 ms per pixel, and the final image was created by amalgamating 20 images for same analysis area. Recorded images and data were processed using a CAMECA WinImage software and OpenMIMS plugin (Gormanns et al., 2012) in ImageJ (Schneider et al., 2012) distribution of Fiji (Schindelin et al., 2012). The different scans of each image were aligned to correct image drift during acquisition. Final images were generated by adding the secondary ion counts of each recorded secondary ion for each pixel over all scans. Intracellular carbon and nitrogen uptake from stable isotope-labeled substrates was calculated by drawing regions-of-interest on $^{12}C^{14}N^-$ and/or $^{32}S^-$ images and calculating $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratio (inferred from the $^{12}C^{15}N/^{12}C^{14}N$ ratio) while having the data from blank filter area for standardizing multiple analysis data.

Metabolic Byproducts

The metabolic byproducts of the supplemented ^{13}C-labeled carbon sources, $^{13}CO_2$, dissolved inorganic ^{13}C, and $^{13}CH_4$, were all measured with IRM–GC–MS using a Thermo/Finnigan Delta Plus XP IRMS instrument (Thermo Electron Corp., San Jose, CA, United States). Total CO_2, dissolved inorganic carbon (DIC), and CH_4 were also measured.

Nucleotide Sequence Accession Numbers

The nucleotide sequences reported in this study have been deposited in the DDBJ/EMBL/GenBank database under accession number DRA007788.

RESULTS

Sample Collection and Cell Counts

Upon arrival at Oregon State University, the produced fluid sample was black in color. The solids at the bottom of each serum bottle (**Figure 1**) served as a relative indicator for the presence of oxygen wherein the anoxic incubations remained black. A sample exposed to oxygen turned orange suggesting oxidation of iron.

Cells were enumerated with automated fluorescence microscopy at 0, 19, 32, and 60 days of incubation. At the beginning of the incubation period (0 day), the cell numbers in the samples varied between 7.9 ($\pm$1.5) × 10^7 cells/mL of the produced fluid. During the incubation period from 0 to 19 days, cell numbers declined to between 1.6 × 10^7 and 3.5 × 10^7 cells/mL and then, between 19 and 60 days, a gradual decline in cell numbers was observed yielding between 1.9 × 10^6 and 3.6 × 10^6 cells/mL by the time the experiment ended.

Metabolic Byproducts

We measured DIC in the incubation samples at 60 days in order to determine the degree to which different substrates were respired under anoxic conditions. Samples containing ^{13}C-labeled acetate, methanol, methane, and no substrate
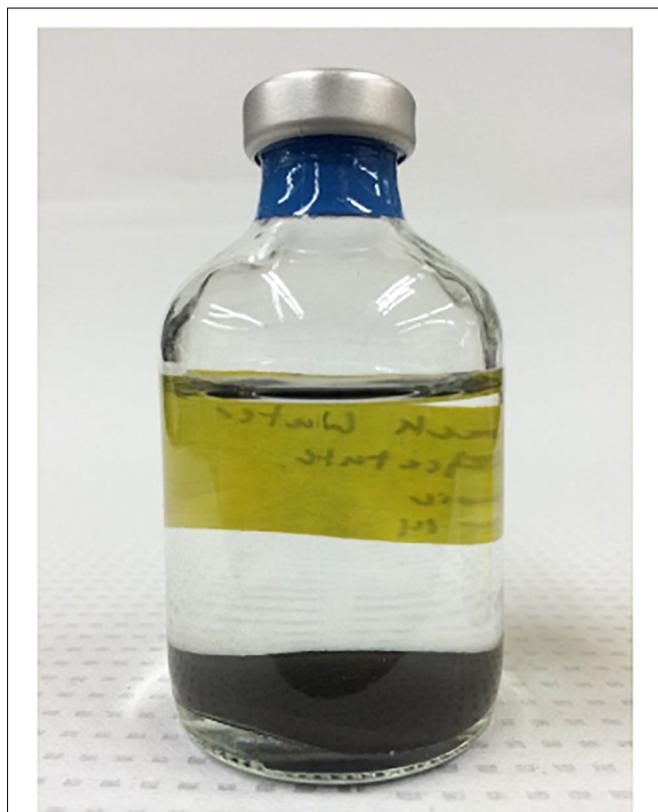
FIGURE 1 | Serum bottle containing produced water as prepared for anoxic incubations.

addition control contained DIC concentrations of 2.8, 2.7, 2.9, and 2.7 mM, respectively, and carbon isotopic composition (atomic % of ^{13}C-DIC) of 1.19, 1.19, 1.12, and 1.10%, respectively. The killed controls all had a lower concentration of DIC with lower ^{13}C (^{13}C-labeled acetate: 0.9 mM, 1.12%; ^{13}C-labeled methanol: 0.9 mM, 1.11%; ^{13}C-labeled methane: 1.0 mM, 1.13%, we did not prepare a killed control for no substrate addition control). Although DIC in the killed control for ^{13}C-labeled glucose was not measured, the sample containing ^{13}C-labeled glucose had the highest concentrations of DIC and atomic % of ^{13}C of 4.8 mM and 1.40%, respectively.

Total CH_4 and carbon isotopic composition of CH_4 (atomic % of ^{13}C) in the incubation samples at 32 days and 60 days were measured to determine if any of the substrates were used for methanogenesis. The total CH_4 at 32 days was between 0.8 and 1.6 μM for all samples. At 60 days the total methane concentrations were more varied with samples containing acetate, methanol, bicarbonate, and glucose showing evidence of 7.5, 0.9, 3.4, and 2.1 μM CH_4, respectively. The killed controls had no detectable methane. Atomic % of ^{13}C-CH_4 values were highest in the incubation containing methanol at both 32 and 60 days with 34.54 and 36.13%, respectively. Atomic % of ^{13}C-CH_4 in the incubation with ^{13}C-bicarbonate at 32 and 60 days were 3.36 and 2.08%, respectively. Atomic % of ^{13}C-CH_4 in the incubations with ^{13}C-acetate or ^{13}C-glucose didn't show high values (0.997–1.002%).

NanoSIMS Analysis

NanoSIMS analysis revealed that microorganisms in the produced fluid actively assimilated carbon and nitrogen provided in the incubations. For carbon, this was indicated by an atomic % ^{13}C value of ≥ 1 in individual cells (see **Table 1** for summary of ^{13}C assimilation in different treatments) and NanoSIMS images that were taken for different carbon sources under anoxic conditions. ^{13}C assimilation was observed in all of the substrates other than methane (**Figure 2** and **Supplementary Figure 1**). The relative abundance of ^{13}C in individual cells is illustrated in **Figure 2** (middle row) by color gradients with warmer colors indicating a higher percentage of ^{13}C incorporated during the incubation and cooler colors indicating a lower abundance of ^{13}C incorporated during the incubation. Glucose and methanol were assimilated favorably in the incubations. The incubation containing bicarbonate and acetate showed very slight ^{13}C assimilation after 60 days. Anoxic methane samples showed no assimilation of ^{13}C (**Table 1**).

To determine the relative preference that substrate-assimilating cells had for carbon or nitrogen, the assimilation ratios of ^{13}C and ^{15}N were plotted (**Figure 3**). In general, cells that were able to assimilate nutrients assimilated more ^{13}C than ^{15}N. Carbon from glucose was preferentially assimilated over nitrogen between 19 and 60 days. Carbon from acetate was preferentially assimilated over nitrogen at 32 and 60 days. Methanol assimilation was only observed at 32 days, but was again preferred over nitrogen. Few of the observed cells assimilated a significant amount of bicarbonate by 60 days.

Microbial Community Characterization

All samples at 19 and 32 days were dominated by the *Clostridia* such that cells from this class of Bacteria composed over 95% of the community (**Figure 4**). Within the *Clostridia*, *Halanaerobium* and *Fusibacter* were the dominant genera (**Figure 5**). While *Halanaerobium* composed 80% or more of the community in most incubations, *Fusibacter* became a larger portion of the community by 32 days, with the exception of samples containing glucose (**Figure 5**). The diversity of the microbial communities varied over time and between carbon substrates (**Table 2**). For example, Chao richness as an indicator of community diversity decreased between 19 and 32 days in incubations that contained acetate or methane, but increased during this same interval in incubations that contained glucose or methanol. Rarefaction curves indicated that the sequencing effort captured part of the diversity in the incubated samples of produced fluid (**Supplementary Figure 2**).

DISCUSSION

Our research on the metabolic activity of cells present in produced fluids is consistent with reports from past investigations that establish the role of microbes in corrosion and gas souring during hydrocarbon recovery (Enning and Garrelfs, 2014), and those specific to hydrofracturing that have characterized the microbial ecology of the produced fluids (Struchtemeyer et al., 2011; Struchtemeyer and Elshahed, 2012;

TABLE 1 | Summary of ^{13}C assimilation in individual cells.

Carbon source	Time (days)	Maximum atomic ^{15}N % assimilation	Maximum atomic ^{13}C% assimilation	Number of cells that assimilated ^{15}N	Number of cells that assimilated ^{13}C	Total number of cells observed	Percent of cells showed substrate incorporation
Acetate	0	–	–	–	–	–	–
	19	–	–	–	–	–	–
	32	0.8	3.9	0	5	33	15
	60	0.8	4.2	0	8	30	27
Bicarbonate	0	–	–	–	–	–	–
	19	0.1	0.8	0	0	85	0
	32	0.8	0.9	0	0	100	0
	60	1.0	1.1	1	2	53	4
Glucose	0	–	–	–	–	–	–
	19	0.9	8.1	0	60	76	79
	32	1.0	6.1	4	11	14	79
	60	1.0	11.4	6	22	30	73
Methane	0	–	–	–	–	–	–
	19	–	–	–	–	–	–
	32	0.9	0.2	0	0	70	0
	60	0.9	0.3	0	0	61	0
Methanol	0	–	–	–	–	–	–
	19	0.2	0.1	0	0	54	0
	32	0.9	4.6	0	6	39	15
	60	0.5	0.4	0	0	29	0

The minimum and maximum atomic ^{13}C% assimilation show the range of assimilation that was observed individual cells. A dash indicates that data was not taken for that sample.
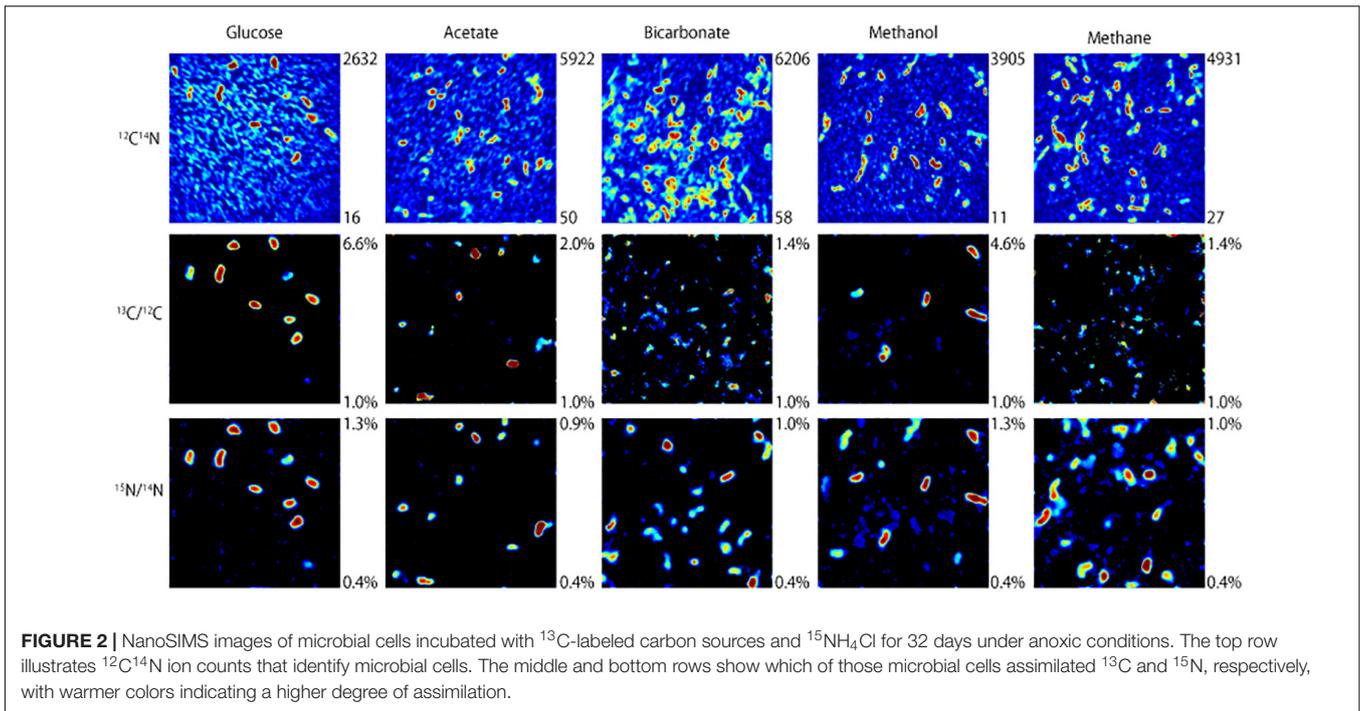
FIGURE 2 | NanoSIMS images of microbial cells incubated with ^{13}C-labeled carbon sources and ^{15}NH$_4$Cl for 32 days under anoxic conditions. The top row illustrates ^{12}C^{14}N ion counts that identify microbial cells. The middle and bottom rows show which of those microbial cells assimilated ^{13}C and ^{15}N, respectively, with warmer colors indicating a higher degree of assimilation.
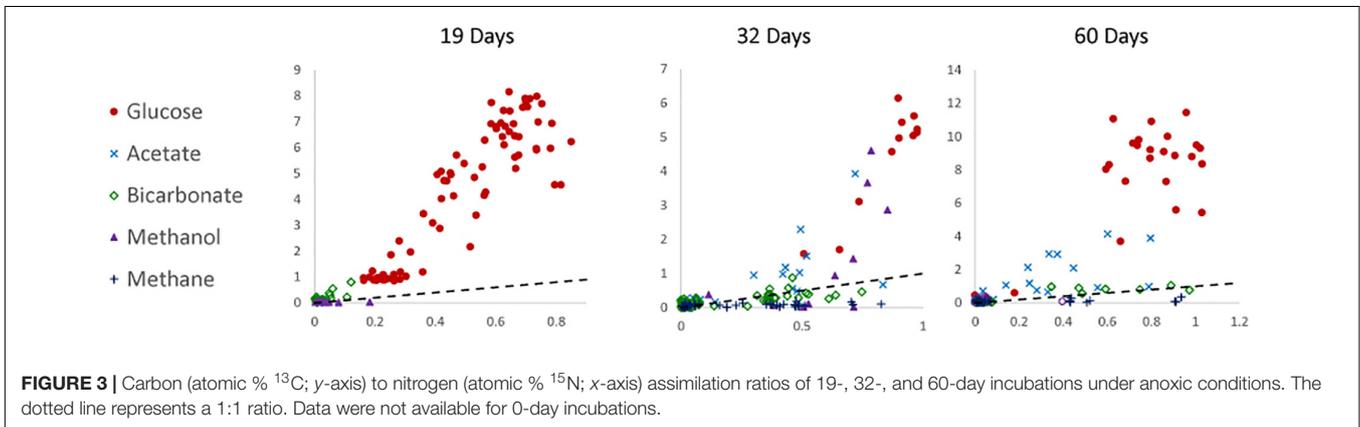
FIGURE 3 | Carbon (atomic % ^{13}C; y-axis) to nitrogen (atomic % ^{15}N; x-axis) assimilation ratios of 19-, 32-, and 60-day incubations under anoxic conditions. The dotted line represents a 1:1 ratio. Data were not available for 0-day incubations.

Mohan et al., 2013b, 2014; Cluff et al., 2014; Akob et al., 2015; Daly et al., 2016). In our research, isotopically labeled nutrients were used to determine the survival and revivability of microorganisms derived from a fluid produced from hydrofractured shales. The carbon compounds used were those that could be available in hydrofractured wells including carbohydrates, short-chain fatty acids, C1 compounds, and inorganic carbon. Glucose, acetate, and methanol are all compounds that may be derived from chemical additives. Methanol is added as a gel stabilizer, acid corrosion inhibitor or winterizing agent (Stringfellow et al., 2014; Daly et al., 2016; FracFocus Chemical Disclosure Registry, 2016). Some hydrofracturing additives can break down into simpler carbon compounds such as glucose or acetate (Stringfellow et al., 2014). Acetate can be an important carbon source in the subsurface if formed by thermal cracking or biodegradation of complex organic compounds found in the subsurface

(Horsfield et al., 2006). Methane and bicarbonate are naturally present in shale and dissolve into the produced fluids. The nitrogen source, ammonium, can be present naturally in shale as a substitute for potassium ions in illite, and present as ammonium chloride which may be added to hydrofracturing fluid as a scale inhibitor (Orberger et al., 2005; Environmental Protection Agency [EPA], 2012). We found that the produced fluid microorganisms, dominated by two genera of halophiles, were alive, revivable, and able to metabolize added compounds anabolically and catabolically under anoxic conditions.

Metabolism of Carbon Sources Under Anoxic Conditions

To determine which carbon compounds were metabolized under anoxic conditions, the concentration and ^{13}C-atomic % of DIC and CH$_4$ were analyzed. DIC concentrations were higher in
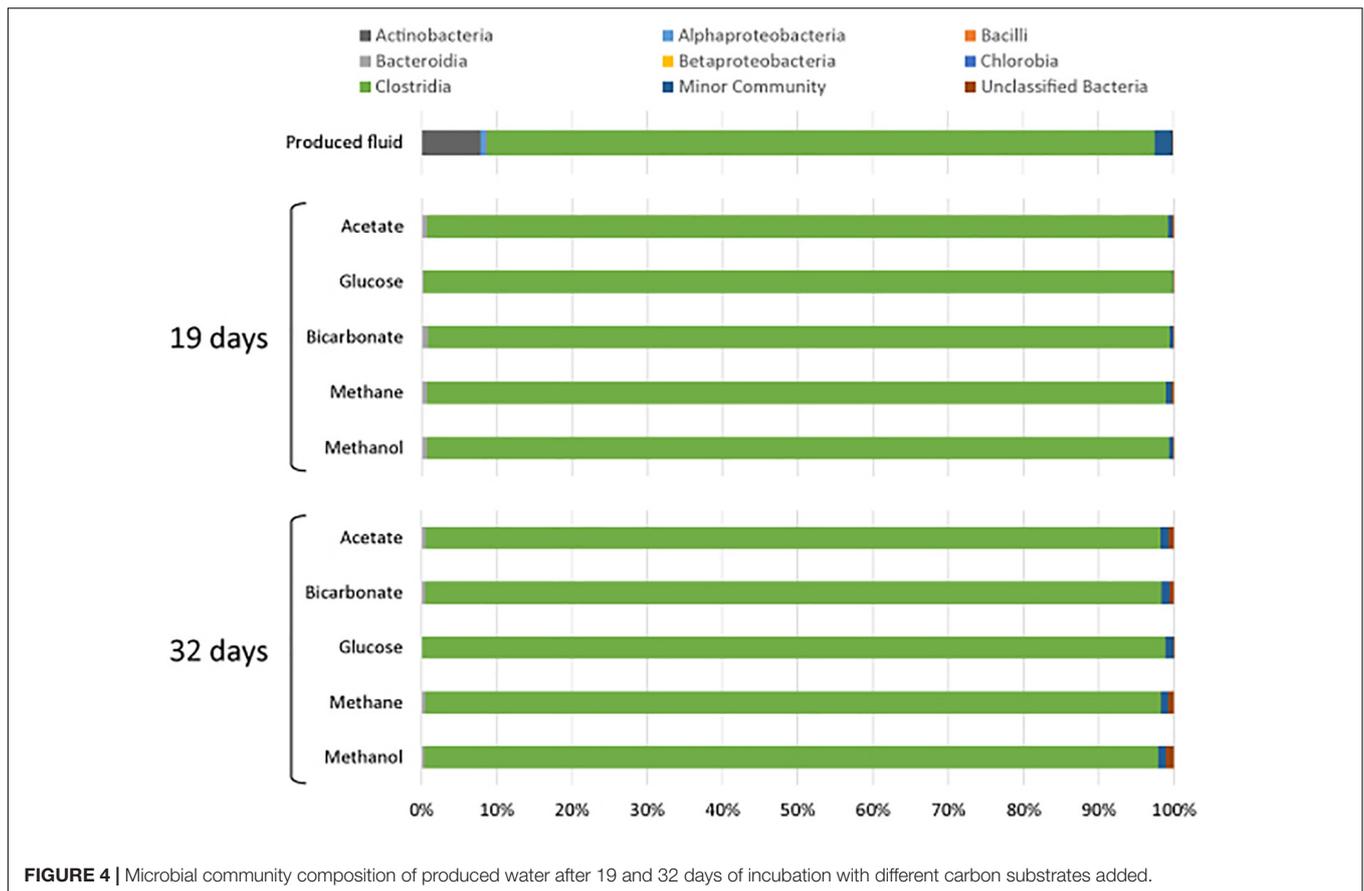
FIGURE 4 | Microbial community composition of produced water after 19 and 32 days of incubation with different carbon substrates added.

the no substrate added incubation (2.7 mM) than all killed control conditions (0.9∼1.0 mM) showing that the organic carbon originally present in produced fluid was metabolized by the microbial community. As discussed above, carbohydrates, short-chain fatty acids, C1 compounds, and inorganic carbon as well as nitrogen compounds such as ammonium salts and amines are all compounds that may be present in produced fluid (Stringfellow et al., 2014; FracFocus Chemical Disclosure Registry, 2016). Also, degradation of guar gum, a viscosifier used in injected fluids, by microorganism of *Halanaerobium* sp. can produce acetate (Liang et al., 2016).

The atomic % of ^{13}C-DIC in the sample containing ^{13}C-labeled glucose (1.40%) demonstrates the ability of microbes in the produced fluid to metabolize glucose. Fermentative glucose consumption was observed in *Halanaerobium* isolate (Booker et al., 2017). It is possible that anaerobic respiration in hydrofractured gas wells is similar to that observed in oil wells where ferrous iron or sulfate are often plentiful and commonly used as electron acceptors (Nazina et al., 1995; Röling et al., 2003; Struchtemeyer and Elshahed, 2012). Sulfate reducers are particularly troublesome in hydrofracturing wells because they produce hydrogen sulfide, which corrodes well casings and equipment (Bottero et al., 2010; Cord-Ruwisch et al., 2013; Booker et al., 2017; Lipus et al., 2017). On the other hand, fermentation has been used to enhance oil recovery through the release of metabolic byproducts such as acids, solvents, or gases

which can increase access to oil and increase its mobility in the formation (Desai and Banat, 1997). We observed a 2.1 mM increase in DIC concentration (to 4.8 mM) over the no substrate addition control (2.7 mM); however, the increase of atomic % of ^{13}C-DIC to 1.40% could not solely be explained by the metabolism of glucose. This suggests that, when added, glucose is metabolized as a carbon source but also may stimulate the activity of microorganisms to metabolize other organic compounds present in the produced fluid during incubation.

Incubations containing ^{13}C-labeled acetate showed a significant increase of DIC concentrations and atomic % of ^{13}C-DIC, which indicate anaerobic metabolism of both added acetate and other organic compounds in the produced fluid. High atomic % of ^{13}C-CH$_4$ was not observed in the incubation. Because acetoclastic methanogens are sensitive to high salinity environments (Waldron et al., 2007), they may be less active in hydrofractured systems than cells that possess other metabolic strategies.

There was a slight increase in atomic % of ^{13}C-DIC in incubations containing ^{13}C-labeled methanol. This is consistent with methanol incorporation into microbial cells, as carried out via methylotrophic metabolisms. Methylotrophs can use methane, methanol and other methylated compounds as their sole carbon and energy source (Chistoserdova, 2011). Incubations containing methanol yielded high levels of atomic % of ^{13}C-CH$_4$ by the end of

FIGURE 5 | Genera of the class Clostridia that were present in produced water after 19 and 32 days of incubations with different carbon substrates added.
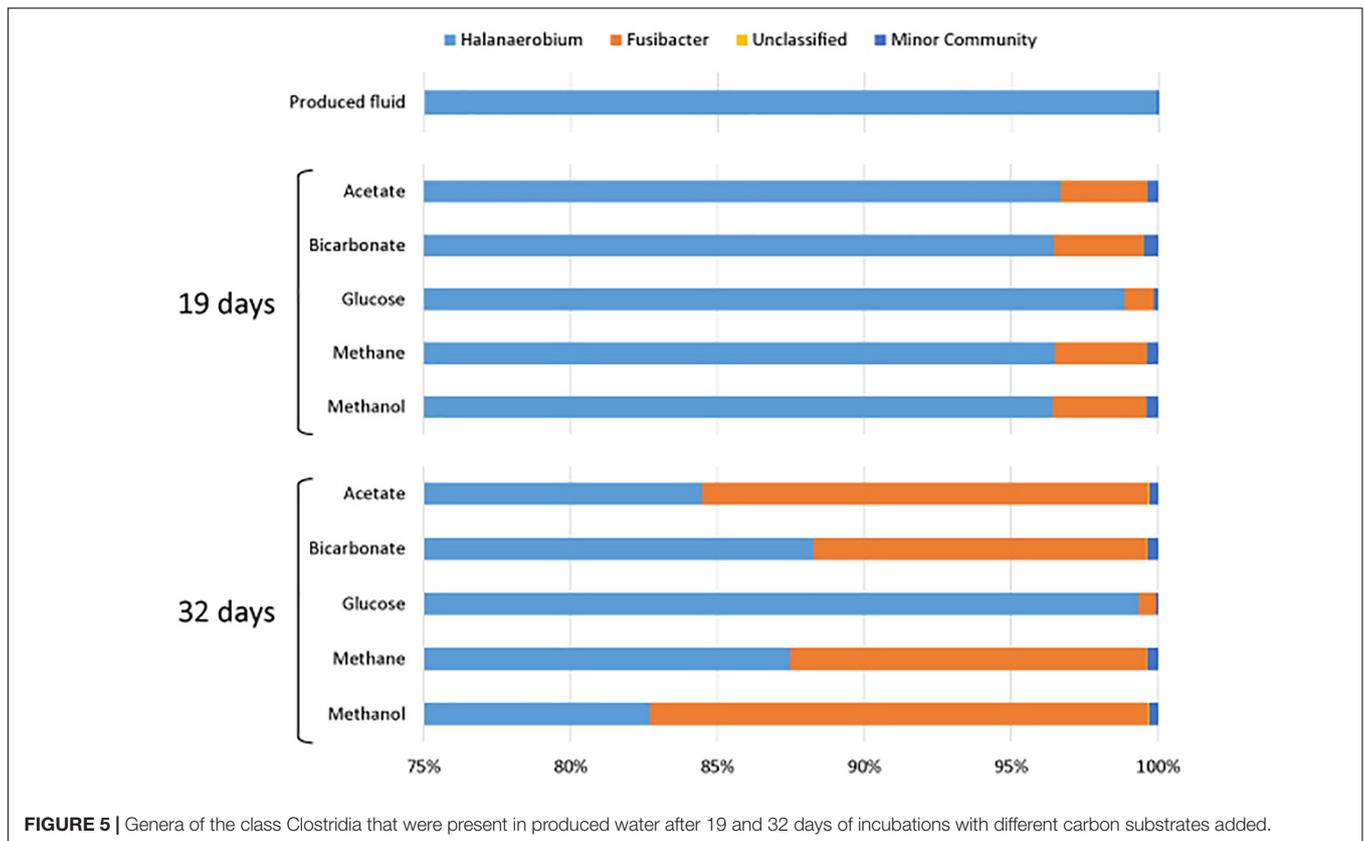
TABLE 2 | Sequencing and diversity estimates of the produced fluids for 19 or 32 days under anoxic conditions.

Carbon source	Time (days)	Number of sequences	Observed OTUs$_{0.03}$	Coverage (%)	Chao richness	ACE	Jackknife	Shannon	Simpson
Acetate	19	96,774	128	95	254	365	337	0.34	0.89
	32	63,893	67	97	100	177	99	0.63	0.69
Bicarbonate	19	98,732	103	97	209	343	221	0.34	0.89
	32	81,528	116	96	209	284	216	0.58	0.74
Glucose	19	89,707	57	97	99	109	104	0.12	0.96
	32	106,533	81	96	357	323	2106	0.13	0.96
Methane	19	110,675	86	96	156	209	163	0.38	0.87
	32	98,699	95	96	136	190	138	0.58	0.73
Methanol	19	84,115	88	97	145	213	154	0.33	0.89
	32	93,031	135	96	222	326	246	0.67	0.66

All diversity estimates were made at a 97% similarity level.

the 60-day incubation period compared to incubations with all other substrates, indicating high methanogenesis activity using methyl moiety.

The high atomic % of ^{13}C-CH$_4$ values in incubations containing ^{13}C-labeled bicarbonate suggest the occurrence of hydrogenotrophic methanogenesis.

Assimilation of Carbon and Nitrogen Under Anoxic Conditions

NanoSIMS was used to view carbon and nitrogen assimilation in individual cells and to compare the utilization of different

carbon sources by the microorganisms in the produced fluids under anoxic incubations. Anaerobic activity is expected as the produced fluid is derived from a highly reduced, anoxic subsurface environment. The produced fluid used in this sample had low oxygen saturation when the experiments were started. Aerobic microbial communities have been identified in hydrofracturing fluid prior to injection into the subsurface; however, facultative and strict anaerobes dominate in the fluids when they return from the subsurface (Mohan et al., 2011, 2013a; Cluff et al., 2014). Glucose was the most readily assimilated carbon source under anoxic conditions. Between 73 and 79% of the microbial cells in

the incubated samples showed assimilation of glucose and the highest assimilation of ^{13}C (~11.4%) was observed. A wide variety of anaerobic microorganisms, particularly fermenters possess phosphoenolpyruvate:carbohydrate transferase systems that can transport glucose into the cell and phosphorylate it, making it directly available for catabolic or anabolic reactions (Roseman, 1969; Romano et al., 1970, 1979). This finding demonstrates the ability of microorganisms in the produced fluids to utilize carbohydrates as their carbon source and is consistent with published evidence of microbes that possess genes related to carbohydrate metabolism or microbes capable of glucose use in produced fluids (Mohan et al., 2014; Daly et al., 2016; Booker et al., 2017). Polymer gels are added as proppant carriers and viscosity modifiers; however, high temperatures, extreme chemical conditions, or biological activity can degrade them to simple carbohydrates (Kahrilas et al., 2015). For example, guar gum is added in high concentrations to hydrofracturing fluid to adjust viscosity, and its degraded byproducts promote microbial activity downhole (Lester et al., 2014).

Acetate was the next most readily assimilated carbon source (**Table 1** and **Figure 3**) and is a common byproduct of fermentation in the subsurface (Lovley and Chapelle, 1995). Acetic acid, which is added to hydrofracturing fluid as a buffer, could also be a source of carbon (Stringfellow et al., 2014). Fermentative production of acetate was observed in a microcosm experiment dominated by *Halanaerobium* (Borton et al., 2018). Acetate can be assimilated by the glyoxylate cycle, alternate glyoxylate pathway, and glyoxylate regeneration cycle (Ensign, 2006) and is used by iron, sulfate, and nitrate reducing bacteria, all of which have been identified in the produced fluids from gas wells (Struchtemeyer and Elshahed, 2012; Mohan et al., 2013a,b). Acetoclastic methanogens can also assimilate acetate, but their presence may be unlikely in this setting because of their sensitivity to high salinity and to changes in salt concentration (Patel and Roth, 1977; Waldron et al., 2007; Winkel et al., 2014).

A particularly interesting result of our study was that microorganisms in the produced fluid assimilated carbon from methanol, a compound sometimes added to hydrofracturing fluid as a winterizer or chemical stabilizer. This result was observed after 32 days of incubation and not again at 60 days; however, such cells may have been present but below the limit of detection using NanoSIMS. This may suggest the presence of methylotrophs, which could also include methanotrophs and methanogenic archaea. We did not observe either anabolic or catabolic metabolism of methane during our study. This may have resulted because $^{13}CH_4$ was added as a gas without pressurization and was therefore less accessible by microorganisms. Methanotrophy should not be ruled out; however, rates of anaerobic oxidation of methane can be quite low (c.f., Orcutt et al., 2013) and may not have been detected during our relatively short incubations. Methane in the subsurface is dissolved into hydraulic fracturing fluid under pressure, increasing its solubility and making it readily available.

Carbon fixation was not significantly observed in cells incubated with bicarbonate. The maximum percentage of cells that were observed to assimilate ^{13}C-bicarbonate was only 1% at 60 days, but cells that assimilated bicarbonate also assimilated small amounts of nitrogen. This may suggest that autotrophy is not an energy efficient metabolism for microorganisms in hydrofracturing wells, at least under the conditions of our experiment. Ample carbonate (as calcium carbonate) often exists as natural fractures in shale, and can dissolve into solution upon the addition of hydrofracturing fluid (Gale and Holder, 2010). Sodium or potassium carbonate may be added to hydrofracturing fluid (Stringfellow et al., 2014) such that concentrations reach tens to hundreds of mg/L (Haluszczak et al., 2013; Lester et al., 2015). Produced fluids from the Marcellus shale contains an average of 165 mg/L of calcium carbonate (Barbot et al., 2013).

The ratios of ^{13}C and ^{15}N assimilation into microorganisms in the produced fluid showed that cells assimilating supplied nutrients incorporated more carbon than nitrogen although DIC measurements clearly showed metabolism of other non-labeled sources of carbon or nitrogen present in the produced fluid. This apparent preference for carbon was particularly noticeable in glucose incubations. This may be because nitrogen is more limiting than carbon in such environments, or alternatively that these microorganisms were incorporating high C/N ratio organic matter rather than ammonium. In our study, it seems unlikely that the produced fluid is carbon limited, considering the high organic content of shale. The C/N ratio of Devonian (Marcellus shale) age organic carbon is between 174.80 and 193.28 atomic C/N, and the C/N ratio of kerogen is even higher, making nitrogen the limiting nutrient (Orberger et al., 2005). Produced fluids from Marcellus shale were reported to have total organic carbon concentrations of between 1.2 and 1530 mg/L (Barbot et al., 2013). Also considering the length of these incubations, the incorporation of ^{13}C may have not come from the original carbon source but rather from cell debris that had previously incorporated the ^{13}C, which may have served as an additional nitrogen source. In this interpretation, there may have been some turnover of the originally incorporated labeled substrates. Heterotrophic bacteria can assimilate amino acids over ammonium as this allows them to conserve energy required to build amino acids (Kirchman et al., 1989; Morono et al., 2011).

Interpretation of microbial activity, including carbon and nitrogen assimilation, can be confounded by environmental conditions such as high salinity, high temperature, high pressure, or non-neutral pH that are common in subsurface environments (Postma and Jakobsen, 1996; Colwell et al., 1997; Fredrickson et al., 1997). Hydrofracturing produces a dynamic environment where these conditions vary depending on the stage of hydrofracturing and the residence time of the fluid downhole. Microbial cells may not be assimilating carbon and nitrogen for growth and replication, but may instead assimilate these elements mainly for cell maintenance. Extreme conditions increase the amount of energy that microorganisms need to repair or replace cellular components even if they are well adapted to the environment

(Hoehler, 2007; Morozkina et al., 2010). Furthermore, slow growth could be advantageous for cells in hydrofractured wells where biocides are used because slow growth and nutrient limitation can reduce microbial sensitivity to antimicrobials, which are commonly used in hydrofracturing (Mah and O'Toole, 2001).

Microbial Communities in Produced Fluid Incubations

Microbial community diversity determinations were conducted on the produced fluid samples incubated under anoxic conditions for 0, 19, and 32 days. The 0 day glucose sample had 50% of the sequences removed and this was also apparent in the microbial community, but less so than the 0 day no substrate control. It is suspected that these samples had poorer DNA recovery and PCR amplification than other samples.

Microbial diversity estimates indicated that the diversity in these samples was lower than what has been previously found in produced fluids and impoundment waters (Struchtemeyer et al., 2012; Mohan et al., 2013a,b). This may have resulted from conditions when the samples were collected, sample handling, or from the placement of these microbial communities into microcosms thereby eliminating the input and output of nutrients and metabolic waste products. Most of the microbial communities in this experiment were very similar, indicating that the addition of substrate did not affect the microbial community. The composition of the microbial community was similar to what has been observed in late stage produced fluids (Mohan et al., 2013a; Cluff et al., 2014; Liang et al., 2016; Booker et al., 2017).

All of the samples were dominated by Clostridia. This is in agreement with past observations wherein late stage produced fluids were dominated by *Clostridia*, specifically *Halanaerobium* (Mohan et al., 2013a; Cluff et al., 2014). Cluff et al. (2014) also reported other indicator genera that we detected including *Flexistipes*, and *Methanohalophilus*. *Halanaerobium* accounted for 80% or more of the *Clostridia* sequences in all of the incubations. *Halanaerobium* are anaerobic, halophilic, alkalophilic, and thermophilic bacteria and thus likely to be adapted to oil and gas wells environments. For example, *H. locusroseus* can grow in 5–30% NaCl, and at 20–50°C (Cayol et al., 1995). *H. congolense* in particular has been isolated from a number of oil wells and implicated in well corrosion due to hydrogen sulfide production by thiosulfate reduction (Magot et al., 2000; Ravot et al., 2006). *Halanaerobium* can use carbohydrates as carbon sources and therefore may have been responsible for the assimilation of glucose under anoxic conditions (Oren, 2002). As a fermenter, *Halanaerobium* produces acetate and CO_2, compounds that may sustain other microorganisms. *Halanaerobium* spp. have previously been detected in hydrofracturing fluids from separators, storage tanks and impoundments (Davis et al., 2012; Mohan et al., 2013a,b; Akob et al., 2015; Liang et al., 2016; Booker et al., 2017; Lipus et al., 2017).

Fusibacter was a prominent member of the microbial communities throughout this experiment. *Fusibacter* are also fermenters and can produce acetate by fermenting glucose (Ravot et al., 1999). This genus has been isolated from many hydrocarbon-rich environments and can produce hydrogen sulfide by reducing thiosulfate or elemental sulfur (Ravot et al., 1999; Agrawal et al., 2010; Ben Hania et al., 2012; Smii et al., 2015). Its role in carbon assimilation is likely similar to that of *Halanaerobium* in that it may use glucose as a carbon or energy source. Interestingly, *Fusibacter* began to increase in percentage of the community between 0 and 32 days, as did the number of sequences. Possibly, *Fusibacter* was less sensitive to conditions (e.g., product inhibition) in the microcosms than *Halanaerobium*. For example, the growth of *H. saccharolyticum* can be inhibited by an excess of acetate (Kivistö et al., 2012). Because *Halanaerobium* is an alkaliphile, the production of hydrogen during fermentation may have caused a reduction in pH, making the produced fluids environment in a closed system less habitable. However, some species of *Fusibacter* are also prone to product inhibition by hydrogen, unless thiosulfate is present (Ravot et al., 1999). The common finding of both *Fusibacter* and *Halanaerobium* in oil and gas reservoirs suggests that additional research should be conducted on their presence and potential interactions in these environments.

Halanaerobium and *Fusibacter* were the most abundant microorganisms, but their presence does not explain metabolic processes observed with carbon sources other than glucose. Minor members of the community may have played a larger part in metabolizing other carbon sources. Appearing in much lower abundance were the genera *Anaerophaga*, *Geotoga*, *Flexistipes*, *Asticcacaulis*, *Carboxydocella*, *Desulfotomaculum*, *Methanohalophilus*, and a number of unclassified microorganisms. Most of these organisms are halophilic, thermophilic, or both, and should be well adapted to gas well conditions. Representatives of *Anaerophaga* and *Geotoga* can ferment glucose and may have also contributed to respiration or assimilation of glucose (Davey et al., 1993; Denger et al., 2002). *Flexistipes*, a thermophile which requires at least 3% NaCl to grow, can utilize acetate as an energy source (Fiala et al., 1990; Nakano and Zuber, 2004). The presence of *Carboxydocella* is intriguing as it is known for its use of carbon monoxide (Sokolova et al., 2002; Slepova et al., 2006) which may be present in shales and may be removed by the produced fluid along with methane to the surface. *Desulfotomaculum* spp. are sulfate reducers often found in oil wells, and one species, *D. kuznetsovii*, is methylotrophic (Nazina et al., 1988; Nilsen et al., 1996). Recent reconstruction of the genome of *Methanohalophilus* from produced shale fluids indicated methanogenesis using methanol via methanol:MtaC co-methyltransferase (*MtaB*) (Daly et al., 2016). *Methanohalophilus* was detected in all of our incubations; however, the highest atomic % of $^{13}C\text{-}CH_4$ was observed in the methanol-amended incubation (34.54 and 36.13% at 32 and 60 days), strongly indicating methanogenesis by *Methanohalophilus* in our incubations. These findings are consistent with evidence presented by others showing a prominent role for microbes of this genus in produced fluids (Daly et al., 2016; Borton et al., 2018) and for their ability to use methylated compounds (Mathrani et al., 1988).

CONCLUSION

We found that microorganisms in incubations of produced fluid from a hydrofractured shale were revivable in laboratory incubations and retained the ability to metabolize added substrates catabolically and anabolically. When incubated anoxically, microbial communities were active and responsive to diverse carbon substrates. Following anoxic incubations, microbial communities were not diverse and many of the genera observed were related to microbes that have previously been detected in hydraulically fractured shale fluids or similar environments and that possess qualities such as resistance to high salinity, heat, and the presence of metals. An ideal sample set to examine microbial properties of shales would involve collecting and analyzing subsurface cores with attention to sampling technique to prevent microbial contamination. Such an effort would verify whether microbes that we studied and that others have detected exist in and are active in these formations. The microbes that we examined in the produced fluid were at least fractionally alive and may have the metabolic potential to affect subsurface geochemistry and to consume organics found in the subsurface or delivered during the hydraulic fracturing process.

AUTHOR CONTRIBUTIONS

JW, MT, CV, and FC designed the project. JW collected the samples. JW, YM, MI, AI, TH, and TT conducted the experiments, analyzed the samples, and interpreted the data. JW, YM, AI, FI, and FC wrote the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00376/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.