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Title: **Changes in microbial communities and associated water and gas geochemistry across a sulfate gradient in coal beds: Powder River Basin, USA**

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Abstract. Competition between microbial sulfate reduction and methanogenesis drives cycling of fossil carbon and generation of CH_4 in sedimentary basins. However, little is understood about the fundamental relationship between subsurface aqueous geochemistry and microbiology that drives these processes. Here we relate elemental and isotopic geochemistry of coal-associated water and gas to the microbial community composition from wells in two different coal beds across CH_4 and SO_4^{2-} gradients (Powder River Basin, Montana, USA). Areas with high CH_4 concentrations generally have higher alkalinity and δ^{13}C-DIC values, little to no SO_4^{2-} , and greater conversion of coal-biodegradable organics to CH_4 (based on δ^{13}C-CH_4 and δ^{13}C-CO_2 values). Wells with SO_4^{2-} concentrations from 2-10 mM had bacterial populations dominated by several different sulfate-reducing bacteria and archaea that were mostly novel and unclassified. In contrast, in wells with SO_4^{2-} concentrations <1 mM, the sequences were predominated by presumptive syntrophic bacteria as well as archaeal *Methanosarcinales* and *Methanomicrobiales*. The presence of sequences indicative of these bacteria in low SO_4^{2-} methanogenic wells may suggest a syntrophic role in coal biodegradation and/or the generation of methanogenic substrates from intermediate organic compounds. Archaeal sequences were observed in all sampled zones, with an enrichment of sequences indicative of methanogens in low SO_4^{2-} zones and unclassified sequences in high SO_4^{2-} zones. However, sequences indicative of *Methanomassiliicoccales* were enriched in intermediate SO_4^{2-} zones and suggest tolerance to SO_4^{2-} and/or alternative metabolisms in the presence of SO_4^{2-}. Moreover, sequences indicative of methylotrophic methanogens were more prevalent in an intermediate SO_4^{2-} and CH_4 well and results suggest an important role for methylotrophic methanogens in critical zone transitions. The presented results demonstrate *in situ* changes in bacterial and archaeal population distributions along a SO_4^{2-} gradient associated with recalcitrant, organic carbon that is biodegraded and converted to CO_2 and/or CH_4.

1. Introduction

In organic-rich formations within sedimentary basins, such as coal beds, complex, methanogenic communities drive the cycling of fossil carbon (coal, shale, oil) and generation of natural gas (methane; CH_4) (Strąpoć et al., 2008). Different coal beds within or between basins contain different amounts of biogenic CH_4, and little is known about the relationship between microbial community dynamics, turnover of recalcitrant carbon, aqueous geochemistry and CH_4 concentrations. Sulfate-reducing bacteria (SRB) are assumed to out-compete methanogens for substrates (*e.g.*, H_2, acetate, formate) in the presence of SO_4^{2-}, while in low SO_4^{2-} conditions (<1 mM), methanogenesis is the terminal process for anaerobic mineralization of organic carbon (Muyzer and Stams, 2008). Yet, little is known about methanogenic activity at marginal SO_4^{2-} levels and/or transition zones in terrestrial environments (Ma et al., 2017). In addition, little is known about methanogens that utilize alternative (non-competitive with SRB) substrates, such as methanol or other methyl-donors, to produce CH_4 potentially under higher SO_4^{2-} conditions (Vinson et al., 2017).

Recent laboratory and pilot field-scale studies have demonstrated that methanogens and associated microbial communities can be stimulated, by addition of nutrients and trace metals, to generate 'new' CH_4 to sustain the lifetime of existing coalbed CH_4 (CBM) wells (*e.g.*, Ulrich and Bower, 2008; Jones et al., 2010; Barnhart et al., 2017; Davis et al., 2018). In order for microbially enhanced CBM (MeCoM) to be advanced, it is important to understand microbial community dynamics during the conversion of complex organic substrates to CH_4 under different *in situ* environmental conditions (Ritter et al., 2015; Davis et al., 2018).

Much of the previous research on microbial methanogenesis in coal beds has focused on single types of analyses, such as aqueous geochemistry (*i.e.*, alkalinity and SO_4^{2-} concentrations) or the isotopic signature of produced gases (*e.g.*, $\delta^{13}C$-CO_2; $\delta^{13}C$-CH_4; δD-CH_4) to infer metabolic pathways of methanogenesis (*i.e.*, hydrogenotrophic, acetoclastic and/or methylotrophic) and bacterial sulfate reduction (*e.g.*, Flores et al., 2008; Rice et al., 2008; McIntosh et al., 2010). Separate studies have

investigated the archaeal and bacterial communities in coal beds (*e.g.*, Green et al., 2008; Klein et al., 2008), while only a few studies have compared microbial communities spatially across basins or between different coal beds (Penner et al, 2010; An et al., 2013; Shelton et al., 2016).

Most studies have identified microbial communities associated with the conversion of organic substrates to CH_4 via formation water or core samples from single boreholes (e.g. Strąpoć et al., 2008; Jones et al., 2010; Penner et al., 2010; Ünal et al., 2012). However, research has suggested that microbial community characterization through the analysis of microorganisms in formation waters does not fully capture subsurface microbial processes (Alfreider et al., 1997; Penner et al., 2010). Moreover, intact core samples are difficult and costly to collect aseptically. For this reason, down-well incubations have been used as an alternative for community characterizations (*e.g.* Alfreider et al., 1997; Griebler et al., 2002; Peacock et al., 2004; Reardon et al., 2004), and the present study utilized a down-well incubation technique with a diffusive microbial sampler (DMS) as previously described (Barnhart et al., 2013).

The current study investigated spatial variability of microbial communities in coals and how this distribution is related to SO_4^{2-} concentrations and other aqueous environmental parameters that can be reflected in water and gas isotopic signatures. The study focused in the Powder River Basin (PRB) in Wyoming and Montana, one of the first large basins to undergo intensive development of microbial CBM. Results from this study highlight unique microbial populations across a terrestrial critical zone transition with respect to subsurface recalcitrant carbon and microbial sulfate reduction and methanogenesis.

2. Background

2.1. Microbial Methanogenesis and Bacterial Sulfate Reduction

Microbial methanogenesis represents the final major step of the biodegradation of organic carbon, which becomes thermodynamically favorable after alternative electron acceptors (*e.g.,* ferric

iron and SO_4^{2-}) have been exhausted (Kuivila et al., 1989). Degradation of organic matter under methanogenic conditions involves microbial consortia that break down complex organic matter into intermediate substrates (e^- and/or carbon) such as acetate, formate, CO_2, and H_2 (Jones et al., 2010; Orem et al., 2010; Strąpoć et al., 2011). Methanogens then convert these simplified compounds to CH_4 and CO_2 by two dominant pathways: 1) CO_2 reduction (hydrogenotrophic methanogenesis), and 2) acetate fermentation (acetoclastic methanogenesis) (Ferry, 1993). CH_4 may also be generated by methylotrophic methanogens which use a range of methylated compounds including methanol and methylamines produced by coal kerogen demethoxylation (Strąpoć et al., 2011). Methanol is a non-competitive substrate that is not utilized by SRB, opening up the possibility that methanogens may co-exist with SRBs in coal beds independent of SO_4^{2-} levels (Barnhart et al., 2013), as has been shown in SO_4-rich marine sediments (Whiticar, 1996; Whiticar et al., 1986). Moreover, the conditions leading to the development of sulfate reduction over methanogenesis are of particular interest to researchers investigating the potential of stimulating microbial methanogenesis for MeCoM as well as for improved understanding of methane emissions from coal formations as high SO_4^{2-} concentrations can be observed in CBM reservoirs (Ritter et al., 2015).

Studies utilizing carbon stable isotope ($\delta^{13}C$) and δD values of CH_4 and $\delta^{13}C$ values of CO_2 in the PRB have suggested that the dominant metabolic pathway for CBM generation is CO_2 reduction (hydrogenotrophic methanogenesis; Flores et al., 2008), whereas some microbial enrichments have shown a predominance and/or mixture of acetoclastic methanogens (Green et al., 2008; Ulrich and Bower, 2008). It is common to observe a shift in the microbial community towards acetoclastic methanogens in laboratory coal enrichment experiments, as acetoclastic methanogens often grow faster compared to hydrogenotrophic methanogens when stimulated with common amendments (*e.g.*, acetate, algal extracts or yeast extracts; Jones et al., 2010; Barnhart et al., 2013).

The relative dominance of different methanogenic pathways in subsurface, terrestrial environments depends upon multiple factors, including nutrient and carbon availability, salinity, and temperature (Alperin et al., 1992; Zinder, 1993; Nakagawa et al., 2002; Warren et al., 2004; Megonigal et al., 2005). Limited organic substrates and longer water residence times have been shown to favor CO_2 reduction, whereas rapid recharge and large supplies of fresh organic matter have been shown to favor acetate fermentation in anoxic wetland sediments (Nakagawa et al., 2002).. In addition, acetoclastic methanogens may be inhibited by the build-up of toxic organic compounds (Warren et al., 2004; Jones et al., 2010) or high salinity (>1 M Cl^-; Waldron et al., 2007). Salinity or temperature limitations for methanogens or SRB are not expected in the PRB as coal waters are relatively dilute (<5,000 mg/L total dissolved solids) and formation temperatures are low (<30°C) (Bates et al., 2011). Rather, we hypothesize that SO_4^{2-} concentrations, which can be variable in the PRB, are the dominant control on methanogenic pathways, and a systematic characterization of bacterial and archaeal communities in PRB coal beds has not been reported in conjunction with changing hydrogeology and geochemistry.

2.2. Powder River Basin Geology

The PRB is a drainage and structural basin located in southeastern Montana and northeastern Wyoming (Figure 1A). The basin is bordered by the Bighorn Mountains to the west, Black Hills to the east, and the Casper Arch, Laramie Mountains, and Hartville Uplift to the south and covers approximately 20,000 km^2 and asymmetrical with the axis near the western edge (Flores et al., 2008). The basin was formed during the Laramide Orogeny, which also uplifted the surrounding mountains (Anna, 1986). The main coal-bearing unit is the Tertiary Fort Union Formation (700-1800 m thick), deposited 66-58 Ma (Anna, 1986). The uppermost Fort Union Formation (the Tongue River Member) contains sandstone, siltstone, shale, some carbonates and conglomerates, and regionally-extensive thick (up to 77 m) coals referred to as the Wyodak-Anderson coal zone (Flores, 2004). Coals were deposited in

rivers, floodplains, and wetlands in the basin (Flores and Ethridge 1985; Flores, 1986; Lillegraven, 1993; Flores, 2004). Samples collected as part of this study are from the Wyodak-Anderson coal zone (Canyon (Monarch/Carney) and Anderson coals) (Figure 1B).

The Wyodak-Anderson coal zone is a regional aquifer within the Fort Union Formation (Daddow, 1986; Lowry and Wilson, 1983; Bartos et al., 2002) and has been a major target of CBM production since the 1990s. In general, groundwater along the northwestern margin of the PRB, in the study area, flows from the Big Horn Mountains towards the northeast, in the same direction as the Tongue River (Lobmeyer, 1985). Local recharge occurs through clinker deposits that form ridges and hilltops, and act as hydrologic conduits to adjacent coals due to high permeability (Heffern and Coates, 2004).

2.3. Aqueous Geochemistry of CBM

Produced waters from PRB coalbeds are primarily Na-HCO_3 type (Lee, 1981; Van Voast, 2003; Brinck et al., 2008; Rice et al., 2008; Bates et al., 2011). Bicarbonate accumulates in CBM systems as a result of the respiration of CO_2 from microbially-mediated redox processes (Lee, 1981; Van Voast, 2003; Brinck et al., 2008). Calcite precipitation and cation exchange on clays lowers Ca^{2+} concentrations and enriches coal waters in Na^+. In areas near the basin margin where methanogenesis is absent, waters can contain significant concentrations of SO_4^{2-}, in addition to Na^+ and HCO_3^- (Brinck et al., 2008).

Nutrients, such as nitrogen and phosphorus, may be limiting in methanogenic environments (Penner et al., 2010; Bates et al., 2011). Total dissolved nitrogen concentrations in the PRB coal waters ranged from 50 to 1000 µM, and PO_4^{3-} concentrations ranged from below the detection limit to 5 µM (Bates et al., 2011). Dissolved organic carbon (DOC) is also important to methanogenesis because intermediate organic substrates, such as long-chain fatty acids, alkanes, and low-molecular-weight aromatics are utilized by syntrophic communities to produce CH_4 (Orem et al., 2010; Strąpoć et al.,

2011). Previous measurements of DOC in PRB coal waters ranged from 0.11 to 0.93 mM (Orem et al., 2014).

2.4. Isotopic tracers of methanogenesis

Vinson et al. (2017) showed that the difference between $\delta^{13}C\text{-}CH_4$ and $\delta^{13}C\text{-}CO_2$ is challenging to apply to field-based studies, such as previously done in the PRB (Flores et al., 2008), where methanogenesis competes with non-methanogenic pathways, such as bacterial sulfate reduction. This is problematic because it is routinely used to infer the apparent fractionation factor (α) of acetoclastic, hydrogenotrophic, or methylotrophic methanogenesis in cultures. One central reason for this difficulty is that $\delta^{13}C\text{-}CH_4$ and $\delta^{13}C\text{-}CO_2$ values reflect not only the methanogenic fractionation factor, but also the proportion of metabolized carbon (*f*) that is routed through methanogenesis. The value of *f* in a system reflects (1) the oxidation state of low-molecular weight (LMW) intermediates (electron balance between CH_4 and CO_2) and (2) the competition between methanogenesis and non-methanogenic processes (*e.g.*, bacterial sulfate reduction). Therefore, *f* records the extent to which LMW is consumed by methanogenesis. In this study, we analyzed $\delta^{13}C\text{-}CH_4$ and $\delta^{13}C\text{-}CO_2$ in methanogenic coal bed waters to calculate *f*, which was compared to aqueous environmental conditions and the microbial community composition to determine controls on coal biodegradation and microbial methanogenesis.

3. Methods

3.1. Sample locations and coal zones

Samplers (DMS) and water samples were collected from 7 monitoring wells operated by the Montana Bureau of Mines and Geology (MBMG) in the PRB (Fig. 1; Table 1). All wells sampled were completed in single coal zones. Four of the monitoring wells (WR-33, WR-48, WR-34 and SH-396) were completed in the Anderson coal zone along Young's Creek in Bighorn County, Montana. These wells were along a linear surface transect ~7 km long. The other 3 monitoring wells (WR-24, CBM02, and

HWC) were completed in the Canyon Coal ~20-35 km apart. Two of the Canyon wells are located on the west side of the Tongue River, with one well along Young's Creek and the other northwest of the Tongue River Reservoir along Highway 314. The third Canyon well is located along Hanging Woman Creek on the east side of the Tongue River. Water and dissolved gas samples from Canyon coal wells were collected in 2011 and 2014, whereas water and dissolved gas samples from Anderson coal wells were collected in 2013 and 2014 (Table 2). Microbial samples were collected in 2010, 2011, 2012, and 2013 from the Canyon coal wells, and 2012 and 2015 from the Anderson coal wells (Table 3). Microbial samples were not collected at the same time as water and dissolved gas samples; however, there was minimal temporal variability in chemical parameters (*i.e.*, major ion chemistry, water stable isotopes) between sampling dates.

3.2. Field Sample Collection

3.2.1 Water Sampling

Water samples were collected with a Grundfos submersible pump after three wellbore volumes were pumped and field parameters stabilized (water temperature, pH, and dissolved oxygen). Temperature was measured using an Oakton temperature probe, pH was measured using an Oakton pH 110 meter and an Orion Ross Combination electrode, and dissolved oxygen was measured using a YSI meter. All water samples for chemical and isotopic analysis were filtered using a 0.45-µm syringe tip nylon filter, except for DOC and nutrients, and stored on ice in the field and in a refrigerator 4°C until analysis. Water samples for DOC were filtered through 0.7-µm pre-combusted glass fiber filters and kept in 30-mL pre-combusted amber glass bottles. Water samples for nutrient (NH_4^+ and PO_4^{3-}) analyses were filtered using syringe tip Whatman Polyethersulfone (PES) 0.2-µm filters, and kept in 30 mL HDPE bottles.

Samples for major cations were collected in 60-mL HDPE bottles with no headspace, and two drops of concentrated Optima Grade nitric acid was added to lower the pH to <2. Samples for anions were collected in DI-washed 60-mL HDPE bottles with no headspace. Samples for carbon stable isotope ($\delta^{13}C$) values of dissolved inorganic carbon (DIC) were collected in glass serum bottles, preserved with mercury chloride, and capped with no headspace. Samples for $\delta^{34}S$-SO_4^{2-} and $\delta^{18}O$-SO_4^{2-} were collected in 1-L HDPE bottles and ten drops of concentrated nitric acid were added for to prevent bacterial sulfate reduction.

3.2.2. Dissolved Gas Samples

Dissolved gas samples were collected from the monitoring wells after purging and at the same time as the water samples for chemical and isotopic analysis described above. Samples for gas isotopes and gas composition were collected by filling a 5-gallon bucket with water. Next, a dissolved gas bottle manufactured by Isotech Laboratories, Inc. was submerged and inverted. A hose from the well was then inserted into the bottle, and water and gas were allowed to flow into the bottle for approximately 5 minutes. The hose was removed, and the bottle was capped upside down and stored inverted on ice until it was sent to Isotech Laboratories, Inc. for analysis. In addition, in order to measure dissolved CH_4 concentration, an additional bottle was filled in a similar manner, but with the submerged bottle remaining upright instead of inverted.

3.2.3. Microbial Samples

Samples for microbial community analysis were collected from monitoring wells in the Canyon and Anderson coal beds using a DMS (Barnhart et al., 2013) and from filtered groundwater samples. The DMS cylinder (12.7 cm long and 6.4 cm in diameter) was filled with 25 g of subbituminous coal (2 mm – 4 mm particle size) from the coal bed to be sampled. Coal particles were enclosed in a mesh within the

DMS cylinder. DMS samplers were lowered into monitoring wells and allowed to incubate for 3 months, after which time samplers were removed from wells using aseptic techniques and returned to the laboratory for analysis. Following retrieval of the DMS, groundwater samples for microbial analysis were obtained by pumping the well with a Grundfos submersible pump until three wellbore volumes were pumped and field parameters stabilized prior to microbial sampling (as described for water chemistry). Water was filtered through a 0.45-µm syringe filter until the filter plugged and no additional water passed through to obtain the maximum concentration of microorganisms for DNA analysis. The filters were immediately stored on dry ice and taken back to the lab for analysis.

3.3. Analytical Methods:

3.3.1. Water and Dissolved Gas:

Alkalinity was titrated in the field within 12 hours of sample collection using the Gran-Alkalinity titration method (Gieskes and Rogers, 1973). Major cations were analyzed with a Perkin-Elmer Optima 5100DV Inductively Coupled Plasma-Optical Emission Spectrometer (precision ±2%), and major anions were analyzed using a Dionex Ion Chromatograph model 3000 with an AS23 analytical column (precision ±2%) in the Department of Hydrology and Atmospheric Sciences at the University of Arizona in Tucson, Arizona. Charge balance error was less than 5% for all measured waters, with the exception of water sampled from well WR-33, where the charge balance error was 13.6%, which persisted even after reanalysis of all major ion components. δ^{13}C-DIC (1-σ precision ±0.3‰), δ^{34}S-SO_4^{2-} (1-σ precision ±0.15‰) and δ^{18}O-SO_4^{2-} (1-σ precision ±0.7‰) were analyzed at the University of Arizona Environmental Isotope Laboratory. Samples were measured on a ThermoQuest Finnigan Delta Plus XL continuous flow gas ratio mass spectrometer.

DOC was determined using a Shimadzu TOC-VCPH analyzer in the U.S. Geological Survey (USGS) Eastern Energy Resources Program Laboratory in Reston, Virginia. The method detection limit was 100 ppb. NH_4^+ and PO_4^{3-} were determined using standard colorimetric methods on a Seal Analytical AQ2

Automated Discrete Analyzer also in the USGS Laboratory. The detection limit was 0.05 mg/L for both NH_4^+ and PO_4^{3-}.

Dissolved gas molecular and isotopic composition was measured at Isotech Laboratories, Inc. (Champaign, Illinois). Dissolved gases in mole % were measured by gas chromatography, and C and H isotopes of CH_4 and C isotopes of CO_2 were measured by isotope ratio mass spectrometry. Select samples were also analyzed for dissolved CH_4 concentrations (reported in mmole/L) by gas chromatography. Correlations between chemical constituents were investigated using Principle Components Analysis (PCAs) (Leps and Smilauer, 2003). When multiple water chemistry analyses were available for wells, average values were used in the PCAs.

3.3.2. Microbiology

DNA was extracted from the coal and slurry from the DMS samplers and the groundwater filters using a FastDNA Spin Kit for Soil (MP Biomedical) and purified using One Step PCR Clean Up (Zymo Research). The bacterial and archaeal SSU rRNA genes were amplified using a universal prokaryotic primer as described in Takahashi et al. (2014). A 0.8% agarose gel in TAE buffer was used to check the PCR products for DNA of the correct size. The purified PCR amplicons were sequenced with an Illumina MiSeq (Illumina, San Diego, CA, USA) following the "16S Metagenomics Sequencing Library Preparation" Illumina protocol for paired end sequencing (support.illumina.com/documents/documentation/). Following PCR clean up, purification, and indexing PCR, DNA concentration was determined using PicoGreen stain (Quant-IT, Invitrogen). DNA concentrations were normalized and pooled with a 12.5% PhiX control library. Forward and reverse reads were joined using QIIME (Caporaso et al., 2010). The sequences were aligned using SILVA (Quast et al., 2013). The aligned reads were quality filtered, chimeras were removed, and OTUs and phylotypes were classified with an 80% confidence using RDP database with Mothur version 1.38.1 (Haas et al., 2011, Wang et al., 2007). Mothur 1.38.1 was used to

calculate species richness using Inverse Simpson Index. Canoco was used to compare the inter-species correlations divided by the standard deviation to generate the principal components analysis (PCAs) (Leps and Smilauer, 2003). The cladograms were created using the Linear Discriminant Analysis Effect Size (LEfSe) analysis following the parameters set by Segata et al. (2011).

The qPCR analysis was performed as previously described in Jones et al. (2010) with the following modifications: parameters were adjusted for the use of high-fidelity Kapa® HiFi HotStart ReadyMixPCR kit according to manufacturers instructions and synthetic DNA (g-Blocks ®) were used to generate the standard curve for absolute quantification.

4. Results

4.1. Geochemistry

The CBM monitoring wells were divided into two groups based on SO_4^{2-} concentration: "high SO_4^{2-} wells" with SO_4^{2-} >2 mM and "low SO_4^{2-} wells" with SO_4^{2-} <1.4 mM (Table 1; Fig. 2), as SO_4^{2-} concentration is known to influence microbial community composition (Muyzer and Stams, 2008). Well HWC contained <0.01 mM SO_4^{2-}, and well SH-396 contained 0.03 to 2.60 mM SO_4^{2-} (1.30 mM average value) (Table 1). These two wells were classified as "low SO_4^{2-} wells". Groundwater samples from the other five monitoring wells (CBM02, WR-34, WR-48, WR-33, WR-24) contained higher SO_4^{2-} concentrations from 2.78 to 11.50 mM (Table 1), and were classified as "high SO_4^{2-} wells".

The two low SO_4^{2-} wells were the only monitoring well samples with substantial concentrations of dissolved CH_4, ranging from 0.75 to 3.74 mM (Table 2; Fig. 2a). The high SO_4^{2-} wells contained low dissolved CH_4 concentrations from 4.67×10^{-5} to 2.37×10^{-3}. Well WR-33 had the lowest dissolved CH_4 concentration, whereas well WR-24 had the highest SO_4^{2-} concentration and second lowest CH_4 concentration. Nearby CBM production wells, reported in Bates et al. (2011), contained 29 to 98 mole % CH_4, and little to no detectable SO_4^{2-} (< 0.1 mM). Dissolved CH_4 was not measured in produced waters from CBM production wells. Thus, we assumed production wells contained at least 10 mM CH_4 in order

to include the reported values (*e.g.*, Figure 2a) at the top of the y-axix for comparison to the shallower groundwater monitoring wells.

Groundwater from all of the monitoring wells contained low dissolved oxygen (DO ≤4% saturation), had pH values from 7.0 to 8.5, and temperatures ranging from 9.7 to 15.5^{o}C (Table 1). Major ion concentrations were consistent between monitoring wells with similar CH_4 concentrations (Table 1). Sodium concentrations were high in all monitoring well samples (16.02-30.95 mM), except for water from well WR-33 (3.03 mM average). In contrast, Ca^{2+}, Mg^{2+}, K^{+} and Cl^{-} concentrations were low (< 1.5 mM) in all water samples, except for WR-33 and WR-24. Calcium in WR-33 water was 3.30 mM (average), and Mg^{2+} in WR-33 and WR-24 was 7.19 and 1.10 mM, on average, respectively.

Alkalinity generally increased with increasing dissolved CH_4 concentrations (Fig. 2b), with the highest alkalinity value (28.97 meq/kg) measured in low SO_4^{2-} monitoring wells and CBM production wells (Bates et al., 2011), except for WR-34, which contained 4.38 mM SO_4^{2-} (high SO_4^{2-} well) and 17.82 meq/kg alkalinity. Alkalinity values of low SO_4^{2-} wells were within the range of CBM production wells (Bates et al., 2011) (Fig. 2b). Nitrate concentrations were below the mean detection limit of the ion chromatograph in all monitoring well samples, except WR-33, which contained 0.17 mM NO_3^{-}. NH_4^{+} concentrations in groundwater from the monitoring wells ranged from <0.03 to 0.46 mM (Table 1), whereas PO_4^{3-} concentrations ranged from below detection (<0.03 µM) to 1.41 µM (Table 1). DOC concentrations in groundwater from monitoring wells ranged from 0.12 to 0.79 mM (Table 1). Values of DOC, and other constituents plotted in Fig. 2 are from dates where dissolved CH_4 was also measured (Table 2). Higher CH_4 wells were correlated to higher PO_4^{3-} and DOC concentrations in the Principal Components Analysis (PCA). However, there was no clear relationship between dissolved CH_4 and NH_4^{+} concentrations, although the monitoring well (WR-33) with the lowest CH_4 and alkalinity concentrations had no detectable NH_4^{+}. Other PCA results, which help to summarize the key geochemical similarities

and differences between the wells across the SO_4^{2-} gradient, are discussed below in relationship to the microbial results.

δ^{13}C-DIC values generally increased with increasing alkalinity (Fig. 3a; Table 4), with the highest δ^{13}C-DIC values observed in groundwater from the CBM production wells, previously reported by Bates et al. (2011), and monitoring well HWC that had the highest dissolved CH_4 and lowest SO_4^{2-} concentration. Well SH-396 had δ^{13}C-DIC values within the range of the low SO_4^{2-} wells. δ^{34}S-SO_4^{2-} values ranged from -0.3‰ to 22.0‰ in groundwater from the monitoring wells, whereas formation water from CBM production wells had δ^{34}S-SO_4^{2-} values ranging from -4.4‰ to 101.2‰ (Bates et al., 2011) (Fig. 3b; Table 4). Wells SH-396, WR-34 and CBM02 had δ^{34}S-SO_4^{2-} values $\geq$19.6 ‰.

Only the two, low SO_4^{2-} monitoring wells (HWC and SH-396) and one of the high SO_4^{2-} wells (CBM02) contained enough CH_4 to measure δ^{13}C-CH_4 (Table 2; Fig. 4). For these samples, δ^{13}C-CO_2 values generally increased with increasing δ^{13}C-CH_4 values and were within the same range as nearby CBM production wells (Bates et al., 2011) (Fig. 4). The highest CH_4 sample (HWC) had the highest δ^{13}C-CH_4 and δ^{13}C-CO_2 values and greatest calculated extent of methanogensis (*i.e.*, *f* value). The 'f' contours in Figure 4 were calculated assuming that the δ^{13}C value of biodegradable organics (*i.e.*, LMW) within the coals was -25‰ (Vinson et al., 2017).

4.2. Microbiology

Fewer microbial operational taxonomic units (OTUs) were detected in the filtered water samples compared to the respective DMS coal slurry samples incubated down-well (Table 3), and there was a more even distribution for each OTU (as shown by the Inverse Simpson index) for the water samples versus the DMS samples. In addition, fewer archaeal sequences were detected in the filtered groundwater samples compared to the DMS slurry samples (6 unique OTUs versus 21 out of 71 versus 294 total sequences, respectively). Moreover, with greater evenness in the sampled water communities,

fewer correlations were observed between the sampled archaeal sequences from the groundwater and associated geochemistry. Conversely, the DMSs showed increased total sampled OTUs and decreased evenness (Table 3). Microbial communities in the filtered water samples showed little or no correlation to SO_4^{2-} levels or other geochemical parameters in the LEfSe analysis (Fig. S1.) Thus, differences in methanogenic communities along the SO_4^{2-} gradient would not have been observed with analysis of groundwater only, and the DMS samples are the focus of discussed microbial results. For archaea, unique sequences were not detected in the groundwater, and for bacteria, the following groups were enriched in groundwater across all samples: Comamonadaceae, Desulfuromonadaceae, Acidobacteriae, Ignavibacteriae (data not shown). In addition, there was not a substantial temporal variability of DMS or filtered water microbial results between sampling dates, or between the two different coal types (Anderson versus Canyon coals) based on the PCA results. Bacterial communities in samples collected in 2010 were most closely related to samples collected from the same well in 2012 in the PCA. This is consistent with the relatively long residence time of coal waters in the study area, on the order of 10^3 to 10^4 years (Pearson, 2002; Frost and Brinck, 2005; Randle, 2014; Ritter et al., 2015). Thus, microbial results based on sampling date or coal zone are not discussed further.

Irrespective of coal type or time sampled, the bacterial communities from the low SO_4^{2-} wells grouped in the PCA, while the high SO_4^{2-} wells formed a distinct, larger grouping (Fig. 5). Sulfate and CH_4 were inversely proportional (Fig. 5). Higher CH_4 wells were also correlated to higher PO_4^{3-} and DOC levels. The coal slurry samples (DMSs) showed 5 dominant bacterial groups associated with the low SO_4^{2-} wells that included families Geobacteraceae, Veillonellaceae, Parachlamydiaceae, Verrucomicrobiaceae, and Oxalobacteraceae (Fig. 5). The bacterial groups Veillonellaceae, Parachlamydiaceae, and Verrucomicrobiaceae were tightly aligned with the CH_4 vector in the PCA for the low SO_4^{2-}, high CH_4 wells (Fig. 5). In contrast, the high SO_4^{2-} wells displayed a larger distribution in the PCA with two sub-groups. One group correlated to low DOC and Na^+ with co-occurrence of Desulfobacteraceae. The

second sub-group appeared to be driven more by NH_4^+ levels and had co-occurrence with Peptococcaceae, Rhodocyclaceae, and Comamonadaceae (Fig. 5). The relative abundance graph for the bacteria showed that Desulfobacterales and Clostridales were the most predominant orders in high SO_4^{2-} wells while Desulfuromonadales and unclassified bacteria were the most dominant orders in low SO_4^{2-} wells (Fig. S2).

In terms of the archaeal populations, the low SO_4^{2-} wells were grouped in the PCA, as were the high SO_4^{2-} wells, with the exception of the low SO_4^{2-}, Anderson coal well (SH-396) that tracked more with the high SO_4^{2-} wells compared to the HWC samples (Fig. 6). All the classified archaeal sequences were members of the Euryarchaeota phylum for both high and low SO_4^{2-} wells. Five archaeal groups tightly aligned with the CH_4 vector for the low SO_4^{2-} wells, and included the genera *Methanolinea*, *Methanospirillum*, *Methanolobus*, *Methanoregula*, and *Methanosaeta* (Fig. 6). In the high SO_4^{2-} wells and SH-396, five archaeal populations were grouped, including genera *Methanomassiliicoccus*, *Methanosphaerula*, and *Methanobacterium,* while one archaeal group, an unclassified archaea, correlated to two high SO_4^{2-} wells (WR-34, WR-48) (Fig. 6). The relative abundance graph for the archaea showed that Methanomassiliicoccales was the most predominant order in high SO_4^{2-} wells while Methanosarcinales was the most dominant order in low SO_4^{2-} wells (Fig. S3).

In order to infer potential for methanogenic and SO_4^{2-} reducing activity in the different wells, qPCR analysis was performed tarketing biomarker genes for SO_4^{2-} reduction (*dsr*B) and methanogenesis (*mcr*A) (Fig. 7). Both *dsr*B and *mcr*A were detected in all the samples, but for the higher sulfate wells WR-24 and WR-28 only *dsr*B was detected. The qPCR results indicated *mcr*A abundance was higher in the low SO_4^{2-} wells and low in the high SO_4^{2-} wells while the *dsr*B appeared to be highest in the CBM02 that had 'interemdiate' levels of sulfate (Fig. 7).

When the SSU rRNA gene sequences for both bacteria and archaea from the DMS coal slurry were used in LEfSe analyses, strong linear discriminant analysis scores for several sequences were

statistically correlated to high or low SO_4^{2-} wells and allowed for further classification from the phylum level to the genus level. Sequences indicative of the PVC super phylum (Planctomycetes, Verrucomicrobia and Chlamydiae) were identified as being prevalent in the low SO_4^{2-} wells and the sequences from the PVC super phylum were further classified at the genus level as *Luteolibacter, Parachlamydia* and *Planctomyces* (Fig. 8). LEfSe analyses indicated the following sequences for the low SO_4^{2-} wells: *Anaerospora (*Veillonellaceae*), Janthinobacterium*, *Massilia*, and *Oxalicibacterium* (Oxalobacteraceae) (Fig. 8). LEfSe identified other sequences indicative of organisms that were not in the PCA and more prevalent in the low SO_4^{2-} wells such as *Desulfuromonas, Acidithiobacillus, Methylomonas*, and *Methylococcus* (Fig. 8).

LEfSe further classified sequences that significantly associated with the high SO_4^{2-} wells such as sequences indicative of *Simplicispira* (Comamonadaceae), *Desulfosalsimonas* (Desulfobacteraceae), *Desulfosporosinus and Desulfitobacterium* (Peptococcaceae) (Fig. 8). The LEfSe analysis showed similar archaeal trends as the PCA with no dominant populations for the high SO_4^{2-} wells and *Methanoregula* and *Methanospirillum* dominated in the low SO_4^{2-} wells (Fig. 6 and Fig. 8). When LEfSe analysis was used on the filtered coal water samples for both bacteria and archaea, unique populations associated with high SO_4^{2-} wells were not identified and few bacteria and no archaea were identified from the low SO_4^{2-} wells (Fig. S1).

5. Discussion

5.1. SO_4^{2-} controls on methanogenesis

Environmental conditions, particularly SO_4^{2-} levels, were a dominant control on the microbial community compositions, and microbial populations did not differ significantly between coal beds or temporally for the same well. SO_4^{2-} is often below detection in methanogenic aquifers, and previous studies in various environments have suggested that SO_4^{2-} concentrations must be <1mM for

methanogenesis to commence (Lovley and Klug, 1983; Phelps et al., 1985; Whiticar et al., 1986; Capone and Kiene, 1988; Hoehler et al., 1998; Löffler and Sanford, 2005; Finke et al., 2007). In estuarine sediments, methanogenesis has been shown to be active across a range of SO_4^{2-} levels (1 to 10 mM) (Sela-Adler et al., 2017), while SO_4^{2-} levels in the mM range impacted hexadecane to CH_4 conversions in contaminated sediments (Ma et al., 2017). Recent work with a methanogenic consortium from coal showed that both methanogens and SRB could be active at SO_4^{2-} levels up to 1 mM (Glossner et al., 2016). Results from our study demonstrate a demarcation of bacterial and archaeal populations around the 1-2 mM SO_4^{2-} level, suggesting that SO_4^{2-} in conjunction with available organic carbon may impact co-existence and competition between SRB and methanogens. The results also suggest that slower rates of coal biodegradation may set a lower threshhold for SO_4^{2-} tolerance by different microbial trophic groups than observed in near-surface environments with higher rates of carbon turnover.

5.2. Microorganisms responsible for coal degradation

Bacterial species are thought to be primarily responsible for coal biodegradation and the fermentation of soluble organics into substrates that are then utilized by methanogens to generate CH_4 (Strąpoć et al., 2011; Ritter et al., 2015). Results in this study showed bacterial diversity in all wells was much greater than archaeal diversity, consistent with previous studies in the PRB (Barnhart et al., 2013; 2016) and other coal basins (Strąpoć et al., 2011). However, bacterial diversity was not significantly different between high and low SO_4^{2-} wells. Since bacteria are vital to several processes that are important for SO_4^{2-} reduction and methanogenesis, diversity of bacterial species is expected to be greater than archaeal diversity in CBM aquifers (Penner et al., 2010; Barnhart et al., 2013), as observed in this study.

In our study, sequences indicative of members from the Peptococcaceae family such as *Desulfitobacterium* and *Desulfosporosinus* grouped more with the vectors that represented NH_4^+ and

DOC (or lower PO_4^{3-}). Known *Desulfitobacterium* can use a variety of electron acceptors including nitrate, sulfite and halogenated organic compounds (Villemur et al., 2006). Known *Desulfosporosinu*s are a well-studied SRB genus that can utilize a wide variety of energy sources and have been observed in both low and high SO_4^{2-} environments (Pester et al., 2012). In terms of the cosmopolitan SRB from the DMS samples, sequences indicative of *Desulfosporosinus* and *Desulfosalsimonas* species were commonly observed. *Desulfuromonas* sequences were observed in most samples, and based upon cultivated isolates, the genus is incapable of reducing SO_4^{2-} (Lonergan et al., 1996). In addition, a recent isolate from a CBM well, *Desulfuromonas carbonis*, was reported to reduce Fe^{3+}, Mn^{4+}, and S^0 (An and Picardal, 2015). Therefore, *Desulfuromonas*-like populations may be responding to geochemical factors other than SO_4^{2-}. In high SO_4^{2-} wells, sequences indicative from the family Desulfobacteraceae, such as *Desulfosalsimonas,* were observed. Known Desulfobacteraceae are *Proteobacteria* that can reduce SO_4^{2-}, sulfites, and S^0 and are observed in different environments including psychrophilic and saline environments (Kuever, 2014). The genus *Desulfatiferula* (in the Desulfobacteraceae) has cultivated isolates shown to be anaerobic alkene degraders (Grossi et al., 2011; Hakil et al., 2013) and suggest the potential role of this genus in carbon turnover in high SO_4^{2-} environments.

Several bacterial sequence groups were correlated with the low SO_4^{2-}, high CH_4 wells. Both LEfSe and PCA identified sequences indicative of Oxalobacteraceae, Verrucomicrobiaceae, and Parachlamydiaceae. PCA correlated Oxalobacteraceae with low SO_4^{2-} wells, and LEfSe analysis identified three genera within this family: *Janthinobacterium*, *Massilia*, and *Oxalicibacterium*. The *Janthinobacterium* genus has commonly been observed in psychrophilic environments but also associated with aromatic contamination (*e.g*., Mojib et al., 2013; Ren et al., 2016). The *Massilia* genus has been isolated from a variety of environmental and human samples (Kampfer et al., 2011), and recent work documented the ability of novel isolates to degrade phenanthrene and herbicides (Wang et al., 2016; Lee et al., 2017). *Oxalicibacterium* are oxalic acid utilizing aerobes (Tamer et al., 2002), and

sequences indicative of *Oxalicibacterium* populations were previously observed in oilfield formation water (Pavlova-Kostryukova et al., 2014). Our results suggest Oxalabacteracaea may be important to the turnover of complex carbon in low SO_4^{2-} environments. The LEfSe analysis also correlated *Planctomyces* sequences with low SO_4^{2-} samples. *Planctomyces* are common to both fresh- and marine water/sediment environments, and *Planctomyces* have been observed at high numbers in both the oxic and anoxic layers of peat bogs and in CH_4-rich cold seep sediments (Ivanova and Dedysh, 2006; Reed et al., 2006).

The LefSe sequence analysis identified the genera *Luteolibacter* within the Verrucomicrobiaceae family that was correlated with low SO_4^{2-} wells. Known *Luteolibacter* are hetertrophs capable of utilizing a wide range of polysaccharides including those from algal biomass (Cardman et al., 2014). Parachlamydiacea sequences were grouped with the low SO_4^{2-} wells, and PCA correlated *Parachlamydia* with low SO_4^{2-} samples. Known *Chlamydia* and chlamydia-like organisms are obligate-intracellular bacteria that can be animal pathogens but also infect and reside in ubiquitous protists (*e.g.*, amoeba; Delafont et al., 2013). Recent work has shown that environmental Chlamydiae have better host-free survival compared to human pathogens, such as *C. trachomatis* (Coulon et al., 2012) and wildlife can be a potential reservoir for chlamydia-like organisms (Regenscheit et al., 2012; Delafont et al., 2013). The observation of chlamydia-like sequences enriched in the low SO_4^{2-} samples may suggest a higher occurrence of protists in these wells, but future work is needed to characterize the distribution of possible eukaryotic populations and the potential importance to the system.

A Veillonellaceae sequence vector aligned with low SO_4^{2-} wells in PCA; however, LEfSe analysis did not correlate this group with low SO_4^{2-} samples with high significance (although the group was identified in the hierarchal clustering of low SO_4^{2-} samples for HWC). The exact role of this potential group is unknown; however, a recent study suggested a role for Veillonellaceae species as corrinoid-providing microorganisms from contaminated groundwater (Men et al., 2017). Corrinoids are cyclic

pyrrole molecules that serve as co-factors for enzymes that typically contain vitamin B_{12} and cobalt, and these enzymes are important for the acetyl-CoA pathway in both bacteria and methanogens (White et al., 2012).

Many of the sequence groups for both high and low SO_4^{2-} samples were indicative of microorganisms capable of using simplified aromatics and/or recalcitrant carbon in energy limited environments and included *Geobacter* (Geobacteraceae) and *Simplicispira*. *Geobacter* are well-studied organisms with some species known to be associated with the break-down of complex organic matter (*e.g*., Zhao et al., 2016; Chen et al., 2016). *Simplicispira* have been commonly observed with activated sludge (Lu et al., 2007).

The LEfSe analysis also identified several bacterial groups not observed in the PCAs and included sequences indicative of *Propionivibrio*, *Anaerospora,* and *Acidithiobacillus*. *Propionivibrio* strains have been shown to degrade quinic acid (hydroaromatic) (Brune et al., 2002) and may contribute to the turnover of intermediate byproducts of coal degradation. The occurrence of *Acidithiobacillus* sequences is typically associated with autotrophic growth with oxidation of reduced sulfur (including S^0) in low pH environments (Nunez et al., 2016); however, the observation of sequences indicative of this group suggests a broader niche space or the existence of micro-niches up-stream.

5.3. Methanogenic communities

The archaeal sequence groups *Methanolinea, Methanospirillum, Methanolobus, Methanoregula*, and *Methanosaeta* that aligned with the low SO_4^{2-} wells (Fig. 6) were suggestive of a mixture of methanogenic pathways. Cultivated representatives of *Methanolinea, Methanospirillum,* and *Methanoregula* are CO_2 reducing (hydrogenotrophic) methanogens (Sakai et al. 2012, Parshina et al. 2014, Yamamoto et al. 2014). Knonw *Methanosaeta* are acetoclastic methanogens and known *Methanolobus* are methylotrophic methanogens (Mori et al. 2012, Doerfert et al. 2009). The

methanogenic community in high SO_4^{2-} wells consisted of *Methanomassiliicoccus, Methanosphaerula*, and *Methanobacterium* based upon representative sequences. Known *Methanosphaerula* and *Methanobacterium* are hydrogenotrophic methanogens (Cadillo-Quiroz et al. 2009, Luo et al. 2002). *Methanosphaerula* sequences were only observed in well WR-33 and *Methanobacterium* sequences were observed in both SH-396 and CBM02.

Interestingly, for archaea, the SSU rRNA gene analysis from the DMS indicated the order *Methanomassiliiccocales* was present all of the wells regardless of SO_4^{2-} levels. While *Methanomassiliicoccales* was more prevalent in high SO_4^{2-} wells, especially well CBM02, the methanogen sequence group was also detected in the low SO_4^{2-} wells (particularly SH-396). Known *Methanomassiliicoccales* belong to the Class Thermoplasmata and classified species are methylotrophic methanogens capable of utilizing methanol to produce CH_4 (Borrel *et al*., 2014). *Methanomassiliicoccales* are evolutionarily distinct from the other detected methanogens and genetic analysis indicates these methanogens may utilize a wide range of methylated compounds (Borrel et al., 2014). Recently, methoxydotrophic methanogens that can utilize coal-derived methoxylated compounds ($R\text{-}OCH_3$) were reported (Mayumi et al., 2016), and the presence of methanogenesis-related genes in archaeal phyla beyond the Euryarchaeota has recently been demonstrated (one of the investigated environments being a coal-seam) (Evans et al., 2015; Vanwonterghem et al., 2016). Therefore, novel (and under studied) methanogens and/or methylotrophic methanogens may be able to co-exist with SRB at relatively high SO_4^{2-} levels because of their ability to utilize non-competitive substrates.

Sequences indicative of methanotrophs (*i.e*., bacterial CH_4 oxidizers) were observed, particularly in the high CH_4 wells, including Methylococcaceae (*Methylococcus* and *Methylomonas),* and Verrumicrobiacea (Ogiso et al., 2012, Kleiveland et al, 2012). For the DMS, *Methylococcus* and *Methylomonas* were more prevalent in low SO_4^{2-} (high CH_4) wells. In addition, sequences indicative of

putative archaeal CH_4 oxidizers were not observed, although it is possible that the primer sets used did not detect these organisms and they may be present in the environment.

5.4. Source of nutrients for microbial communities

Previous studies have hypothesized that microbial communities in coal beds and shales may acquire nutrients (*i.e*., nitrogen and phosphorus) from groundwater recharge transporting in nutrients from near-surface environments (Bates et al., 2011; Schlegel et al., 2011). Thus, nutrient concentrations would be expected to be higher near recharge areas, at basin margins, and decrease as they are consumed by *in situ* microbial communities as water moves downgradient. The dominant N-species in coal waters is NH_4^+, and NH_4^+ concentrations were similar across all of the wells sampled in this study, except for WR-33, which had no detectable NH_4^+ or PO_4^{3-}, and the lowest CH_4 and alkalinity concentrations. Well WR-33 is located along a fault and contains relatively young, tritiated water, indicating modern waters and recent recharge (Ritter et al., 2015). Bates et al. (2011) also found lower concentrations of N species in PRB CBM wells near recharge areas. The correlation between PO_4^{3-} and CH_4 in the PCA, and higher NH_4^+ values away from recharge zones, supports the hypothesis that nutrients (N and P) in coal-associated waters are primarily released from organic matter during coal biodegradation or via water-rock reactions, rather than being transported in with groundwater recharge. Consistent with this, Pashin et al. (2014) observed a correlation between NH_3-NH_4^+ and total dissolved solids in the Black Warrior Basin, a similar microbial CBM area that could be the result of ion exchange between silicate minerals and formation water. There was no clear pattern between DOC concentration and wells with higher CH_4 concentrations (Fig. 2d), although DOC and CH_4 were somewhat correlated in the PCA (Fig. 5), suggesting a complex relationship between available carbon and the extent of methanogenesis. DOC concentrations in all monitoring and production wells were similar to

concentrations observed in produced waters from the PRB and other coal basins (Orem et al., 2007; Bates et al., 2011; Orem et al., 2014).

5.5. Chemical and isotopic signatures of microbial activity

The observed increase in alkalinity with increasing CH_4 concentrations (Fig. 2b) was expected as CO_2 is a byproduct of both bacterial SO_4^{2-} reduction and methanogenesis (Lee, 1981; Van Voast, 2003; Brinck et al., 2008), and although hydrogenotrophic methanogenesis consumes CO_2, the increase in alkalinity shows that more CO_2 is produced by coal biodegradation than is consumed. δ^{13}C-DIC values generally increased with increasing alkalinity and CH_4 concentrations (Fig. 3a), consistent with methanogenesis. The well with the highest CH_4 concentration (HWC) appears the most 'methanogenic' in terms of elevated alkalinity and δ^{13}C-DIC values (Martini et al., 2004). It also has the greatest 'extent of methanogenesis' (*f* value), in terms of the proportion of LMW converted to CH_4 (Vinson et al., 2017).

Following traditional methods for interpreting gas isotopes (Whiticar et al., 1986), the δ^{13}C values of dissolved CH_4 and CO_2 from well HWC ($\alpha^{13}C_{CH4\text{-}CO2}$ = 1.076) would be interpreted to represent hydrogenotrophic methanogenesis, yet the microbial sequence results show evidence indicative for multiple methanogenic pathways, including hydrogenotrophic, acetoclastic, and methylotrophic methanogenesis. This further supports the conclusion by Vinson et al. (2017) that δ^{13}C values of CO_2 and CH_4 cannot be simply applied to infer methanogenic pathways in subsurface systems where multiple methanogenic and non-methanogenic processes may impact $\alpha^{13}C_{CH4\text{-}CO2}$. Interestingly, well SH-396, which had a lower CH_4 concentration, higher SO_4^{2-} concentration, and lower 'extent of methanogenesis' (*f* value) compared to HWC, seemed to group with higher SO_4^{2-} wells in terms of the archaeal population. Both SH-396 and HWC appear to contain acetoclastic methanogens, but HWC (the other low SO_4^{2-} well) contained *Methanosaeta* which is thought to be better suited for scavenging lower acetate concentrations (Jetten et al., 1992).

Sulfur isotopes of SO_4^{2-} can provide evidence of bacterial SO_4^{2-} reduction, as bacteria preferentially remove ^{32}S, enriching the residual pool of SO_4^{2-} in ^{34}S (Clark and Fritz, 1997). Most of the methanogenic samples that were analyzed for $\delta^{34}S$-SO_4 had non-detectable SO_4^{2-}, although the high CH_4, low SO_4^{2-} well (HWC) had a similar $\delta^{34}S$-SO_4 value as several of the high SO_4^{2-} wells. Most of the variability in $\delta^{34}S$-SO_4 values occurs in samples containing SO_4^{2-} at or below 5 mM (Fig. 3b). Previous studies suggest that high SO_4^{2-} concentrations in parts of the PRB are the result of terrestrial evaporite (gypsum) dissolution and/or pyrite oxidation (Lee, 1981; Van Voast, 2003; Brinck et al., 2008). For samples with substantial concentrations of SO_4^{2-} (>5 mM), $\delta^{34}S$-SO_4 values ranged from -0.3‰ to 6.5‰ and terrestrial evaporite (gypsum) or pyrite oxidation could be a possible source for the SO_4^{2-} (Clark and Fritz, 1997). Well SH-396, which had low, but variable SO_4^{2-} (0.03 to 2.6 mM), two of the high SO_4^{2-} wells (CBM02 and WR-34), and a previous CBM production well sample, have higher $\delta^{34}S$-SO_4 values ($\geq$19.6‰), which likely indicates the influence of bacterial SO_4^{2-} reduction. This further confirms that well SH-396 represents a transition zone between bacterial SO_4^{2-} reduction and the early stages of methanogenesis, where methylotrophic methanogens appear to predominate the sampled communities.

6. Conclusions

The natural redox gradient from sulfate reducing to methanogenic of some PRB coal beds creates a model environment to study microbial community interactions and controls of microbial cycling of fossil carbon and CH_4 generation in the terrestrial subsurface. In addition, implementation of the downhole sampling device, DMS, enabled better characterization of *in situ* microbial populations and comparisons between planktonic microbial communities and biofilms on coal surfaces.

CH_4 concentrations, alkalinity, SO_4^{2-}, and PO_4^{3-} concentrations were closely related for samples collected in this study. Water from wells with relatively high CH_4 concentrations (0.75-3.7 mM) had high alkalinity concentrations (>9 meq/kg) and high $\delta^{13}C$-DIC values (>5‰). Wells with high SO_4^{2-}

concentrations (>2 mM) were predominated by sequences indicative of presumptive SRB (*Desulfosporsinus*, *Desulfosalsimonas*) with some enrichment for Peptococcaceae, Rhodocyclaceae, and Comamonadaceae, and the latter populations likely contribute to overall coal degradation coupled to bacterial sulfate reduction. Under low SO_4^{2-} conditions (<1.4 mM) with greater extents of methanogenesis (*i.e.*, increased $\delta^{13}C$-CH_4 and $\delta^{13}C$-CO_2 values), bacterial and archaeal populations were more diverse, and sequences were predominated by Oxalobacteraceae, Methanomicrobia, Planctomycetes, Methylococcaceae, and Verrucomicrobiaceae. Archaeal sequences were observed in both SO_4^{2-} zones and were predominated by novel unclassified members in the high SO_4 wells and *Methanosarcinales* and *Methanomicrobiales* in low SO_4^{2-} wells. Sequences indicative of *Methanomassiliicoccales*, a methylotrophic methanogen, were present throughout both low and high SO_4^{2-} wells.

Archaeal diversity showed a decline as SO_4^{2-} levels increased with a drastic decrease in OTUs of known methanogens that correlated to lower CH_4 levels (and lower abundane of *mcr*A). The significance of different carbon cycling pathways involved in the turnover of recalcitrant carbon under various redox conditions is still a topic of debate, and unknown CH_4 cycling pathways are still being discovered from a variety of environments. Redox transitions exist along gradients of increasingly recalcitrant carbon in many environments and microbial community dynamics coupled with hydrogeochemistry could help determine redox control on microbial processes at the genotypic/ecological level.

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Figure and Table Legends

Figure 1. Map showing location of monitoring wells sampled in the Anderson and Canyon coal seams in the Powder River Basin, near the border of Wyoming and Montana, as part of this study. A) Location of monitoring wells in the Anderson(■) and Canyon (●) coal beds. B) Schematic diagram highlighting depth of Anderson and Canyon coal bed wells and land surface elevation. Monitoring wells are divided into two groups based on SO_4^{2-} concentrations: (1) "High SO_4 wells" with [SO_4^{2-}] >2 mM (red), and (2) "Low SO_4 wells" with [SO_4^{2-}] <1.4 mM (green).

Figure 2. Dissolved CH_4 in selected groundwater samples from monitoring wells in the Anderson and Canyon coal beds versus (A) SO_4^{2-}, (B) alkalinity, (C) NH_4^+, and (D) dissolved organic carbon (DOC) concentrations. Nearby coal-bed methane (CBM) production wells reported in Bates et al. (2011) are shown for comparison at the top of the graphs, as CBM production wells contained detectable CH_4 (measured as mole %, rather than dissolved CH_4 concentration). Thus, for comparison purposes the CBM production well data is at the top of the graph so that the range of other values in the CBM production wells could be compared to the groundwater monitoring well data.

Figure 3. Carbon and sulfur isotope indicators of microbial processes. A) $\delta^{13}C$ value of dissolved inorganic carbon (DIC) versus alkalinity. B) $\delta^{34}S$ value of SO_4^{2-} versus SO_4^{2-} concentration. The symbols are the same as in Figure 2. CBM production well data are from Bates et al. (2011).

Figure 4. Dissolved and produced gas isotope ($\delta^{13}C$-CO_2 and $\delta^{13}C$-CH_4) values. Only three of the monitoring wells contained enough dissolved CH_4 in groundwater to analyze for $\delta^{13}C$-CH_4. Results for nearby coal-bed CH_4 production wells are shown for comparison. Dotted lines represent values of *f*, which represents the proportion of coal carbon converted to CH_4, assuming $\delta^{13}C$ of metabolizable coal C

is -25‰. Therefore, *f* records the relative favorability of methanogenesis vs. non-methanogenic pathways, such as sulfate reduction (Vinson et al., 2017).

Figure 5. Principal components analysis of low (green) and high (red) SO_4^{2-} samples from Anderson (■) and Canyon (●) coal seams based upon the sampled bacterial populations from DMSs. Blue vectors represent aligned bacterial groups, and red vectors represent selected geochemistry.

Figure 6. Principal components analysis of low (green) and high (red) SO_4^{2-} samples from Anderson (■) and Canyon (●) coal seams based upon the sampled archaeal populations from DMSs. Blue vectors represent aligned bacterial groups, and red vectors represent selected geochemistry.

Figure 7. Quantitative PCR for *dsr*B and *mcr*A for selected DMS samples listed (left to right) in order of decreasing SO_4^{2-} and increasing CH_4 concentrations.

Figure 8. Comparison between high SO_4^{2-} (red) and low SO_4^{2-} (green) bacterial and archaeal community composition shown with a phylogenetic cladogram created using LEfSe analysis. Circles in gold are organisms in the cladagrom that were determined to likely not explain differences between high and low SO_4^{2-} wells based on biological consistency and relevance. Samples were obtained using a DMS, with SO_4^{2-} concentrations determined from water samples prior to deployment of DMS.

Figure S1. Comparison of 0.45 µm filtered groundwater samples between high SO_4^{2-} and low SO_4^{2-} (red) bacterial and archaeal community composition shown with a phylogenetic cladogram made from LEfSe. Only definitive biomarkers for low SO_4^{2-} wells were identified.

Figure S2. Relative abundance of detected bacterial populations for the different samples. All of the detected bacterial taxa are represented at the order level. The larger cicles are representative of an overall higher percentage of the relative abundance according to the given scale.

Figure S3. Relative abundance of detected archaeal populations for the different samples. All of the detected archaeal taxa are represented at the order level. The larger cicles are representative of an overall higher percentage of the relative abundance according to the given scale.

Table 1. Coal bed monitoring well sample locations, depth, field parameters, major ion chemistry, and nutrient analyses. Dissolved oxygen (DO) was only measured in 2014. NA is not analyzed.

Table 2. Dissolved methane concentration and dissolved gas (molecular and isotopic) composition of groundwater samples from coal bed monitoring wells. Cells with asterisks indicate methane concentrations were too low for isotopic analysis. ND is not detected, while NA is not analyzed.

Table 3. Field samples for community analyses from both filtered groundwater and diffusive microbial samplers (DMSs) incubated down-well in coal bed monitoring wells. Number of sequences is the number of sequences analyzed for each sample post-filtering. Coverage is the estimated coverage of possible diversity, the observed OTUs (species richness) are empirically determined, and Chao estimates the probable species richness based upon the sampled diversity. Inverse Simpson is a diversity index

Table 4. Stable isotopic composition of water, dissolved inorganic carbon (DIC), and sulfate (asterisks

indicates sulfate concentrations were too low for isotopic analysis) in groundwater samples from coal bed monitoring wells.

Table1

Well Name	Latitude	Longitude	Land Surface Elevation (m)	Depth Below Land Surface (m)	Date Sampled	Temp (^oC)	DO %	pH	Alkalinity (meq/kg)	Na^+ (mM)	K^+ (mM)	Ca^{2+} (mM)	Mg^{2+} (mM)	Cl^- (mM)	NO_3^- (mM)	SO_4^{2-} (mM)	DOC (mM)	NH_4^+ (mM)	PO_4^{3-} (µM)
HWC	45.1254	-106.4836	1193	71	09/10/2011	13.5		8.5	28.97	29.33	0.13	0.11	0.09	0.52	<0.01	<0.01	0.21	0.28	NA
HWC					30/04/2014	13.0	1.3	8.0	25.38	27.49	0.16	0.16	0.10	0.50	<0.01	0.00	0.39	0.08	1.41
SH-396	45.0490	-107.0088	1205	86	14/08/2013	14.6		7.4	9.87	16.43	0.09	0.06	0.04	0.17	<0.01	2.60	0.12	0.03	0.43
SH-396					01/05/2014	13.1	2.4	7.8	13.55	16.96	0.12	0.14	0.11	0.08	<0.01	0.03	0.13	0.08	0.33
CBM02	45.1801	-106.8906	1080	80	09/10/2011	13.9		8.5	10.64	16.16	0.12	0.19	0.10	0.12	<0.01	2.78	0.29	0.21	NA
CBM02					30/04/2014	13.7	4.0	8.0	8.70	16.02	0.14	0.22	0.13	0.13	<0.01	2.91	0.15	0.04	0.63
WR-33	45.0067	-106.9760	1141	50	14/08/2013	11.7		7.0	5.42	3.15	0.38	3.44	7.64	NA	NA	NA	NA	NA	NA
WR-33					29/04/2014	11.7	3.6	7.0	6.02	2.92	0.35	3.17	6.74	2.16	0.17	5.09	0.37	<0.03	<0.03
WR-34	45.0027	-106.9700	1154	160	01/05/2014	15.5	3.0	7.7	17.82	26.13	0.16	0.16	0.12	0.24	<0.01	4.38	0.79	0.07	0.80
WR-48	44.9939	-106.9660	1130	51	14/08/2013	12.3		7.6	11.64	30.95	0.19	0.25	0.28	NA	NA	NA	NA	NA	NA
WR-48					28/04/2014	12.3	3.9	7.7	11.05	26.64	0.18	0.22	0.23	0.23	<0.01	7.97	0.21	0.11	0.91
WR-24	45.0210	-106.9885	1155	45	05/10/2011	10.7		7.4	12.86	28.83	0.25	0.89	0.97	0.24	<0.01	10.35	0.16	0.46	<0.03
WR-24					29/04/2014	9.7	1.4	7.6	9.97	29.16	0.29	0.90	1.22	0.28	<0.01	11.50	0.17	0.19	<0.03

ND, concentration was below detection limit of the instrument

NA was not measured

Table2

Well Name	Date Sampled	Dissolved CH_4 (mM)	Dissolved Gas Composition (mole %)						$\delta^{13}C$-CO_2 ‰	$\delta^{13}C$-CH_4 ‰	$\delta^{2}H$-CH_4 ‰
			Ar	O_2	CO_2	N_2	CH_4	C_2-C_6			
HWC	########	NA	0.083	0.460	1.310	2.560	95.540	0.038	2.2	-69.4	-293.0
HWC	########	3.74	0.097	0.770	0.860	3.740	94.490	0.038	2.0	-69.1	-304.3
SH-396	########	0.748	0.941	1.04	3.05	52.74	42.13	0.104	-19.3	-85.8	-328.2
CBM02	########	NA	1.440	4.470	0.920	92.920	0.212	0.000	-17.4	-57.2	NA
CBM02	########	0.00237	1.570	3.420	1.460	93.350	0.196	ND	-17.0	ND	ND
WR-33	########	0.0000467	1.13	6.91	8.54	83.42	0.005	ND	-20.4	ND	ND
WR-34	########	0.00231	1.52	2.50	4.04	91.74	0.202	ND	-20.3	ND	ND
WR-48	########	0.00150	1.02	2.99	3.50	92.37	0.123	ND	-16.7	ND	ND
WR-24	########	NA	1.330	4.810	3.910	89.830	0.101	0.000	-17.8	NA	NA
WR-24	########	0.00112	1.380	3.290	5.100	90.140	0.088	ND	-17.7	ND	ND

ND, concentration was below detection limit of the instrument

NA was not measured

Table3

Well Name	Year Sampled	Coal Seam	Sample Type	Number of Sequences	Coverage	Observed OTUs	Chao	Inverse Simpson
HWC	2010	Canyon	Filtered Water	9295	1	94	94	17.4
HWC	2011	Canyon	Filtered Water	14696	1	95	95	14.8
HWC	2011	Canyon	DMS Samplers	29838	1	149	149	10.9
HWC	2012	Canyon	DMS Samplers	49672	1	150	150	8.4
HWC	2013	Canyon	DMS Samplers	69229	1	126	126	11.2
HWC	2013	Canyon	DMS Samplers	27188	0.99	99	99	8.29
SH-396	2015	Anderson	DMS Samplers	116254	1	187	187	15
CBM02	2010	Canyon	Filtered Water	33734	0.99	69	69	3.83
CBM02	2011	Canyon	Filtered Water	30797	1	62	62	3.54
CBM02	2011	Canyon	DMS Samplers	181382	1	124	124	8.85
CBM02	2012	Canyon	DMS Samplers	40123	0.99	103	103	3.81
CBM02	2013	Canyon	DMS Samplers	17934	0.99	73	73.1	5.86
WR-33	2015	Anderson	DMS Samplers	134643	1	145	145	6.06
WR-34	2012	Anderson	DMS Samplers	238496	0.99	123	123	3.39
WR-48	2015	Anderson	DMS Samplers	154220	1	128	128	4.25
WR-24	2010	Canyon	Filtered Water	9882	0.99	59	59	10.5
WR-24	2011	Canyon	Filtered Water	18055	1	91	91	10.5
WR-24	2010	Canyon	DMS Samplers	410620	1	96	96	1.6
WR-24	2012	Canyon	DMS Samplers	57963	1	97	97	5.37

Table4

Well Name	Date Sampled	$\delta^{2}H$-H_2O ‰	$\delta^{18}O$-H_2O ‰	$\delta^{13}C$-DIC ‰	$\delta^{34}S$-SO_4^{2-} ‰	$\delta^{18}O$-SO_4^{2-} ‰
HWC	09/10/2011	-131.5	-17.4	12.6	2.7	15.1
HWC	30/04/2014	-136.3	-17.9	11.6	7.7	ND
SH-396	14/08/2013	-163.1	-21.2	-8.8	22.0	5.4
SH-396	01/05/2014	-169.4	-21.7	-8.4	ND	ND
CBM02	09/10/2011	-164.7	-20.9	-7.2	20.9	11.2
CBM02	30/04/2014	-164.6	-21.4	-7.5	21.1	-1.4
WR-33	29/04/2014	-144.4	-18.8	-11.2	-0.3	-3.5
WR-34	01/05/2014	-164.9	-21.1	-10.4	19.6	1.3
WR-48	28/04/2014	-156.0	-20.1	-7.8	2.3	-7.5
WR-24	05/10/2011	-163.8	-21.1	-7.6	6.5	2.4
WR-24	29/04/2014	-164.4	-21.2	-8.3	6.5	2.7

ND, [SO_4] too low (insufficient) to measure isotopes

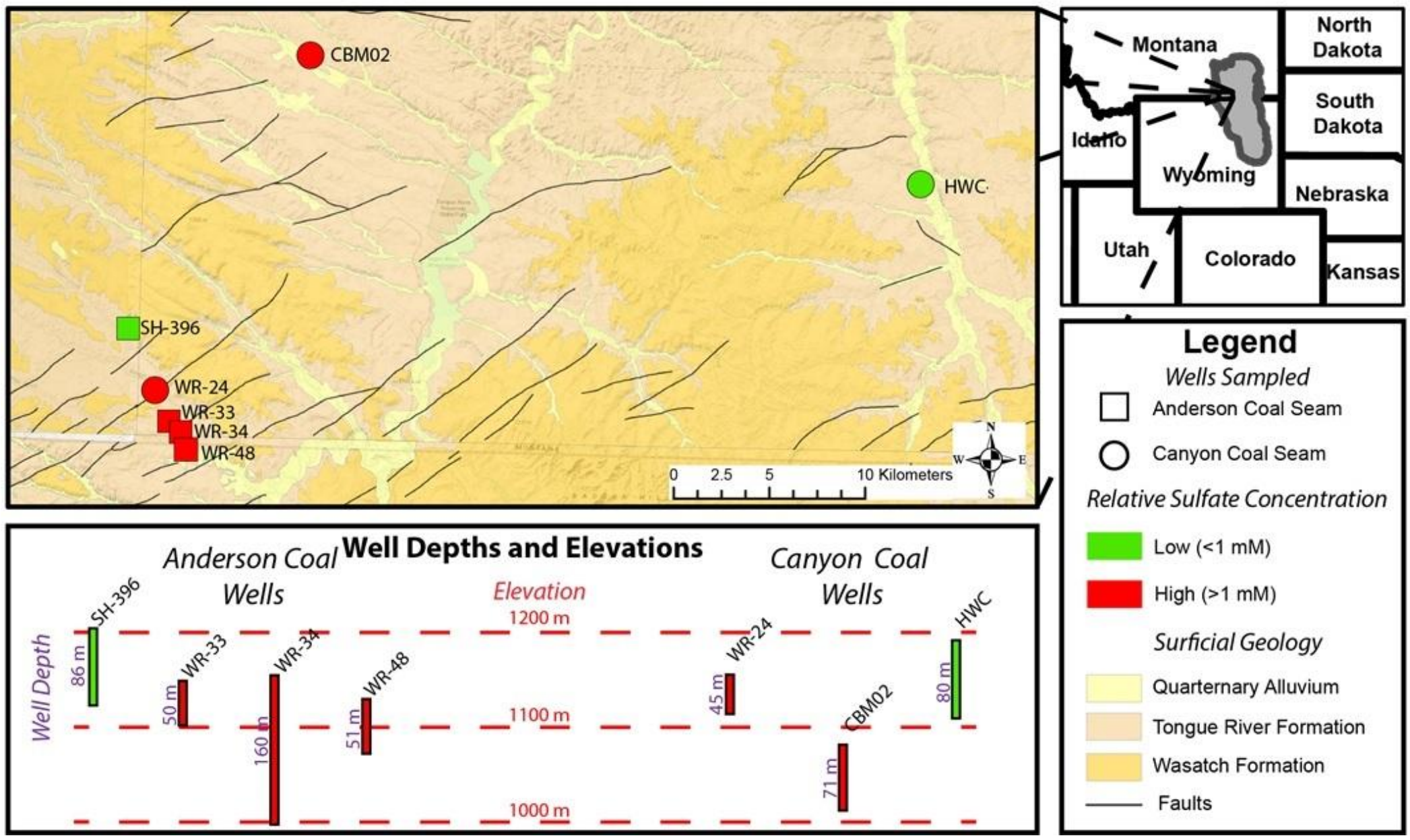

CBM02
HWC
SH-396
WR-24
WR-33
WR-34
WR-48
0 2.5 5 10 Kilometers
N
W
E
S
Montana
North Dakota
South Dakota
Idaho
Wyoming
Nebraska
Utah
Colorado
Kansas
Legend
Wells Sampled
Anderson Coal Seam
Canyon Coal Seam
Relative Sulfate Concentration
Low (<1 mM)
High (>1 mM)
Surficial Geology
Quarternary Alluvium
Tongue River Formation
Wasatch Formation
Faults
Well Depths and Elevations
Anderson Coal Wells
Canyon Coal Wells
Elevation
1200 m
1100 m
1000 m
Well Depth
SH-396
86 m
WR-33
50 m
WR-34
160 m
WR-48
51 m
WR-24
45 m
CBM02
71 m
HWC
80 m

Figure 1.

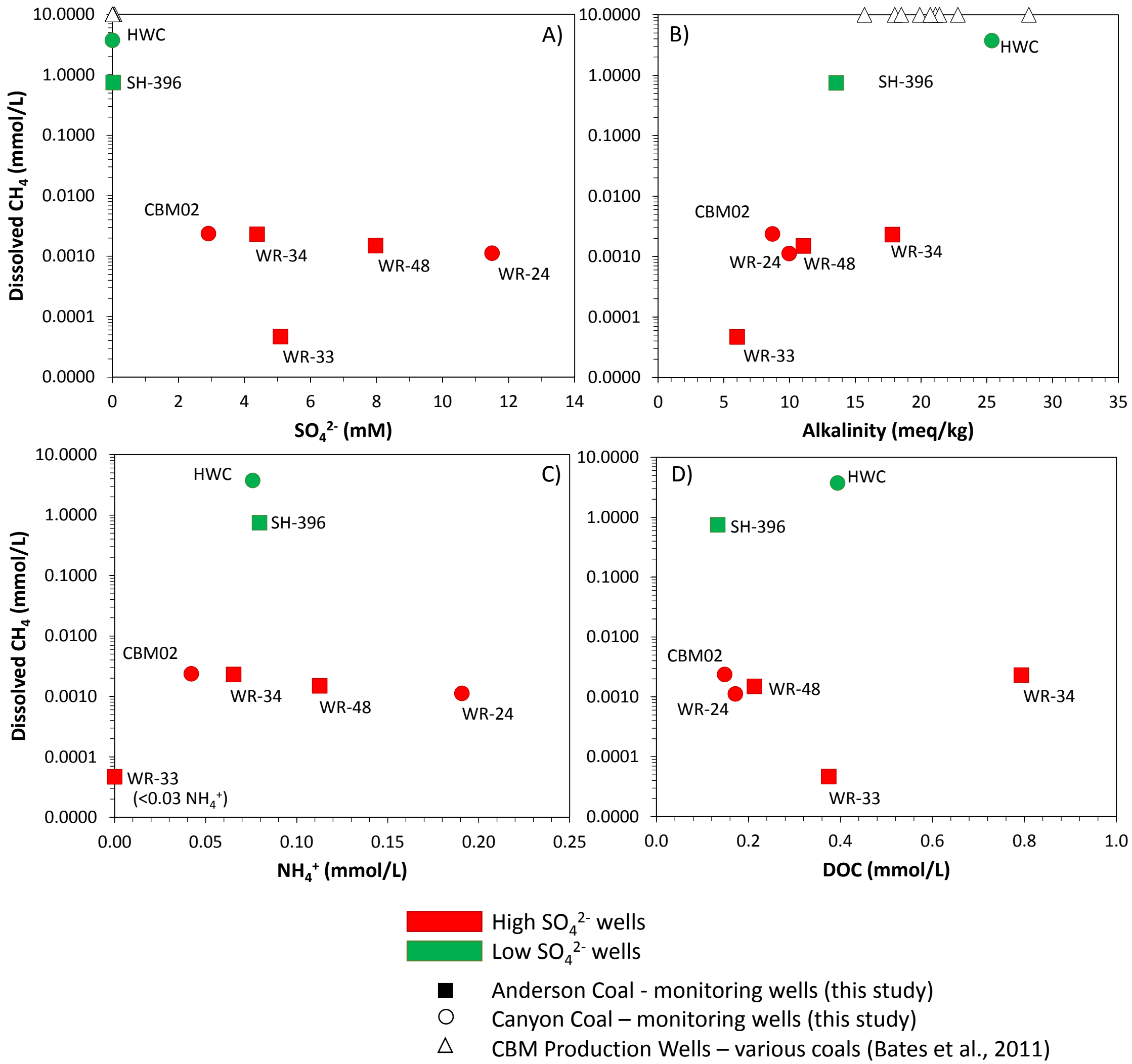

A)
B)
C)
D)
HWC
SH-396
CBM02
WR-34
WR-48
WR-24
WR-33
WR-33 (<0.03 NH4+)
Dissolved CH4 (mmol/L)
SO42- (mM)
Alkalinity (meq/kg)
NH4+ (mmol/L)
DOC (mmol/L)
High SO42- wells
Low SO42- wells
Anderson Coal - monitoring wells (this study)
Canyon Coal – monitoring wells (this study)
CBM Production Wells – various coals (Bates et al., 2011)

Figure 2.

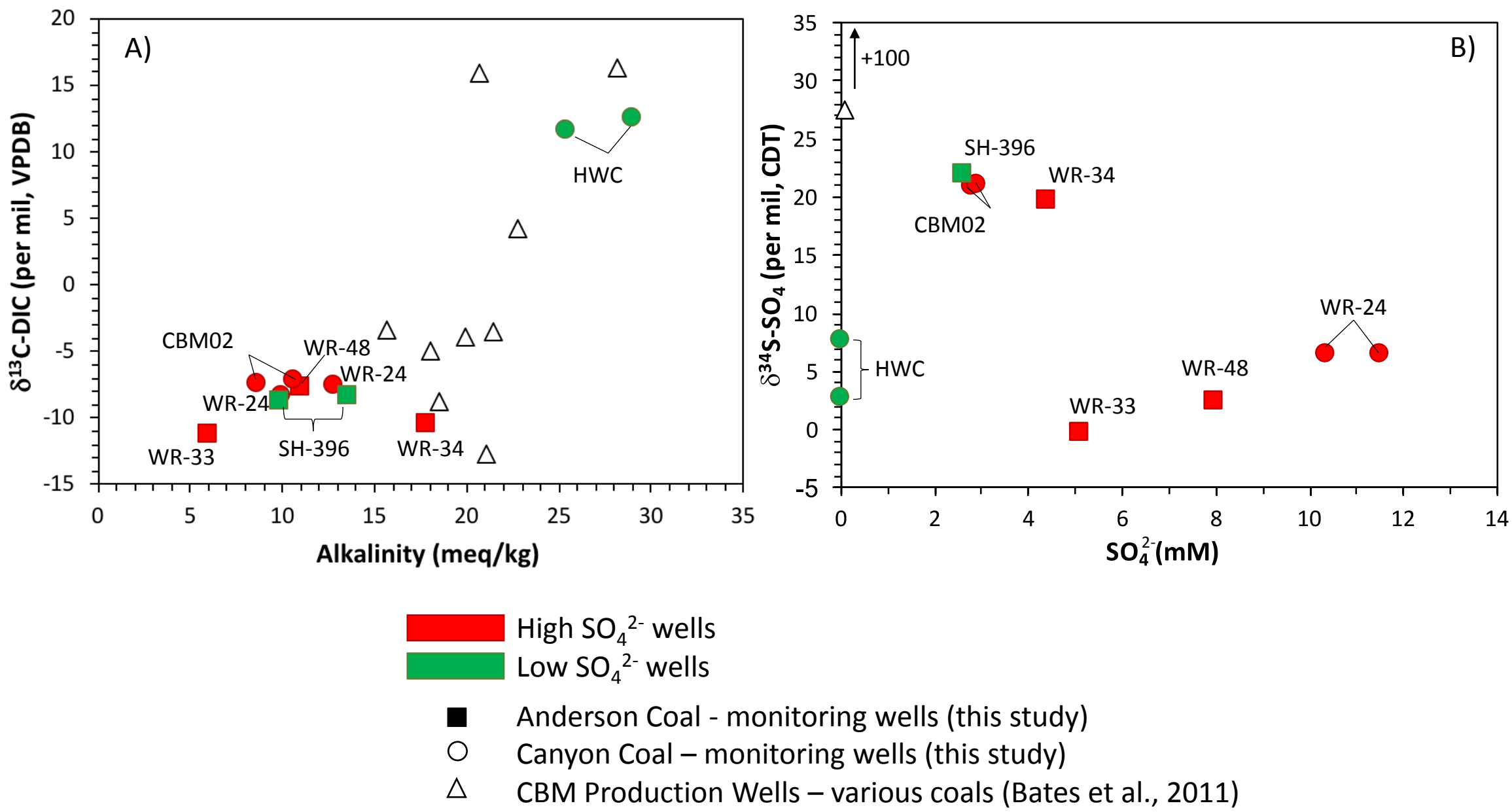
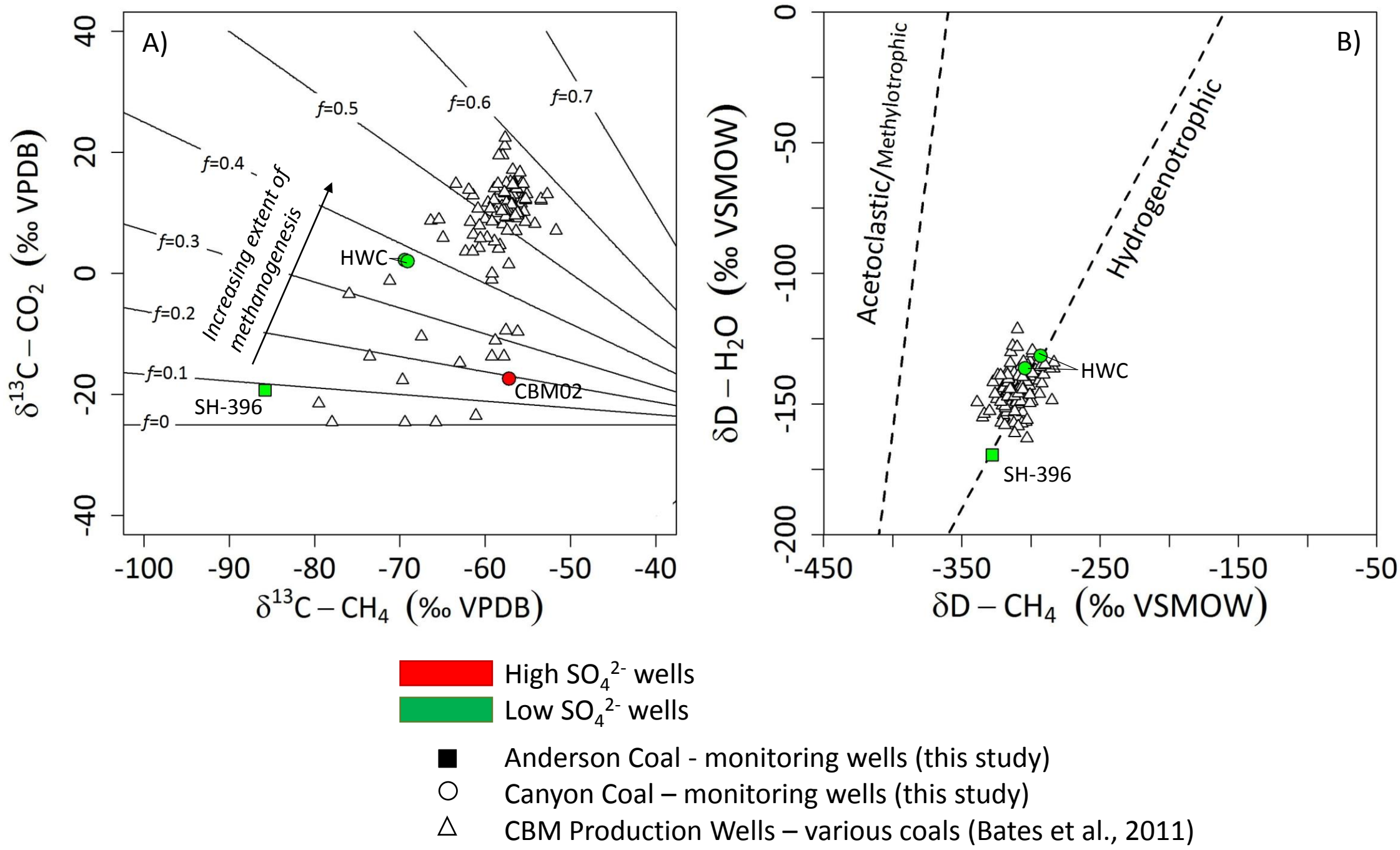

A)
B)
HWC
CBM02
WR-48
WR-24
WR-33
SH-396
WR-34
+100
SH-396
WR-34
CBM02
WR-24
HWC
WR-48
WR-33
δ13C-DIC (per mil, VPDB)
Alkalinity (meq/kg)
δ34S-SO4 (per mil, CDT)
SO42-(mM)
High SO42- wells
Low SO42- wells
Anderson Coal - monitoring wells (this study)
Canyon Coal – monitoring wells (this study)
CBM Production Wells – various coals (Bates et al., 2011)

Figure 3.


A)
B)
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f=0.7
f=0.4
f=0.3
f=0.2
f=0.1
f=0
Increasing extent of methanogenesis
HWC
CBM02
SH-396
δ13C – CO2 (‰ VPDB)
δ13C – CH4 (‰ VPDB)
Acetoclastic/Methylotrophic
Hydrogenotrophic
HWC
SH-396
δD – H2O (‰ VSMOW)
δD – CH4 (‰ VSMOW)
High SO42- wells
Low SO42- wells
Anderson Coal - monitoring wells (this study)
Canyon Coal – monitoring wells (this study)
CBM Production Wells – various coals (Bates et al., 2011)

Figure 4.

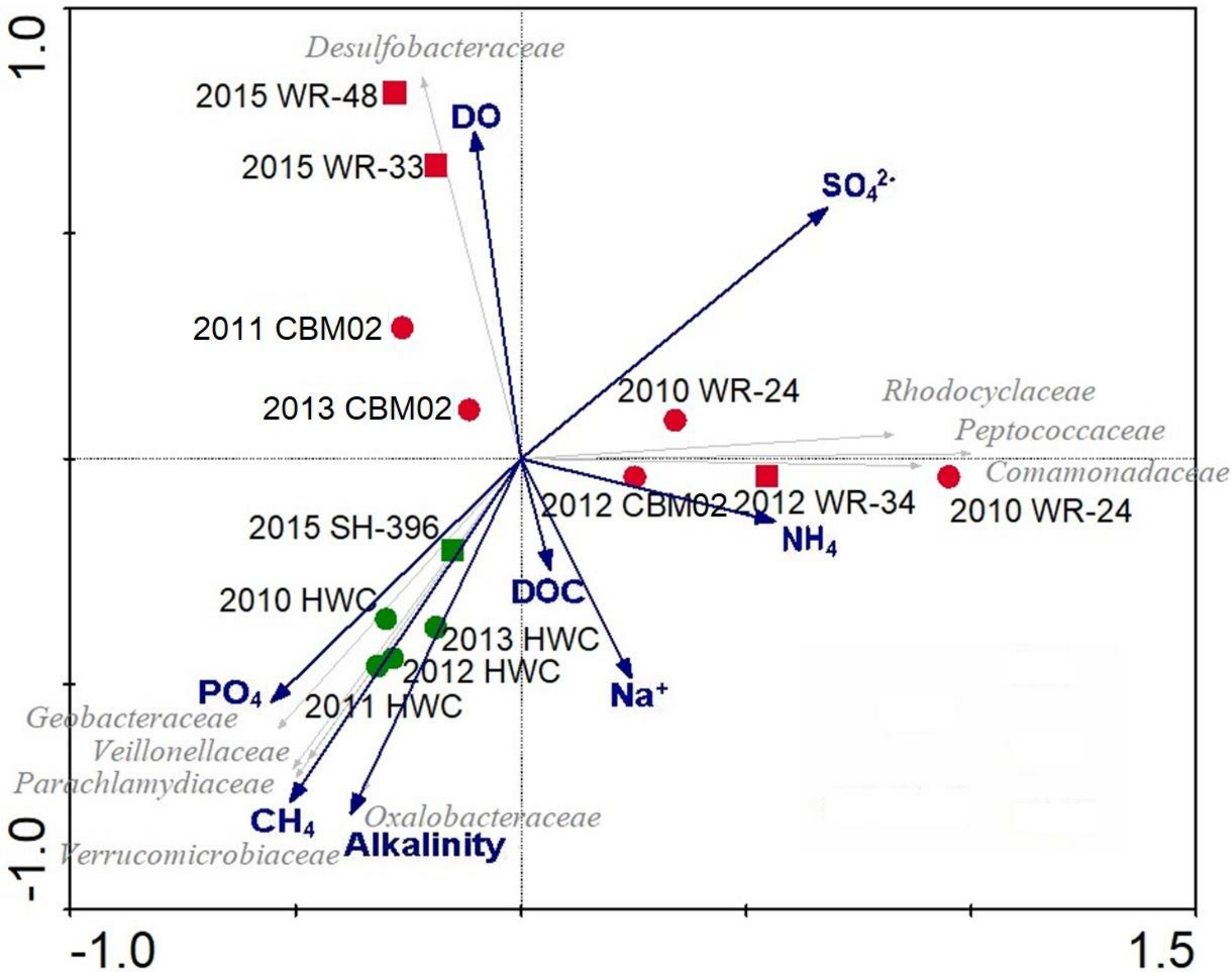
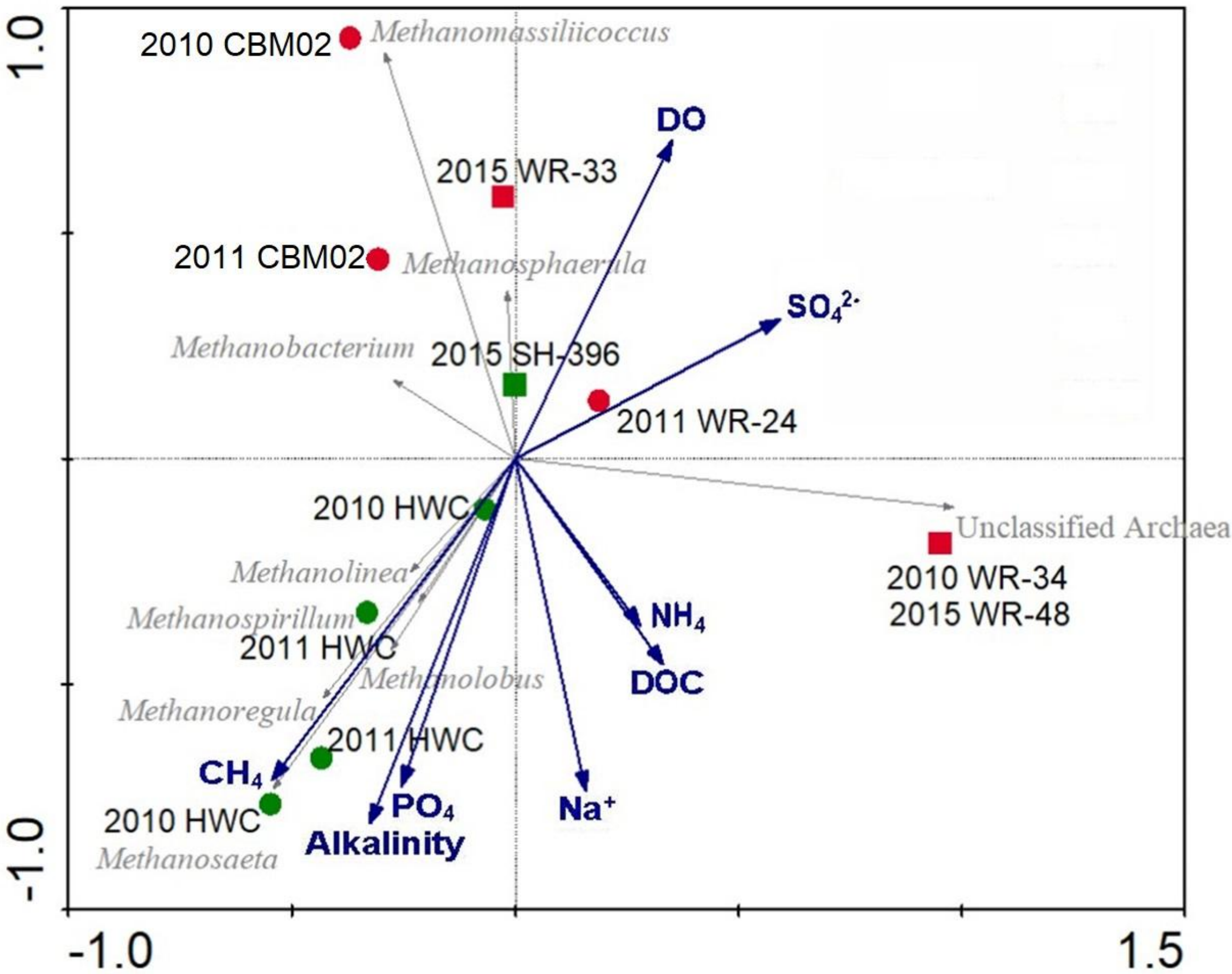

1.0
-1.0
-1.0
1.5
Desulfobacteraceae
2015 WR-48
DO
2015 WR-33
SO_4^{2-}
2011 CBM02
2013 CBM02
2010 WR-24
Rhodocyclaceae
Peptococcaceae
Comamonadaceae
2012 CBM02
2012 WR-34
2010 WR-24
NH_4
2015 SH-396
DOC
2010 HWC
2013 HWC
2012 HWC
PO_4
2011 HWC
Na^+
Geobacteraceae
Veillonellaceae
Parachlamydiaceae
CH_4
Oxalobacteraceae
Alkalinity
Verrucomicrobiaceae

Figure 5.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-1.0
1.5
2010 CBM02
Methanomassiliicoccus
DO
2015 WR-33
2011 CBM02
Methanosphaerula
SO_4^{2-}
Methanobacterium
2015 SH-396
2011 WR-24
2010 HWC
Unclassified Archaea
2010 WR-34
2015 WR-48
Methanolinea
Methanospirillum
2011 HWC
NH_4
DOC
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Methanoregula
2011 HWC
CH_4
2010 HWC
Methanosaeta
PO_4
Alkalinity
Na^+

Figure 6.

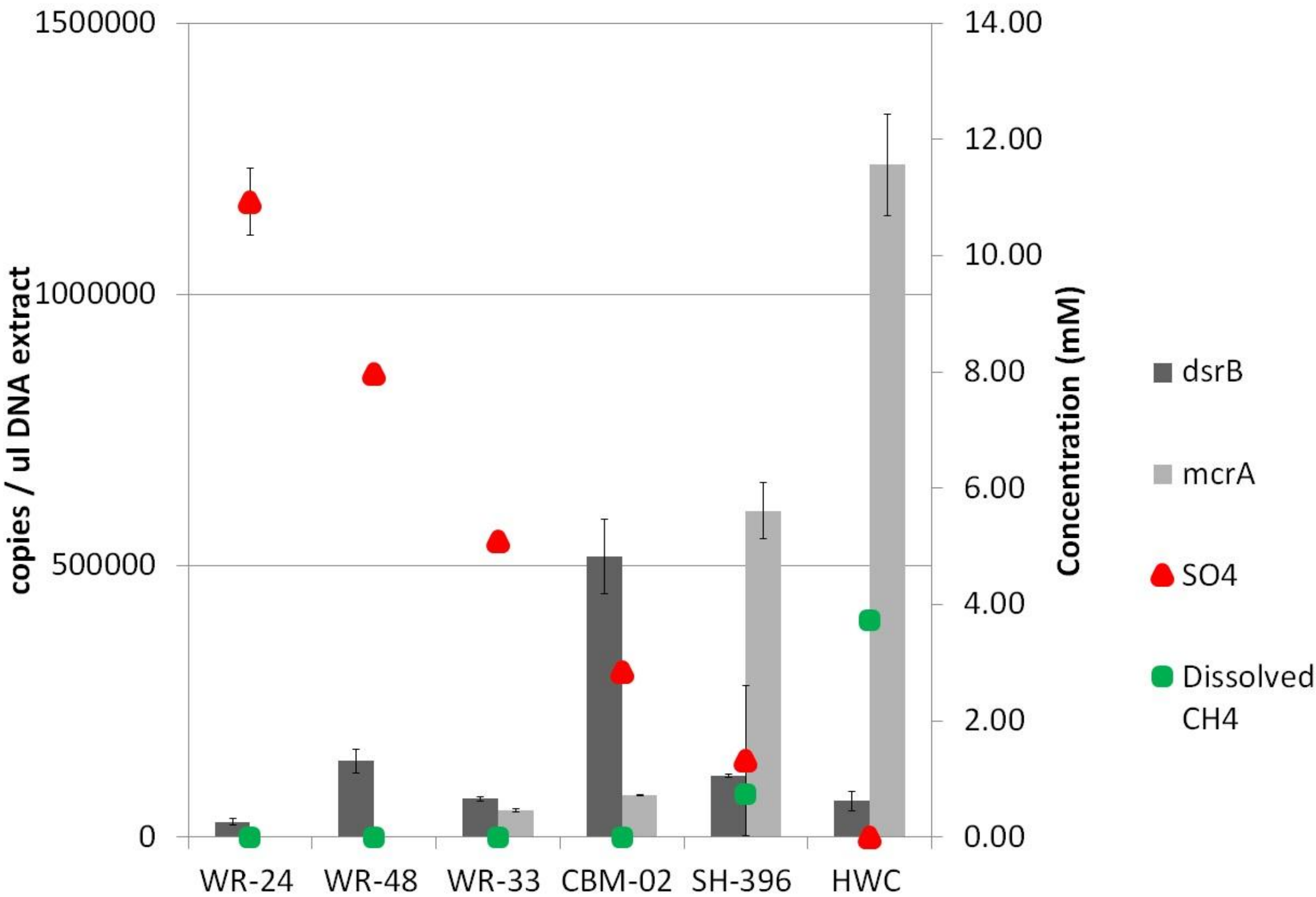
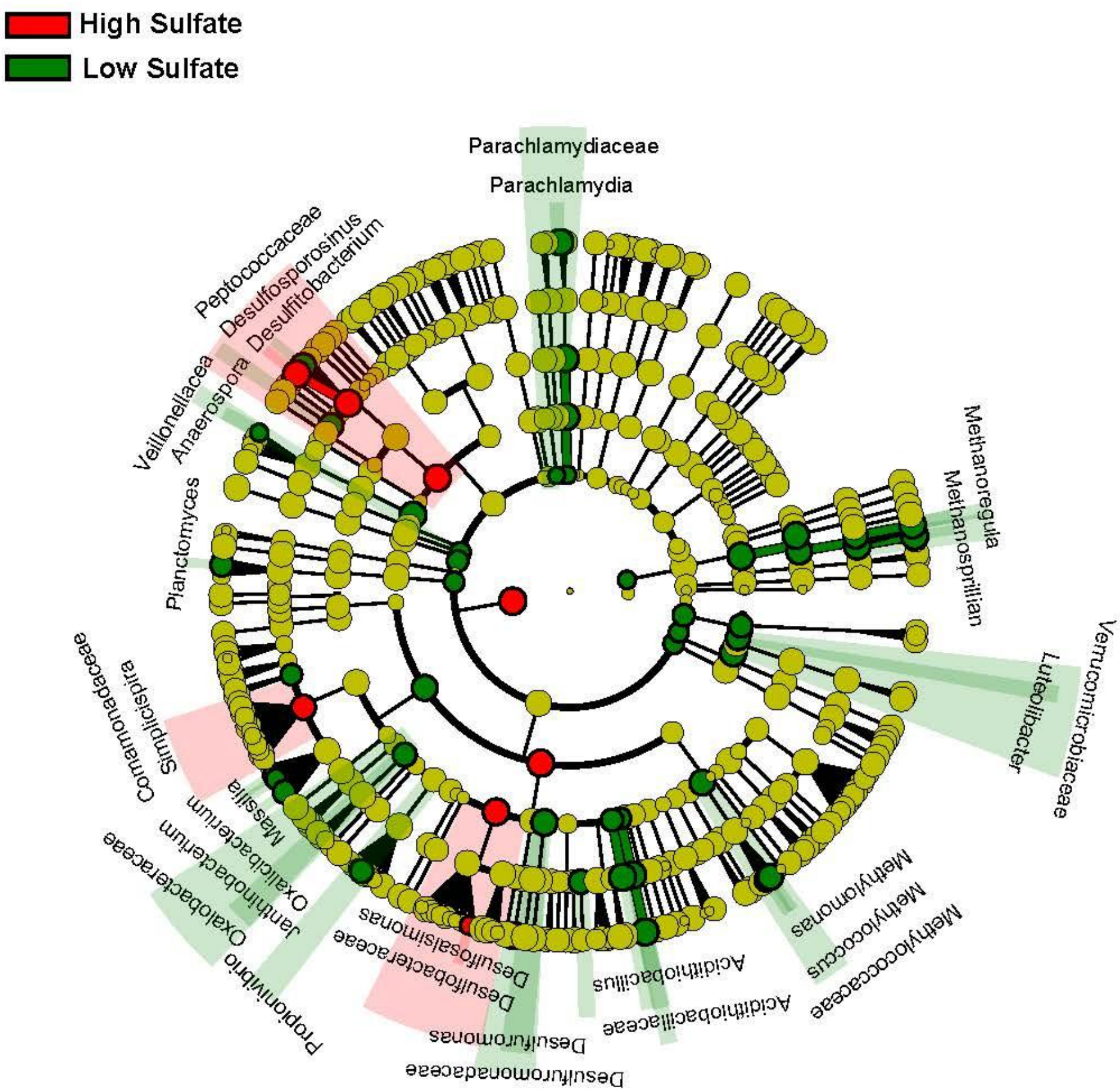

1500000
1000000
500000
0
copies / ul DNA extract
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Concentration (mM)
WR-24
WR-48
WR-33
CBM-02
SH-396
HWC
dsrB
mcrA
SO4
Dissolved CH4

Figure 7.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Low Sulfate
Parachlamydiaceae
Parachlamydia
Peptococcaceae
Desulfosporosinus
Desulfitobacterium
Veillonellacea
Anaerospora
Planctomyces
Methanoregula
Methanosprillian
Verrucomicrobiaceae
Luteolibacter
Comamonadaceae
Simplicispira
Massilia
Oxalicibacterium
Janthinobacterium
Oxalobacteraceae
Propionivibrio
Desulfosalsimonas
Desulfitobacteraceae
Desulfuromonas
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Acidithiobacillus
Acidithiobacillaceae
Methylomonas
Methylococcus
Methylococcaceae

Figure 8.

Supplemental Figures

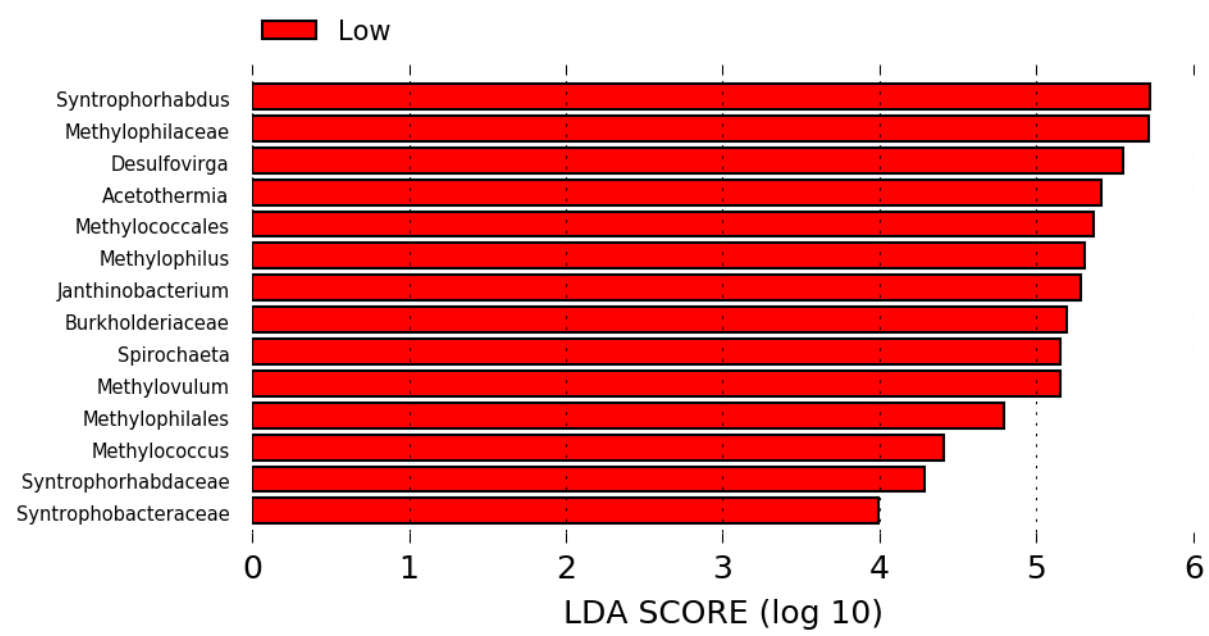
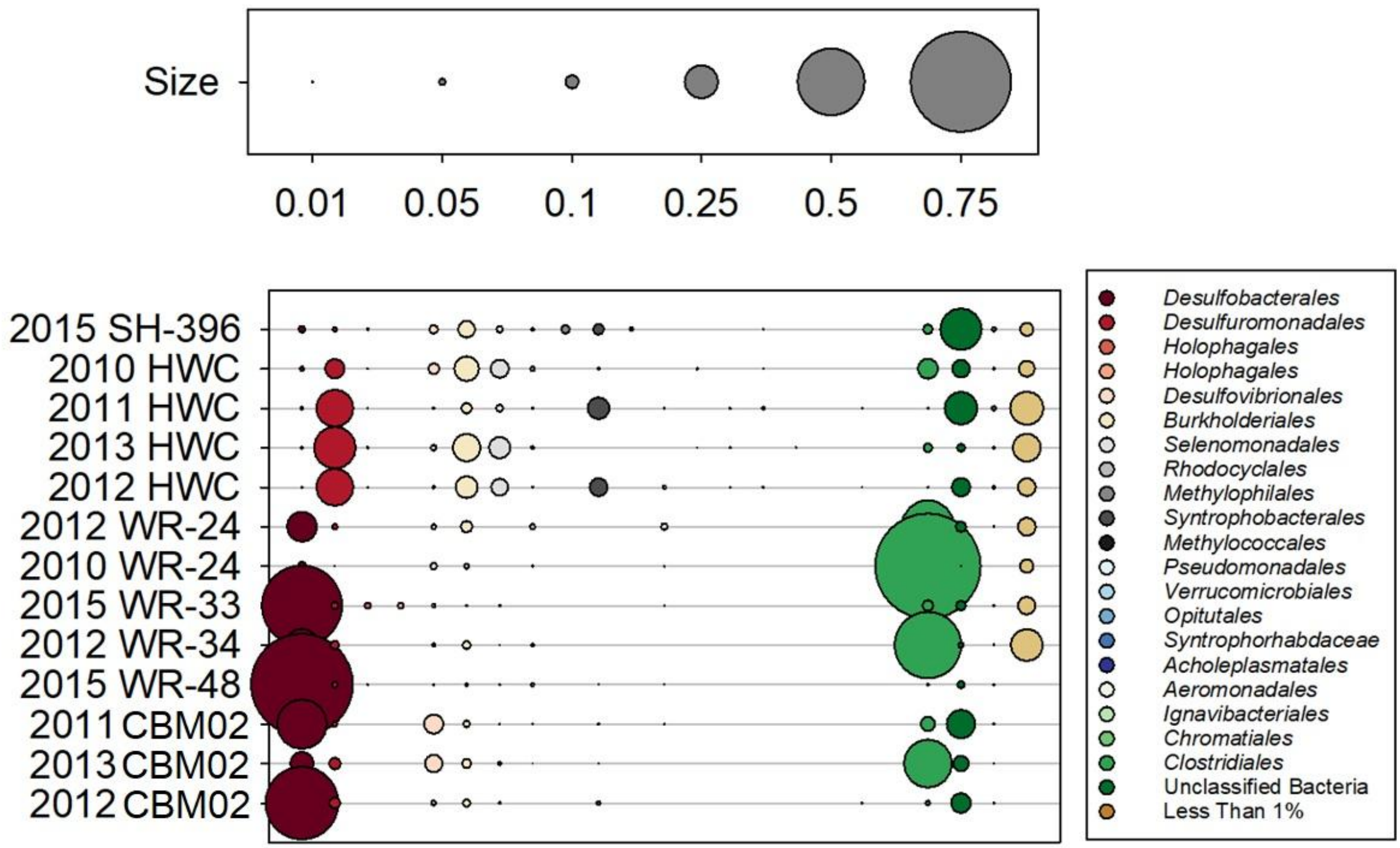

Low
Syntrophorhabdus
Methylophilaceae
Desulfovirga
Acetothermia
Methylococcales
Methylophilus
Janthinobacterium
Burkholderiaceae
Spirochaeta
Methylovulum
Methylophilales
Methylococcus
Syntrophorhabdaceae
Syntrophobacteraceae
0
1
2
3
4
5
6
LDA SCORE (log 10)

Figure S1.


Size
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0.05
0.1
0.25
0.5
0.75
2015 SH-396
2010 HWC
2011 HWC
2013 HWC
2012 HWC
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2010 WR-24
2015 WR-33
2012 WR-34
2015 WR-48
2011 CBM02
2013 CBM02
2012 CBM02
Desulfobacterales
Desulfuromonadales
Holophagales
Holophagales
Desulfovibrionales
Burkholderiales
Selenomonadales
Rhodocyclales
Methylophilales
Syntrophobacterales
Methylococcales
Pseudomonadales
Verrucomicrobiales
Opitutales
Syntrophorhabdaceae
Acholeplasmatales
Aeromonadales
Ignavibacteriales
Chromatiales
Clostridiales
Unclassified Bacteria
Less Than 1%

Figure S2.

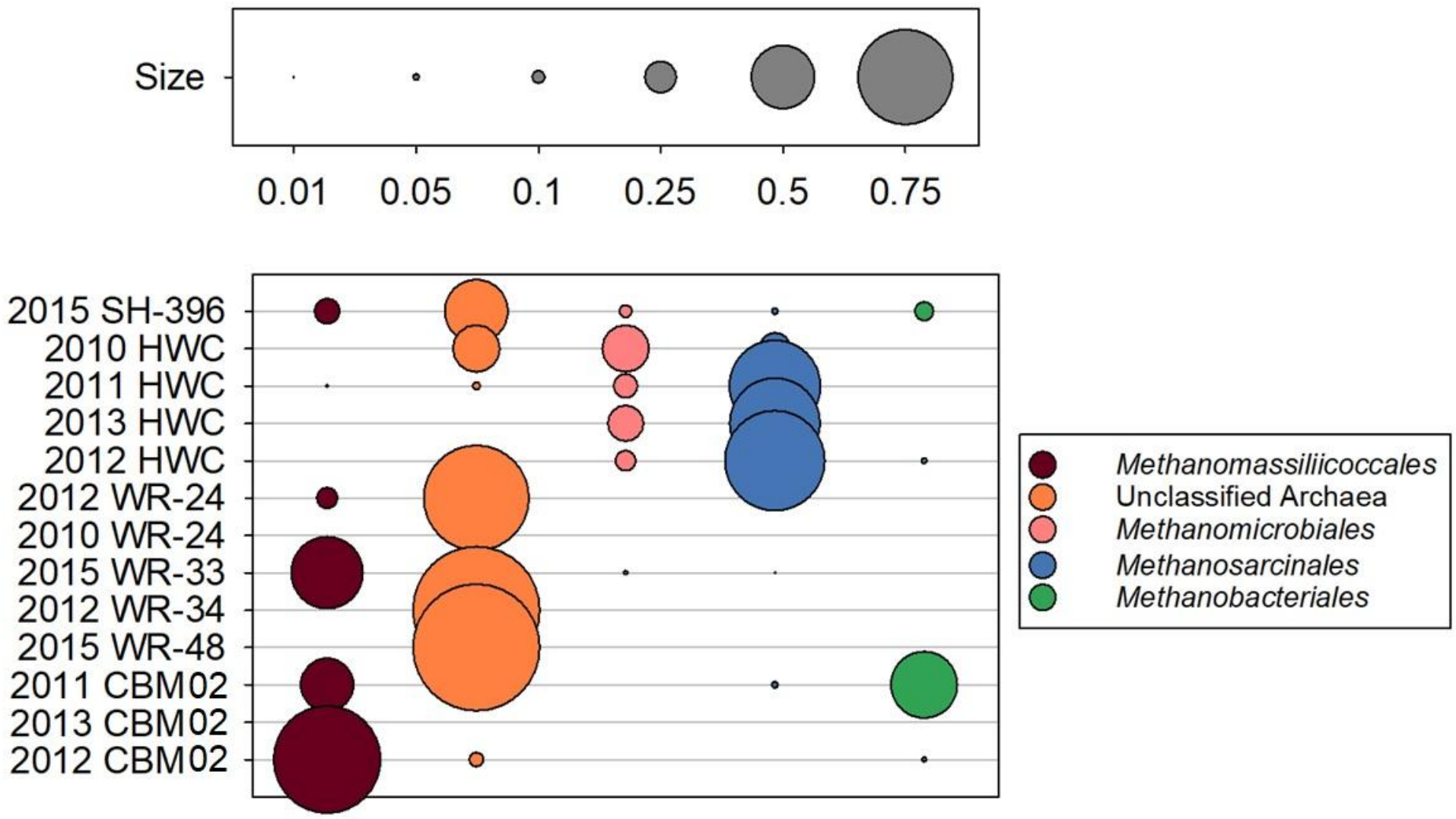
Size
0.01
0.05
0.1
0.25
0.5
0.75
2015 SH-396
2010 HWC
2011 HWC
2013 HWC
2012 HWC
2012 WR-24
2010 WR-24
2015 WR-33
2012 WR-34
2015 WR-48
2011 CBM02
2013 CBM02
2012 CBM02
Methanomassiliicoccales
Unclassified Archaea
Methanomicrobiales
Methanosarcinales
Methanobacteriales

Figure S3.