Microbial communities in flowback water impoundments from hydraulic fracturing for recovery of shale gas

Arvind Murali Mohan1,2, Angela Hartsock1, Richard W. Hammack1, Radisav D. Vidic1,3 & Kelvin B. Gregory1,2

1National Energy Technology Laboratory, Pittsburgh, PA, USA; 2Department of Civil and Environmental Engineering, Carnegie Mellon University, Pittsburgh, PA, USA; and 3Department of Civil and Environmental Engineering, University of Pittsburgh, Pittsburgh, PA, USA

Abstract

Hydraulic fracturing for natural gas extraction from shale produces waste brine known as flowback that is impounded at the surface prior to reuse and/or disposal. During impoundment, microbial activity can alter the fate of metals including radionuclides, give rise to odorous compounds, and result in biocorrosion that complicates water and waste management and increases production costs. Here, we describe the microbial ecology at multiple depths of three flowback impoundments from the Marcellus shale that were managed differently. 16S rRNA gene clone libraries revealed that bacterial communities in the untreated and biocide-amended impoundments were depth dependent, diverse, and most similar to species within the taxa c-proteobacteria, a-proteobacteria, d-proteobacteria, Clostridia, Synergistetes, Thermotogae, Spirochetes, and Bacteroidetes. The bacterial community in the pretreated and aerated impoundment was uniform with depth, less diverse, and most similar to known iodide-oxidizing bacteria in the a-proteobacteria. Archaea were identified only in the untreated and biocide-amended impoundments and were affiliated to the Methanomicrobia class. This is the first study of microbial communities in flowback water impoundments from hydraulic fracturing. The findings expand our knowledge of microbial diversity of an emergent and unexplored environment and may guide the management of flowback impoundments.

Introduction

Commercial development of natural gas and petroleum liquids from deep shale formations requires engineered permeability through hydraulic fracturing (Arthur et al., 2008; Veil, 2010). Hydraulic fracturing of shale is most commonly performed through the introduction of large volumes (c. 5000–20 000 m³) of aqueous fracturing fluid at sufficient rate and pressure to break the hydrocarbon-bearing formation and increase its permeability for optimal flow rates of gas and liquids into the well bore (Arthur et al., 2008; Gregory et al., 2011). After hydraulic fracturing, the pumping pressure is decreased, and fluid in the formation returns to the surface through the well casing (GWPC & ALL Consulting, 2009; Gregory et al., 2011). This water is referred to as ‘flowback’, and the period of flowback is variable but commonly lasts 2 weeks. The flowback is captured and temporarily impounded at the surface for durations ranging from a week to several months prior to treatment and disposal or reuse for subsequent hydraulic fracturing. The limited availability of flowback disposal options such as deep well injection (Arthur et al., 2008; Gregory et al., 2011) and the high costs and technical limitations associated with advanced flowback treatment technologies such as reverse osmosis, thermal distillation, and crystallization (Kargbo et al., 2010; Gregory et al., 2011) have made flowback water impoundments an important water management strategy during development of natural gas from the Marcellus shale.

Flowback from the development of shale gas contains fracturing fluid additives as well as suspended and dissolved constituents from the shale formation including high concentrations of salts and metals (Gregory et al., 2011; Barbot et al., 2013), dissolved and nonaqueous hydrocarbons, and in some locations naturally occurring...
radioactive material (NORM; Hill et al., 2004; Soeder & Kappel, 2009). In the Marcellus region, pretreatment of flowback water is frequently performed before impoundment and reuse to remove solids and precipitate divalent cations and sometimes to remove organic hydrocarbons. During impoundment, biocides and aeration are commonly utilized to control deleterious biogeochemical activity such as the production of reduced sulfur compounds (Kermani & Harrop, 1996; Little & Lee, 2007) and fermentation of hydrocarbons that produce organic acids (Roberge, 2000). These biogeochemical changes can lead to aesthetic concerns and health hazards to on-site workers due to toxic gas production (Davidova et al., 2001) and result in corrosion of infrastructure (Little et al., 2000; Roberge, 2000) that can complicate the water management strategy, increase production costs, and potentially limit disposal, treatment, or reuse options for flowback water.

There is little information on the microbial ecology of flowback water from hydraulic fracturing and none on the impoundments that are used for storage and treatment of flowback. Studies on microbial communities associated with fossil reservoir environments have largely focused on communities in well-head samples of production water recovered during oil extraction (Dahle et al., 2008; Gittel et al., 2009; Pham et al., 2009; van der Kraan et al., 2010; Ren et al., 2011). Only a limited number of studies have examined the microbiology associated with hydraulic fracturing operations. What is known is that bacteria in flowback may originate in the geologic formation, infrastructure and equipment for water handling, fracturing fluid amendments, or source water used to create the fracturing fluid (Fichter et al., 2008). Recent work by Struchtemeyer et al. (2011) determined that drilling fluid (a.k.a. drilling mud) utilized during drilling of horizontal wells in a deep shale reservoir can be a potential source of microorganisms in flowback. A related study of microbial communities in well-head samples from hydraulic fracturing of the Barnett Shale in Texas reported that some bacterial cells survived biocide amendments commonly utilized in hydraulic fracturing fluids (Struchtemeyer & Elshahed, 2012).

Regardless of the source of microorganisms, during impoundment, the microbial community will evolve in a way that reflects the water chemistry of the initial flowback (e.g. pH, TDS, electron donor, and acceptor concentrations), any pretreatment of the flowback (e.g. suspended solids removal, precipitation of divalent metals, and removal of organics), surface conditions (e.g. wind, rain, and sunlight), and any in situ treatment of the impounded water (e.g. biocide addition, aeration, and dilution). Despite the importance of microbial communities in the management of flowback water impoundments, there are no prior studies on the ecology of flowback water impoundments.

This research aims to introduce new understanding of the microbial communities that develop in flowback water impoundments from hydraulic fracturing for unconventional oil and gas recovery. Samples were collected from multiple depths in three impoundments having different pretreatment and management strategies. The bacterial and archaeal communities were characterized using 16S rRNA gene clone libraries and were enumerated using quantitative PCR (qPCR) and 4′, 6-diamido-2-phenylindole (DAPI) cell counts. Water quality parameters in the impoundments and at each depth are presented. Phylogenetic information was utilized to show relationships between the communities by UniFrac analysis and determine richness and diversity in each impoundment.

Materials and methods

Sample collection

Water samples were collected from three flowback water impoundments from hydraulic fracturing of the Marcellus shale in October 2010. The source water for hydraulic fracturing of each natural gas well was a mix of freshwater from a common source (c. 80–90%) and recycled flowback water (c. 10–20%). In the case of each of the wells, the fracturing fluid was a mixture of the source water, propellant (sand; 99.5% by volume), corrosion inhibitor, friction reducer, HCl (0.47% by volume), and glutaraldehyde (0.03% by volume for microbial control). Impoundments were about 4 m deep and contained c. 3 million liters of flowback water. The first impoundment received untreated flowback water (untreated impoundment). Flowback in the second impoundment was treated with glutaraldehyde (c. 150 mg L⁻¹) for microbial control 3 weeks prior to sampling (biocide-treated impoundment). The third impoundment received flowback that was pretreated for suspended solids removal, amended with sulfate for precipitation of barium and strontium, subjected to oxidation for iron removal, pH-adjusted, and was regularly aerated on-site (pretreated and aerated impoundment). Water was stored in the impoundments for c. 80 days prior to sampling.

Samples were collected in sterile, 1-L polypropylene bottles from the geometric center of each impoundment at 3 depths: the bottom, middle, and surface using a battery-powered pump connected to sterile tubing. Sampling bottles were filled without headspace and sealed using screw caps to prevent oxygen intrusion and were stored on ice during brief transportation and frozen at −80 °C within 5 h of sampling.
Analytical methods

Cations were analyzed by inductively coupled plasma optical emissions spectroscopy (ICP-OES) on a Perkin Elmer Optima 3000 Radial View spectrometer (Perkin Elmer, Waltham, MA) according to US EPA method 6010C. Sulfate, bromide, and nitrate were analyzed using a Dionex ICS-3000 ion chromatogram with an IonPac AS18 column (Sunnyvale, CA) according to EPA method 300.1 with 28 mM KOH as eluent at a flow rate of 1 mL min⁻¹. Iodide was measured at Columbia Analytical Services, Inc. (Rochester, NY) according to EPA method 300.0. Acetate, butyrate, and propionate were measured using HPLC techniques described elsewhere (Li et al., 2011).

Molecular analyses

Unfiltered samples were centrifuged at 6000 g for 30 min in an Avanti J-E centrifuge (Beckman Coulter, Brea, CA) to pellet the cells. DNA was extracted from cell pellets using a slight modification of a previously described method (Holmes et al., 2004). Briefly, cells were resuspended in TE/sucrose buffer (6.7% sucrose, pH 8), 10% SDS, proteinase K (20 mg mL⁻¹), and lysozyme solution (60 mg mL⁻¹) with incubation steps for 30 min and 1 h at 37 °C. Finally, DNA was extracted once with chloroform/isoamyl alcohol (25 : 24 : 1) and resuspended in 1 mL min⁻¹. DNA was extracted once with chloroform/isoamyl alcohol (25 : 24 : 1) and resuspended in PCR grade water.

16S rRNA gene fragments were amplified using the universal primer sets for Bacteria and Archaea: BAC 338F/907R (Lane, 1991; Amann et al., 1995) and ARCH 344F/915R (Casamayor et al., 2002). The composition of the PCR mixture and thermal cycling conditions are detailed in the Supporting Information. Clone libraries were constructed from PCR-amplified 16S rRNA gene fragments using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) using the M13F primer. Raw sequences were aligned to bacterial and archael SILVA databases using the Mothur program (http://www.mothur.org/; Schloss et al., 2009) and were checked for chimeras using the UCHIME program (Edgar et al., 2011) implemented within Mothur. Chimeric sequences and those with > 2.5% of ambiguous bases and/or < 450 bps were discarded. A total of 512 clones (Bacteria – 364, Archaea – 148) were selected for further analysis. Mothur was used to group sequences with ≥ 97% identity into operational taxonomical units (OTUs) and calculate the Chao and ACE estimates of species richness and Shannon–Weaver and Simpson measures of diversity. One representative sequence from each OTU was selected to assign taxonomical affiliations using the Naïve Bayesian Classifier tool (Wang et al., 2007) available in the Ribosomal Database Project (http://rdp.cme.msu.edu/) and the BLASTN algorithm in the NCBI rRNA database (Altschul et al., 1997).

Microscopy and cell counting

Cells were stained with 10 μg mL⁻¹ of DAPI (Sigma-Aldrich, St. Louis, MO) for 20 min in the dark at room temperature. Cells were filtered (127 mm Hg) onto 0.2 μm, 25-mm black polycarbonate membranes (Millipore, Isopore). Cellulose nitrate filters (0.6 μm, 25 mm; Whatman) were used as membrane support filters. Membrane filters were mounted in 4 : 1 Citifluor (AF-1; Ted Pella)/Vectashield (Vector Laboratories, Burlingame, CA) on glass microscope slides. Cells were viewed using a
Leitz Diaplan epifluorescent microscope equipped with a 100x PL FLUOTAR (N.A. 1.32) objective and a filter cube for DAPI fluorescence. For each sample, filtered volumes were adjusted to achieve 20–100 cells per field of view, and up to 500 cells or 20 distinct fields of view, whichever came first, were counted.

**Results**

**Aqueous geochemical characteristics**

Results presented in Table 1 reveal that the concentrations of cations and anions in the impoundments were similar to prior reports for flowback from hydraulic fracturing in the Marcellus (Blauch et al., 2009; Gregory et al., 2011; Barbot et al., 2013; Haluszczak et al., 2013), but higher than those observed in produced water from other reservoirs (Grabowski et al., 2005; Pham et al., 2009). Within the untreated and biocide-amended impoundments, concentrations of cations and anions were greater at the middle and bottom depths as compared to the surface depths, suggesting density separation. However, concentrations of ions in the impoundment that was pretreated and aerated were uniform with depth, likely a result of mixing during aeration. This is also supported by greater coefficient of variability values for ion concentrations across different depths in the untreated and biocide-amended impoundments as compared to the pretreated and aerated impoundment (Table S1). Acetate was detected at all depths of the biocide-amended impoundment (39–62 mg L\(^{-1}\)) and deeper depths of the untreated impoundment (32–76 mg L\(^{-1}\)). These values were comparable to concentrations reported in produced fluids from petroleum development (Grabowski et al., 2005). Acetate was not detected in the pretreated and aerated impoundment. The pH at all depths of the biocide-amended impoundment and the deeper depths of the untreated impoundment were slightly more acidic (pH 6.5–6.8) than the surface depth of the untreated impoundment and all depths of the pretreated and aerated impoundment (pH 7.2).

**UniFrac clustering, microbial abundance, and diversity**

Clustering by the UniFrac metric grouped samples by impoundment and depth with strong jackknife support (Fig. 1). Within the untreated and biocide-amended impoundments, sequences from the middle and bottom depths clustered more closely to each other than to sequences at the surface. In the untreated impoundment, quantification of bacterial 16S rRNA gene copy numbers using q-PCR indicated an increase in bacterial gene concentration with depth (\(10^5\)–\(10^9\) copies of 16S rRNA gene mL\(^{-1}\); Table 2). Results from qPCR enumeration correlated well with the trend in total cell counts determined using DAPI, which indicated an increase in microbial concentration with depth (\(10^6\)–\(10^8\) cells mL\(^{-1}\); Table 2). Archaeal gene copy numbers (\(10^6\) copies of 16S rRNA gene mL\(^{-1}\)) in the bottom depth of the untreated impoundment were three orders of magnitude lower than the corresponding bacterial gene concentration. In the biocide-amended impoundment, bacterial gene concentrations (\(10^9\) copies of 16S rRNA gene mL\(^{-1}\)) were uniform throughout the impoundment and were greater than the corresponding archaeal gene concentrations (\(10^6\)–\(10^9\) copies of 16S rRNA gene mL\(^{-1}\); Table 2). DAPI cells counts also estimated uniform microbial concentrations

<table>
<thead>
<tr>
<th>Element</th>
<th>Concentrations in mg L(^{-1})</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Untreated impoundment</td>
</tr>
<tr>
<td></td>
<td>Surface</td>
</tr>
<tr>
<td>Ba(^{2+})</td>
<td>277</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>6150</td>
</tr>
<tr>
<td>Total Fe</td>
<td>0.3</td>
</tr>
<tr>
<td>K(^+)</td>
<td>190</td>
</tr>
<tr>
<td>Na(^+)</td>
<td>14 250</td>
</tr>
<tr>
<td>SO(_4^{2-})</td>
<td>894</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>35 100</td>
</tr>
<tr>
<td>Br(^-)</td>
<td>371</td>
</tr>
<tr>
<td>I(^-)</td>
<td>5.6</td>
</tr>
<tr>
<td>NO(_3^-)</td>
<td>ND*</td>
</tr>
<tr>
<td>SO(_4^{2-})</td>
<td>15.1</td>
</tr>
<tr>
<td>Acetate</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ND, non detectable concentrations.
throughout the biocide-amended impoundment (10⁶ cells mL⁻¹). In the pretreated and aerated impoundment, results from qPCR and DAPI cell counts indicated uniform microbial concentrations across all depths (10⁶ copies of 16S rRNA gene mL⁻¹ and 10⁶ cells mL⁻¹; Table 2). Cell numbers calculated from DAPI were compared with qPCR equivalent cell counts estimated using 4.2 copies of the 16S rRNA gene per cell for Bacteria and 1.7 for Archaea (http://rrndb.mmg.msu.edu/index.php). Cells numbers from both techniques were within an order of magnitude at all depths in each of the impoundments.

In the untreated and biocide-amended impoundments, the overall bacterial diversity was greater than the corresponding archaeal diversity estimates (Table 3). Archaeal sequences were not recovered from the pretreated and aerated impoundment. Within each of the three impoundments, bacterial diversity increased with depth (Table S2). In the untreated and biocide-amended impoundments, Good’s estimates indicated a greater coverage for the archaeal clone library (98–99%) than the bacterial clone library (78–87%; Table 3). The relatively low bacterial coverage (78%) in the untreated impoundment compared to the other impoundments (87–93%) suggests that further screening of clones may have resulted in a greater number of OTUs and an even higher diversity and species richness estimates (Table 3). However, the majority of the bacterial sequences recovered in each of the impoundments could be grouped under a small number of OTUs, suggesting that the PCR clone library identified the dominant bacterial communities.

Microbial community analysis

Distinct bacterial and archaeal communities were identified at different depths of the flowback impoundments...
A detailed description of bacterial and archaeal 16S rRNA gene sequences identified in these impoundments is presented in Tables S4 and S5. Sequence similarity of clones recovered in this study to previously characterized species is reported at the 98–100% level unless specifically mentioned. Also, it is interesting to note that there were no OTUs shared between all three impoundments (Fig. 4). The untreated impoundment shared seven and five different species with the biocide and pretreated and aerated impoundments, Table 3.

Table 3. Diversity and richness estimates* of bacterial and archaeal OTUs* in the three impoundments

<table>
<thead>
<tr>
<th>Impoundment and domain</th>
<th>No. of sequences†</th>
<th>OTU&lt;sub&gt;0.03&lt;/sub&gt; Coverage (%)</th>
<th>Chao richness</th>
<th>Abundance-based coverage estimation richness</th>
<th>Simpson diversity index</th>
<th>Shannon diversity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria</td>
<td>116</td>
<td>32</td>
<td>78</td>
<td>332</td>
<td>259</td>
<td>0.18</td>
</tr>
<tr>
<td>Archaea</td>
<td>51</td>
<td>4</td>
<td>98</td>
<td>4</td>
<td>4.53</td>
<td>0.32</td>
</tr>
<tr>
<td>Biocide-amended</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria</td>
<td>111</td>
<td>22</td>
<td>87</td>
<td>52</td>
<td>113</td>
<td>0.23</td>
</tr>
<tr>
<td>Archaea</td>
<td>97</td>
<td>4</td>
<td>99</td>
<td>4</td>
<td>4.83</td>
<td>0.84</td>
</tr>
<tr>
<td>Pretreated and aerated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria</td>
<td>137</td>
<td>19</td>
<td>93</td>
<td>34</td>
<td>42</td>
<td>0.48</td>
</tr>
</tbody>
</table>

*All diversity and richness estimates are based on ≥ 97% sequence identity. The 95% confidence interval values for diversity and richness estimates are presented in Tables S6 and S7.
†Sequences from surface, middle and bottom depths for bacteria and archaea were pooled together to analyze diversity and richness estimates.
‡Archaea were detected only in the untreated and biocide amended impoundments.

(Figs 2 and 3). A detailed description of bacterial and archaeal 16S rRNA gene sequences identified in these impoundments is presented in Tables S4 and S5. Sequence similarity of clones recovered in this study to previously characterized species is reported at the 98–100% level unless specifically mentioned. Also, it is interesting to note that there were no OTUs shared between all three impoundments (Fig. 4). The untreated impoundment shared seven and five different species with the biocide and pretreated and aerated impoundments,
respectively. Between the untreated and pretreated and aerated impoundment, the shared species include the abundant *Halanaerobium* sequences (discussed below).

**Untreated Impoundment**

This impoundment received untreated flowback water and was not subjected to any pretreatment prior to sampling. The bacterial community at the surface depth was dominated (83%) by the class *α*-proteobacteria (Fig. 2). The majority of sequences (90% of the *α*-proteobacteria; 75% of the total bacterial community) were affiliated to the genus *Roseovarius* and were highly similar to aerobic iodide-oxidizing bacteria (IOB) *Roseovarius* sp. 2SS-5 previously isolated from seawater (GenBank accession no. AB114422) and *Roseovarius* sp. IOB-12 isolated from iodide-rich brines (GenBank accession no. JF417974; Table S4). *α*-proteobacteria constituted a minor fraction of the bacterial community (3%) at deeper depths. Sequences similar to *γ*-proteobacteria constituted the major bacterial class in the middle (60%) and bottom (46%) depths. The majority of sequences (91–95%) of the...
γ-proteobacteria; 42–57% of the total bacterial community) identified in these depths were most similar to species within the genus *Marinobacterium* isolated from oil field produced fluids (GenBank accession no. HM041920). The closest related cultured strain was *Marinobacterium georgiense* strain KW-40, a strict aerobe capable of utilizing a wide range of carbon sources including carbohydrates, amino acids, aromatic compounds, and methanol (Gonzalez et al., 1997). Although previously thought to be a strict aerobe, *Marinobacterium* species have been reported in anaerobic cultures from oil field produced water (Pham et al., 2009). Sequences similar to the class Clostridia were identified only at the middle (8%) and bottom (15%) depths. The majority of sequences (63% of the Clostridia; 6% of the total bacterial community) identified in the middle depth were highly similar to the species *Halanaerobium congolense* SEBR 422 (GenBank accession no. NR_026044), isolated from an African oil field (Ravot et al., 1997). Members of the genus *Halanaerobium* are halophilic fermentative organisms known for their ability to ferment saccharides to H₂, CO₂, and acetate (Ollivier & Cayol, 2005). Additionally, the type strain *H. congolense* is known to reduce thiosulfate and elemental sulfur to sulfides (Ravot et al., 1997). Clostridial sequences identified at the bottom depth were highly similar to species from five genera including *Fusibacter*, *Alkalibacter*, *Geosporobacter*, *Halanaerobium*, and Clostridium.

Sequences affiliated to *Thermotoga* were identified only in middle (9%) and bottom (6%) depths. These clones had the greatest similarity to *Geotoga petraea* (GenBank accession no. HM037999) and an uncultured *Thermotoga* (GenBank accession no. EU721761), identified in an Alaskan oil well (Pham et al., 2009). *Geotoga petraea* is a moderately thermophilic heterotroph that grows over a broad range of salt concentrations up to 10% NaCl (Ollivier & Cayol, 2005). Species within *Thermotoga* can ferment complex hydrocarbons to H₂ and acetate and reduce thiosulfate and sulfur to sulfide (Youssef et al., 2009). Sequences affiliated to *Spirochetes* (12%) were detected exclusively at the bottom depth of the impoundment and were similar to uncultured species previously identified in oil wells and associated produced fluids (GenBank accession nos GU179808, HM041923, AY800103). Sequences that were unclassifiable at the class/phyla level were identified at the middle (6%) and bottom (15%) depths. The majority of these sequences representing only 3–9% of the total bacterial community were 93% similar to uncultured bacteria identified in sediments from the Sea of Japan (GenBank accession no. AB121107).

*Archaea* were detected only at the bottom depth of the impoundment. A majority of the archaeal sequences recovered (41%; Fig. 3) were affiliated with the genus *Methanoplanus* and had the greatest similarity to *Methanoplanus limicola* (GenBank accession no. AB546259; Table S5). *Methanoplanus limicola* is a hydrogenotrophic methanogen with an obligatory acetate requirement for growth (Wildgruber et al., 1982). A strain of *M. limicola* was isolated previously from a corroded natural gas pipeline (Mori et al., 2010). Sequences affiliated to the genus *Methanohalophilus* constituted 26% of the archaean community in the untreated impoundment. These sequences were highly similar to the species *Methanohalophilus mahii*, a methylotrophic halophile isolated from anaerobic sediments in the Great Salt Lake, Utah (Pater & Smith, 1988; GenBank accession no. CP001994).

**Biocide-amended impoundment**

Flowback in this impoundment was treated with 150 mg L⁻¹ of glutaraldehyde for microbial control 3 weeks prior to sampling. Sequences affiliated with *Clostridia* constituted a major fraction of the bacterial community (42–53%) at all depths of the impoundment (Fig. 2). The majority of these sequences (88–100% of the Clostridia; 37–53% of the total bacterial community) were highly similar to the species *M. congolense* SEBR 4224 (Table S4) that was also detected in deeper depths of the untreated impoundment. The widespread identification of this species in high-salinity environments (van der Kraan et al., 2010; Davis et al., 2012) may be due to its energy-efficient strategy of accumulating K⁺ to maintain osmotic balance (van der Kraan et al., 2010; Oren, 2010). Sequences affiliated with the γ-proteobacteria constituted the major bacterial class at the surface cline of the impoundment (44%) and decreased with depth (Fig. 2). The majority of these sequences (68% of the γ-proteobacteria; 30% of the total bacterial community) at the surface depth were similar to *Marinobacter hydrocarbonoclasticus* P721(1) isolated from seawater (Gauthier et al., 1992; GenBank accession no. GU370116). The type strain of *M. hydrocarbonoclasticus* is a facultative anaerobe that utilizes hydrocarbons as a sole source of carbon and energy over a broad range of salinity (0.08–3.5 M NaCl; Gauthier et al., 1992). δ-proteobacteria were identified only in middle and bottom depths (12–13%). Most of the δ-proteobacterial sequences (62–75%) recovered were similar to the *Desulfobacter halotolerans* DSM 11383, an anaerobic sulfate-reducing organism that oxidizes acetate and H₂ (Brandt & Ingvorsen, 1997; GenBank accession no. AB121107).
NR_026439). Synergista-related sequences were detected only at the bottom depth of the impoundment (11%), and all sequences shared relatively low similarity (90%) to the strictly anaerobic fermentative organism Aminobacterium colombiense, an amino acid-fermenting organism isolated from an anaerobic lagoon of a wastewater treatment plant (Chertkov et al., 2010; GenBank accession no. CP001997). Sequences that were unclassifiable at the class/phyla level accounted for 19–21% of the total bacterial community in the middle and bottom depths. The majority of these sequences (14–18%) were similar at a 93% level to an uncultured bacterium identified in sediments from the Sea of Japan (GenBank accession no. AB121107).

Archaea were detected only in the middle and bottom depths of the biocide-amended impoundment. Sequences were similar to species within the genera Methanolobus, Methanohalophilus, Methanoplanus, and Methanoebacterium that were also detected in the untreated impoundment. The majority of the archaeal sequences (89–94%; Fig. 3) were most similar to the halotolerant, methylotrophic species M. mahii (GenBank accession no. CP001994; Table S5). Sequences similar to the species M. limicola constituted 2% of the archaeal community at the middle depth, but increased to 9% at the bottom depth.

Pretreated and aerated impoundment

Flowback water in this impoundment was pretreated for removal of metals and suspended solids, pH-adjusted, and regularly aerated on-site. The clone library analyses reveal that unlike the untreated and biocide-amended impoundments, the bacterial community was homogeneous with depth and dominated (80–91%) by sequences with the greatest similarity to α subdivision of the Proteobacteria (Fig. 2). The majority of these sequences (79–85% of the α-proteobacteria; 65–74% of the total bacterial community) were closely affiliated with the genus Roseovarius and were most similar to aerobic, IOB such as Roseovarius sp. 2S5-2 and Roseovarius sp. IOB-12 isolated from seawater (GenBank accession no. AB114422) and iodide-rich brines (GenBank accession no. JF417974; Table S4). Sequences similar to the gamma subdivision of the Proteobacteria were identified at all depths of the impoundment and ranged from 7% to 16% of the community. Most of the γ-proteobacterial sequences (36–71%) were similar to an uncultured γ-proteobacterium identified in gas field produced water (GenBank accession no. HM755873). Sequences related to Bacteroidetes and Planctomycetes constituted a minor fraction of the bacterial community in this impoundment. Archaea were not detected at any depth in the pretreated and aerated impoundment.

Discussion

Results from the clone library analyses reveal that the dominant microbial communities in impoundments were similar to halotolerant species among α- and γ-subdivisions of the Proteobacteria and Firmicutes previously identified in oil and gas brines (Pham et al., 2009; van der Kraan et al., 2010; Davis et al., 2012; Zhao et al., 2013). This correlates well with the estimated impoundment salinity range of 3.1–5.4% (based on Na+ and Cl− concentrations), comparable to flowback and produced waters from other oil and gas wells (van der Kraan et al., 2010; Davis et al., 2012; Barbot et al., 2013). The finding of robust communities of halotolerant species suggests that salinity was likely an important selective pressure for emergence of specific microbial groups as observed in other saline environments (Ahn et al., 2009). High salinity in the impoundments may preclude significant colonization by organisms that originate in freshwater makeup for hydraulic fracturing fluids or those that are transported by deposition from other surface sources (e.g., from air particulates or runoff). It is worth noting that the makeup water for hydraulic fracturing fluids that fed impoundments considered in this study contained between 10% and 20% flowback water from previous fracturing operations. The reuse of flowback for subsequent hydraulic fracturing is now common practice in the Marcellus region (Gregory et al., 2011). It is likely that the microbial community in the recycled flowback served as a seed for these impoundments. Furthermore, the recycling of flowback may lead to the selection of organisms that are resistant to certain biocide treatments (Russel et al., 1997; Chapman, 1998; Struchtemeyer & Elshahed, 2012) and ultimately increase costs associated with microbial control.

Geochemical and ecological stratification was observed in the untreated and biocide-amended impoundments. Distinct microbial communities were observed in each depth of these impoundments. Results from UniFrac grouping (Fig. 1) and stratified diversity data (Table S2) also support this observation. In addition, UniFrac groupings also showed that microbial communities within the middle and bottom depths in each of these impoundments were more similar to each other than the surface. This stratification is likely reflective of a stratification of metabolic capabilities driven by variable energy sources, nutrients, and carbon available in each cline. Further, the microbial diversity in impoundments increased with depth, similar to the trend observed in other stratified saline aqueous environments such as the Clipperton Atoll (Galand et al., 2012). However, the aerated and pretreated impoundment had uniform geochemical concentrations and was dominated by aerobic α-proteobacteria.
(Roseovarius sp.) at all depths; a finding most likely the result of mixing in the water column during aeration (Symons et al., 1967).

A majority of the sequences recovered from the aerated and pretreated impoundment and the surface cline of the untreated impoundment were highly similar to species isolated in the genus Roseovarius. These species are known for their heterotrophic, iodide-oxidizing ability to produce both free and organic iodine in the presence of oxygen (Amachi et al., 2005). Previous studies of microbial communities in iodide-rich natural gas brines revealed populations of IOB (Arakawa et al., 2012). The iodide concentrations in the impoundments (4.4–10.3 mg L⁻¹) were comparable to concentrations reported in the above study. Related studies have attributed the dominance of IOB in natural gas brines to iodine production by these organisms (Amachi et al., 2005; Arakawa et al., 2012). The iodine forms that are produced by Roseovarius have bactericidal and sporicidal properties (McDonnell & Russell, 1999). The finding of relatively low bacterial diversity and species richness in the pretreated and aerated impoundment (Table 3) may be the result of iodide-oxidizing activity by Roseovarius, but this hypothesis remains to be examined. Additionally, their potential role in the corrosion of iron and steel infrastructure (Wakai et al., 2008) suggests that water management strategies that may lead to the enrichment of iodide-oxidizing populations should be avoided.

Taxonomical analysis of the untreated and biocide-amended impoundments revealed multiple sulfidogenic taxa that may utilize sulfate, sulfur, thiosulfate, and sulfite; a finding similar to a previous report on populations in produced water from the Barnett Shale in Texas (Davis et al., 2012). Clones recovered from the untreated and biocide-amended impoundments included populations most similar to known sulfidogenic organisms such as H. congolense (Ravot et al., 1997), Thermotogae (Youssef et al., 2009), and D. halotolerans (Brandt & Ingvorsen, 1997); all of which may produce sulfide from nonsulfate oxidants. In the untreated and biocide-amended impoundments, fermentative species within the classes Clostridia, Thermotogae, and Synergistia capable of producing organic acids (Ravot et al., 1997; Youssef et al., 2009; Chertkov et al., 2010) were also identified. Control of sulfidogenic and acid-producing organisms is of great interest due to aesthetic and human health concerns associated with sulfide production and microbial corrosion of metal infrastructure associated with acid production (Roberge, 2000). Standard assays used in the oil and gas industries to determine sulfidogenic potential of produced waters target sulfate-reducing bacteria and miss organisms capable of producing sulfide from other sulfur sources (Duncan et al., 2009). Further, media used in these culturing-based assays might not be suitable for growth of bacteria from these complex environments (NACE standard TM0194, 2004), thereby underestimating the sulfidogenic and acid-producing potential of flowback water impoundments.

Archaeal sequences recovered from the untreated and biocide-amended impoundments were similar to halophilic, methylotrophic, and hydrogenotrophic methanogens identified in a variety of oil and gas environments. Although acetate was detected in the lower depths of these impoundments, acetoclastic methanogens were not identified. Acetoclastic methanogenesis can be inhibited in high-chloride waters (1.02–2.27 M Cl⁻; Waldron et al., 2007) such as those in the impoundments. The majority of the archaean community (89–94%) in the biocide-amended impoundment and a significant fraction of the community in the untreated impoundment (26%) were similar to M. mahii, capable of converting methanol and methylamines to methane (Paterek & Smith, 1988). The abundance of these sequences may be attributable to their energy-efficient strategy of accumulating K⁺ ions in the cytoplasm to balance osmotic stress (Spring et al., 2010) and the addition of methanol as a fracturing fluid additive. In the untreated impoundment, hydrogenotrophic methanogens such as M. limicola and Methanocalculus halotolerans formed a significant fraction of the archaean community (43%); however, their relative abundance decreased in the biocide-amended impoundment (6–9%). This might be due to the presence of putative sulfate-reducing δ-proteobacterial populations in the lower clines of the biocide-amended impoundment that compete with hydrogenotrophic methanogens for hydrogen (Oremland & Polcin, 1982). The presence of fermentative organisms that metabolize hydrocarbons to acetate, H₂, and CO₂ and of hydrogenotrophic methanogens that convert H₂/CO₂ to methane suggests that syntrophic interactions may be important for hydrocarbon degradation in flowback impoundments (Grabowski et al., 2005).

Treatment of flowback water to limit deleterious microbial activity and reduce concentrations of divalent cations is common in the Marcellus region to sustain recycling and reuse of flowback water (Gregory et al., 2011). Aeration is also commonly used in the industry as a means of microbial control (Tischler et al., 2010). Aeration and mixing of the environment is likely to reduce the number of niches in the impoundment by preventing the establishment of dissolved oxygen and redox gradients and by removing other nutrient gradients through mixing (Symons et al., 1967; Fast, 1973). Aeration and pretreatment of the impoundment resulted in uniform concentrations of anions, cations, microbial biomass, and the emergence of a homogenous aerobic microbial community with depth. The aerated impoundment also lacked
anaerobic species that are commonly detected in produced and flowback waters (Li et al., 2007; Pham et al., 2009; Davis et al., 2012; Struchtemeyer & Elshahed, 2012). Pretreatment for metal removal likely lowered concentrations of iron and barium relative to prior reports on water quality characteristics of flowback from the Marcellus (Blauch et al., 2009; Chapman et al., 2012; Barbot et al., 2013). The high sulfate concentrations observed in the pretreated and aerated impoundment (c. 235 mg L\(^{-1}\)) are indicative of residual sulfate following precipitation for barium removal.

Biocides are an important component of microbial control during hydraulic fracturing (Fichter et al., 2008). The effect of glutaraldehyde addition on the microbial community in the biocide-amended impoundment was not apparent at the time of sampling, c. 20 days after biocide addition. Results from q-PCR and DAPI enumeration show that the biocide-amended impoundment had the highest average biomass across different depths. Glutaraldehyde kills cells by attaching to amino groups and cross-linking the cell wall (Gorman et al., 1980; Russell, 1994). However, glutaraldehyde is deactivated by reaction with nucleophilic groups including thiols, alcohols, amines, and sulfides (Williams & Mcginley, 2010). Further, glutaraldehyde is considered readily biodegradable in freshwater environments as defined by OECD 301A test and also exceeds the passing criteria for biodegradability in seawater environments as defined by OECD 306 test (Leung, 2001). The high microbial concentrations in the biocide-amended impoundment may be partially explained by reduced concentration and/or bioavailability of glutaraldehyde for microbial control. The high cell numbers in biocide-amended impoundment may also be the result of additional soluble carbon provided by the dead cells to heterotrophs that survived treatment. A previous study of flowback water revealed that commercial biocides did not completely eliminate the microbial population (Struchtemeyer & Elshahed, 2012).

This study characterizes the geochemistry and microbial ecology of flowback water impoundments from natural gas extraction operations in the Marcellus shale. Elevated concentrations of divalent metals, bromide, and chloride detected in impoundments emphasize the need for safe handling and disposal of flowback water to avoid environmental contamination. Similarly, treatment to remove divalent cations before reuse may prevent scaling in equipment that reduces gas production rates (Gregory et al., 2011). Results from microbial characterization showed the presence of a depth-dependent and diverse microbial community in the untreated and biocide-amended impoundments with populations that resembled those in other oil and gas environments, including fermentative and sulfidogenic bacteria, and methanogenic archaea. The variation in microbial community structure in impoundments emphasizes the need for sampling at multiple depths, rather than simple surface grab samples, for a thorough understanding of the microbial populations. High microbial counts in the biocide-amended impoundment underscore the importance of better understanding biocide efficacy in flowback and produced water environments. Reuse of flowback water for subsequent hydraulic fracturing is an emerging water management strategy. Given the shortcomings of biocides in these complex solutions, there is a significant risk for seeding deleterious microbial populations in the formation when reusing impounded water. Results suggest that mixing and aeration homogenizes the microbial community and chemistry and prevents establishment of the deleterious anaerobic microorganisms. Identification of diverse, sulfidogenic, and acid-producing populations emphasizes the need to reconsider standard culture-based techniques used by the oil and gas industries that target only a small group of bacteria. Detection of sequences that could not be assigned class-level affiliations, at the confidence level of interest, in the untreated and biocide-amended impoundments suggests that novel bacteria whose ecological significance and metabolic capabilities are not well understood may exist in these environments. Identification of no common, dominant species between the three impoundments located in the same county suggests that microbial communities emerging in these engineered environments may be based on factors other than regional environmental ones, including treatment strategy, surface conditions, composition of the hydraulic fracturing fluid, and/or the microbial community present in formation water.

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### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Data S1.** Materials and methods.

**Table S1.** Mean, standard deviation (Std Dev) and coefficient of variation (CV) values for element concentrations across the different depths of all three impoundments.

**Table S2.** Diversity and richness estimates\(^a\) of bacterial OTUs at different depths of the three impoundments.

**Table S3.** Diversity and richness estimates\(^b\) of archaeal\(^a\) OTUs at different depths of the biocide amended and untreated impoundments.

**Table S4.** Phylogenetic affiliations, sequence similarity, isolation source and relative abundance of bacterial OTUs (≥ 97% sequence identity) representing the bacterial community at surface (0 m), middle (1.5–1.8 m) and bottom (3.4–4 m) depths of the three impoundments.

**Table S5.** Phylogenetic affiliations, sequence similarity, isolation source and relative abundance of archaeal\(^b\) OTUs (≥ 97% sequence identity) at the middle (1.8 m) and bottom (3.4–4 m) depths of the untreated and biocide amended impoundments.

**Table S6.** Chao, ACE, Shannon and Simpson estimates of richness and diversity for bacterial sequences (combined from all three depths) in the three impoundments with 95% confidence measures.

**Table S7.** Chao, ACE, Shannon and Simpson estimates of richness and diversity for Archaeal sequences in the biocide amended and untreated impoundments with 95% confidence measures.