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Aerobic biodegradation of organic compounds in hydraulic fracturing fluids

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Abstract Little is known of the attenuation of chemical mixtures created for hydraulic fracturing within the natural environment. A synthetic hydraulic fracturing fluid was developed from disclosed industry formulas and produced for laboratory experiments using commercial additives in use by Marcellus shale field crews. The experiments employed an internationally accepted standard method (OECD 301A) to evaluate aerobic biodegradation potential of the fluid mixture by monitoring the removal of dissolved organic carbon (DOC) from an aqueous solution by activated sludge and lake water microbial consortia for two substrate concentrations and four salinities. Microbial degradation removed from 57 % to more than 90 % of added DOC within 6.5 days, with higher removal efficiency at more dilute concentrations and little difference in overall removal extent between sludge and lake microbe treatments. The alcohols isopropanol and octanol were degraded to levels below detection limits while the solvent acetone accumulated in biological treatments through time. Salinity concentrations of 40 g/L or more completely inhibited degradation during the first 6.5 days of incubation with the synthetic hydraulic fracturing fluid even though communities were pre-acclimated to salt. Initially diverse microbial communities became dominated by 16S rRNA sequences affiliated with *Pseudomonas* and other *Pseudomonadaceae* after incubation with the synthetic fracturing fluid, taxa which may be involved in acetone production. These data expand our understanding of constraints on the biodegradation potential of organic compounds in hydraulic fracturing fluids under aerobic conditions in the event that they are accidentally released to surface waters and shallow soils.

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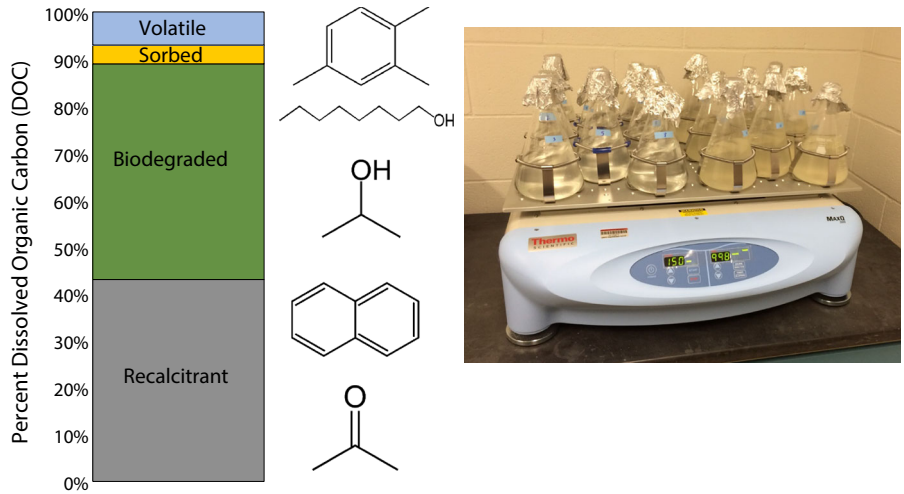
Graphical Abstract



Keywords Hydraulic fracturing fluid · Organic additives · Acetone · Shale energy development · Aerobic biodegradation · *Pseudomonas*

Introduction

Hydraulic fracturing is a method used to stimulate production of unconventional oil and gas wells through the pressurized injection of fluid that propagates a network of interconnected fractures within subsurface hydrocarbon reservoirs (Soeder 2010). The volume of fluid injected varies by reservoir, but typically requires 12–19 million liters of a carrier fluid containing water (90 %), sand proppant (9 %), and added chemicals (1 %) which may include biocides, friction reducers, gelling agents, corrosion inhibitors and other additives (King 2012; Jiang et al. 2013; Vidic et al. 2013). The chemicals aid delivery of proppant into induced fractures to open channels for hydrocarbon flow and to protect well infrastructure against microbial fouling and mineral scaling that may constrict flow paths and decrease well productivity (Arthur et al. 2008). A portion of the injected fluid (10–15 %) returns to the surface as flowback (Jiang et al. 2013) and may initially contain a signature of injected compounds until wells mature (Abualfaraj et al. 2014; Strong et al. 2014). Flowback fluids become increasingly enriched in salt with longer residence time in the formation (Cluff et al. 2014; Rowan et al. 2014) and may be re-used to fracture new wells after treatment and dilution or disposed (Jiang et al. 2013; Rahm et al. 2013). One environmental concern regarding these activities is the risk that injected or flowback fluids could be spilled at the surface or released from malfunctioning well casings to the shallow subsurface, which could degrade drinking water resources through chemical contamination (USEPA 2012). However, some of the added organic compounds can serve as carbon and energy sources to support microbial growth (Cluff et al. 2014); thus, the ability of microorganisms to degrade such compounds and the speed at which this degradation occurs will in part control the persistence of organic compounds in surface spills.

Two factors that hinder our ability to constrain bioattenuation rates of organic compounds used in injected fluids are their compositional variability and chemical complexity. In the United States, field crews completing unconventional oil and gas wells typically develop reservoir-specific injection formulas from more than 2500 different commercial products (Waxman et al. 2011). The disclosed chemicals in these products include more than two dozen organic compounds with potential health hazards such as ecological toxicity, human carcinogenicity, or other risks for which they are regulated chemicals or candidate contaminants under the Safe Drinking Water Act or Clean Air Act (USEPA 2010; Waxman et al. 2011; Stringfellow et al. 2014). Despite measures to contain surface releases during fluid mixing and well

completion, such as lining well pads and installing perimeter containment barriers, releases of hydraulic fracturing fluid to surface waters and shallow soils have been documented (Brantley et al. 2014). For example, in the Marcellus shale region of Pennsylvania, more than 132,000 liters of blended injection fluid or flowback fluids were reportedly released to streams, farmland, and forests as a result of accidental surface spills between 2010 and 2013 (PA 2014). Several other large releases have been described for the Utica and Bakken formations, including an explosion in eastern Ohio that impacted 5 miles of surface water and killed an estimated 70,000 fish (Gebeloff and Sontag 2014; Woods 2014). Understanding the bioattenuation potential of organic chemicals in these fluids within surface environments will help frame migration rates as parameters of transport models, risk assessments, and remediation technologies for impacted water resources.

Some of the organic compounds used in fracturing fluids are relatively biodegradable on an individual basis (Stringfellow et al. 2014) while others may lack evidence of biodegradability or persist under certain environmental conditions (Kahrilas et al. 2015). Ethylene glycol, commonly used as a scale inhibitor in hydraulic fracturing fluids (Gregory et al. 2011), is readily biodegradable across a range of conditions in aqueous and soil environments (Evans and David 1974; Klecka et al. 1993; Mrklas et al. 2004), generally reaching full removal over short timespans (Staples et al. 2001). Similarly, isopropanol, which is used as a surfactant, and common biocides formaldehyde and glutaraldehyde can be easily degraded by aerobic and anaerobic microbial consortia (Adroer et al. 1990; Bustard et al. 2000; Leung 2001; Kahrilas et al. 2015). However, when not the sole substrate for microbial growth, the biodegradation efficiency of these and other additives may be dramatically reduced. Competitive inhibition can occur during biodegradation of mixtures of BTEX (benzene, toluene, ethylbenzene, and xylenes) compounds (Arvin et al. 1989; Bielefeldt and Stensel 1999; Reardon et al. 2000; Littlejohns and Daugulis 2008) or polycyclic aromatic hydrocarbons (PAHs) (Bouchez et al. 1995; Guha et al. 1999), which may be components of petroleum distillates added to fracturing fluids as friction reducers. Additionally, the ionic strength of injected fluids containing recycled flowback diluted to salinities between 20 and 150 g/L total dissolved solids (TDS) (Mauter and Palmer 2014; Strong et al. 2014; Mouser et al. in review) may inhibit biodegradation activity (Lefebvre and Moletta 2006). For example, biodegradation of simple carbon compounds (e.g. acetate) and complex polysaccharides (e.g. guar gum, a gelling agent used in hydraulic fracturing fluid) is slowed by salinities consistent with those used in Marcellus injected fluids (Ng et al. 2005; Lester et al. 2014). Studies evaluating the biodegradation potential of individual or structurally-related compounds can therefore inform only incomplete predictions of chemical persistence. Furthermore, evaluations of chemical biodegradability within surface water and shallow soil should consider salinity conditions typical of injected hydraulic fracturing fluids.

As shale gas development is primarily occurring in rural forests and agricultural areas which contain low-order streams with high rates of surface water transport, the goal of this study was to assess the degradability of organic carbon additives in injected fracturing fluids under idealized aqueous aerobic conditions. To accomplish this goal, we evaluated the biodegradability of a chemical mixture that is representative of blended injected fluid used in the Marcellus shale region of Pennsylvania and Ohio, USA across a range of characteristic substrate concentrations (25–250 mg/L DOC) and salinities (0–60 g/L NaCl). We hypothesized that a substantial fraction of added carbon could be respired or assimilated by aerobic municipal wastewater sludge or by aerobic microorganisms inhabiting an Ohio lake. Based on previous studies of similar individual compounds, we anticipated a negative correlation between salinity and degree of biodegradation (Ng et al. 2005; Lester et al. 2014). We also sought to relate changes in bulk organic carbon concentrations to microbial growth kinetics, microbial taxonomy, and concentrations of individual organic compounds in order to identify potentially persistent chemicals, thereby constraining aerobic biotransformation rates of specific organic compounds used in hydraulic fracturing fluids for use in chemical fate models.

Materials and methods

Synthetic hydraulic fracturing fluid

Formulas for hydraulic fracturing fluid injected into Marcellus shale wells in Pennsylvania and Ohio were

obtained from FracFocus and used to develop a solution for repeatable laboratory experiments. A recipe was compiled from the most common additives reported in the formulas, and additives were obtained from chemical manufacturers supplying Marcellus field crews. The composition of the synthetic fracturing fluid (SFF) was developed to be statistically similar in carbon, nitrogen, and phosphorus concentrations to injected fluid samples obtained from Marcellus shale wells (Mouser et al. in review). The SFF solution was made with freshwater from Senecaville Lake, OH, a characteristic sand proppant (100 mesh, Unimin Corporation, New Canaan, CT), and 13 chemical additives (Table 1). These included a corrosion inhibitor, surfactant, clay stabilizer, gelling agent, and biocide provided by chemical manufacturers and hydrochloric acid, citric acid, ethylene glycol, boric acid, ethanolamine, potassium hydroxide, ammonium persulfate, and potassium carbonate purchased from Thermo Fisher Scientific (Waltham, MA). Lake water was collected in a 10-L HDPE carboy, transported on ice to Ohio State University (OSU), refrigerated at 4 °C, and used within 7 days. Chemicals were added to lake water, mixed overnight before addition of sand, and used in experiments within 24 h (see Supplementary Information).

Substrate effect on organic carbon removal

Degradation of organic compounds in the SFF at two substrate concentrations was evaluated using OECD Method 301A (OECD 1994) designed for testing the ready biodegradability of manufactured polymers. The suite of OECD 301 methods is internationally recognized as a standard and adaptable metric of chemical degradation. Method 301A is specifically designed to evaluate polymer biodegradability at near-optimal aerobic growth conditions in a mineral solution of trace nutrients including: potassium and sodium phosphates, ammonium chloride, calcium chloride, magnesium sulfate, and iron (III) chloride (OECD 1994). The method also incorporates a reference chemical (e.g., acetate) degraded under the same conditions as the test material to ensure microbial growth is possible at the conditions selected and to enable kinetic comparisons. The three experimental units used in this study included: (1) test solutions of SFF; (2) reference solutions of acetate; and (3) abiotic controls of SFF. Raw SFF was diluted with the mineral solution (OECD 1994) in ratios of 1:15 or 5:3 to two target concentrations of 25 or 250 mg/L DOC. Calculated quantities of sodium acetate were added to mineral solution to reach similar target concentrations. The lower concentration was used because it fell within the range recommended by the method, while the higher was one order of magnitude closer to reported concentrations for field-used injected fluids (median 226 mg/L TOC [n = 19]; mean 414 mg/L DOC [n = 3]) (Hayes 2009; Cluff et al. 2014) and for flowback and produced water samples (median 449 mg/L TOC [n = 28]; mean 346 mg/L TOC [n = 31]) (Ny 2011; Orem et al. 2014). Test solutions were prepared in triplicate and reference solutions in duplicate at both DOC concentrations. All biotic treatments were inoculated with 25 mg/L total suspended solids (TSS) of sieved activated sludge microorganisms. The sludge was collected from the aeration tank of a municipal wastewater treatment plant (Alum Creek Water Reclamation Facility, Lewis Center, OH). Abiotic volatilization controls were prepared in duplicate at both DOC concentrations and received no sludge, but were treated with sodium azide to inhibit the growth of microorganisms from the base fluid (Senecaville Lake water) and mineral solution. All solutions were mixed in Erlenmeyer flasks (1 L), adjusted to pH 7.4 ± 0.2 with 1 M NaOH, covered loosely with aluminum foil, shaken continuously at 150 RPM in the dark at 24 °C, and sampled over 7 days with sterile syringes. Fluid was tested daily for dissolved oxygen (DO) and pH using an Orion 5 Star Meter (Thermo Fisher Scientific) calibrated before each use and cleaned between measurements.

Salinity effect on organic carbon removal

The effect of salinity on degradation of organic compounds in SFF was also considered using three experimental units: (1) test solutions containing sludge microorganisms; (2) test solutions containing lake water microorganisms; and (3) killed sludge controls. All units contained SFF diluted with mineral solution (OECD 1994) to a target concentration of 250 mg/L DOC. Sludge treatments received granular NaCl to final concentrations of 0, 20, 40, or 60 g/L salt (near the lower bound of Marcellus shale injected fluid salinity) (Mauter and Palmer 2014) and were prepared in triplicate at each salt concentration. Sludge treatments were inoculated with sieved activated sludge (25 mg/L TSS) previously acclimated in shaking flasks to the same salinities (0, 20, 40, or 60 g/L

Table 1 Chemical additives and respective masses or volumes comprising the synthetic fracturing fluid (SFF) used for biodegradation experiments

Chemical additive	Disclosed ingredients	Mass (g) or volume (ml)/L SFF
Carrier/base fluid	Source water (collected from Atwood Lake in Senecaville, OH)	896 ml
Proppant	Sand (100 mesh sand produced by Unimin)	99 g
Acid	HCl (15 % by mass)	3.5 g
Fe control	Citric acid	0.014 g
Corrosion inhibitor AI600[1]	Ethylene glycol, dimethyl formamide, decanol, isopropanol, octanol, 2-butoxyethanol, ethoxylated nonylphenols, cinnamaldehyde, tar bases, quinoline derivatives, benzyl chloride (quaternized), triethyl phosphate	0.007 ml
Friction reducer WFR-61LA[1]	Petroleum distillate, sodium chloride, alcohol ethoxylated $C_{12–16}$, quaternary ammonium chloride	0.12 ml
Surfactant Revert Flow[1]	Alcohol ethoxylated, isoporopanol, citrusturpenes, alcohol ethoxylated C_6–C_{12}, DB-964 (polyoxyethylene-polyoxypropylene block polymer)	0.47 ml
Clay stabilizer CC-120[1]	Proprietary non-hazardous salt	0.44 ml
Gelling agent WGA 15L[1]	Petroleum distillate	0.52 ml
Biocide EC6110A[2]	Glutaraldehyde, quaternary ammonium compound, ethanol	0.05 ml
Cross linker	Ethylene glycol	0.008 g
	Boric acid	0.004 g
	Ethanolamine	0.002 ml
Breaker	Ammonium persulfate	0.005 g
pH adjustor	K_2CO_3	0.018 g
	KOH	0.007 g

[1] Weatherford

[2] NALCO

NaCl) over a period of 4 weeks through aeration, carbon amendment, and incremental (10 g/L) salt addition. Duplicate lake water treatments received no salt and no sludge but contained Senecaville Lake water. Duplicate killed controls received sludge microorganisms (25 mg/L TSS) inactivated by a 4-h exposure to sodium azide (1 M) to estimate volatilization and adsorption. All solutions were mixed in Erlenmeyer flasks (1 L), adjusted to pH 6.7 ± 0.3 with 1 M NaOH, and handled as described above.

Organic carbon analysis

Fluid samples for DOC measurement were syringe filtered (0.22 μm pore size, EMD Millipore, Billerica, MA) into pre-baked borosilicate volatile organic analysis vials with Teflon-lined septa, preserved with HCl (pH < 3), and stored at 4 °C for less than 48 h before analysis. DOC was determined following EPA Method 415.1 using a TOC-V CSN Total Organic Carbon Analyzer (Shimadzu Corporation, Kyoto, Japan). One raw SFF sample prepared for the salinity experiment and duplicate solutions of 0 and 20 g/L salt sludge treatments and killed controls containing SFF were analyzed for purgeable volatile organic compounds (VOCs), C_1–C_{10} linear alcohols, and isopropanol using a gas chromatograph with a mass spectrometer following EPA Method 8260B by MASI Labs, Dublin, OH (see Supplementary Information, Table S1 for a list of analytes tested). SFF treatments were sampled at three times during the salinity experiment (0, 2.5, and 6.5 days). Unfiltered samples were stored without headspace in pre-baked amber borosilicate volatile organic analysis vials with Teflon-lined septa, preserved with 10 % $Na_2S_2O_3$ (sodium thiosulfate), and stored at 4 °C for less than 2 weeks before analysis.

Epifluorescent cell staining, enumeration, and kinetic calculations

Unfiltered samples were immediately fixed in glutaraldehyde (1 % final concentration) and stored at

4 °C for less than 2 weeks. Prior to cell counting, samples were vortexed and diluted 1:100 with RNA-free water (Fisher Scientific, Waltham, MA) before addition of 0.1 % SYBR® Gold (Life Technologies, Carlsbad, CA) then held in the dark for 20 min for stain binding. Live and dead cells with intact membranes were enumerated on a Guava EasyCyte flow cytometer (EMD Millipore, Billerica, MA) (Stingl et al. 2007). Cell concentrations were converted to biomass using a conversion factor of 5.8×10^{-14} g dry mass per cell (Frossard et al. 2013). Specific yield (Y) was calculated as biomass produced (ΔX) per substrate mass degraded (ΔS) during a given time by $Y = \Delta X/\Delta S$. Specific growth rate (μ) was calculated as the biomass produced (ΔX) per unit time elapsed (Δt) normalized to the total biomass (X) at the end of a given period by $\mu = 1/X^{*}\Delta X/\Delta t$.

DNA extraction and sequencing analysis

For analysis of the microbial community dynamics, 20 mL of fluid from lake water treatments and from 0 to 20 g/L salt sludge treatments were collected and centrifuged in sterile 50-mL tubes at 5000 RPM for 45 min to concentrate biomass. Tubes were decanted, flash frozen, and stored at −80 °C. Nucleic acids were extracted using the PowerSoil DNA Isolation kit (MoBio, Carlsbad, CA) and quantified using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Samples for DNA sequencing were collected from these three treatments in duplicate (n = 6) at the start of the experiment (day 0) and from the three treatments in duplicate (n = 6) at the end of the experiment (day 6.5). DNA was amplified in duplicate PCR reactions on a S1000 Bio-Rad Thermal Cycler (Foster City, CA) using protocols described previously (Cluff et al. 2014) with primers 515F and 806R covering the V4 region of the 16S rRNA gene that target both Bacteria and Archaea (Bates et al. 2011). After pooling duplicate PCR reactions, amplicons were purified, quantified, multiplexed, and unidirectionally pyrosequenced on a Roche Genome Sequencer FLX Titanium System (Roche Diagnostics, Indianapolis, IN) at the OSU Plant Microbe Genomics Facility. Sequences have been deposited at the National Center for Biotechnology Information under Bioproject #PRJNA259375.

16S rRNA gene sequence processing and microbial community analysis

Raw sequences were trimmed to 180–610 bp, quality filtered by removing primer mismatches and demultiplexed using the QIIME pipeline (Caporaso et al. 2010) adapted with the QIIME toolset (http://github.com/smdabdoub/qiime-tools). Operational taxonomic units (OTUs) were assigned at a 97 % similarity threshold using the Blastn algorithm (Altschul et al. 1990) and aligned to the GreenGenes database version 13.8 (DeSantis et al. 2006). Low-level OTUs (<0.01 % of total sequences) were pruned. Chimeras were removed using ChimeraSlayer (Haas et al. 2011). Data was rarefied (evenness = 1190) to compute richness and diversity. The UniFrac method was used to calculate the beta-diversity (Lozupone and Knight 2005), and samples were clustered and tested for similarity using ANOSIM. Principal coordinates analysis (PCoA) plots were generated with jackknifed samples (n = 250).

Results and discussion

Substrate effect on organic carbon removal

Biodegradation of organic compounds in SFF was first demonstrated by analysis of DOC trends (Fig. 1). In dilute treatments (25 mg/L target DOC concentration), degradation of carbon in the SFF began immediately, with the majority of DOC (70 ± 4 %) removed from solution in less than 2 days and nearly all (91 ± 1 %) within 6.5 days. In contrast, biodegradation of carbon in concentrated SFF (250 mg/L target DOC concentration) showed a lag period of several days, after which more than half the added DOC (59 ± 4 %) was removed within 6.5 days. Although degradation of acetate was more rapid in dilute solutions, near complete DOC removal (>96 %) was observed at both the low and high acetate concentrations by day 4. DOC in the four abiotic controls decreased 7 ± 3 % during the experiment, suggesting that loss of carbon through volatilization was small relative to biological removal. Average dissolved oxygen measurements for the treatments indicated the solutions remained aerobic during the experiment, with decreased values corresponding to periods with greatest carbon removal (Table S2, Supporting Information). Average

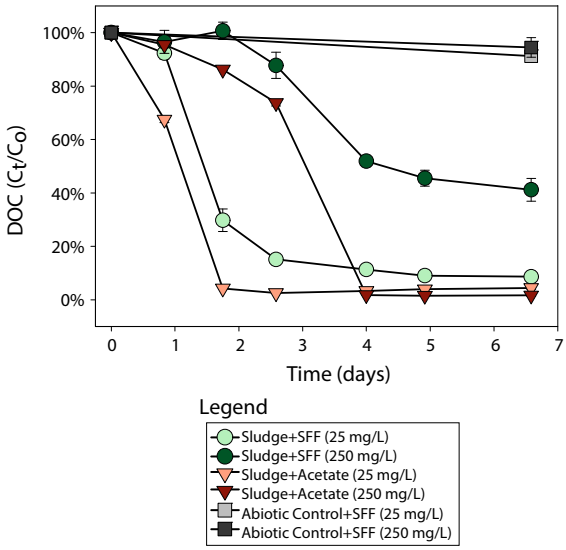


Fig. 1 Dissolved organic carbon (DOC) removal by activated sludge microorganisms as a function of time, reported as a percent of starting concentration (C_t/C_o) in solutions of (*circle*) synthetic fracturing fluid (SFF), (*inverted triangle*) acetate and (*square*) abiotic controls at dilute (25 mg/L target DOC, *light colors*) and concentrated (250 mg/L target DOC, *dark colors*) substrate concentrations

pH values remained neutral to slightly basic (7.2–8.0) throughout the duration of the experiment (Table S2).

Salinity effect on organic carbon removal

Biodegradation of organic compounds in SFF was next evaluated in solutions containing DOC and salinities representative of field-injected hydraulic fracturing fluids (Fig. 2). Sludge with no added salt degraded more than half (56 ± 1 %) of added carbon in concentrated SFF treatments (250 mg/L DOC) within 6.5 days, while sludge that was acclimated to 20 g/L salt removed an equivalent amount (57 ± 3 %). Carbon removal efficiency was similar to that observed for concentrated SFF treatments during the first experiment (Fig. 1), supporting the reproducibility of these data in separate experiments with different sludge seeds. However, salinities above this threshold significantly inhibited biodegradation, with just 3 ± 2 % DOC loss in solutions receiving 40 g/L salt and 1 ± 5 % loss for the 60 g/L salt treatment over 6.5 days. We were initially concerned that the salt-acclimated sludge was not as healthy as the sludge without added salt, therefore we repeated experiments multiple times, mixing the starting sludge seed to remove this potential effect. We also collected samples 40 days into the concentrated SFF salinity experiment, at which point 64 ± 4 % (0 g/L salt), 73 ± 3 % (20 g/L salt), 43 ± 3 % (40 g/L salt), and 25 ± 7 % (60 g/L salt) of carbon had been removed from solutions, with the highest salt treatments showing the lowest overall removal. Finally, we tested the effect of salinity on low concentration SFF (25 mg/L DOC) and found little difference in removal efficiency (63 ± 4 %) over 6.5 days for concentrations of 10 g/L salt and 60 g/L salt (data not shown). These observations indicated that: (1) activated sludge microbes survived salinity acclimation, but that

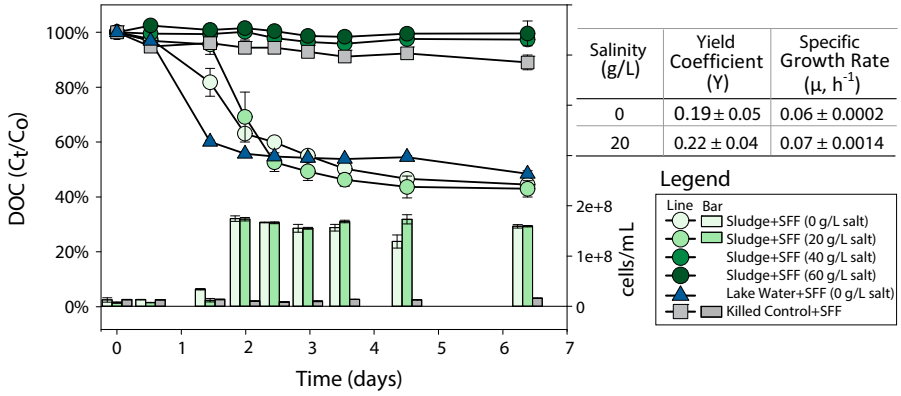


Fig. 2 Dissolved organic carbon (DOC) removal by activated sludge microorganisms or by lake water microorganisms as a function of time, reported as a percent of starting concentration (C_t/C_o) in solutions of (*circle*) synthetic fracturing fluid (SFF), (*square*) killed controls, and (*triangle*) lake water. The ability of sludge microorganisms to degrade organic compounds in SFF was tested at a range of salt concentrations between 0 and 60 g/L, with low concentrations shown in *light colors* and high shown in *dark*. Cell numbers in solutions containing activated sludge microorganisms with SFF and 0 or 20 g/L salt, and in killed controls containing SFF, are displayed as columns. Maximum observed yield coefficients and specific growth rates are also shown

growth was initially inhibited in concentrated SFF treatments during a long lag period that did not occur at low SFF concentrations, and (2) removal efficiency over a long time period was much poorer at high salinity, suggesting the combined effect of high salinity (40 to 60 g/L salt) and concentrated SFF (250 mg/L DOC) was difficult for sludge microorganisms to overcome.

In lake water treatments, microorganisms reduced bulk DOC of the SFF by more than half (52 ± 1 %) within 6.5 days, almost as much as microorganisms in activated sludge (Fig. 2). In killed adsorption and volatilization controls, DOC decrease was minor (11 ± 3 %) but marginally greater than that observed in abiotic volatilization controls, possibly due to the addition of killed sludge cells providing greater surface area for sorption. Again, the magnitude of abiotic loss was insufficient to explain the DOC removal observed in biotic flasks. Solution DO (3.2–4.3 mg/L) and pH (7.2–8.0) remained near the ranges reported in the first experiment with sufficient oxygen to support aerobic respiration (See Table S3, Supporting Information).

Biodegradation of alcohols and loss of volatile organic compounds

Selective metabolism of SFF chemical additives was indicated by organic compound analyses (Table 2). In total, 11 of 71 tested VOCs, 2 of 11 tested alcohols, and one tentatively identified compound were detected in the raw SFF and in experimental solutions (Table S4, Supplemental Information). With the exception of toluene and n-butyl benzene, all VOCs and alcohols detected in the raw SFF were also detected in day 0 test solutions (Table S2). Median values for compounds detected at concentrations of 20 μg/l or higher are summarized in Table 2. Collectively, the total carbon mass calculated from individual VOC and alcohol concentrations accounted for only a small fraction (3–6 %) of initial bulk DOC (Table 2; note that most of the recalcitrant compounds should be GC amenable, whereas most of the organic compounds in the SFF are hydrophilic surfactants that are not amenable to the GC techniques deployed). The two alcohols 2-propanol (isopropanol) and 1-octanol (octanol) together accounted for 2–4 % of initial bulk DOC, thus comprising the majority of total carbon mass detected. Alcohol concentrations decreased to below detection limits within 6.5 days in all biotic flasks analyzed, while no such loss was observed in killed controls (Table 2). At the same time, acetone concentrations increased up to two orders of magnitude in biotic treatments, but did not increase in killed controls (Table 2). These data suggest acetone was generated during the biological breakdown of the SFF and represented an incomplete transformation product within residual DOC.

In both biotic and killed controls, the cycloaliphatic compound limonene was tentatively identified at initial concentrations comparable to the two detected alcohols (1500–3200 μg/L), but these concentrations decreased substantially within 2.5 days and were below detection in all samples within 6.5 days (Table 2). Naphthalene concentrations decreased by half in both biotic and killed controls over the course of the experiment (Table 2), likely due to volatilization from the aerobic reactors. Other aromatic compounds, including BTEX compounds, were also identified in initial samples but were below detection for all treatments later in time (See Table S4, Supplemental Information). Similarly, two trimethyl-substituted benzene compounds were detected in initial samples at tens to hundreds of μg/L, while concentrations decreased to below 10 μg/L in all samples after only 2.5 days (Table S4). These trends suggest that loss of purgeable VOCs was substantial for the detected aromatic and cycloaliphatic compounds. Considering a 7 % reduction in DOC due to abiotic volatilization losses during the first experiment (approximately 17 mg/L DOC), and assuming this accounts for the purgeable organic portion of the SFF, we detected a total loss of 6.6 mg/L carbon based on the sum of individual chemical analyses in the killed control (Table 2), representing over a third (39 %) of the volatilized fraction. This suggests that other VOCs were stripped from the system that were not detected using EPA method 8260B, which is perhaps unsurprising considering the complexity of hydraulic fracturing fluids and the limited list of analytes interrogated by the standard method.

Microbial enumerations and calculated growth kinetics

Biodegradation of organic compounds in SFF occurred concurrently with significant increases in microbial cell numbers (Fig. 2). A decrease in DOC for solutions containing activated sludge and in

Table 2 Median values of duplicate experimental samples analyzed for organic compounds detected at concentrations greater than 20 μg/L from SFF treatments under no salt or 20 g/L salt

Time	Treatment	Isopropanol	Octanol	Acetone	Limonene	Naphthalene	1,2,4-trimethyl-benzene	n-butyl-benzene	Total alcohols + VOCs (as mg/L-C)	DOC (mg/L-C)	Porportion alcohol + VOCs to DOC (%)
Day 0	Synthetic fracturing fluid	10,400	7100	171	9500	71	410	<1			
	Sludge, 0 g/L salt	3150	4550	154	2250	29	233	43	7.8	239	3.2
	Sludge, 20 g/L salt	4550	4300	117	3200	29	249	54	9.3	249	3.7
	Lake water	5050	5250	109	4550	26	221	40	9.3	194	4.8
	Killed control	5650	7800	163	3800	31	302	69	13.8	240	5.8
Day 2.5	Sludge, 0 g/L salt	1250	1250	2175	27	23	7	<1	3.1	142	2.2
	Sludge, 20 g/L salt	3650	<200	1305	<1	19	<1	<1	3	132	2.3
Day 6.5	Sludge, 0 g/L salt	<500	<200	6510	<1	7.5	<1	<1	4	106	3.8
	Sludge, 20 g/L salt	<500	<200	10025	<1	<1	<1	<1	6.2	105	5.9
	Lake water	<500	1000	14350	<1	19	5	<1	9.6	93	10.4
	Killed control	5450	5200	149	<1	8.5	<1	<1	7.2	211	3.4

In the case of non-detects, medians were calculated from reported detection limits. Numbers shown for the synthetic fracturing fluid is based on one sample. Individual VOCs and alcohol concentration units are reported as μg/L. Total VOCs + alcohols are calculated after conversion of individual compound concentrations to carbon mass based on their molecular weight. Measured DOC concentrations are also shown, with the proportion of VOCs + alcohols to DOC calculated from these values. Concentrations of all detected analytes for individual samples can be found in Table S2, Supporting Information

solutions with sludge acclimated to 20 g/L salt corresponded to significant increases in cell concentrations compared to killed controls. Cell concentrations peaked at day 2, after which DOC loss slowed (Fig. 2). During the growth phase, the concentration of cells increased more than an order of magnitude from $1.3 \pm 0.4 \times 10^7$ cells/mL on day 0 to $1.7 \pm 0.05 \times 10^8$ cells/mL on day 2 in solutions with no added salt. Notably, a similar magnitude of increase was observed in solutions containing 20 g/L salt (from $0.7 \pm 0.1 \times 10^7$ cells/mL on day 0 to $1.7 \pm 0.04 \times 10^8$ cells/mL on day 2), suggesting that the added salinity had little effect on growth. Cell concentrations in both solutions were relatively stable after this point (between 1.3×10^8 and 1.7×10^8 cells/mL), and remained significantly greater than values observed in killed controls (Fig. 2). Although the SFF contained a commercially-used biocide at concentrations reported by industry in the bulk injected fluid, it had little observable effect on growth.

Maximum observed specific yields (Y) and specific growth rates (μ) occurred between day 1 and 2, representing the exponential growth phase (Fig. 2). Yield coefficients describing biomass produced per substrate mass degraded were similar for sludge treatments with 0 and 20 g/L salt (0.19 ± 0.05 and 0.22 ± 0.04, respectively). By these calculations, approximately 20 % of carbon removed from solution was assimilated while the remainder was likely mineralized to CO_2. Specific growth rates were marginally greater in solutions with 20 g/L salt compared with solutions without salt (0.070 ± 0.001 and 0.0600 ± 0.0002, respectively). Specific growth rates of 0.0305 h^{-1} have been reported for microbial consortia growing on 1 % isopropanol (Bustard et al. 2000), suggesting that degradation of organic compounds in SFF may allow microbial growth to occur about twice as fast as dilute isopropanol.

Microbial community dynamics and diversity

A total of 38,922 16S rRNA gene sequences were obtained from solutions exhibiting significant biodegradation. After filtering and pruning, 22,909 (59 %) of these sequences were considered to be high quality and retained for analysis (Table 2). Microbial communities in SFF test solutions inoculated with sludge had similar richness, diversity and evenness at the start of the experiment regardless of salinity (0 or 20 g/L salt). Communities in test solutions with only lake water microorganisms at T0 contained higher diversity and richness, with values about double those inoculated with sludge (Table 3). Starting communities were significantly more diverse than the communities that developed by the end of the experiment in all treatments. After 6.5 days of incubation with SFF, we observed a significant decrease in the number of OTUs ($p < 0.001$), richness ($p < 0.0001$), diversity ($p < 0.0001$), and evenness ($p < 0.0001$) regardless of treatment. Using Anosim, samples were combined

Table 3 Summary of richness and diversity estimates (at a 97 % OTU similarity cutoff) for samples collected from starting SFF solutions (day 0) containing sludge with no salt (0 g/L) or 20 g/L salt and lake water treatments, and these same treatments after 6.5 days incubation (day 6.5) based on 454-pyrosequencing of the 16S rRNA gene

Time	Treatment	Sample ID	No. sequences	OTU^{003}	Chao richness	Shannon diversity	Simpson evenness
Day 0	Sludge, 0 g/L salt	A	1230	172	401	5.17	0.93
		B	1375	156	290	5.08	0.93
	Sludge, 20 g/L salt	A	1479	191	338	6.1	0.97
		B	4089	191	372	5.59	0.94
	Lake water	A	1315	430	811	8	0.99
		B	1195	416	732	8	0.99
Day 6.5	Sludge, 0 g/L salt	A	1748	63	109	4.01	0.91
		B	1738	59	86	3.93	0.91
	Sludge, 20 g/L salt	A	1829	41	66	3.62	0.89
		B	3162	44	111	3.61	0.89
	Lake water	A	1791	39	55	3.61	0.89
		B	1958	40	77	3.64	0.89

Headers show experimental unit (treatment) and replicate name (sample ID)

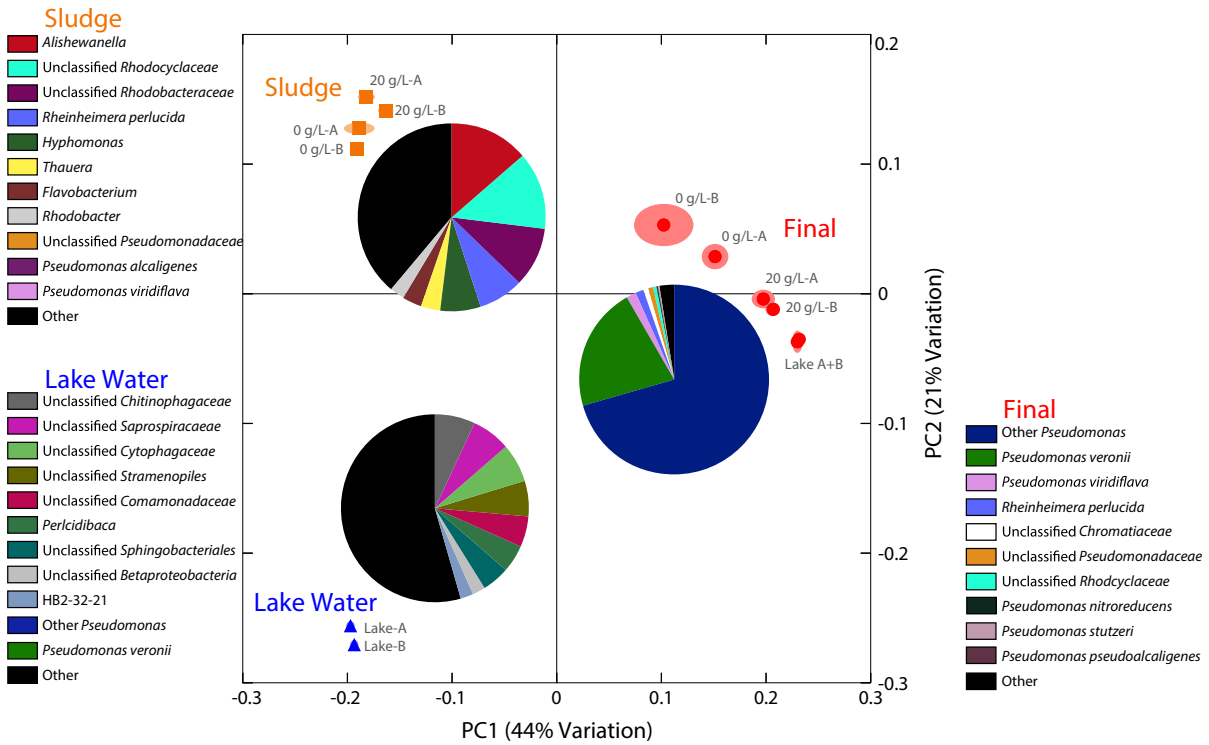


Fig. 3 Two-dimensional principal coordinates analysis plot of jackknifed (n = 250) microbial communities using unweighted UniFrac analysis for samples collected from starting treatments containing sludge and SFF at no salt (0 g/L) and 20 g/L salt (Sludge, in *orange*) and lake water treatments (Lake, in *blue*). Samples were collected after 6.5 days from these same treatments (Final, *red*). Pie charts for Sludge (n = 4), Lake (n = 2), and Final (n = 6) show the average relative abundance of dominant taxa from each statistically distinct group

into three distinct sample groups ($p < 0.01$) representing the sludge communities on day 0; the lake water communities on day 0; and all of these communities after 6.5 days of incubation in SFF (Fig. 3). Samples were separated across the x-axis (PC1, 44 % variance explained) based on sample time, with day 0 communities to the left and day 6.5 communities to the right. The day 0 samples were further separated across the y-axis (PC2, 21 % variance explained), with activated sludge treatments plotting above the x-axis and lake water treatments plotting below (Fig. 3).

The day 0 microbial communities in sludge treatments were dominated by taxa associated with *Proteobacteria* including *Alishewanella* and unclassified species of *Chromatiaceae* in the *γ-proteobacteria* along with *Thauera* and other unclassified species within *Rhodocyclaceae* of the *β-proteobacteria* (See Table S5, Supplemental Information). Sequences with similarity to two *α-proteobacteria* families were also high in relative abundance at the start of the experiment, including *Hyphomonas* and other *Hyphomonadaceae* along with *Rhodobacteria* and other *Rhodobacteraceae*. *Flavobacterium* within *Bacteroidetes* was also prevalent in day 0 sludge communities (Fig. 3). Many of these taxa have been described for other activated sludge communities (Weissbrodt et al. 2014), particularly those acclimated to higher salinities (Li et al. 2013). All of these taxa were also detected in background controls containing only lake water microorganisms, but at significantly lower relative abundance (Table S5). The day 0 microbial communities in lake water treatments were dominated by the *Bacteroidetes* including unclassified species within families *Chitinophagaceae*, *Saprospiraceae*, and *Cytophagaceae* as well as members of the *Sphingobacteriales* genus (Fig. 3). Unclassified species within the *β-proteobacteria* and specifically the

family *Comamonadaceae* were detected at relatively high abundance, as were *Perlucidibaca* spp. within *γ-proteobacteria*. Day 0 lake water treatments also contained a high abundance of sequences within the euykaryotic *Stramenopiles* phylum, which could include a diversity of algae or diatoms.

The microbial communities of all samples were dramatically altered after incubation with SFF (Fig. 3). After 6.5 days, communities were dominated by sequences with similarity to multiple species of *Pseudomonas* and other unclassified *Pseudomonadaceae*. Specifically, unclassified species within genus *Pseudomonas* species were detected at low abundance (<1 %) in initial sludge and lake communities, but increased in relative abundance to comprise an average 70 % of observed sequences by the end of the experiment (Table S5). Similarly, sequences affiliated with *Pseudomonas veronii* increased from less than 0.1 % at the start of the experiment to an average 21 % of the community after 6.5 days. After SFF incubation, sequences associated with the *Chromatiaceae* family of *γ-proteobacteria* and specifically *Rheinheimera perlucida* persisted in sizeable abundance, as did unclassified species within *Rhodocyclaceae* of the *β-proteobacteria*. Both *Chromatiaceae* and *Rhodocyclaceae* were also detected in starting sludge communities.

Implications for surface releases

Lake water microbial communities were found to be as efficient as municipal activated sludge in biodegrading a synthetic hydraulic fracturing fluid created from reported field formulas, suggesting that impacts to water resources resulting from surface spills of injection fluids can be mitigated in part by natural microbiological processes, and that dilution of saline fracturing fluids with fresh water could further facilitate microbial activity. From a bulk mass balance perspective, the fate of carbon in SFF at field-used concentrations (250 mg/L DOC) included about 7 % volatilization, 4 % sorption to cells or glassware, 46 % mineralization or microbial assimilation (biodegradation), and 43 % persisted in solution for more than 6.5 days. Biodegradation at high SFF concentrations was substantially lesser in rate and extent than that of the reference compound acetate, which was degraded by more than 96 % in only 4 days. Salt concentrations of 20 g/L, representing the lower bound of reported salinity of injected fluids, had no effect on degradation. However, salinities above this threshold completely inhibited degradation for at least 6.5 days in treatments with high DOC, after which degradation proceeded only slowly and to a much lesser extent. SFF was efficiently converted to microbial biomass, with 1 mol organic carbon assimilated for every 4 mol respired.

The use of a commercial glutaraldehyde-based biocide as part of the SFF did not appear to inhibit growth of activated sludge or lake water microbial consortia to any substantial degree in treatments receiving or lacking salt amendment. Glutaraldehyde is known to biodegrade at sub-inhibitory levels in aerobic and anaerobic aquatic systems (Kahrilas et al. 2015). However, dosages at or above this level are effective in slowing growth of *Pseudomonas fluorescens* (Vikram et al. 2014), a bacterium phylogenetically similar to taxa that dominated the SFF treatments. Interestingly, *P. fluorescens* cells remained viable in solutions containing organic matter and salts from Marcellus-produced fluids despite being given a biocide dosage that was lethal when applied in a buffer solution (Vikram et al. 2014). Since biocides such as glutaraldehyde can be easily neutralized by other organic and inorganic constituents in injected and flowback fluids (Struchtemeyer et al. 2012), the timing of application may play an important role in the effectiveness and persistence of such compounds within these fluids and on the response of the microbial community.

Although two alcohols detected in this study (isopropanol and octanol) were efficiently degraded and several trimethyl-substituted benzene and BTEX compounds appeared to be stripped during mixing, a substantial fraction (43 %) of recalcitrant chemical additives and biodegradation byproducts could not be mineralized by surface water microorganisms or by sludge bacteria within 1 week, which suggests environmental persistence of certain compounds may be expected following a surface spill. However, other environmental conditions (e.g., differing freshwater chemistry, microbial seeds, or temperatures) may yield different results. With the exception of acetone, which comprised a small portion of residual DOC, the identity and concentration of recalcitrant organic compounds remained poorly characterized in the SFF, but likely included glycol polymers, amino alcohols, ethoxylated alcohols, polysaccharides (e.g.

guar), quaternary ammonium compounds, and petroleum distillates; biotransformation products from degradation of these compounds; and other VOCs tentatively identified in this report (e.g. cycloalkanes). Preliminary analyses of the diesel range organic compounds (DRO) in the SFF by liquid–liquid extraction preparation techniques presented analytical challenges due to the complex mixture of surfactants and petroleum distillates. In particular, a thick emulsion layer formed between the aqueous and organic layers, and we hypothesize that this could explain the wide variability of DRO measurements we observed. In the absence of such an emulsion layer, the authors routinely achieve standard compound recoveries between 90 and 100 %. Nevertheless, the recalcitrant organic compounds might reside in the semi-volatile, non-purgeable range, compared to the volatile compounds (i.e., >284 ppb DRO remaining after 270 h of aerobic degradation). Further characterization of the organic compounds in the SFF will help to identify which of these additives persist to a greater degree under idealized (and non-ideal) conditions.

The microbial production of acetone (C_3OH_6) observed here can occur by the dehydrogenation of isopropanol (C_3OH_8) by mixed microbial consortia (Bustard et al. 2000; Songa et al. 2010) and other aerobic isolates (Bustard et al. 2002; Kotani et al. 2007), and is thought to involve one or more NAD(P)-dependent alcohol dehydrogenases: *adh1*, *adh2*, and *adh3* (Reid and Fewson 1994). Certain *Pseudomonas* species are among the bacteria known to contain *adh*-like genes that can dehydrogenate isopropanol for acetone production (Levin et al. 2004; Tasaki et al. 2006). Acetone can also be produced by the decarboxylation of acetoacetate ($C_4O_3H_6$) as a byproduct of pyruvate fermentation. This process is well-documented for *Clostridium acetobutylicum* which employs the acetoacetate decarboxylase pathway in acetone-butanol-ethanol (ABE) solvent production from starches under anaerobic conditions (Jones and Woods 1986). In order to determine which microbial process might be responsible for acetone production in this study, samples were analyzed for butanol and ethanol at the start and end of the experiment, but these compounds were not detected. Except for a very small proportion of unclassified *Clostridiaceae* (<0.01 % average relative abundance), 16S rRNA gene sequences contained no taxa associated with *Clostridia*. The dissolved oxygen measurements further demonstrated that solutions remained aerobic for the majority of the experiment, conditions under which ABE fermentation would be unlikely. Based on this evidence we believe *Pseudomonas* spp. may be involved in acetone production via isopropanol dehydrogenation, as taxa within this genus dominated microbial communities (>90 % average relative abundance) in all samples after 6.5 days. However, analyzing the molar concentrations of isopropanol and acetone indicates that significantly more acetone was produced than isopropanol was initially present (Table 2); therefore, biodegradation of other unidentified constituents, likely including polypropylene glycols (PPGs), may have contributed to observed acetone production. Acetone concentrations are likely to increase in solution until the parent compound is consumed (Bustard et al. 2000), which suggests that significant PPGs may remain after 6.5 days. In a related study employing sediment and groundwater microorganisms and this same SFF (Mouser et al. in review), only low-level acetone was detected after 25 days in batch microcosm experiments (<20 µg/L) even though it was detected at higher concentrations in earlier samples. Therefore, the analyses in this study may reflect an incomplete biodegradation process or a process specific to aqueous solutions, where acetone degradation can occur more fully during longer incubation periods or more efficiently in the presence of sediment, both being conditions relevant to a release of hydraulic fracturing fluid into surface water, soil, or shallow aquifers.

It is notable that *Pseudomonas* thrived in SFF treatments, as certain species of this genus are well-documented hydrocarbon degraders (Williams and Sayers 1994) and have been detected in studies profiling microbial communities in injected fluid and produced water from shale gas wells (Struchtemeyer and Elshahed 2012; Mohan et al. 2013a; Cluff et al. 2014; Strong et al. 2014). Specifically, several *Pseudomonas* isolates including two species closely associated with 16S rRNA gene sequences detected in this study (*P. stuzeri,* and *P. alcaligenes*) have demonstrated abilities to degrade PAHs such as naphthalene and phenanthrene, linear and branched alkanes, and diesel range compounds (Takizawa et al. 1994; Ramos et al. 1995; Whyte et al. 1997; Beal and Betts 2000; Bosch et al. 2000; Filonov et al. 2000; Barathi and Vasudevan 2001; Tian et al. 2003; Yeo et al. 2003; Kaczorek et al. 2011; Rocha et al. 2011; Tang et al.

2011; Gai et al. 2012; Zhang et al. 2012). *Pseudomonas* was one of the dominant genera encapsulated in gels from Bakken shale wells and reintroduced to flowback fluids in laboratory experiments, which substantially improved organic carbon degradation rates (Aukema et al. 2014; Strong et al. 2014). *Pseudomonas* and other *Pseudomonadaceae* were also enriched in sediment microcosms amended with this same SFF (Mouser et al. in review). They appear to tolerate elevated salinities and biocide treatment (Vikram et al. 2014) based on their presence in flowback, produced fluids, and storage ponds of shale gas wells (Mohan et al. 2013b; Cluff et al. 2014; Strong et al. 2014). These findings support their importance as key members of the aqueous and sediment microbial communities involved in the attenuation of fracturing fluids following a surface release. Sequences associated with *Thauera* and *Rheinheimera* genera were also enriched after SFF incubation. These bacteria are capable of degrading certain hydrocarbons (*Thauera*) (Shinoda et al. 2004), have some salinity tolerance (*Rheinheimera)* (Romanenko et al. 2003), and, in the case of the genus *Thauera,* have been observed in Marcellus shale well fluids (Strong et al. 2014).

Conclusion

Raw hydraulic fracturing fluids have been accidentally released to surface waters and soil, where they pose unknown risks to water resources, human health, and the environment (PA DEP 2014). This study suggests that much, but not all, of the organic matter in HFF could be degraded aerobically via activated sludge if there were some pre-treatment to reduce the salinity. Without such primary treatment or substantial dilution, the organic load in these fluids could survive biological treatment trains and be discharged to receiving waters. This study investigated the degradability of potentially harmful organic compounds in these fluids using a standard biodegradability test employing activated sludge and surface water microorganisms to frame the fate of bulk carbon in an aquatic aerobic system. Within about 1 week, 57 % to more than 90 % of added DOC is removed in high to low concentration SFF treatments, respectively, with a small portion of this percentage volatilizing (7 %) or sorbing (4 %). The remaining carbon mass (43 % or less) persists and degrades at a much slower rate after this time. It is evident that compounds known to be individually biodegradable to a great extent (Stringfellow et al. 2014) may not degrade as efficiently when combined in the complex chemical mixtures that are hydraulic fracturing fluids. They may also produce intermediate metabolites that persist until parent compounds are removed; in this case, a substantial amount of acetone was produced from incomplete degradation of organic compounds in SFF. Further analysis into the composition of hydraulic fracturing fluids and their degradability in the environment will be aided by the development of analytical methods for quantifying unknown compounds in these mixtures and identifying their attenuation processes across a range of environmental conditions.

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