ANALYSIS OF METHANE PRODUCING COMMUNITIES WITHIN UNDERGROUND COAL BEDS

by

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The journey has been long and I would not have made it this far without your help. My only hope is that the adventure continues and I have a feeling that it will, in fact, I see a mountain up ahead.

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ABSTRACT

The Powder River Basin in southeastern Montana and northeast Wyoming is the largest source of coal mined in the United States but most of the coal contained in the basin is buried too deeply to be economically accessible. These remote coal beds are dynamic zones where biogeochemical processes work to sustain a microbial ecosystem. Previous work has shown that a direct byproduct of these microbial processes is biogenic methane that can be harvested and utilized as an energy source. Methane is the principle component of natural gas and this can be used as an energy source for electricity generation, heat and transportation fuel producing only carbon dioxide and water when burned in the presence of oxygen. The only known organisms on the planet able to produce methane are classified as *Archaea*, microorganisms termed methanogens. However, little is known about the responsible methanogens, the conditions conducive to coal-associated methane production, nor the microbial community interactions that promote methane production. Advances in subsurface sampling and molecular techniques have provided a route to capture active microbial consortia from coal beds, but methods need to be refined in order to deal with the unique attributes of coal. Microorganisms involved in coal bed methane (CBM) formation were investigated by applying molecular methods in combination with cultivation techniques with and without nutrient supplementation to maximize methane production in batch, bench-scale incubations. Our research suggests that *Clostridium* species are involved with the breakdown of coal and *Acetobacterium* species are able to utilize substrates produced by the coal degradation. Coal and yeast extract each appear to contribute important nutrients that stimulate coal degrading communities. A better understanding of this microbial system and the biotic and abiotic parameters that control activity may permit microbially enhanced CBM production *in situ* to become an industrially sustainable process through the application of suitable methane stimulation strategies.

INTRODUCTION

The subsurface is the largest, yet least understood microbial ecosystem on the planet (Whitman et al., 1998). Low rank coal beds offer an optimal subsurface microbial habitat due to their large pore volume and carbon content. The Powder River Basin (PRB) contains one of the largest, low-rank (sub-bituminous) coal deposits in the world, and common mining practices are not feasible for most of the coal due to the coal bed depth. These coals are considered low-rank coal because of their low heating value (between 8,300 and 13,000 BTUs-per-pound) compared to bituminous and anthracite coals. Recent geological research has revealed active methanogenesis occurring within the depths of the Canyon coal seam of the PRB (Figure 1) (Wheaton, et al. 2005).

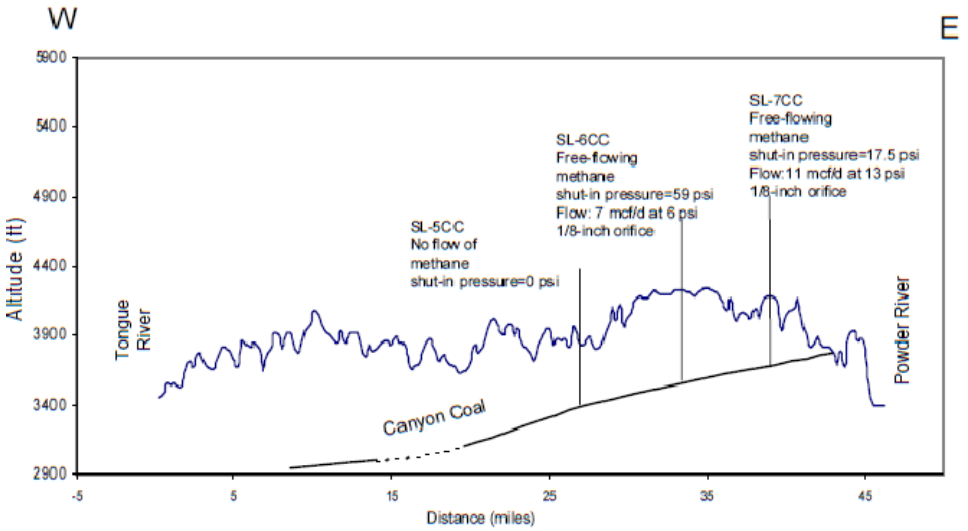

Figure 1: Active Methanogenesis: This figure indicates that methane generation is happening in real time and is generated biologically. The three wells were outside the influence of producing fields. Analysis of these wells revealed that gas pressure decreased toward the outcrop and gas flow rate increased toward the outcrop. This indicated that gas is actively flowing toward the outcrop and venting to the atmosphere from this coal seam. This has been occurring for a long time and if the methane was created thermogenically it would have depleted long ago (Wheaton et al. 2006).

This shallow coal seam is not mature enough to be generating thermogenic methane; therefore data indicates that microorganisms (*Bacteria* and *Archaea*) are mediating this process (Strapoc et al., 2011). The ever-increasing need for energy and this living community's unknown *in situ* methane-producing potential has made understanding these microorganism's *in situ* physiology and community structure attractive.

In recent years, the role of microorganisms in coal bed methane (CBM) formation has been the subject of an increasing number of investigations. These studies have relied on traditional formation water and/or coal core samples to access the coal-utilizing microbial community. Research suggests groundwater samples do not reflect microbial densities and activity in the subsurface and currently, it is not technically possible to aseptically collect core samples from methane-producing coal beds (Alfreider et al., 1997; Budwill et al., 2010). Down-well incubation of sterile substrate has emerged as an alternative method for sampling subsurface microorganisms (Alfreider et al., 1997; Griebler et al., 2002; Marxsen, 1982; Peacock et al., 2004 Reardon et al., 2004). This sampling strategy focuses on retrieving active microbial colonizers of specific sediments from the subsurface (Reardon et al., 2004).

A down-well incubation technique was utilized in the present study with several modifications to minimize contamination and target a coal-utilizing microbial population. The diffusive microbial sampler (DSM) only exposed the sediment at the bottom of the well where the coal seam is open, water flows through and methanogenesis presumably takes place. Sediment within the sampler was composed of sub-bituminous coal particles from the Powder River Basin (PRB), which allowed the diffusive sampler to essentially

close the gap in the coal seam created by the bore hole and also provide a substrate for *in situ* microbial growth (Figure 2).

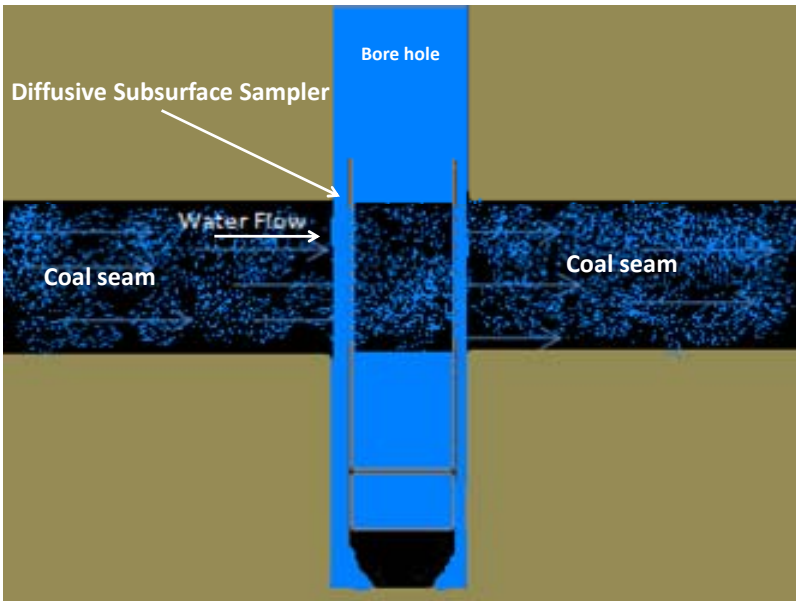

Figure 2: Sampling strategy used in this study: Schematic outlining the deployment of the DSM in CBM boreholes/wells. Sterile coal from the Decker Coal Mine (Powder River Basin) was loaded into the sampler and allowed to inoculate for three months. Upon retrieval the DSM closes to avoid contamination. At the surface slurry contained within the DSM was used to inoculate media and for community analysis.

Cell counts were compared between the *in situ* inoculum from the sampler and water pumped to the surface from several wells that accessed the deep subsurface PRB coal seams (Figure 3).

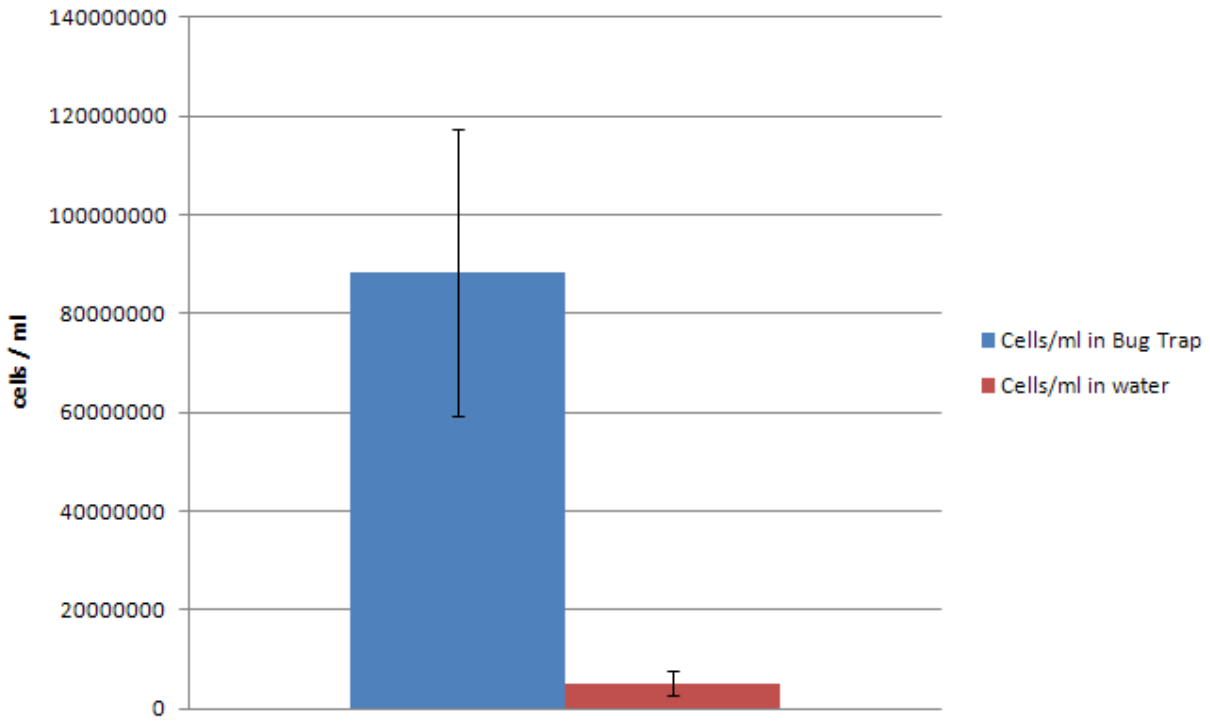

Figure 3: Water vs Diffusive Microbial Sampler (Bug Trap) Samples: Epifluorescent microscopy images of DAPI-stained cells collected from groundwater and slurry samples to estimate *in situ* microbial cell counts. Slurry samples were collected from down-well coal columns.

Although the PRB coal seams contain high levels of carbon, the environment can still be considered oligotrophic and planktonic cells may experience nutrient limitations. In this area it would be advantageous for microorganisms to form biofilms on surfaces where nutrients are more readily available. Therefore, we hypothesize biofilms compose a major microbial community structure within the coal seams of the PRB making coal-associated analysis an important tool in understanding subsurface microbial community structure. The next goal was to examine the individual microbial communities involved in converting coal to methane.

With the creation of desired functional environments, previous enrichment studies have demonstrated that coal beds contain the requisite microbial consortia capable of methanogenesis and that these consortia are able to utilize some coal components (Shumkov et al., 1999; Thielemann et al., 2004; Green et al., 2008; Harris et al., 2008; Kruger et al., 2008; Penner et al., 2010; Unsal et al., 2010; Wawrik et al., 2010; Stropoc, 2011). These studies suggest that coal beds are generally colonized by an array of bacterial taxa that commonly include members of the *Proteobacteria* (Li et al., 2008; Shimizu et al., 2007; Midgley et al., 2010; Penner et al., 2010). In addition to *Proteobacteria*, numerous *Firmicutes*, mostly from the order *Clostridiales* have been detected (Green et al., 2008; Shimizu et al., 2007; Strapoc et al., 2008). *Archaea* have also been detected in several core and water samples originating from coal beds but the diversity is much lower compared to the bacterial diversity detected (Green et al., 2008; Li et al., 2008; Shimizu et al., 2007; Stropoc et al., 2008; Klein et al., 2008; Midgley et al., 2010; Penner et al., 2010). *Methanosarcinaceae* was found to be dominant in the PRB but the analysis was based on SSU rDNA sequences from enrichment cultures and not in situ analysis (Green et al., 2008).

This study combined enrichment techniques with molecular biology techniques to investigate the individual microbial populations involved in specific parts of microbial conversion of coal to methane. Understanding the role of potentially important community members involved in converting coal to methane provides insight into how this processes and mechanisms *in situ*. With a basic understanding of the microbial

community structure and ecology, appropriate enrichment strategies were investigated that could make coal bed methane production sustainable.

Subsurface microbial communities have produced vast amounts of methane within the less-developed Montana portion of the Powder River Basin (Figure 4), but the productive life of a typical well in the PRB is only 7-8 years (Flores et al., 2001).

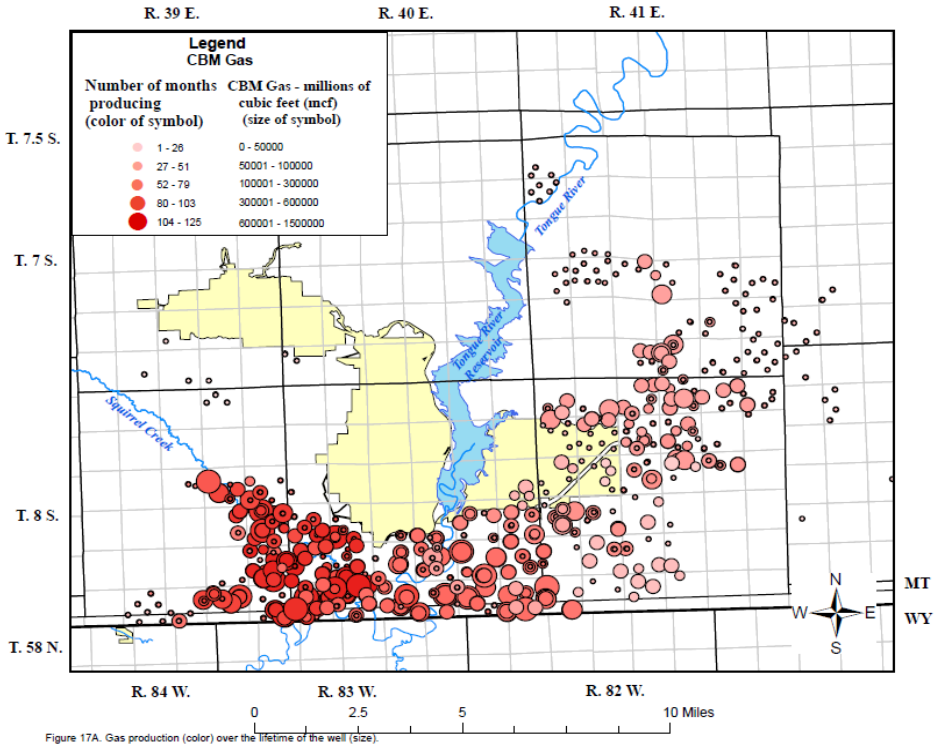

Figure 4: Current CBM development in Montana: This figure illustrates the large amounts of methane produced by subsurface microorganisms and the short lifespan of a CBM well. Each of the dark red larger circles represent over 1.0 trillion moles of methane given that 1.0 cubic foot of methane equals approximately 1.2 moles of methane. Figure courtesy of the Montana Bureau of Mines and Geology

Researchers have found that the subsurface organisms produce methane in real-time, but not fast enough to keep up with current coal bed methane extraction techniques (Green et

al. 2008) (Figures 1 and 4). In order to make this energy source industrially sustainable the subsurface microorganisms need to convert the coal to methane at a faster rate. Enrichment studies were utilized to investigate methods to stimulate microbial CBM formation. In the current study, CO_2 was added to enrichments to stimulate the archaeal population because all known methanogenic *archaea* possess the ability to reduce CO_2 to CH_4. Yeast extract and urea were added to other enrichments because they contain nutrients proven to stimulate bacterial populations (Sato et al., 1992). Further enrichment studies were carried out that investigated less complex amendments and the effect of coal particle size on methane production.

This research was aimed at enhancing the fundamental understanding of the ecology and physiology of methane-producing, coal-associated communities with the intent of identifying strategies for enhancement of in situ coal bed methane production.

METHODS AND MATERIALS

Sampling Sites and Collection

The study sites were located near the Decker coal mine in southeastern Montana. The wells WR-53A and HWC-O1 are monitoring wells used by the Montana Bureau of Mines and Geology to monitor the effect of methane development on regional groundwater tables. Complete geochemical analysis and well logs the can be obtained at http://mbmggwic.mtech.edu. Prior to sampling, the wells were flushed by pumping at least five well volumes of water until pH and conductivity were stable. For the estimation of bacterial abundances in the water, 1 mL was removed from a sterile sampling device and fixed with formaldehyde (2% final concentration) in the field in a sterile microcentrifuge tube.

A Diffusive Microbial Sampler (DMS) was designed to colonize native coal material with an indigenous microbial consortium from the Powder River Basin (PRB) coal seams. The DSM was filled with sterile DI water and sterile coal which is the proposed substrate for methane generation within the PRB. It was deployed to the bottom of a well where the coal-water interface was exposed. Upon retrieval, the slurry samples were fixed with formaldehyde (2% final concentration) in the field. The water and the slurry samples were stained with DAPI in the lab to perform cell counts on each sample. The slurry samples were also fixed with paraformaldehyde and glutaraldehyde in the field for FISH analysis.

Coal and Metabolite Enrichment Studies

The DMS was retrieved from well WR-53A using aseptic techniques and 3 mL of slurry was immediately added to anaerobic coculture medium (CCM) containing 30mM sodium acetate and (per liter) 2.17 g NaCl, 5.5 g $MgCl_2 \cdot 6H_2O$, 0.14 g $CaCl_2 \cdot 2H_2O$, 0.5 g NH_4Cl, and 0.335 g KCl. The medium was buffered using 1.1 mM K_2HPO_4 and 30 mM $NaHCO_3$ with 1 ml of 1000X per liter nonchelated trace elements and 1 ml of 1000X per liter vitamin solution amended with 2.0 g/liter choline chloride added as growth supplements. L-Cysteine • HCl (1 mM) and sulfide (1 mM as $Na_2S \cdot 9H_2O$) were added as reducing agents. Resazurin (1 mg/liter) was added as a redox indicator. Stock solutions of K_2HPO_4 (1 M), $NaHCO_3$ (6.0 M), L-cysteine • HCl (1 M), $Na_2S \cdot 9H_2O$ (1 M), and the nonchelated trace element and vitamin mixtures were prepared under anoxic conditions (Walker et al., 2009).

The DMS was retrieved from well HWC-01 using aseptic techniques and 3 mL of slurry was immediately added to three enrichment media that contained CO_2 in the headspace, N_2 in the headspace and N_2 in the headspace with 5g/L urea and 5g/L yeast extract added as amendments. The anaerobic CCM media contained concentrations of ions that reflected the chemistry of the well: (per liter) 3.86 mg $MgCl_2 \cdot 6H_2O$, 5.21 mg $CaCl_2 \cdot 2H_2O$, 0.5 g NH_4Cl, and 5.0 mg KCl. The medium was buffered using 1.1 mM K_2HPO_4 and 12.37 mM $NaHCO_3$ with 1 ml of 1000X per liter nonchelated trace elements and 1 ml of 1000X per liter vitamin solution amended with 2.0 g/liter choline chloride added as growth supplements. L-Cysteine • HCl (1 mM) and sulfide (1 mM as $Na_2S \cdot 9H_2O$) were added as reducing agents. Resazurin (1 mg/liter) was added as a redox

indicator. Stock solutions of K_2HPO_4 (1 M), $NaHCO_3$ (6.0 M), L-cysteine • HCl (1 M), Na_2S • $9H_2O$ (1 M), and the nonchelated trace element and vitamin mixtures were prepared under anoxic conditions (Walker et al., 2009).

Methane production was monitored by a direct injection SRI 8610C gas chromatograph (GC) with a thermal conductivity detector (TCD) and a S.S. Molecular Sieve 13X Packed Column. After significant methane production was detected in the enrichments inoculated from well WR53-A, 1 mL was inoculated to fresh CCM media with 5 g coal added in place of sodium acetate as a carbon source and CCM media with 30mM sodium acetate along with an 80:20 H_2/CO_2 headspace. After significant methane production was detected in the 5 g/L yeast extract and 5 g/L urea enrichments inoculated from well HWC-01, 1 mL of inoculated media was used to inoculate fresh CCM media amended with 1 g/l yeast extract and media with 1 g/l urea along with controls with and without 1 g of coal. Further enrichments were also carried out with enrichments from well HWC-01 that had not been exposed to yeast extract or urea by adding 1 mL of inoculated media from top methane producing cultures to fresh CCM media that contained yeast extract constituents and enrichments that contained differing amounts of coal and differing coal particle sizes. The different coal particle sizes were obtained by screening the coal through a sieve to ensure even particle distribution. The coal used in the experiments was obtained from the Decker Coal Mine in the Powder River Basin (MT) by the Montana Bureau of Mines and Geology. The enrichments were incubated in the dark at 25°C and methane production was monitored.

DNA Extraction and Analysis

Top methane-producing cultures were filtered onto a 0.2 μM filter and both the filter and the coal were included in a DNA extraction. Total community DNA was extracted using the BIO 101 FAST DNA spin kit (MP Biomedical, Solon, OH). In order to account for heterogeneity in community diversity and structure as well as variability in DNA extraction efficiency the culture was extracted in triplicate using half of a 0.2 μM filter and 0.25g coal per extraction with negative controls according to the manufacturer's instructions with three modifications: 460 μL of sodium phosphate buffer and 250 μL of phenol pH 6.6 were added in the lysis step in order to dissolve the filter. Also the silica matrix bound DNA was allowed to settle for 30 min and the SPIN Filter was allowed to air-dry in a sterile biosafety cabinet for 30 min following the salt-ethanol wash. An equal amount of each triplicate reaction was combined and quantified through spectrophotometric assessment at 260 nm on a NanoDrop ND-1000 spectrophotometer (NanoDrop, Willmington, DE). Approximately 2.0-2.5 ng of DNA was used to perform polymerase chain reactions (PCR) with universal bacterial primers FD1 (5'-AGAGTTTGATCC TGGCTCAG-3') and 1540R (5'-GGAGGTGWTCCARCCGC-3') and universal archaeal primers 21F (5'-TTCYGGTTGATCCYGCCRGA-3') and 1494R (5'-CGGTTACCT TGTTACGACTT-3'). The thermal profile used in the bacterial PCR reaction included initial denaturation at 94˚C (2 min) followed by 30 cycles of denaturation at 94˚C (30 sec), annealing at 58˚C (1 min), primer extension at 72˚C (1 min). The thermal profile used in the archaeal PCR reaction included: initial denaturation at 94˚C (5 min) followed by 25 cycles of denaturation at 94˚C (30 sec), annealing at 55˚C

(30 sec), primer extension at 72˚C (2 min). The final reaction mixture (20 µL) contained 10 µL 2X EconoTaq PLUS (Lucigen, Madison, WI) and 1.0 µM of each forward and reverse primer. A 2% agarose gel in TAE buffer was used to check the PCR products for DNA of the correct size. Triplicate bands were excised and cleaned using the Promega Wizard SV Gel and PCR Clean-UP System according to the manufacturer's instructions (Promega Inc. Madison WI). Cleaned products were cloned using the Invitrogen TOPO vector (pCR 2.1 – TOPO) Kit according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). Forty archaeal and 90 bacterial clones were picked and submitted for sequencing to Functional Biosciences (Madison, WI) using Sanger sequencing methods with bacterial primer 529R (5'-GGCAGATTCTTTGCCTTCTG-3') and archaeal primer 519R (5'-ACAATGTCATTGGTCAATTCATT-3'). Sequences were extracted from chromatograms using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned, clustered and dereplicated using the pyro pipeline of the ribosomal database project (http://rdp.cme.msu.edu/). Dereplicate sequences were identified using the BLASTn algorithm by NCBI (http://ncbi.nlm.nih.gov).

Microscopy and FISH Analysis

Samples for FISH were collected in sterile microcentrifuge tubes, stored on ice or at 4°C, and fixed within 24 h after collection. Slurry was fixed on slides with a 2% agarose solution. Hybridizations were carried out in buffer containing 0.9 M NaCl, 20 mM Tris/HCl (pH 7.4), 0.01% sodium dodecyl sulfate (SDS), and 25 to 50 ng of each oligonucleotide probe, with 35% formamide concentrations. Oligonucleotide probes

EUB338 and Arch 915 were synthesized and labeled at the 5’ end with fluorescent dyes (Cy3 and Cy5). The slides were incubated overnight in sealed chambers equilibrated with the hybridization buffer. The slides were immersed for 10 min at 48°C in wash buffer (20 mM Tris/HCl [pH 7.4], 0.01% SDS, 5 mM EDTA, and NaCl concentrations determined by Lathe’s formula [29]). The slides were then rinsed with distilled water, air dried, and counterstained with 4’,6’diamidino-2-phenylindole (DAPI).

RESULTS

Direct Microscopic Observations

Subsurface microbial communities that produce methane were evaluated by comparing samples obtained from pumped groundwater and a coal-filled Diffusive Microbial Sampler (DMS). Pumped groundwater from a coal seam yielded approximately 100-fold fewer microorganisms per volume than coal slurry from the DMS (106 cells/ml versus 108 cells/ml slurry) (Figure 5). The slurry contained a negligible amount of coal that could be pulled through a 22 gauge needle. The increased cell numbers resulted from sterile coal particles being exposed to in situ conditions down-well for twelve weeks which suggested the colonization and growth of indigenous microbial populations. The utilization of coal as a substrate would require microorganisms to be closely associated with the coal particles due to the low solubility of coal components in groundwater. Therefore, we attempted to observe microbial populations associated with coal in DMS incubated in situ.

Coal slurry was immediately removed from the DMS in the field and fixed for FISH analysis, and epifluorescent visualization demonstrated subsurface coal-associated biofilms (Figure 6).

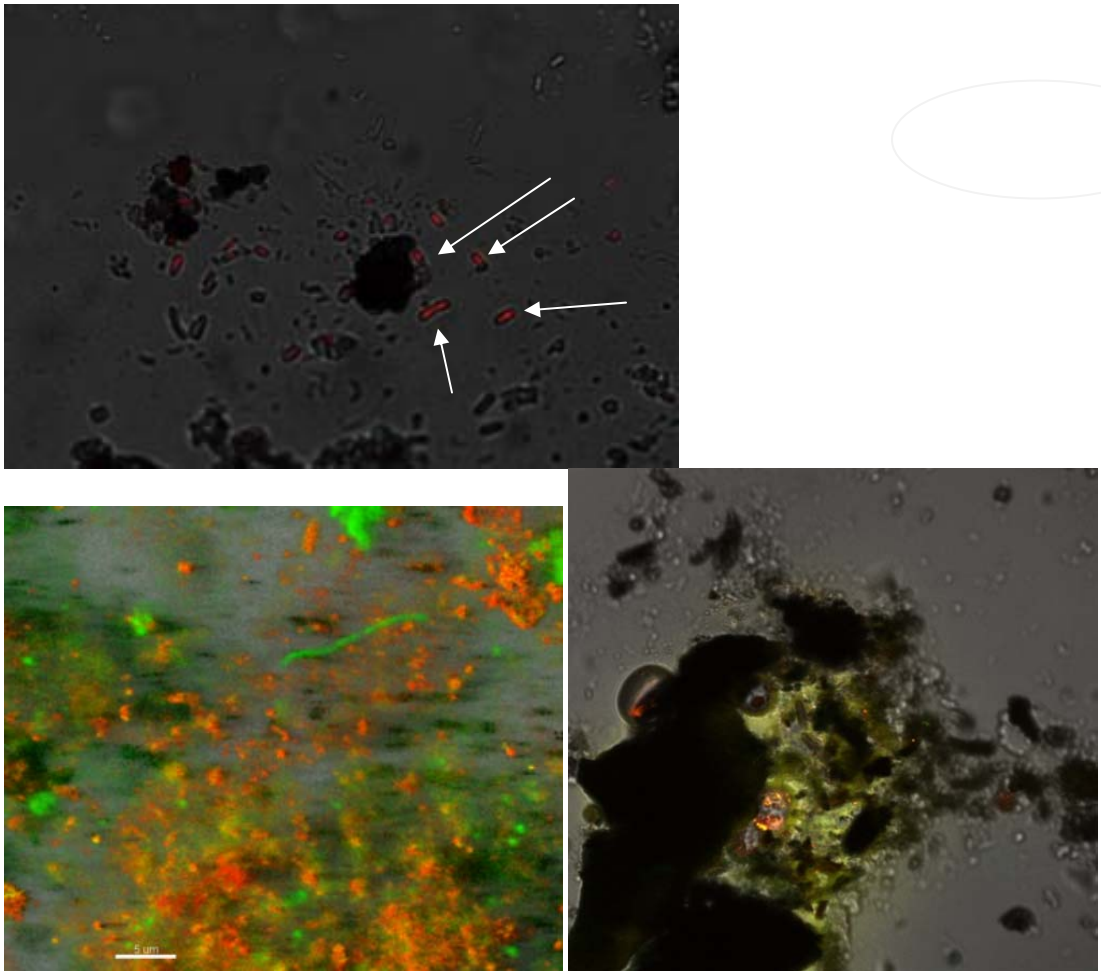

Figure 6: FISH Images of *in situ* DMS Sample: FISH Samples were fixed in the field for FISH analysis and appear to be congregated around coal particles. FISH analysis shows the community consists of both *archaea* and *bacteria*. The white arrows point at *bacteria* detected and the green fluorescence indicates the presence of *archaea*.

Diffusive sampling techniques allowed this enviornmental biofilm time to form. Archaea and bacteria were detected and bacteria appeared to outnumber archaea within the in situ community. Microorganisms appeared to be concentrated in the proximity of coal particles, and aggregates around coal particles contained both Bacteria and Archaea. Understanding the diversity of microorganisms contained within this biofilm community and the roles they serve in the conversion of coal to methane is vital in order to

effectively engineer this microbial system for maximal methane production in a sustainable manner.

Ability of the Consortium to Produce Methane from Coal and Metabolites

The DMS was raised from each well and used to inoculate anaerobic media with different amendments (i.e., coal or acetate/H_2). These amendments were used to determine differences in community composition and structure related to coal-dependent methane production. Methane production was monitored and clone libraries were created to compare the different communities from different amendments. Significant methane production was evident within several weeks in cultures that were provided acetate, but the coal-only cultures required more time before methane was detected (2 months compared to 2 weeks).

A total of 40 archaeal clones (SSU rDNA genes sequences) from the acetate and H_2-amended culture were sequenced and consisted of two predominant archaeal OTUs and 44 clones from the coal-only culture were sequenced and divided into three predominant OTUs (Figure 7). The coverage of the clone library was high due to the low diversity. Phylogenetic analysis revealed that 97% of the clones in the acetate-amended and 80% in the coal-only culture library were closely related to the genus Methanosarcina. Another OTU detected in both enrichments had 95% sequence identity to Methanospirillum hungatei, a methanogen that uses H_2 and CO_2 for methanogenesis. An OTU most closely related to a Methanothrix, an acetogenic methanogen (Huser, 1982), was detected in the coal-only enrichment but not the acetate-amended culture.

The methanogen specific, mcrA gene yielded similar results, detecting low archaeal diversity. The dominant organisms were identical in both libraries and 3 distinct archaea were detected in the coal enrichment and 2 in the metabolite enrichment (Figure 7).

A total of 76 bacterial clones (SSU rDNA gene sequences) from the coal-only culture and 82 bacterial clones from the acetate-amended culture were sequenced (Figure 7). The bacterial diversity was greater in the coal-only enrichment compared to the acetate-amended enrichment (24 versus 7 OTUs at the genera-level). The most abundant OTU in the coal-only enrichment was closely related (98% sequence identity) to the SSU rDNA gene of Clostridium (Sato, 1992), a well-known fermenter. An OTU closely related (99% sequence identity) to the SSU rDNA gene of Acetobacterium, a homoacetogen, comprised a significant fraction of both libraries. The presence of sequences indicative of homoacetagens suggests potential competition between acetogens and hydrogenotrophic methanogens for H_2 and that indirect shuttling of reductants from fermenters to acetoclastic methanogens may go through homoacetogens.

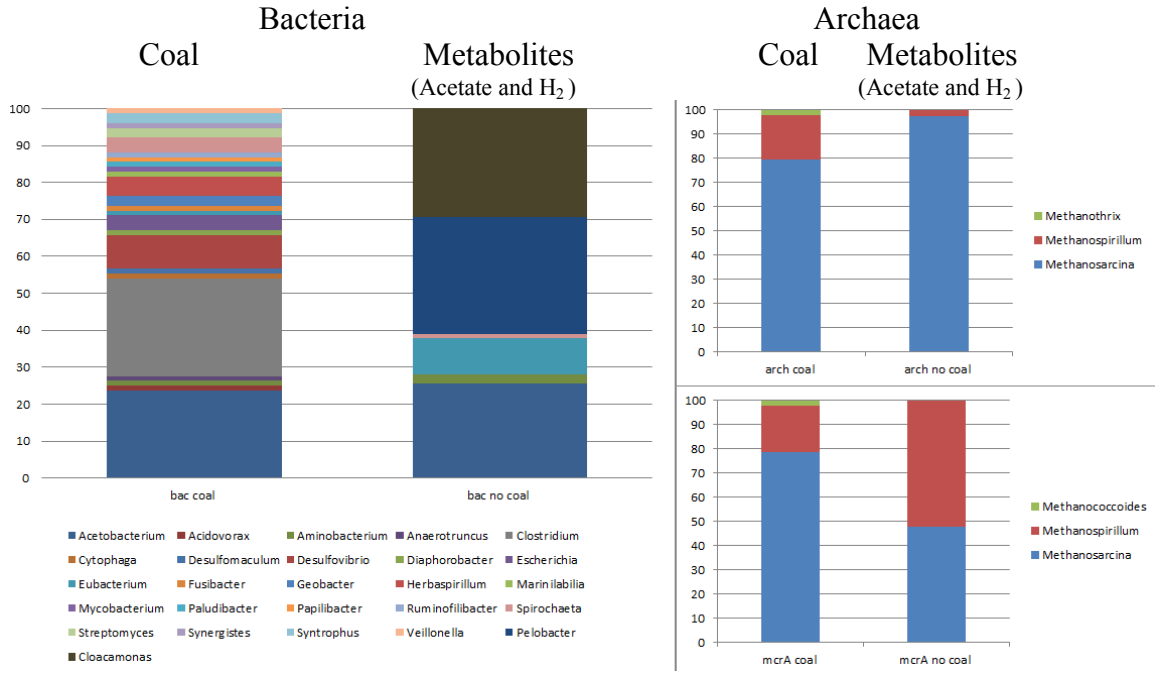

Figure 7: Ability of the Consortium to Produce Methane from Coal and Metabolites: Incubated slurry from the DSM was used to inoculate enrichment cultures with coal as the only energy source and compared to enrichments with acetate and H_2. DNA was extracted from these enrichments and clone libraries were constructed to compare the communities.

Biostimulation of the Coal-Dependent Community

Methane production was detected in all inoculated coal-containing microcosms while methane was not observed in uninoculated microcosms, indicating that methane did not desorb from the coal in significant rates or amounts. Methane was detected microcosms after 40 d of incubation, and the production slowed after approximately 70 d of incubation (Figure 8). The addition of CO_2 did not significantly increase methane production compared to a coal-only control, but the addition of a mixture of 5 g/L yeast extract and 5 g/L urea caused an approximate 10-fold increase in methane (15 µmol/mL

culture) compared to the non-stimulated culture. After methane production ceased, coal (starting amount of 1 g) was still present in the media indicating that only a small fraction of the coal may be utilized for methane production or an essential nutrient had been depleted in the batch incubations. Therefore, these microcosms were enriched further to investigate the role coal and nutrients had on stimulating methane production.

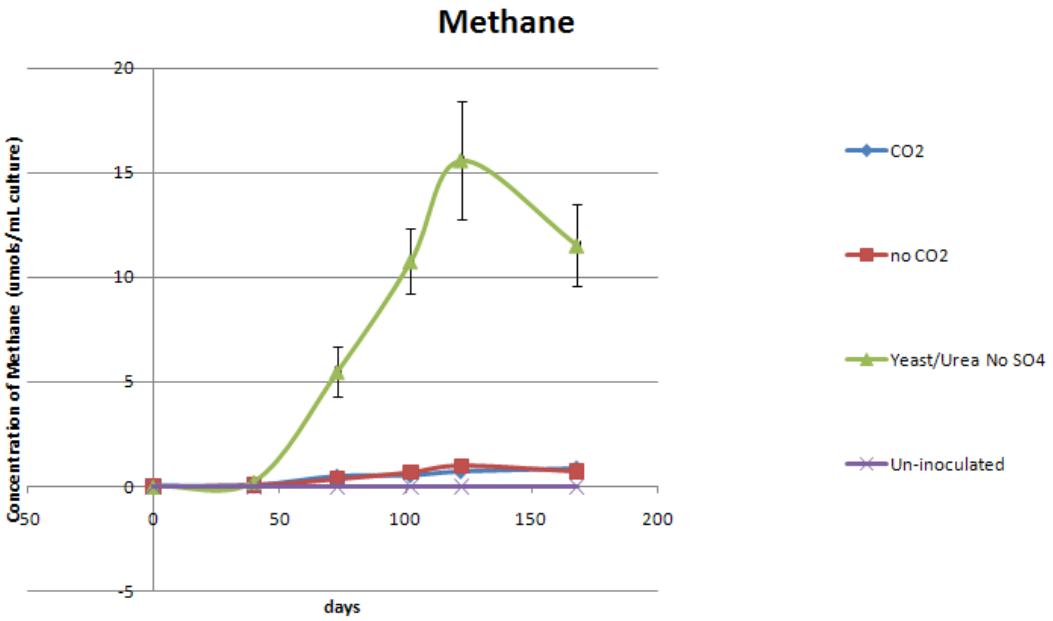

Figure 8: Microcosm Methane Production: The DSM sampled a wells in the PRB known to produce methane. Slurry from the DSM was used to inoculate media containing a control media, media with additional CO_2 and media with 1 g/l yeast extract and urea added. Methane production was monitored in the headspace of the microcosms to determine if the added constituents stimulate the physiology of the microbial community.

Enrichments from the Microcosms

Aliquots removed from a top methane-producing microcosm enriched with yeast extract and urea were used to inoculate eight different enrichments in triplicate which contained constituents of the microcosm believed to be involved in stimulating methane production (e.g., yeast extract, urea, bicarbonate). After five weeks of incubation the

enrichments that contained coal and yeast extract produced the most methane compared to coal-only, urea plus coal, and bicarbonate (approximately 4.5-fold) (Figure 9). Enrichments amended with yeast extract alone produced less methane than the coal-only enrichments, indicating that the increase in methane production was coal-dependent and not solely from yeast extract utilization. Current work is underway to elucidate the differences in microbial populations between coal-only and coal amended with yeast extract. In addition, yeast extract contains many nutrients that could possibly stimulate methane production; therefore, different factors were tested for stimulation of methane production with the microbial consortium in the presence of coal (e.g., peptone, vitamins, glutamate, and yeast extract).

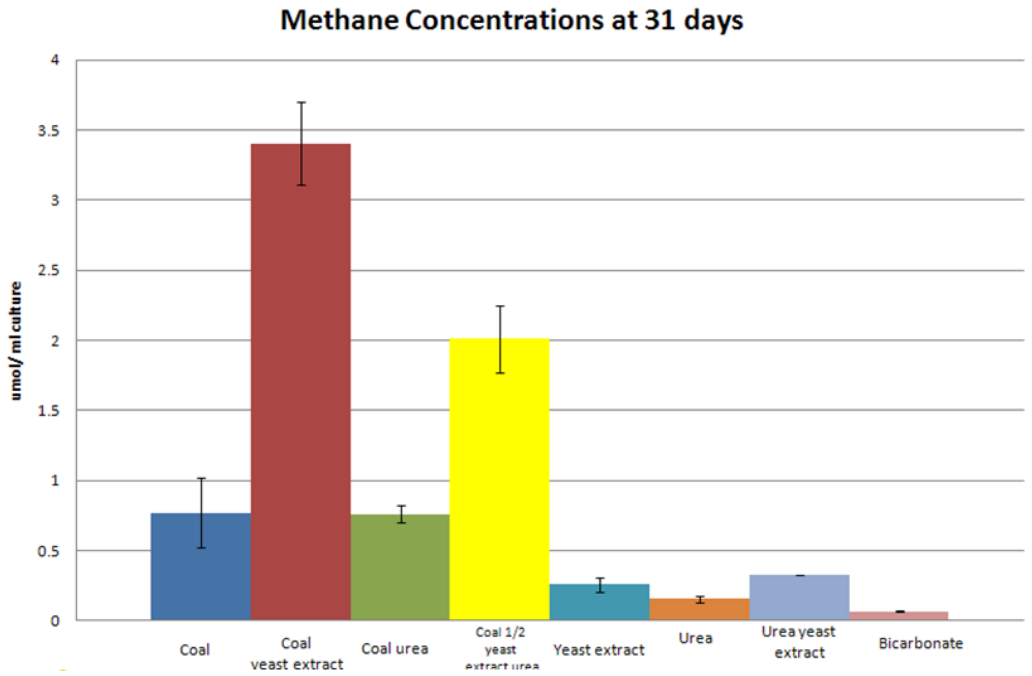

Figure 9: Enrichments from HWC-01 Microcosms: In order to determine if the urea or yeast extract was promoting methane formation samples were removed from a top methane producing microcosm HWC and used to inoculate sterile enrichments containing yeast extract and urea with and without coal. One sample was analyzed from each enrichment after 31 days of incubation with gas chromatography.

Yeast Extract Constituents

Complex proteins and sodium glutamate are two known constituents of yeast extract. Aliquots removed from a top methane-producing microcosm that had not previously been exposed to yeast extract were added to enrichments that contained individual components of yeast extract with and without coal. Methane was measured in the headspace of these enrichments after 60 days, which was the approximate time methane production was stimulated in the original microcosms. Sodium glutamate, peptone, mixed vitamins, and yeast extract all increased methane production in the presence of coal, while the additives alone did not produce more methane than the coal-only enrichment (Figure 10). Mixed vitamins had a marginal effect on methane production, and while peptone and glutamate increased methane production, yeast extract had the highest increase (almost 2-fold over peptone and glutamate) (Figure 10). These results indicated that peptides and individual amino acids could stimulate methane production, but the stimulation with yeast extract was highest.

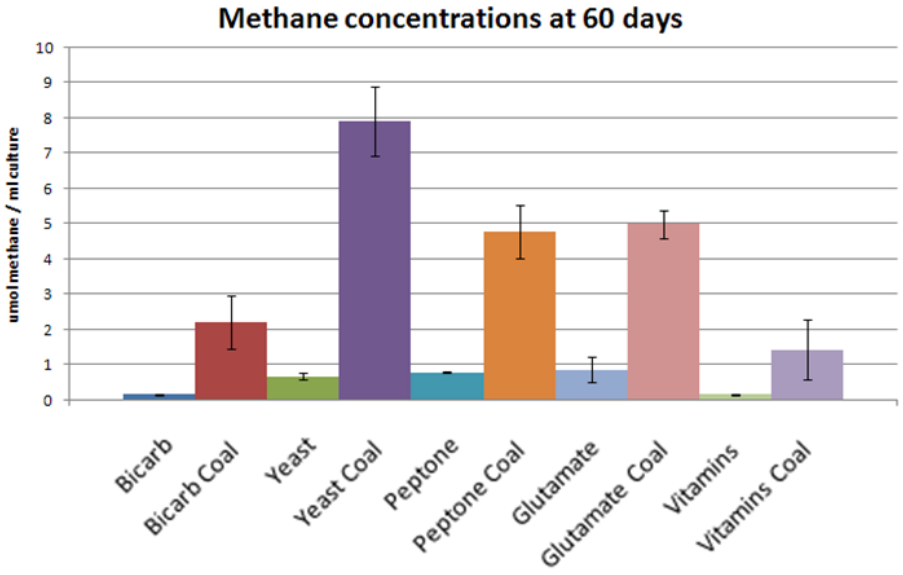

Figure 10: Enrichments Investigating Components of Yeast Extract: Different components of yeast extract were added to enrichments with and without coal to investigate the effect of the yeast extract constituents on methane production. One sample was analyzed at 6 days of incubation with gas chromatography.

Coal and Methane Production

We were able to demonstrate that methane production is directly linked to the amount of coal in the medium, and methane production increased as the coal mass available to the organisms increased (Figure 11). The methane production increased untll approximately 50 to100 days then leveled out, indicating stimulation of the methane producing population occurred during that time. The methane production also appeared to be coal-dependent although methane production was observed at a slower rate in enrichments with 1 g/l yeast extract with no coal added or low coal levels (0.001 and 0.01 g coal).

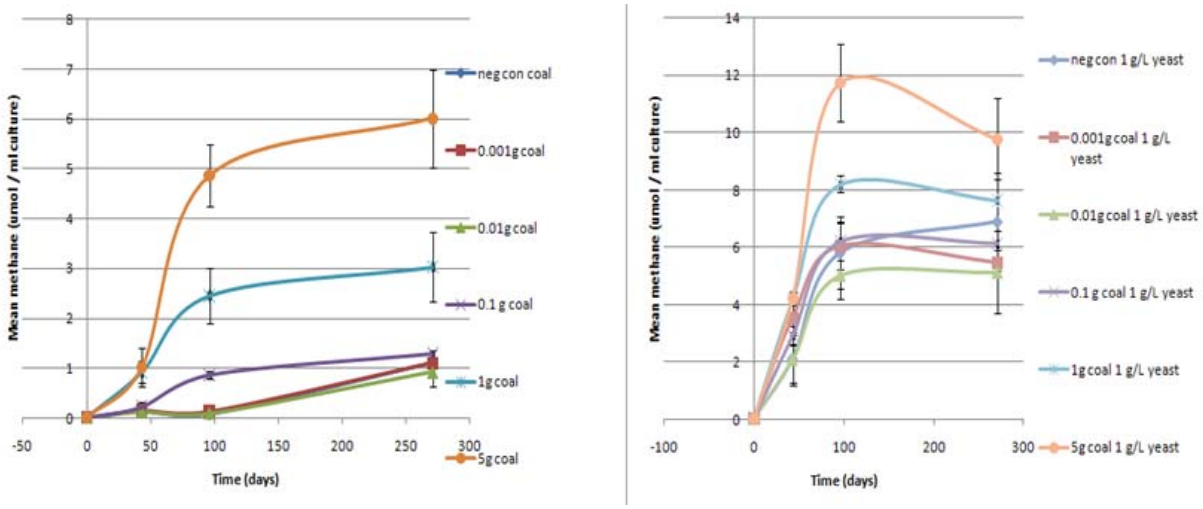

Figure 11: Enrichments Investigating Amounts of Coal: Different masses of coal were added to enrichments to investigate its impact on methane generation. Headspace of the enrichments were monitored for the presence of methane of stimulated media with 1 g/l yeast extract and non-stimulated enrichments with different masses of coal.

Interestingly, methane production was not significantly stimulated when the surface area of the coal was increased (Figure 12). Coal particles were fractionated based upon size in the ranges of <0.85 mm, <1.1 mm, and >2.0 mm, and then added to medium with or without yeast extract (1 g/L). The addition of 1g/L yeast extract stimulated methane production but the increased surface area of the coal did not significantly affect the methane production in long-term stimulated enrichments. Similar results were observed with and without yeast extract, indicating that increased surface area does not increase coal-dependent, methane production for the particle size range tested in the presence of yeast extract. While this is somewhat unexpected, the data suggest that coal surface area is not a significant factor in coal-degradation when microbial populations are stimulated with a nutritive such as yeast extract.

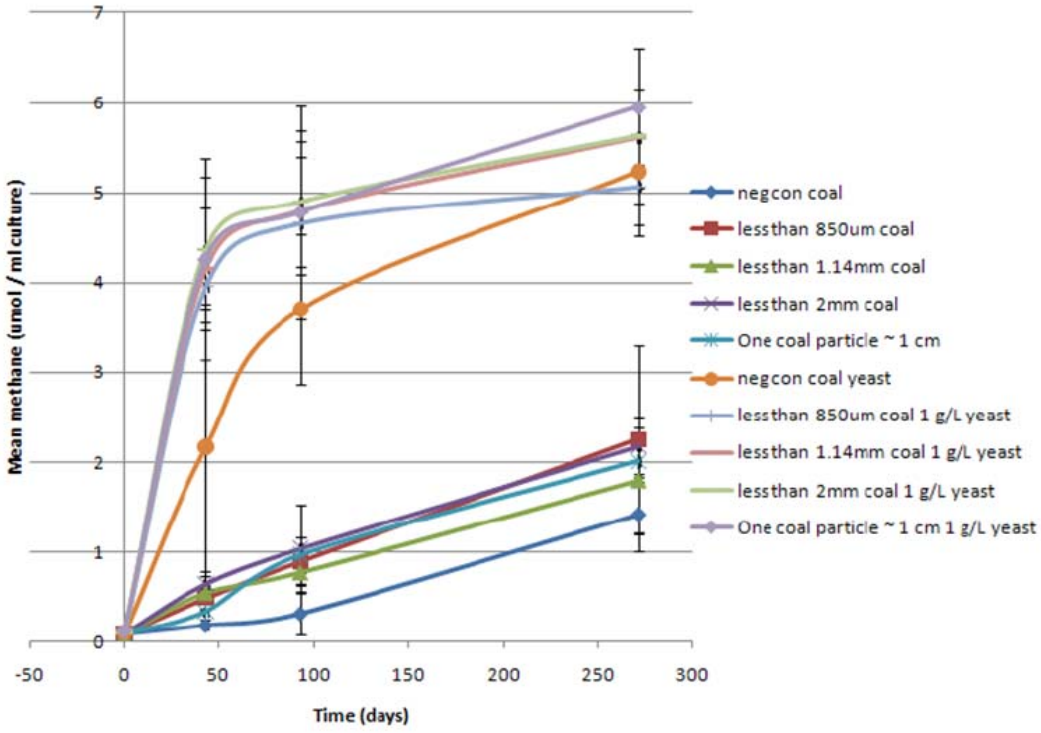

Figure 12: Enrichments Investigating Surface Area of Coal: The same mass of coal per mL culture was added to enrichments with significantly different surface areas to investigate the effect of surface area of coal on methane production. Headspace of the enrichments were monitored for the presence of methane of stimulated media with 1 g/l yeast extract and non-stimulated enrichments. *Neg con coal* did not contain any coal in the media and *neg con coal* yeast did not contain coal but did contain yeast extract and these two negative controls were inoculated.

DISCUSSION

Our study was comprised of three main objectives. The first was to compare structure and quantity of subsurface coal bed methane-producing communities with diffusive sampling techniques and microscopic observations. The second objective was to compare community composition and structure of microbial communities enriched from coal-beds that could produce methane in a coal-dependent fashion. In this step, enrichment techniques were combined with molecular biology techniques to help separate and identify relevant microorganisms. The third objective investigated the role of coal in methane production and potential nutrients that could stimulate subsurface communities. This comprehensive study began by investigating cell numbers obtained from traditional produced water samples versus slurry from the diffusive microbial sampler (DMS).

Sampling is the process whereby information is obtained from selected parts of an entity, with the aim of making general statements that apply to the entity as a whole. Traditionally, produced water and/or core samples were used to investigate coal-associated microorganisms within the subsurface. However, recent research has found that there can be significant differences between the microbial communities contained within these two standard sampling platforms when comparing subsurface matrix material and groundwater (Klein, 2008). These differences could be due to the observation that the biologically active components in aquifer ecosystems are attached to the sediment (Hazen, 1991; Alfreider, 1997). Therefore, sampling water at the surface, far from the active community will not likely result in a representative sample of the

microorganisms that utilize the coal in situ. Core samples could overcome this problem but this sampling strategy is very expensive and the samples can easily become contaminated with drilling fluid. Coring can also impact subsurface hydrology and connectivity.

Subsurface microbiologists have begun to address these problems in other deep subsurface environments by allowing the colonization of sterilized sediments with diffusive sampling devices (Hirsch and Rhades-Rohkohl, 1990; Marxsen et al., 1982; Alfreider et al., 1997). We utilized this sampling technique in our study and compared cell counts retrieved from slurry within the DMS to produced water samples. Our results agree with other studies investigating deep subsurface systems: a significant portion of active microorganisms in aquifers are attached to aquifer material, and only a small fraction is suspended in the groundwater (Holm et al., 1992; Hazen et al., 1991).

Fluorescent in situ hybridization (FISH) analysis of the active *in situ* structure of subsurface microbial communities within methane producing areas of the Canyon coal seam revealed microorganisms associated with the coal surfaces, suggesting presence of coal associated biofilms. The observation of coal-associated biofilms in the subsurface may explain the differences reported between the microbial community observed in coal core samples and produced water samples (Klein et al., 2008). An attached microbial community would not necessarily be represented in water pumped to the surface through a well bore hole. Hence, there would be a potential bias toward planktonic microbes, as opposed to sessile microbes that exist on the coal surfaces. With an understanding of the structure of microbial communities within active methane-producing regions of the

Powder River Basin, we investigated the individual community members that composed enrichments from *in situ* formed biofilm on native coal material.

In the past, microbial identification required the isolation of pure cultures (or simplified enrichments) followed by testing for multiple physiological and biochemical traits. These methods selected for certain microorganisms that could be transported from their natural environment into a laboratory setting and be grown on nutrient media. Consequently, this method was not ideal for subsurface microorganisms that live in the absence of oxygen and are difficult to access. Therefore, methods were developed for identification and phylogenetic characterization without cultivation. This technology came about with the discovery of the conserved nature of SSU rDNA, PCR amplification, and DNA sequence determination (Weisburg, et al. 1985).

Molecular techniques have been applied to coal-bed microbial systems in an attempt to detect dominant microbial community members present in produced water and coal core samples (Shimizu et al. 2001; Li et al. 2008; Strapoc et al; 2008; Fry et al. 2009; Penner et al. 2010; Midgley et al. 2010). Results from these studies generally exhibit a very diverse bacterial community with several genera of archaea that include members of the phylum Euryarchaeota. The vast diversity of the bacterial communities makes it difficult to infer any physiology based solely on bulk extraction and sequencing of nucleic acids. Therefore we applied enrichment techniques in an attempt to limit the bacterial diversity to the substrates provided in the medium.

Methanogenesis can be viewed as the end product of a complex series of trophic interactions in which several groups of bacteria work together to oxidize and reduce

carbon, leading to the production of methane (Chauhan et al., 2004). The precursors of biogenic methane formation are limited to simple compounds, primarily acetate, methanol, CO_2 and H_2 (Jones et al., 2010). Acetate has been observed to be the first organic compound released from coal in enrichments, presumably by fermentation of coal hydrocarbons (Jones, 2010). Based on these findings we set up two enrichments: bicarbonate media with coal as the only energy source and bicarbonate media with potential metabolites that result from coal degradation (acetate and carbon dioxide) along with an electron donor, hydrogen. Methane production first occurred in the metabolite enrichments indicating coal degredation was likely the rate-limiting step in methane production from coal. Molecular biology techniques can be combined with enrichment studies to begin to understand the physiological and biochemical basis of coal methanogenesis and relevant microorganisms involved in the conversion to coal to methane (Strapoc et al., 2011).

Enrichments revealed higher bacterial SSU rDNA gene sequence diversity (>3-fold) in the coal-only culture compared to the metabolite culture (24 versus 7 unique OTUs). The SSU rDNA archaeal gene sequences were nearly identical between the two enrichments and this suggested that *Methanosarcina* and *Methanospirillum* populations predominated irrespective of the presence of coal in enrichment cultures from our tested samples. The mcrA functional gene analysis also suggested low methanogenic functional diversity within the enrichments. Based upon enrichment cultures, coal promoted bacterial diversity but not archaeal diversity, and the diversity of bacterial populations may play an important role in coal degradation (e.g. cross-feeding of acetogens and/or

methanogens). Stimulation of the microbial communities involved in supplying the archaeal population with metabolites could make CBM sustainable.

Each OTU generated in this study represents a phylotype and may be representative of a bacterial species or population. Nearly thirty percent of the bacterial OTUs had a low similarity (less than 90% identity) to any known cultured bacteria published on the NCBI website (June, 2011) and may represent novel species specifically adapted to a coal-bed ecosystem. Further isolation studies from coal-beds are needed to better understand indigenous organisms and function. However, the phylogenetic similarity of some clones to cultured species with known physiology is consistent with the different trophic groups required to convert complex organic matter to methane (Whiticar et al., 1999). It is generally thought that coal is converted to methane by groups of fermentative bacteria that are syntrophically associated with methanogenic archaea (Strapoc et al., 2011). Our research suggests that Clostridia could be involved with the breakdown of coal and *Acetobacterium* are able to utilize substrates produced by coal degradation. However, it is unclear if *Acetobacterium* species aid in the cross-feeding process with methanogens or if they compete with methanogens for substrates. Many homoacetogens like *Acetobacterium* possess the ability to reduce CO_2 with hydrogen to form acetate, whereas in the presence of methanogens they oxidize sugars and lactate to acetate coupled with hydrogen formation (Winter et al., 1980; Cord-Ruwisch et al., 1986). Therefore, *Acetobacterium* species could use or form hydrogen depending on the prevailing conditions. If coal degradation is the limiting step in this

process as our research suggests stimulating the *Clostridium* populations and other hydrocarbon degraders *in situ* may lead to industrially sustainable CBM development.

The metabolic potential of subsurface microorganisms is often higher than the *in situ* conditions allow due to nutrient limitations. Methane production can be stimulated by the addition of many different nutrients. In our study, enrichments were generated from the sampled subsurface communities and methane production was monitored. Methane production was determined to be coal-dependent and complex constituents, such as yeast extract and peptone, significantly increased methane production. Interestingly, when coal and yeast extract were not added in combination very little methane was produced (Figure 9). Our research has revealed the presumptive presence of fermentative bacteria (e.g., *Clostridium*) in these environments to be associated with coal. Other studies indicate that *Clostridium* species growth and production of fermentation products are stimulated in the presence of yeast extract (Sato et al., 1992). Therefore, both coal and yeast extract appear to contribute important nutrients that stimulate methane-producing, microbial communities.

Yeast extract comprises the water soluble components of the lysed yeast cell, and is primarily amino-acids, peptides, carbohydrates and salts. Enrichments were used to evaluate the methane stimulating contribution of different components in yeast extract. Peptone enrichments represented the peptides and carbohydrates in yeast extract, glutamic acid enrichments represented a significant amino acid in yeast extract, and mixed vitamins signified the contribution of added trace elements/vitamins. Glutamic acid and peptone stimulated methane production but not as much as yeast extract.

Stimulation efforts could be impeded if rich complex stimulants such as yeast extract are added to the subsurface. Thick subsurface biofilms could form and cause plugging of subsurface formations when supplied with complex nutrients (Komlos et al., 1998), and this plugging could impede further stimulation. Therefore, even with a lower initial stimulating ability, it is important to investigate simple nutrients that could enhance methane production in situ without significant hindrance to hydrology.

Enrichment experiments demonstrated that as the amount of coal in the medium increased the rate and extent of biological methane production also increased. Interestingly, under our tested growth conditions, increased surface area of coal particles did not significantly increase the rate or final level of methane produced. This trend was observed in both stimulated and non-stimulated enrichments and these results indicated that the methane production potential from the billions of tons of coal buried within the Powder River Basin (USGS) is significant. The chemistry of the existing coal surface is also likely to be involved (responsible) in stimulating methane production even without the need to further fracture the existing coal deposits.

CONCLUSIONS

There is currently enormous interest in the question as to what extent the *in situ* microbial conversion of coal into methane can contribute towards satisfying the growing energy needs of the world as more sustainable and renewable sources are developed. The results of this study indicate that the largest portion of the methane-producing consortia in the PRB are attached to coal particles in the subsurface, and the sampling strategy developed in this study targeted an active coal-utilizing microbial consortium that was composed of a diverse bacterial community associated with a less diverse methanogenic community. Direct analysis of microbes within the DMS revealed that the coal was actively colonized through biofilm formation. Enrichment techniques were combined with molecular biology methods to infer potentially important organisms involved in producing methane metabolites from coal and microorganisms that utilize the metabolites.

Many of the bacterial OTUs had a low similarity (less than 90% identity) to any known cultured bacteria contained in the NCBI database, indicating further isolation studies from coal beds are needed to better understand the microbial processes in this environment. However, our research suggests that *Clostridium* species are involved with the breakdown of coal and *Acetobacterium* species are able to utilize substrates produced by the degradation of coal. The archaeal OTUs were not very diverse and did not change significantly when coal was removed from the medium.

Enrichment techniques were employed to investigate the importance of certain nutrients and coal in methane production. This study showed that both coal and yeast

extract appear to be important contributors. The yeast extract components, glutamic acid and peptone stimulated methane production but not as much as yeast extract. Finally, several enrichment experiments demonstrated that as the amount of coal in the culture medium increased, the rate and extent of biological methane production increased as well. This research will help create accurate subsurface microbial models that begin to understand one of the largest ecosystems on Earth.

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