Factors controlling the co-occurrence of microbial sulfate reduction and methanogenesis in coal bed reservoirs

Authors: Andrew W. Glossner^a, Lisa K. Gallagher^b, Lee Landkamer^b, Linda Figueroa^b, Junko Munakata-Marr^b, Kevin W. Mandernack^{a,1*}

a. Department of Chemistry and Geochemistry, Colorado School of Mines, Golden, Colorado, USA 80401 andy.glossner@gmail.com

b. Department of Civil and Environmental Engineering, Colorado School of Mines, Golden, Colorado, USA 80401 l.landkam@mines.edu, jmmarr@mines.edu, lfiguero@mines.edu

1. Present address: Department of Earth Sciences, Indiana University~Purdue University, 723 West Michigan Street, SL118, Indianapolis, Indiana, USA 46202
kevinman@iupui.edu

*Corresponding author: Kevin W. Mandernack

Postal address: Department of Earth Sciences, Indiana University~Purdue University, 723 West Michigan Street, SL118B, Indianapolis, Indiana, USA 46202

Telephone: (317) 274-8995

kevinman@iupui.edu

Abstract

Sulfate-reducing microorganisms (SRM) and methanogenic archaea have been previously observed in coal bed methane reservoirs, suggesting that the model for separation of these organisms based on sulfate concentration may not apply to such reservoirs. Using a methanogenic consortium enriched from coal, microcosm experiments showed simultaneous activity of methanogens and sulfate reducers at sulfate concentrations ranging from 50 to 1000 μM when coal was the sole substrate. These experiments revealed no apparent correlation between methanogenic potential and sulfate concentration. In other microcosm experiments with varying acetate amendments, concentrations of the phospholipid fatty acids (PLFAs) 14:0, 16:1 ω 5, 16:1 ω 7*cis*, 16:1 ω 7*trans*, and *cy*17:0 correlated strongly with the initial acetate concentration in microcosms with 500 μM sulfate, while *i*17:0 correlated strongly in microcosms with 200 μM sulfate. A significant portion of the acetate in these experiments went to microbial metabolisms other than dissimilatory sulfate reduction or methanogenesis, suggesting that some of these PLFAs were likely produced by some other unknown acetate-consuming microorganisms. Copies of the *dsrA* gene increased at least 10-fold over initial levels in samples without molybdate (MoO_4^{2-}) across all experiments, indicating that SRM were active when not inhibited by MoO_4^{2-} . In experiments with < 300 μM acetate, copies of the *mcrA* gene increased over 49 days regardless of sulfate concentration. These results suggest that both SRM and methanogens are active at low acetate concentrations and may compete for available acetate with other acetate-consuming bacteria in coal bed methane reservoirs.

Keywords: Sulfate reduction; methanogenesis; coal; microbial; Powder River Basin

1. Introduction

Coal bed methane (CBM) has become an important energy resource, comprising roughly 7% of natural gas production (Annual Energy Outlook 2014), with approximately 40% of this gas being microbial in origin (Strapoć et al., 2011). From laboratory and field studies, some basins, such as the Powder River Basin (PRB) in Wyoming and Montana, USA, have been shown to harbor active microbial communities capable of ultimately converting coal to methane (Harris et al., 2008; Jones et al., 2010; Ulrich and Bower, 2008). There is much commercial interest in developing microbial consortia or other technologies to enhance this process (Ritter et al., 2015), but doing so requires a fundamental understanding of the interactions of the entire microbial community and its metabolisms in coal bed reservoirs.

The process of microbial methanogenesis from coal is complex, but it is thought to be enhanced with introduction of microbes and nutrients through meteoric water recharge (Strapoć et al., 2011). Such recharge is a common feature at many of the world's largest reserves of microbial CBM (Flores et al., 2008; Martini et al., 1998, 1996; McIntosh et al., 2008; Schlegel et al., 2011a, 2011b; Scott et al., 1994; Tseng, 1997; Walvoord et al., 1999; Zhou and Ballentine, 2006). Strapoć et al. (2011) provide a thorough review of the pathways of methanogenesis from coal. Basically, fermentative, anaerobic microorganisms degrade the large geopolymers to form long chain organic acids, which are then further broken down to monomers and oligomers and ultimately to the substrates necessary for methanogenesis, mainly hydrogen and acetate. In many anoxic environments, sulfate-reducing microorganisms (SRM) and methanogenic archaea compete for these latter substrates.

The competition between SRM and methanogens in anoxic environments is governed mainly by sulfate, hydrogen, and acetate concentrations. However, at both

freshwater (Lovley and Klug, 1986, 1983) and marine sulfate concentrations (Middelburg et al., 1993; Mitterer, 2010), sulfate reducers are known to outcompete methanogens due to their higher affinity and lower threshold concentrations for hydrogen and acetate (Muyzer and Stams, 2008; Schönheit et al., 1982). In marine sediments the commonly accepted model for the distribution of these two processes holds that methanogenesis will not emerge as the dominant terminal electron accepting process until the sulfate has been depleted (Jørgensen and Kasten, 2006; Martens and Berner, 1974; Oremland and Taylor, 1978). Because the concentration of sulfate in marine sediments is typically much higher (10-50 mM) than in freshwater sediments (10-500 μ M), sulfate reducers often account for the majority of carbon mineralized under high sedimentation rates in marine sediments (Canfield et al., 2005). Under freshwater sulfate conditions SRM can still thrive at the lower sulfate concentrations by having much higher affinity for sulfate (Ingvorsen et al., 1984) and therefore rapidly deplete the available sulfate in freshwater sediments (Canfield et al., 2005; Lovley and Klug, 1983).

While freshwater bogs, lake sediments, and surface waters illustrate the fundamental relationships between SRM and methanogens, deep subsurface basins are different because physicochemical parameters, such as groundwater recharge or mineral dissolution, result in limited amounts of sulfate and low rates of organic matter degradation due to the recalcitrant nature of coal (Lovley and Chapelle, 1995). Subsurface coal bed methane reservoirs can be viewed as essentially closed systems over short time periods because the rates of groundwater recharge and organic matter degradation are slow relative to surface sites (Bates et al., 2011). Schlegel et al. (2011) argue that in parts of the Illinois Basin sulfate reduction occurred at some point in the past 10 ka, imparting a lighter isotopic signature on the DIC and enriching the remaining sulfate pool in ^{34}S . The authors argue that methanogenesis occurred subsequent to sulfate reduction and that methanogenesis had not yet overprinted the isotopic signature of the

DIC that sulfate reduction had generated. In this model sulfate reduction and methanogenesis are mutually exclusive processes separated by time rather than geochemical zonation as occurs in sediments. Other studies have found 16S rRNA gene sequences indicative of SRM in coal bed methane reservoirs (Green et al., 2008) as well as in abandoned mines with active methanogenic populations (Beckmann et al., 2011). It is therefore possible that sulfate reduction and methanogenesis may occur contemporaneously in such reservoirs, albeit at very low rates due to the limiting amount of labile carbon.

The availability of substrates necessary for both sulfate reduction and methanogenesis in CBM reservoirs will also influence their relative activities. The produced water from the Wyodak-Anderson coal zone of the PRB generally has low sulfate concentrations of around 1.6 μM (Rice et al., 2008), though localized regions within the basin can have much higher concentrations up to 40 mM (Ulrich and Bower, 2008). Acetate is another potential limiting substrate for SRM and methanogens in coal beds, and is generally at low levels in produced waters from the PRB ($< 2 \mu\text{M}$) (Ulrich and Bower, 2008). Acetate is an important intermediate in the degradation of complex organic matter in environments such as peat bogs (Metje and Frenzel, 2007), oil reservoirs (Bonch-Osmolovskaya et al., 2003), lacustrine sediments (De Graaf et al., 1996; Winfrey and Zeikus, 1979), and various organic-rich shales, clays, and mudstones (Jones et al., 1989; McMahon et al., 1992; Routh et al., 2001). In these environments, it is generally maintained at very low concentrations ($<10 \mu\text{M}$) by active microbial consumption, including methanogenesis. Acetate has been shown to be an important intermediate in coal degradation in abandoned coal mines (Beckmann et al., 2011) and in the Forest City Basin CBM reservoir (McIntosh et al., 2008). Given the variability of sulfate concentrations and the limited metabolic activity of the microbial community in

CBM wells, it is possible that sulfate reduction and methanogenesis co-occur in CBM reservoirs like the PRB, with both being limited by the availability of acetate.

The goal of this study was to determine the effect of varying sulfate and acetate concentrations on the competing processes of sulfate reduction and methanogenesis in coal from the PRB. To investigate this, a series of microcosm experiments was undertaken with a microbial consortium enriched and maintained on PRB coal. These experiments were conducted with variable concentrations of sulfate (50 - 1000 μM) and acetate (250 - 1000 μM) that might be expected in produced waters in CBM reservoirs (Orem et al., 2007; Rice et al., 2008). The effects of these variations on the bacterial community structure were analyzed using microbial membrane phospholipid fatty acids (PLFAs) and real-time quantitative polymerase chain reaction (qPCR). PLFA analysis is a commonly accepted method for determining both living microbial biomass (Balkwill et al., 1988; Boschker et al., 1998; Mills et al., 2010) as well as community structure when PLFAs can be putatively assigned to specific metabolic groups (Dowling et al., 1986; Vainshtein et al., 1992). To better understand the energy and carbon constraints in each microcosm experiment, we measured sulfate and acetate concentrations, methane production, and concentrations of PLFAs. Functional genes for methanogens and sulfate reducers, methyl coenzyme-M reductase (*mcrA*) and dissimilatory sulfite reductase (*dsrA*), respectively, were measured by qPCR to further assess how SRM and methanogens in these microcosm experiments responded to changing sulfate and acetate concentrations.

2. Methods

2.1 Experimental design

Coal was collected in September 2009 by straining cuttings from the effluent of working drill rigs in the PRB. Three seams were sampled from the Wyodak-Fort Union formation, including the Big George, Smith, and Felix seams at depths ranging from 240

to 610 meters. Coal cuttings were immediately rinsed with sterile deionized water in the field to remove drilling fluids, placed in sterile whirl-pak bags, sealed in vacuum bags with chemical oxygen scrubbing packets (OxyFree 504), and stored at 4°C until use. The coal was rinsed again in the lab in the anaerobic chamber with sterile, anoxic deionized water (described below) and then crushed using a sterile mortar and pestle before being rinsed again over a sterile 80 mesh (0.177 mm) sieve prior to use.

2.2 Preparation of Enrichment Culture and Lab Microcosm Experiments

A mixed consortium of microorganisms, whose growth was dependent on coal provided as a substrate and which contained both SRM and methanogens, was enriched from the coal cuttings (Gallagher et al., 2013). The enrichment consortium was incubated at 30°C and continually maintained by transferring every 60 days inocula from the microcosms that previously produced the most methane to new microcosms with fresh coal. The anoxic nutrient medium was modified (Tanner, 2006) to exclude sulfate by replacing MgSO_4 with MgCl_2 , and sulfate was added to the desired concentration as Na_2SO_4 . Medium was prepared by flash-autoclaving deionized water to reduce oxygen saturation, then sparging with 4:1 N_2 : CO_2 for 15 minutes before adding 1 g/L NaHCO_3 just before sealing under N_2 : CO_2 and autoclaving. The trace-vitamin, -mineral and -metal solutions (Tanner, 2006) were filter-sterilized (0.22 μM pore size) and added to the anoxic bicarbonate solution after it had cooled in an anaerobic chamber.

All experiments utilized the microbial consortium noted above and were prepared in an anaerobic chamber with an atmosphere of 5% H_2 , 5% CO_2 , and a balance of N_2 . Experiments were initiated by adding 10 g coal and 50 mL of the sterile, anoxic medium to sterile 200 mL serum bottles. All microcosms were inoculated with 0.5 mL (1%) of the microbial consortium. Upon sealing with butyl rubber stoppers, the headspace of each serum bottle was purged with 4:1 N_2 : CO_2 for at least 5 minutes and pressurized to 1.1 atm. Prior to the initiation of the sulfate and acetate experiments described here, a series

of inhibition experiments were conducted to test whether or not hydrogenotrophic methanogenesis was a major source of methane for our consortium. Microcosms that contained 0.2 mM 4-nitrophenol as an inhibitor of acetotrophic methanogenesis (Bhattacharya et al., 1995) showed no significant difference in methane production over autoclaved control microcosms (unpublished data), suggesting that H_2/CO_2 was not a significant source of methane in our experiments.

Three different sets of experiments were undertaken to examine the competition between SRM and methanogens for substrates derived from coal (see Table 1 for summary). The first experiment, hereafter referred to as var.- SO_4 , included variable concentrations of sulfate from 100 μM to 1000 μM without added acetate to determine if sulfate limited the competition between the two metabolic groups. Two additional experiments were conducted with variable acetate concentrations by amending microcosms with 0, 250, 500 and 1000 μM acetate, with each series of amendments made at 200 μM or 500 μM sulfate. Hereafter, these experiments are simply referred to as 200- SO_4 and 500- SO_4 . These sulfate concentrations were chosen to represent high and low examples of what may be expected in the PRB coal seams. Due to the long incubation times and the time needed to collect and evaluate data from each experiment, the 200- SO_4 and 500- SO_4 experiments were initiated seven to ten months after the initiation of the var.- SO_4 experiments. Consequently, it is reasonable to assume that some changes occurred in the microbial enrichment culture used in the experiments. The background levels of acetate in coal microcosms after inoculation were $49 \pm 20 \mu M$ and $110 \pm 50 \mu M$ in the 200- SO_4 and 500- SO_4 experiments, respectively. Every tested sulfate or acetate condition also included corresponding controls utilizing 5 mM Na_2MoO_4 as an inhibitor for sulfate reducers (Oremland and Capone, 1988).

2.3 Chemical Analyses

The concentration of acetate was monitored by high performance liquid chromatography (HPLC) on an Agilent 1100 Series with a BioRad HPX-87H column (300 x 7.8 mm) and UV/VIS detector set at 210 nm. Concentrations of methane and carbon dioxide in the headspace of microcosms were measured using a GC-17A gas chromatograph (Shimadzu, Kyoto, Japan). Methane was separated using a HaysepQ packed column (2m; Supelco) and carbon dioxide was separated using a molecular sieve (5Å pore size, 2m) held at 100°C with a thermal conductivity detector (200°C) and flame ionization detector (200°C) connected in series for quantification. The calibration of the instrument was checked periodically with authentic methane and carbon dioxide standards obtained from Scott Specialty Gases (Plumsteadville, PA).

Determining the rate of microbial sulfate reduction in mixed cell suspensions or sediment slurries is generally accomplished by amending experiments with $^{35}\text{SO}_4^{2-}$ and trapping the resulting radiolabelled $^{35}\text{S}^{2-}$. This method was not feasible in this work due to the presence of coal that actively scavenged the sulfide. Control experiments were conducted in which coal slurries were amended with sulfide that was subsequently measured as acid volatilized sulfide and titrated iodometrically (49, 50). These experiments demonstrated that sulfide could not be recovered quantitatively in this manner (Glossner, 2013). Therefore, rates of sulfate reduction were determined by measuring the change in sulfate concentration using a Dionex AS50 ion chromatograph with IonPac AS-14A (4mm x 150mm) ion exchange, AG-14A (4mm x 50mm) guard column and a CD25 conductivity detector. The eluent was 10.0 mM sodium carbonate and the calibration done daily with a five point sulfate calibration curve.

2.4 Microbial Community Analysis by PLFAs

All solvents used for PLFA analysis were HPLC grade, and aqueous solutions were stored over chloroform to remove trace organic contamination. All glassware used in the preparation of PLFAs was heated at 460°C for six hours. Once experiments were

completed 1.0 mL subsamples were taken for DNA analysis and frozen at -80°C . The remaining contents of each experiment were then frozen at -80°C and the entire sample lyophilized ($T = -80^{\circ}\text{C}$, $P = 0.08$ mTorr). Samples were then transferred to glass centrifuge tubes and extracted using a modified Bligh-Dyer extraction (White and Ringelberg, 1998). The sample was then re-extracted for archaeal phosphoether lipids (PEL) by adding 75 mL methanol, 37.5 mL chloroform, and 30 mL 10% trichloroacetic acid (wt:vol) (Nishihara and Koga, 1987). The supernatant from this second extraction was phase separated overnight by adding 30 mL water and 37.5 mL chloroform. The total lipid extracts were then combined and the volume reduced under a N_2 stream. The total lipid extract was then separated into neutral, glyco- and polar lipid classes using silica solid phase extraction columns (0.5 g silica, Alltech Extract Clean). SPE columns were prepared by washing with 5 mL methanol followed by 10 mL chloroform. Following loading of the columns with the total lipid extract, neutral lipids were eluted in 10 mL chloroform, glycolipids in 10 mL acetone, and polar lipids in 10 mL methanol. The methanol portion of the lipid extract contained the intact PLFAs and PELs. Fatty acid methyl esters (FAMES) were prepared from bacterial PLFAs by mild alkaline methanolysis (White and Ringelberg, 1998). An internal FAME standard (C13:0, CAS# 1731-88-0) was added for quantification. The position of the double bond in monounsaturated PLFAs was determined by making their dimethyl disulfide adducts (Dunkelblum et al., 1985). Two ubiquitous FAMES were commonly seen in blanks (C16:0 and C18:0) and were generally on the order of 2 nmol total.

Intact polar lipids from archaea were separated from bacterial FAMES using silica gel chromatography by eluting FAMES in 5 mL chloroform and eluting intact PELs in 10 mL methanol. Since amino-containing lipids are not affected by HCl-methanolysis, intact PELs were heated at 140°C for 18h with 2.0 mL 3:2 acetic acid:acetic anhydride (v/v) (Renkonen, 1965) followed by extraction of the mixture with chloroform. The remaining polar head groups

(glycosidic groups) and acetylated core lipids from amino-containing lipids were liberated using 2.0 mL 5% HCl-methanol (wt/vol) at 110°C for 2h (Nishihara and Koga, 1987). Following extraction of the liberated core lipids using 4:1 hexane:chloroform and concentration under a N₂ stream, the resulting alcohols were derivatized using 30 µL 1% trimethylchlorosilane in N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and 30 µL pyridine at 70°C for 2h. Samples were then analyzed by GC-MS within 24h of silylation.

FAMES were analyzed on an Agilent 7890A gas chromatograph with a DB-1MS column (60m x 0.25 mm I.D.) split to a flame ionization detector (FID) for quantification, and an Agilent 5975C Inert XL mass selective detector for aid in identification. The identity of each FAME was preliminarily assigned based on comparison of retention times with those of FAMES from a standard mixture (Sigma Aldrich #47785-U). The injector temperature was 280°C, the FID set to 280°C, and the ion source temperature set to 250°C. The oven temperature program began at 50°C, then increased as follows: 150°C at 20°C min⁻¹, 210°C at 1.5°C min⁻¹, 280°C at 10°C min⁻¹ and held for a final 8 min at 280°C. The flow rate in the column was 1.5 mL min⁻¹. FAME identity was confirmed after electron ionization at 70 eV. Silylated PELs were analyzed on a Restek Rxi-5sil MS column (30 m x 0.25 mm I.D. x 0.25 µm; Restek, Bellefonte, PA) with a flow rate of 1.5 mL min⁻¹. The oven temperature program began at 70°C, increasing to 130°C at 25 C° min⁻¹, to 190°C at 6°C min⁻¹, to 320°C at 25°C min⁻¹ where it was held for a final 20 min at 320°C. The injector, FID, and ion source temperatures were the same as previously specified.

2.5 qPCR analyses

Quantitative (real-time) polymerase chain reaction (qPCR) analysis was used to enumerate methanogens and SRM based on the presence of functional genes unique to those organisms. For both groups of organisms, bulk deoxyribonucleic acid (DNA) was extracted from samples using the MoBio Powersoil® Kit (Carlsbad, CA). Quantification of copy numbers was performed using a LightCycler 480 II instrument (Roche, Branford, CT).

Methyl coenzyme reductase, subunit alpha (*mcrA*) genes were amplified using the primer set mlas/*mcrA*-rev (Steinberg and Regan, 2008) (mlas 5'-GGTGGTGTGTTGGTTTCACMCARTA-3' / *mcrA*-rev 5'-CGTTCATBGCCTA GTTVGGRTAGT-3'). Each reaction (20 μ L) contained 10 μ L Perfecta SYBR Green Supermix (Quanta Biosciences, Gaithersburg, MD), 7 μ L nuclease-free water, 1 μ L primer (10 μ M each), and 2 μ L template DNA. Cycling conditions were as follows: initial denaturation step at 95 °C for 3.5 min, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 7 min. Specificity of product formation was confirmed by melt curve analysis (55 °C to 95 °C with acquisition every 0.5 °C). *Methanosarcina mazei* was used as a standard.

Dissimilatory sulfate reductase, subunit alpha (*dsrA*) genes were amplified using the primer set DSR1F/Del1075R (Gittel et al., 2009) (DSR1F 5'-AC(GC)CACTGGAAGCACG-3' / Del1075R 5'-G(CT)TC(ACG)CGGTTCTT(GAT)C-3'). Each reaction (20 μ L) contained 10 μ L Perfecta SYBR Green Supermix (Quanta Biosciences), 3 μ L nuclease-free water, 1 μ L primer (100 pmol μ L⁻¹ each), and 5 μ L template DNA. Cycling conditions were as follows: initial denaturation step at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min. Specificity of product formation was confirmed by melt curve analysis (58 °C to 95 °C with acquisition every 0.5 °C). *Desulfovibrio vulgaris* was used as a standard.

DNA concentrations were calculated based on triplicate measurements on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). The copy number was calculated based on this concentration, then a standard curve based on 10-fold serial dilutions was constructed to determine gene copy numbers in unknown samples. Samples, standards and controls were run in duplicate. Raw data were exported to LinRegPCR (Ramakers et al., 2003; Ruijter et al., 2009), which estimates fluorescence

baseline using an algorithm that constructs the log-linear phase from the plateau stage downward. This software was used to calculate an average efficiency per amplicon, a common window of linearity, fluorescence threshold, and starting concentrations. Average efficiencies ranged from 1.792 to 1.808 and 1.783 to 1.812 for *mcrA* and *dsrA*, respectively. The detection limits for *mcrA* and *dsrA* were 10^3 copies mL⁻¹ and 10^2 copies mL⁻¹, respectively.

2.6 Data statistical analysis

To test the significance of differences observed in either methane production or biomass, a t-test was performed using all data from the relevant individual microcosms at the final time point. Linear correlation coefficients for individual PLFAs were calculated using the average PLFA quantities (nmole) reported in the tables (see Appendix). Principal component analysis (PCA) was conducted using Minitab (version 17) software and the relative abundance (mole %) of each PLFA measured in individual microcosms from each experiment. In cases where a PLFA was not detected in all microcosms an abundance of 0.001% was used instead.

3. Results

3.1 Variable sulfate experiments

In both the inhibited and uninhibited microcosms of the var.-SO₄ experiments the sulfate concentration did not significantly affect methane production (Figure 1). Each microcosm produced between 0.8 and 2 μ moles CH₄ (g coal)⁻¹, with the lowest methane production observed in microcosms with 500 μ M sulfate. Methane production was similar between the uninhibited and 5 mM MoO₄²⁻ control microcosms, except for microcosms at 50 μ M sulfate, in which the MoO₄²⁻ control produced more methane than the uninhibited microcosms (t-test, 2 tailed, $p < 0.05$). Methane production was also greater in the var.-SO₄ experiments than in the variable acetate experiments (both 200-

SO₄ and 500-SO₄) at all initial acetate concentrations except 1000 μ M (Figure 1 and 4).

While it is possible that significant methane desorbed from coal following exhumation from the subsurface (Stricker et al., 2006), several lines of evidence support the archaeal origin of methane in this study: (1) methane production correlates strongly with acetate concentration in the 200-SO₄ and 500-SO₄ experiments ($R^2 = 0.71$ and 0.89 , respectively), (2) methane production consistently ceased after complete consumption of acetate in all experiments, and (3) *mcrA* gene copies increased in the var.-SO₄ experiments (described later in Results).

Figure 1: Methane production in the var.-SO₄ experiments. Open symbols represent 5 mM MoO₄²⁻ controls (error bars represent 1 standard deviation of triplicate incubations).

Acetate was detected in all microcosms in the var.-SO₄ experiments, to which no acetate was added. Initial acetate concentrations ranged from 100 to 250 μ M, showing a general increase from T-0 to T-1, followed by consumption to below the detection limit (10 μ M) in most samples, except the MoO₄²⁻-amended microcosms with no sulfate and 50 μ M sulfate (Figure 2). The addition of 5 mM MoO₄²⁻ inhibited sulfate reduction in the var.-SO₄, 200-SO₄, and 500-SO₄ experiments (Figures 3 and 5, respectively). In the var.-SO₄ experiments without MoO₄²⁻, sulfate decreased by approximately 90 μ M over the

first 21 days, regardless of the initial concentration, after which it remained unchanged (Figure 3).

Figure 2: Acetate concentrations in the var.-SO₄ experiments. Open symbols represent 5 mM MoO₄²⁻ controls.

Figure 3: Sulfate concentrations in var.-SO₄ experiments. ♦ 50 μM sulfate; ● 100 μM sulfate; ■ 250 μM sulfate; ▲ 500 μM sulfate; ✕ 1000 μM sulfate. Open symbols correspond to 5 mM MoO₄²⁻ controls.

3.2 Variable acetate experiments

Because sulfate amendments did not discernibly affect methane production in the variable sulfate experiments, acetate production from the fermentation of coal was hypothesized to control the relative dominance of methanogens or SRM in coal-based microcosms. This

hypothesis was further tested in microcosms amended with coal, at either 200 or 500 μM sulfate and variable acetate concentrations ranging from 50 to 1000 μM . Methane production was significantly greater in MoO_4^{2-} -inhibited samples relative to those without MoO_4^{2-} for the following microcosms: 200- SO_4 + 1000 μM acetate and 500- SO_4 at both 500 μM acetate and 1000 μM acetate (t-test, 2 tailed, $p < 0.05$) (Figure 4). The 1000 μM acetate plus 5 mM MoO_4^{2-} treatment exhibited the greatest methane production in both the 200- SO_4 ($2.1 \pm 0.3 \mu\text{moles CH}_4$ (g coal) $^{-1}$) and 500- SO_4 ($2.8 \pm 0.1 \mu\text{moles CH}_4$ (g coal) $^{-1}$) experiments (Figure 4).

Figure 4: Methane production in 200- SO_4 (A) and 500- SO_4 (B) experiments. $\dagger$ No acetate; $\blacklozenge$ 250 μM acetate; $\blacksquare$ 500 μM acetate; $\bullet$ 1000 μM acetate. Open symbols correspond to 5 mM MoO_4^{2-} controls.

In the 200-SO₄ and 500-SO₄ experiments, the extent of acetate consumption depended on the sulfate concentration. After 17 days acetate was consumed to below detectable levels in all of the 500-SO₄ experiments (Figure 5B) but was still detectable beyond 30 days in the 200-SO₄ experiments without MoO₄²⁻ and amended with 1000 μ M acetate (Figure 5A). Sulfate concentrations remained unchanged in microcosms with 5 mM MoO₄²⁻, but decreased over time in the 200-SO₄ and 500-SO₄ experiments without MoO₄²⁻ (Figure 5 C and D, respectively).

Figure 5: Acetate concentrations in 200-SO₄ (A) and 500-SO₄ (B), and sulfate concentrations in the 200-SO₄ (C) and 500-SO₄ (D) experiments with variable amended acetate concentrations. + No acetate; $\blacklozenge$ 250 μ M acetate; $\blacksquare$ 500 μ M acetate; $\bullet$ 1000 μ M acetate. Open symbols correspond to 5 mM MoO₄²⁻ controls.

3.3 Microbial community analyses by PLFAs

Total PLFA quantities extracted from the var.-SO₄ experiments ranged from 213 to 852 nmole PLFA (average 489 ± 160 nmole PLFA; Table S1). Total biomass was estimated based on the conversion factor of 2.4×10^4 cells pmole⁻¹ PLFA⁻¹ (Balkwill et al., 1988) and ranged from 5.0×10^9 to 2.1×10^{10} cells (average $1.2 \times 10^{10} \pm 4 \times 10^9$ cells). No correlation was observed between total biomass measured at the end of each experiment and the amended sulfate concentration, and no significant differences were observed between samples with MoO₄²⁻ versus those without it.

The total PLFA quantities extracted from the 200-SO₄ experiment ranged from 477 ± 120 nmole to 917 ± 260 nmole, corresponding to total biomass between $1.1 \times 10^{10} \pm 3 \times 10^9$ and $2.2 \times 10^{10} \pm 6 \times 10^9$ cells (Table S2). For a given acetate concentration, the total final biomass was consistently higher in the experiments without MoO₄²⁻ relative to those with added MoO₄²⁻, however, the difference was not significant (t-test, 2 tailed, unpaired, $p > 0.05$). The biomass estimates for microcosms amended with 250 μ M acetate were not significantly different from the 50 μ M acetate (unamended) controls (t-test, 2-tailed, unpaired, $p > 0.05$). However, microcosms amended with 500 μ M and 1000 μ M acetate, both with and without MoO₄²⁻, showed statistically higher biomass than the unamended controls (t-test, 2-tailed, unpaired, $p < 0.05$).

Total PLFA quantities extracted from the 500-SO₄ experiments ranged from 798 ± 320 nmole to 1742 ± 790 nmole (Table S3), corresponding to $1.9 \times 10^{10} \pm 8 \times 10^9$ to $4.2 \times 10^{10} \pm 2 \times 10^{10}$ cells. Figure 6C shows that, in general, PLFA quantities in the 200-SO₄ and 500-SO₄ experiments increased with increasing acetate concentrations. In the 200-SO₄ experiments, at each of the acetate concentrations tested (250-1000 μ M), the total estimated biomass was consistently higher in the experiments without MoO₄²⁻ than those with it, but this difference was small and not significantly different (t-test, 2-tailed, unpaired, $p > 0.05$). Although the 1000 μ M acetate-amended experiments ($\pm$ MoO₄²⁻) again showed the highest estimated biomass, similar to the 200-SO₄ experiments, the difference was not significantly higher than the experiments at 250

μM and 500 μM acetate. There also was no significant difference in biomass between the acetate-amended microcosms and the unamended controls (t-test, 2-tailed, unpaired, $p > 0.05$).

Fifteen to 16 PLFAs were detected at measurable concentrations in the microcosms of all three experiments. 16:0 and 18:0 were relatively abundant in all samples and are ubiquitous in most bacterial and eukaryotic organisms. Other than 16:0 and 18:0, the most abundant PLFA observed in the var.-SO₄ experiments was 16:1ω7*cis* (Table S1). Similarly, the most abundant PLFAs observed in the 200-SO₄ and 500-SO₄ experiments were 16:1ω7*cis* and 18:1ω7 (Tables S2 and S3, respectively). Every experiment also included lesser amounts of the branched fatty acids *i*15:0 and *ai*15:0, unbranched PLFAs 14:0, 15:0, and 17:0 as well as the monoenic PLFAs 16:1ω5, 16:1ω7*trans*, and 18:1ω9*cis*. The branched PLFA 10Me16:0 was observed in all samples in the var.-SO₄ experiment, varying between 8 and 42 nmole (Table S1). The PLFA 10Me16:0 was also observed in the 500-SO₄ experiment, ranging between 7.6 and 42.9 nmole (Table S3). The PLFA *cy*17:0 was observed in all experiments, with greater amounts observed in 200-SO₄ and 500-SO₄ experiments. Linear correlation coefficients were calculated for average PLFA abundances at the final time point of each experiment and initial acetate concentrations to determine if acetate stimulated specific microbial PLFAs. The PLFA *i*17:0 was correlated with initial acetate concentrations in the 200-SO₄ experiments ($R^2 = 0.92$, Figure 6A). The 16:1ω7*cis* also increased strongly with acetate concentration up to 500 μM acetate (Figure 6A). The PLFAs showing the strongest correlation to initial acetate concentration in the 500-SO₄ experiment were 14:0, 16:1ω7*cis*, 16:1ω5, 16:1ω7*trans*, and *cy*17:0 ($R^2 = 0.83, 0.99, 0.94, 0.98$, and 0.82 , respectively; Figure 6B).

Figure 6: A) Correlations between individual PLFAs (nmole) and initial acetate concentration (μM) in 200-SO₄ experiment. B) Correlations between individual PLFAs (nmole) and initial acetate concentration (μM) in 500-SO₄ experiment. C) Relationship between initial acetate concentration (μM) and total nmole PLFA recovered at final time point from 200-SO₄ and 500-SO₄ experiments. (▲ 200-SO₄ experiment; ◆ 500-SO₄ experiment. Open symbols represent 5 mM MoO₄²⁻ controls).

Because methanogens are archaea and do not produce ester-linked PLFAs, our analysis was insensitive to their growth. We attempted to recover intact archaeal phosphoether lipids from the 500-SO₄ experiments (see Methods) but they were not detected in any of the samples with the highest methane production, suggesting that methanogen biomass was too low to be detected by our phosphoetherlipid method.

3.4 Microbial community analyses by qPCR

In the var.-SO₄ experiments, initial *dsrA* copy numbers were approximately 10^5 copies mL⁻¹ and increased to 10^6 to 10^7 copies mL⁻¹ in experiments without MoO₄²⁻ but remained relatively constant at 10^5 copies mL⁻¹ in the MoO₄²⁻ control experiments (Figure 7). The var.-SO₄ experiments initially contained approximately 10^5 - 10^6 *mcrA* copies mL⁻¹ and increased to 10^6 - 10^7 copies mL⁻¹ by the end of the experiment (Figure 7). No correlation between sulfate concentration and *mcrA* copy number was observed for these experiments. In the acetate-amended 500-SO₄ experiment, initial *mcrA* copy numbers ranged from 1.2×10^6 to 3.9×10^6 copies mL⁻¹ and remained relatively constant in all microcosms except those with 1000 μM acetate + MoO₄²⁻ in which *mcrA* increased to 1.1×10^7 copies mL⁻¹ (Figure 8). Copy numbers of the *dsrA* gene increased from 10^6 to

10^7 copies mL^{-1} in the 500-SO₄ experiments without MoO₄²⁻ but remained constant at approximately 10^5 copies mL^{-1} in microcosms with MoO₄²⁻ (Figure 8). However, Baumler et al. (2008) observed that the amount of extractable DNA from the acidophilic sulfate reducer *F. acidarmanus* decreased with time in the presence of molybdate.

Figure 7: qPCR of the *dsrA* gene (A) and *mcrA* gene (B) for var.-SO₄ experiments. (Gray: Day 0; Black: Day 49).

Figure 8: qPCR of the *dsrA* gene (A) and *mcrA* gene (B) for 500-SO₄ experiments at variable acetate concentrations. (Gray: Day 0; Black: Day 49). Initial *dsrA* copies mL⁻¹ for 500 μM acetate sample was below detection.

4. Discussion

16S rRNA gene sequences associated with sulfate-reducing microorganisms have been seen in produced water from coal bed methane wells alongside sequences for methanogens (Green et al., 2008; Gründger et al., 2015; Klein et al., 2008; Shimizu et al., 2007; Ulrich and Bower, 2008). However, the reason for the co-occurrence of sequences for methanogens and SRM in coal bed methane reservoirs has not previously been determined. Our results show the co-occurrence of sulfate reduction and methanogenesis in coal microcosm incubation experiments, with both metabolic groups competing for acetate at sulfate and acetate concentrations similar to those expected in coal bed methane aquifers.

4.1 Substrate consumption in microcosms

In the var.-SO₄ experiments, the complete consumption of acetate after ~21 days corresponded with the cessation of both methanogen and SRM activity (Figures 1, 2 and 3), suggesting that both microbial processes were limited by acetate. We expected less methane to be produced in the var.-SO₄ experiments as the sulfate concentration increased, though this was not observed. Based on mass balance calculations, the initial acetate added was insufficient to account for the total amount of sulfate that was reduced and methane produced. This was not observed in the 200-SO₄ and 500-SO₄ experiments, suggesting that some acetate may have been produced in the var.-SO₄ microcosms after they were set up, as observed in Figure 2. The relative recalcitrance of organic matter is an important control of methane generation in sedimentary reservoirs, as is the metabolic activity of organisms breaking down the organics in coal to provide substrates needed for methanogenesis (Rice and Claypool, 1981; Schlegel et al., 2013). The primary factors

contributing to the bioavailability of organic matter in coals and shales are not well understood, though burial history and thermal maturity may be important variables that lead to the release of small molecular weight organic substrates utilized by microbes (Formolo et al., 2008; Jones et al., 2008; Strapoć et al., 2010). However, evidence of fermentation in buried shales also suggests that it is an important process for producing organic substrates, including acetate, that can fuel SRM, methanogens and other fermenters (Krumholz et al., 1997; Routh et al., 2001).

By initially choosing the most productive microcosms as inoculum sources for future experiments, both fermentative organisms and methanogens were expected to be enriched. Acetate was not added to the var-SO₄ experiments, however they had higher initial acetate concentrations than the 200-SO₄ and 500-SO₄ experiments, suggesting that fermentative organisms were active in these microcosm experiments. This might also explain the variability in initial acetate concentrations (Figure 2). When the experiments with no added acetate in the 200-SO₄ and 500-SO₄ experiments subsequently began, fermentative organisms may not have been as active, thus explaining the lower initial acetate concentrations (<100 µM). Results from our experiments are consistent with previous field observations (Routh et al., 2001) and suggest that fermentative organisms may generate acetate from coal that in turn can control the activity of both acetoclastic methanogens and SRM.

4.2 Fate of acetate in 200-SO₄ and 500-SO₄ experiments

Measuring the extent of sulfate reduction and methanogenesis, as well as bacterial biomass generated, in the acetate-amended microcosms (200-SO₄ and 500-SO₄) aided our understanding of carbon and energy constraints in these experiments. Acetate was assumed to be the primary electron donor for sulfate reduction and methanogenesis. For sulfate reduction this is suggested by the correlation between total acetate consumed (µmole) and total sulfate reduced (µmole) [$R^2 = 0.93$ (200-SO₄); $R^2 = 0.92$ (500-SO₄)].

Based on simple mass balance calculations, methanogenesis accounted for less than 20% of the acetate consumed in the 200-SO₄ and 500-SO₄ experiments when no MoO₄²⁻ was present (Figure 9 and Table S4). The same was true for experiments containing less than 1000 µM acetate in the presence of MoO₄²⁻, which indicates that methanogens accounted for a relatively minor portion of acetate consumption at almost all acetate concentrations. However, at acetate concentrations of 1000 µM with 5 mM MoO₄²⁻, methanogenesis accounted for 43% and 68% of the consumed acetate in the 200-SO₄ and 500-SO₄ experiments, respectively (Table S4). The percentage of acetate consumed via sulfate reduction ranged from 18% to 58% and 28% to 51% in the 200-SO₄ and 500-SO₄ experiments, respectively, across all acetate concentrations without MoO₄²⁻ (Table S4). Lovley and Klug (1986) proposed that the relative importance of methanogenesis and sulfate reduction in freshwater lake sediments was determined by the rate of organic matter decomposition supplying acetate to both processes rather than by the porewater sulfate concentration (i.e., each is primarily limited by the electron donor). Methanogenesis becomes more important in freshwater sediments as the rate of organic matter decomposition and available acetate increases, causing sulfate reducers to consume sulfate in the uppermost sediment until it is below 30 µM (Lovley and Klug, 1986). In the acetate-amended 200-SO₄ and 500-SO₄ experiments, a higher initial acetate concentration of 1000 µM seems to mimic conditions of higher rates of organic matter decomposition.

Figure 9: Relationship between initial acetate concentration and % of acetate utilized for methanogenesis (closed symbols) and sulfate reduction (open symbols) in 200-SO₄ and 500-SO₄ experiments without MoO₄²⁻ (▲ 200-SO₄ experiment; ◆ 500-SO₄ experiment).

The results from the 200-SO₄ and 500-SO₄ experiments with initial acetate concentrations < 1000 μM, however, are not entirely consistent with Lovley and Klug's (1986) model. The microcosms used here were not designed to test the Lovley and Klug (1986) model as they lack a continuous supply of sulfate from the water column and acetate from relatively rapid organic matter breakdown. Mass balance calculations that assumed acetate to be the primary electron donor for both sulfate reduction and methanogenesis showed that increasing the initial acetate concentration up to 1000 μM decreased the relative proportion of acetate accounted for by both processes (Figure 9). The remainder of the acetate consumed in these experiments may have been utilized by other acetotrophic organisms. The results from the 200-SO₄ and 500-SO₄ experiments showed that total nmoles of PLFA recovered generally increased as the acetate concentration increased (Figure 6C). It seems likely that under coal bed conditions, neither sulfate reduction nor methanogenesis would become dominant unless conditions changed significantly, making acetate much more available.

In the 200-SO₄ and 500-SO₄ experiments without MoO₄²⁻, 25-75%, and 33-63% of available acetate, respectively, was consumed by processes other than dissimilatory electron flow via sulfate reduction or methanogenesis. Much of this acetate may have gone into anabolic processes in other acetotrophs. Therefore, we calculated whether the

amount of acetate added could explain the increase in measured biomass, as determined by PLFA analysis. The general biosynthetic pathway for the fatty acids of PLFAs involves a malonyl-acyl carrier protein (malonyl-ACP) complex initiating the reaction with an acetyl-ACP complex forming a 4 carbon, diacyl-ACP intermediate and releasing CO₂ at each elongation step. Chain elongation continues in this general manner, growing by 2 carbons with each acetyl unit added in each cycle, growing to a chain length of 16 carbons. By setting aside the energy requirements and assuming every carbon in the malonyl-ACP and acetyl-ACP could be provided by the available acetate in our experiments, we estimated the amount of PLFA that could be synthesized in the 200-SO₄ and 500-SO₄ experiments. These estimates were simplified further by assuming a 16 carbon fatty acid (the weighted average chain length of PLFAs observed), that there should be two 16C fatty acids per PLFA, and that PLFAs represented 9.1% of cell mass (Madigan et al., 2012). The estimated PLFA yields ranged from 2 to 139 nmole of 16C PLFA in the 200-SO₄ experiments, and 7 to 89 nmole of 16C PLFA in the 500-SO₄ experiments. These estimates are small compared with our measured PLFA amounts (Tables S2 and S3), yet agree with the order of magnitude of differences in PLFA amounts observed between amended and unamended experiments. This finding suggests that acetate utilization by anabolic processes may have accounted for the remainder of available acetate in these experiments.

Because the headspace gas in these experiments was 20% CO₂, we were unable to detect any increase in headspace CO₂ as a result of heterotrophic growth. However, the correlation between the bacterial PLFAs 14:0, 16:1 ω 7*cis*, 16:1 ω 5, 16:1 ω 7*trans*, and *cyclic*17:0 with the initial acetate concentrations in the 500-SO₄ experiments (Figure 6B), and similar correlations for the i17:0 and 16:1 ω 7*cis* in the 200-SO₄ experiment (Figure 6A), suggest that the added acetate indeed supported bacterial cell growth. The response of the 16:1 ω 7*cis* to added acetate was particularly strong in both the 200-SO₄ and 500-

SO₄ experiments, which suggests that the acetate-consuming bacteria produced significant amounts of this PLFA. As previously noted, relative to the non-acetate amended control, there was a statistically significant increase in bacterial PLFAs in the 200-SO₄ experiments with 500 and 1000 μM acetate, with and without MoO₄²⁻ (Figure 6C, Table S2). Although not statistically significant, increasing acetate may have also resulted in increased biomass in the 500-SO₄ experiments (Figure 6C). PELs were not measured in these experiments, but the lack of a significant correlation between T_{final} *mcrA* copy numbers and initial acetate concentrations suggests that methanogens were not a major sink of acetate. Alternatively, a large portion of acetate appears to have been consumed by bacteria other than SRM that also produce the 16:1ω7*cis* PLFA.

4.3 Microbial community structure

The functional gene for sulfate reducers, *dsrA*, increased significantly in the 500-SO₄ experiments without MoO₄²⁻ (Figure 8). Taken together, the correlation of 16:1ω7*cis* and *cyl*17:0 with acetate amendment, along with the increase in *dsrA* gene copies, suggests that SRM were stimulated by acetate amendment in the 500-SO₄ experiments. It should be noted, however, that none of these PLFAs can be definitively assigned to a specific group of SRM as they may be synthesized by other organisms as well (Hinrichs and Boetius, 2002). These same PLFAs were also detected in samples with MoO₄²⁻ in the 500-SO₄ experiments, but did not correlate with acetate concentrations. This finding suggests that either SRM were not solely responsible for the synthesis of these PLFAs, or that the addition of acetate did not stimulate growth of these organisms in the presence of MoO₄²⁻ but they were still viable at the time of PLFA extraction.

The ~10X increase in *mcrA* copy numbers in all var.-SO₄ microcosms without added acetate is consistent with varying sulfate concentrations having no discernible effect on methane production or methanogen growth, and is consistent with the

supposition that methanogenesis and sulfate reduction are more limited by the electron donor than the electron acceptor. In contrast, in the 500-SO₄ experiments, *mcrA* copy numbers only increased in microcosms with 1000 μM acetate plus MoO₄²⁻ (Figure 8). The acetate concentration did not exceed 300 μM in any of the var.-SO₄ experiment microcosms, therefore, the increase in *mcrA* copy numbers cannot be attributed solely to acetate availability. The differences in methane production between the two acetate-amended experiments and the var.-SO₄ experiments are likely the result of different microbial communities in each. The var.-SO₄ experiments produced more methane than the 200-SO₄ and 500-SO₄ experiments at comparable acetate concentrations. Both the 500-SO₄ and var.-SO₄ experiments contained similar numbers of *mcrA* copies of ~10⁵ – 10⁶ *mcrA* copies mL⁻¹ at the start of each experiment. With the differences observed between the PLFA profiles in the three experiments, and given the clustering observed on the PCA score plot (Figure 10), the most likely reason for the observed differences in methane production is different microbial communities in these experiments. It is possible that the var.-SO₄ experiment consortium included methanogens that were better able to utilize acetate at lower concentrations.

The PLFA profiles of the three different sets of experiments described herein should be compared carefully as each set was conducted at different times with an ever-evolving inoculum source. However, the PLFA profiles of samples collected from all three experiments had similarities, with some notable exceptions. For example, 18:1ω7 was absent from profiles in the var.-SO₄ experiments, but represented 7 – 15% of the total PLFAs in the 200-SO₄ and 500-SO₄ experiments (Tables S1, S2, and S3). The presence or absence of PLFAs in samples from the different experiments may explain the observed clustering of samples on the PCA score plot (Figure 10).

Figure 10: PCA of PLFA profiles from the var.-SO₄ (▲), 200-SO₄ (■), and 500-SO₄ (●) experiments, showing clustering of samples based on experiment.

The branched PLFA 10Me16:0 and the PLFA *cy*17:0 have been observed in *Desulfobacter* spp. grown on acetate (Dowling et al., 1986). The 10Me16:0 was observed in all microcosms in the var.-SO₄ and 500-SO₄ experiments, and the *cy*17:0 was observed in all samples in this study at amounts between 7 - 21 nmole, 63 – 95 nmole and 21 – 232 nmole in the var.-SO₄, 200-SO₄ and 500-SO₄ experiments, respectively (Tables S1, S2, and S3). However, there was no apparent correlation between either of these PLFAs with either acetate or sulfate concentration. In addition, the monoenoic PLFA 16:1 ω 7*cis*, identified in the sulfate reducer *Desulfotomaculum acetoxidans* (Dowling et al., 1986), was observed in all samples for all three experiments. This PLFA has been observed to be an important component of the PLFA profiles of sulfate-reducing communities consuming acetate (Boschker et al., 1998; Pombo et al., 2005). However, 16:1 ω 7*cis* is produced in many types of bacteria (Ratledge and Wilkinson, 1988), and therefore cannot be considered a reliable indicator of SRM. As previously noted, evidence presented here suggests that this PLFA may also be produced by acetotrophic bacteria that are not SRM (Figure 6A and B).

4.4 Implications for coal bed methane reservoirs

The median sulfate concentration in produced waters of the PRB is 1 μM , but the maximum is $\sim 500 \mu\text{M}$ (Rice et al., 2008). Our data suggest that sulfate may persist in areas of the basin with the highest sulfate concentrations because acetate concentrations less than 200 μM are typical of some CBM systems (Schlegel et al., 2013). Because coal is an inherently recalcitrant material that resists decomposition under anoxic conditions (Fakoussa and Hofrichter, 1999), any terminal microbial process will be limited by microbial activities, such as fermentation, occurring “upstream” in this biochemical chain. Acetate has been shown to be an important intermediate in the bioconversion of coal to methane (Beckmann et al., 2011; Green et al., 2008; Jones et al., 2010). Our data suggest that sulfate reduction in coal beds is also limited by the availability of acetate, with both sulfate reduction and methanogenesis potentially occurring simultaneously in coal bed reservoirs with low acetate concentrations ($< 1000 \mu\text{M}$). It is, however, important to note that microcosm experiments such as these are not uniform cell suspensions, and that the various metabolic groups competing for acetate may have occupied microsites within coal particles, potentially affecting the amount of methane produced. For example, the increase in acetate concentrations observed in the var.- SO_4 experiments (Figure 2) may have been due to diffusion of acetate from within coal particles into the liquid medium. In the subsurface we would expect porewater to be in constant contact with coal particles, limiting the possibility of rapid changes in acetate available to SRM, methanogens, or other acetotrophs.

Schlegel et al. (2011a) state that in portions of the Illinois Basin with a high $\delta^{34}\text{S}_{\text{SO}_4}$ and low sulfate concentration ($< 0.02 \text{ mM}$), microbial sulfate reduction occurred prior to methanogenesis and that methanogenesis has now taken over as the dominant terminal electron accepting process. Other portions of the basin contain waters with high $\delta^{34}\text{S}_{\text{SO}_4}$, low $\delta^{13}\text{C}_{\text{CO}_2}$ ($< -40\text{‰}$), and low $\alpha_{\text{CO}_2\text{-CH}_4}$ values (< 1.06) with high methane concentrations, suggesting that sulfate reduction occurred prior to the onset of

methanogenesis but that methanogenesis has not yet been sufficiently active to change the $\delta^{13}\text{C}_{\text{CO}_2}$ value (Schlegel et al., 2011a). However, our data suggest that sulfate reduction and methanogenesis may co-occur when both sulfate and acetate are maintained at low concentrations ($< 1000 \mu\text{M}$). Acetate in produced waters of the Illinois Basin ranges between <0.1 and $200 \mu\text{M}$ (Schlegel et al., 2013), conditions that might prevent either SRM or methanogens from dominating its consumption. Strapoc et al. (2008) did not detect SRM in microbial enrichments from Illinois Basin coal, though their enrichment strategy targeted methanogens. Under the model proposed here, the main determining factor whether SRM or methanogens are dominant is not the sulfate concentration but the metabolic activity of fermentative organisms supplying acetate, and perhaps also the consumption of acetate by bacteria other than SRM and methanogens. This finding may explain why appreciable sulfate concentrations are observed in methane-producing coal and shale basins such as the Illinois basin and PRB. Based on the results of this study, the co-occurrence of sulfate reduction and methanogenesis in anaerobic coal basins depends on the presence of low acetate and sulfate concentrations ($<1000 \mu\text{M}$) together with metabolically active SRM and methanogenic archaea in the formation. Our data also suggest that under such conditions a significant portion of available acetate could be consumed by microbial processes other than dissimilatory sulfate reduction and methanogenesis, including biosynthesis reactions. This model of coalbed methanogenesis does not require methanogens to be the dominant consumers of acetate at any given time, and despite their competition for acetate with SRM and other acetotrophs, methane can accumulate in reservoirs as long as the geochemical and hydrogeologic conditions are favorable. The “resource ratio competition model” proposed by Tilman (1977) perhaps best explains a mechanism by which SRM and methanogens may coexist in coalbeds. In hydrologic zones where acetate is relatively more abundant, SRM would be limited by the sulfate concentration, allowing

methanogens to compete for excess acetate. Conversely, when sulfate is more abundant, both metabolic groups would be limited by the available acetate and neither group may establish a large enough population to outcompete the other for the limited acetate. The Powder River Basin may be the most prominent example of such a system, where low sulfate and acetate concentrations, negligible dissolved oxygen or other electron acceptors, long residence times for reservoir fluids, and a reservoir structure conducive to gas trapping have resulted in significant gas accumulations (Bates et al., 2011). From a practical standpoint, any strategy to enhance methanogenesis from coal via increased acetate production from fermentation should consider the possibility of the coexistence of SRM and methanogens *in situ* before proceeding.

Conclusion

The microcosm experiments conducted in this study showed that microbial methanogenesis and sulfate reduction can occur simultaneously in coal microcosms with low sulfate concentrations ($<1000\ \mu\text{M}$) and low acetate concentrations ($<1000\ \mu\text{M}$). Methane production was significantly higher in microcosm experiments amended with at least $500\ \mu\text{M}$ acetate when SRM were inhibited with MoO_4^{2-} . The relative importance of methanogenesis compared to sulfate reduction decreased in acetate-amended microcosms as the acetate concentration increased up to $1000\ \mu\text{M}$, regardless of sulfate concentration, indicating that SRM were able to out-compete methanogens when acetate was more available. The primary control on microbial sulfate reduction in coal-degrading microcosms was the acetate concentration. Our data indicate that both processes may occur at low rates in sedimentary basins with limited labile carbon and acetate production. Under low acetate and low sulfate conditions, we would expect to find a significant portion of acetate consumed by other microbial processes besides sulfate reduction or methanogenesis.

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Tables

Table 1: Summary of experimental conditions.

Experiment	Sulfate concentration	Acetate concentration
Var.-SO ₄	0 to 1000 μ M	None added, up to 300 μ M initial acetate
200-SO ₄	200 μ M	50 to 1000 μ M
500-SO ₄	500 μ M	100 to 1000 μ M

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