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Type and amount of organic amendments affect enhanced biogenic methane production from coal and microbial community structure

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ABSTRACT

Slow rates of coal-to-methane conversion limit biogenic methane production from coalbeds. This study demonstrates that rates of coal-to-methane conversion can be increased by the addition of small amounts of organic amendments. Algae, cyanobacteria, yeast cells, and granulated yeast extract were tested at two concentrations (0.1 and 0.5 g/L), and similar increases in total methane produced and methane production rates were observed for all amendments at a given concentration. In 0.1 g/L amended systems, the amount of carbon converted to methane minus the amount produced in coal only systems exceeded the amount of carbon added in the form of amendment, suggesting enhanced coal-to-methane conversion through amendment addition. The amount of methane produced in the 0.5 g/L amended systems did not exceed the amount of carbon added. While the archaeal communities did not vary significantly, the bacterial populations appeared to be strongly influenced by the presence of coal when 0.1 g/L of amendment was added; at an amendment concentration of 0.5 g/L the bacterial community composition appeared to be affected most strongly by the amendment type. Overall, the results suggest that small amounts of amendment are not only sufficient but possibly advantageous if faster *in situ* coal-to-methane production is to be promoted.

1. Introduction

Coalbed methane (CBM) is an unconventional natural gas resource formed in subsurface coal seams by thermogenic and biogenic processes. In 2015, the United States had 12,520 billion cubic feet of proven CBM reserves, and CBM production provided approximately 4% of the total annual natural gas requirement [1,2]. The coal beds of the Powder River Basin (PRB) in southeastern Montana and northeastern Wyoming accounted for 16.3% of the CBM produced in the U.S. in 2015 [1,2], and the PRB CBM has been shown to be primarily or completely of biogenic origin [3–5].

Biogenic CBM is the result of coal-to-methane conversion by a diverse, natural microbial community [3,6–8]. Methanogenic archaea produce biogenic methane through a limited number of pathways utilizing simple substrates (H_2/CO_2, acetate, and methyl-compounds). However, generation of these simple substrates from coal requires a diverse microbial consortium, containing both archaeal and bacterial members, with interactive metabolic strategies for sequential

fermentative processes to degrade coal to simpler fermentation by-products [3,6,7]. Biogenic methane is produced continuously in active coal basins, and methods have been proposed for increasing the rate and volume of microbially-produced methane [9].

Rates of commercial biogenic gas removal often exceed the rates of microbial production, resulting in reduced gas extraction. Thus, gas production from many wells is no longer economically viable [10], especially with decreased prices for natural gas due to increases in shale gas production in the 2000s. This has increased the cost-to-profit ratio for CBM retrieval, and many wells have been abandoned. The existing infrastructure creates an ideal opportunity for microbially-enhanced CBM (MeCBM) methods to increase coal-to-methane conversion rates and CBM volume thus extending the lifespan of current and future wells.

Ritter et al. summarized laboratory and *in situ* commercial applications of MeCBM techniques using various amendment methods [9]. While some studies have shown the potential for increased biogenic methane production with coal pre-treatment for enhanced

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bioavailability [11–13], significant potential exists for MeCBM strategies that stimulate microbial populations through nutrient addition [3,9,14,15]. Some previous CBM stimulation studies have used simple carbon substrates, such as acetate [8] or formate [16] to enhance methane production. While adding simple carbon substrates alone does increase methane production, it is unclear whether these additions enhance the coal-to-methane conversion or merely supply a more easily metabolized substrate for bacteria and/or methanogens. Inorganic limiting nutrients for biogenic coal-to-methane conversion include nitrogen, phosphorus, trace elements, and/or vitamins, and addition of these inorganic nutrients resulted in increased biogenic methane production in coal microcosms [14,17]. Determination of the specific nutrients needed for each potential *in situ* application could be costly and time-consuming.

A review of microbial life under extreme energy limitations suggested that microbial communities in subsurface environments can meet organic and inorganic nutritional requirements through biomass turnover [18]. Using this principle, it can be assumed that additions of complex nutrient sources (such as biomass) to the coal environment could provide the combination of nutrients necessary to encourage microbial growth and enhance coal degradation without the need for determining the exact amounts of specific nutrients required. Previous studies have supplemented limiting nutrients and successfully enhanced CBM production through the addition of yeast extract in conjunction with other organic or inorganic nutrient additions [9,14,19,20]. Barnhart et al. introduced the use of yeast and algal extracts as sole amendments to stimulate biogenic methanogenesis from coal and demonstrated a production increase with both amendments [21]. Biostimulation with yeast extract or other biomass-derived nutrient sources could provide the limiting nutrients needed for both the bacterial and archaeal populations. This could result in increased coal-to-methane conversion with potentially reduced cost and without the need to determine the exact nutrient additions needed for *in situ* CBM stimulation.

The goals of this study were as follows: (1) assess the potential of using four different biologically produced multi-nutrient amendments (algae, cyanobacteria, yeast cells, and granulated yeast extract) to enhance methane production from coal, (2) track carbon inputs and outputs to determine whether amendments are indeed stimulating coal-to-methane conversion or merely providing an alternative carbon source for methane production, and (3) determine whether amendment addition causes significant shifts in the microbial community involved in coal-dependent methanogenesis.

2. Materials and methods

2.1. Site and sample collection

The sampling site, located near Birney (Montana, USA) in the Powder River Basin, was thoroughly described by Barnhart et al. [22]. Water from the subbituminous Flowers-Goodale (FG) coalbed was pumped and retrieved in July 2014 from the FG-11 well. Six-gallon plastic storage jugs were rinsed twice with formation water before being filled and stored at 4 °C upon return to the laboratory (Montana State University, Bozeman, MT) until microcosm set up. Coal cores were collected during the July 2013 drilling of FG monitoring wells (FGM-13, FGP-13). The 2-inch diameter cores were cut into approximately 12-inch long sections and placed in polyvinyl chloride (PVC) tubes. These tubes were completely filled with formation water pumped from the FG-11 well, and sealed with flexible rubber caps to allow room for gas desorption. A detailed description of the FG coal bed samples was given by Barnhart et al. [22]. Microbial cultures were collected from two FG wells (FGM-13 and FGP-13) in November 2014 using the diffusive microbial samplers (DMS) described by Barnhart et al. [8]. The slurry (formation water with high suspended solids) from the FGP-13 DMS (13 mL) and FGM-13 DMS (7 mL) were added to a serum bottle prepared with 5 g FG coal and 45 mL anoxic FG formation water before

being allowed to incubate at room temperature in the dark for 5 months prior to being used to inoculate the studies described here.

2.2. Amendment growth and analysis

The microalga, *Chlorella* sp. strain, SLA-04 (isolated from Soap Lake, WA, USA), was cultured for biomass accumulation at 20 °C in Bold's Basal Medium [23] in tube photobioreactors using methods previously described [24]. *Anabaena cylindrica* strain UTEX 1611, a nitrogen-fixing cyanobacterium, was cultured using methods similar to SLA-04 cultivation using Blue-Green Medium (BG-11) [25] without the nitrogen source. For both SLA-04 and UTEX 1611, daily cell counts were used to determine stationary phase when the cell counts were highest, 6.0×10^7 and 4.0×10^7 cells/mL respectively. A yeast, *Saccharomyces cerevisiae* strain EtOH-Red, was cultured in 100 mL of Yeast Extract Peptone Dextrose (YPD) medium [26] in 250 mL flasks at 37 °C and shaken at 100 rpm to keep cells in suspension. Optical density (OD) at 600 nm was measured daily. Yeast culture (5 mL) was collected in 26 mL Balch tubes, and OD was measured with Unico 1100RS tube spectrophotometer (Dayton, NJ, USA). OD increased from an initial OD of 0.77–1.97 OD at stationary phase. The biomass from all three cultures was concentrated by centrifugation, dried by lyophilization, and stored at −20 °C. SLA-04, UTEX 1611, and EtOH-Red biomass as well as granulated yeast extract (EMD Millipore Corporation) (known hereafter as algae, cyanobacteria, yeast, and YE, respectively) were sent to the Iowa State University Soil and Plant Analysis Laboratory (Ames, Iowa) for elemental analysis (Supplementary Table S1) [27].

2.3. Microcosm set up

All microcosms were set up anoxically in 26 mL Balch tubes with butyl rubber stoppers and aluminum crimp seals (Supplementary Fig. S1). The FG coal core (depth 374–375′) was opened in an anaerobic glove bag where it was dried, crushed, and sieved to an effective size range of 0.85–1.19 mm. The prepared coal was stored in oxygen-free glass bottles until microcosm set up. Borosilicate glass beads (GB) (1 mm diameter) were autoclaved for controls and used in lieu of coal to provide a carbon-free solid substrate. Each Balch tube received 1 g of prepared coal or GB. The formation water was sparged for 5 h with anoxic nitrogen gas and reduced with sulfide (1 mM as $Na_2S \cdot 9H_2O$). Resazurin (1 mg/L) was used as a visual redox indicator. The amendments (algae, cyanobacteria, yeast, and YE) were ground to a fine powder with a ceramic mortar and pestle. Two concentrations of each amendment were prepared at $10\times$ desired concentration (0.1 and 0.5 g/L final concentration) in degassed FG formation water and sealed anaerobically in serum bottles. All amended treatments received 1 mL of this prepared amendment concentrate as appropriate. The headspace of all tubes was replaced with 5% CO_2, 95% N_2. pH was tested to ensure a range of 7.5–8.5 as observed in the FG formation water [22] and adjusted with 1 M HCl as necessary. All inoculated treatments received 1 mL of the inoculum described above; 3 mL of the inoculum slurry was stored at −80 °C for microbial community analysis. All microcosms were incubated at room temperature (21 ± 1 °C) in the dark for 111 days, and headspace gas was sampled and analyzed approximately every 2 weeks.

2.4. Gas analysis

Methane production was monitored using an SRI Instruments (Torrance, CA, USA) Model 8601C GC equipped with a thermal conductivity detector (TCD) and interfaced with PeakSimple Chromatography software. A Supelco Molecular Sieve 13X packed stainless steel column (6 feet × 1/8″ O.D.) was used with ultra-high purity helium carrier gas for separation using the following conditions: manual injection, oven temperature 40 °C, TCD temperature 150 °C, and carrier gas pressure 18 psi. Gas samples (1 mL) were taken from the

Table 1

Treatment conditions, final amount of methane produced per g of coal or glass beads, and maximum methane production rates. Variation is expressed as 1 standard deviation for three replicates of each treatment.

Treatment	Solid Substrate (1 g)	Amendment	Amendment concentration (g/L)	Methane produced in 111 days (µg CH_4/g coal or glass beads)	Maximum methane production rate (µg CH_4/g coal or glass beads/day)
1	Coal	–	0	676.2 ± 323.9	16.3 ± 13.0
2	Glass beads	–	0	0.0 ± 0.0	0.0 ± 0.0
3	Coal	Algae	0.1	1370.8 ± 19.4	45.8 ± 0.7
4	Glass beads	Algae	0.1	110.6 ± 48.5	2.7 ± 1.1
5	Coal	Cyanobacteria	0.1	1390.0 ± 17.7	45.7 ± 0.5
6	Glass beads	Cyanobacteria	0.1	169.2 ± 12.5	3.9 ± 1.0
7	Coal	Yeast	0.1	1434.6 ± 18.7	49.5 ± 0.9
8	Glass beads	Yeast	0.1	144.0 ± 35.4	3.8 ± 0.6
9	Coal	Yeast extract	0.1	1456.0 ± 53.9	50.2 ± 1.5
10	Glass beads	Yeast extract	0.1	147.4 ± 21.0	5.1 ± 1.7
11	Coal	Algae	0.5	1959.9 ± 33.5	56.2 ± 0.4
12	Glass beads	Algae	0.5	63.7 ± 36.2	1.6 ± 0.3
13	Coal	Cyanobacteria	0.5	2185.4 ± 96.7	64.7 ± 2.8
14	Glass beads	Cyanobacteria	0.5	91.0 ± 14.5	2.2 ± 0.2
15	Coal	Yeast	0.5	2118.0 ± 263.1	61.3 ± 1.7
16	Glass beads	Yeast	0.5	930.4 ± 66.9	18.8 ± 13.7
17	Coal	Yeast extract	0.5	2125.0 ± 6.1	63.1 ± 2.7
18	Glass beads	Yeast extract	0.5	608.6 ± 237.0	22.8 ± 1.4

microcosm headspace for GC injection. To prevent creating a negative pressure in the tubes, 1 mL of anoxic 5% CO_2 in N_2 gas was injected prior to withdrawing samples.

2.5. DNA extraction and microbial community analysis

On day 111, one replicate of each treatment was destructively sampled for DNA analysis. The coal or GB and liquid fractions were separated by decanting the liquid into a 15 mL Falcon conical centrifuge tube. The coal/GB was transferred to a separate 15 mL Falcon tube. One mL of 10% sodium dodecyl sulfate (SDS) was added to the coal/GB and the tube was placed in a 70 °C water bath for 30 min. The liquid fraction was centrifuged and the supernatant decanted and discarded to leave approximately 2 mL with the pellet. Both sample fractions were stored at −80 °C until extraction.

The coal/SDS mixture was heated in a 70 °C water bath for an additional 30 min just before extraction. Total sample DNA was extracted using the FastDNA Spin Kit for Soil (MP Biomedical, Solon, OH) according to the manufacturer's instructions with minor modifications. Instead of 500 mg of soil, 200 µL of the centrifuged liquid fraction and 200 µL of the pre-treated coal/SDS fraction were used. Times for homogenizing (60 s), binding matrix (20 min), and air drying after the SEWS/ethanol wash (25 min) were extended to increase DNA recovery. After extraction, the DNA was prepared for PCR amplification using the OneStep™ PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA).

Extracted DNA was quantified using a Qubit fluorometer and dsDNA HS Assay Kits (Thermo Fisher Scientific). The 16S rRNA genes were PCR-amplified for thirty cycles with DreamTaq PCR Master Mix (Thermo Fisher Scientific) and annealing temperature of 55 °C for 30 s using the universal prokaryotic primers Pro341F (5′-CCTAC GGGNBGCASCAG-3′) and Pro805R (5′-GACTACNVGGGTATCTAATCC-3′), which amplify the V4 region of the 16S rRNA gene of bacteria and archaea [28]. Amplicons were checked by agarose gel electrophoresis with GelRed DNA stains (Biotium). Library preparation for Illumina MiSeq sequencing was carried out following Illumina's standard protocol "16S Metagenomic Sequencing Library Preparation" prior to being loaded for sequencing on the MiSeq v.3 platform. Sequence reads were analyzed using the MiSeq standard operating procedure of the Mothur software package [29]. In brief, paired reads were joined into contigs. The resulting contigs were screened for ambiguous base pairs, amplicon size, alignment positions, and chimeric sequences. The qualified unique contigs were classified with the Mothur formatted version of the Ribosomal Database Project (RDP; https://rdp.cme.msu.edu/)

training sets with cutoff value of 80. Chloroplast-, mitochondria- and eukarya-like sequences were removed from the analysis. Remaining sequences were binned into phylotypes according to taxonomic classification, and a relative abundance plot for each library was generated. Amplicon sequences from this study were uploaded to National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra) under accession numbers SRR5342596 to SRR5342613.

2.6. Statistical analyses

Differences in microbial community composition were examined through principal component analysis of non-transformed phylotype relative abundances using the CANOCO 4.5 software package (Microcomputer Power, Ithaca, NY). A three-way ANOVA with interaction and Tukey comparisons were used to test for the effects of coal, amendment type, and concentration using the statistical software Minitab v17. A *p*-value less than 0.05 determined significance.

3. Results

3.1. Effects of coal and amendments

To assess the effects of the four amendments on coal-dependent biogenic methane production, 18 treatments, each in triplicate, were utilized (Table 1). Produced methane was detectable by day 19 for all coal treatments except for the unamended coal and the 0.1 g/L algae and cyanobacteria amended coal treatments. By day 35, methane was detected in all coal-containing treatments, and the 0.1 g/L algae and cyanobacteria amended coal treatments had methane production comparable to the other 0.1 g/L coal treatments at all subsequent sampling time points (Fig. 1). Coal treatments produced significantly ($p < 0.0005$) more gas overall than the corresponding GB treatments (i.e., amendment-only glass bead controls). The 0.5 g/L yeast and yeast extract (YE) GB treatments had detectable methane on day 19, but methane was detected later for all other GB treatments: day 63 for 0.1 g/L amended GB treatments and day 54 for 0.5 g/L algae and cyanobacteria amended GB treatments. The unamended GB treatment produced no detectable methane during the 111-day duration of the study (Fig. 1).

The total methane produced by the unamended coal treatments was 676 µg CH_4/g coal after 111 days. For amended coal treatments with the same amendment concentration, regardless of amendment type,
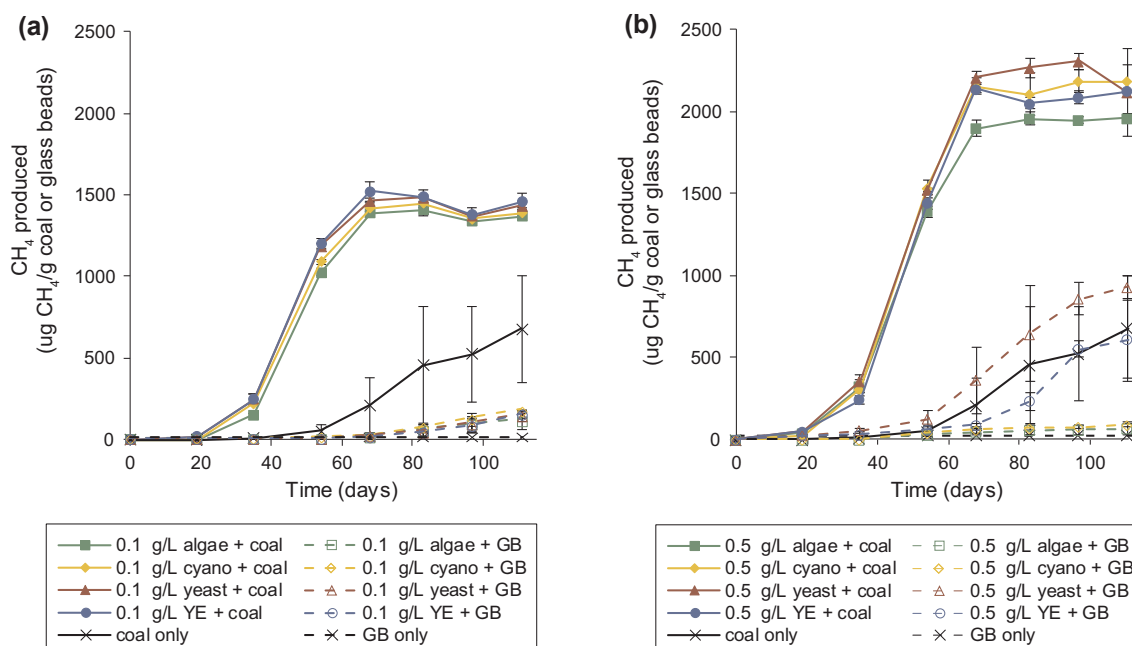
Fig. 1. Methane production over time for (a) unamended and 0.1 g/L amended treatments and (b) unamended and 0.5 g/L amended treatments showing the cumulative methane produced at each sampling day. The methane produced is the sum of methane measured in the headspace and what can be assumed to be dissolved according to Henry's law. It does not include methane that may be sorbed to the coal or glass beads. Error bars represent one standard deviation for triplicates of each treatment.

final amounts of methane produced were not significantly different for either 0.1 g/L ($p = 1$) or 0.5 g/L ($p > 0.608$) amendment concentration. The final methane concentrations for all 0.1 g/L amended coal treatments ranged from 1371 to 1456 µg CH_4/g coal whereas the 0.5 g/L amended coal treatments ranged from 1960 to 2185 µg CH_4/g coal. These results indicate that all four amendments increase methane production similarly in microcosms containing coal. All four 0.1 g/L amended GB treatments produced methane ranging from 111 to 169 µg CH_4/g GB ($p = 1$). However, the 0.5 g/L yeast and YE coal-free GB treatments began producing significantly more ($p < 0.006$) methane by day 60 than the algae and cyanobacteria amended GB treatments. Overall, the 0.5 g/L yeast and YE GB treatments produced 930 and 609 µg CH_4/g GB, respectively, significantly more than the 0.5 g/L algae or cyanobacteria amended GB treatments at 64 and 91 µg CH_4/g GB ($p < 0.001$). Statistically significant groupings for the total methane produced for all treatments can be found in the Supplementary Information (Table S2).

All coal treatments produced more methane than the corresponding GB treatments, suggesting coal was an important carbon source for biogenic methane production. This observation was supported by previous studies in which coal treatments produced more methane than coal-free controls [15,21]. The total methane production on day 111 was similar for coal treatments with equal amendment amount regardless of amendment type. However, while the increase in methane production observed in the 0.5 g/L amended coal treatments was not statistically different between amendments, the variation between the corresponding amended GB treatments suggests that the yeast and YE amendments slightly better promote microbial activity that may be supportive of coal-to-methane conversion. This could be due to a higher bioavailability of yeast and YE in general or due to higher concentrations of macronutrients, such as phosphate (Supplementary Table S1) or micronutrients in yeast or YE amended treatments.

The average rate of methane production was calculated for each treatment for every time-period between samplings (Supplementary Table S3). Maximum production rates are shown in Table 1. The maximum rate for all amended coal treatments was observed between days 35 and 54 at 45.7–50.2 µg CH_4/g coal/day for 0.1 g/L amended treatments and 56.2–64.7 µg CH_4/g coal/day for 0.5 g/L amended

treatments. Statistically significant groupings of the maximum rate similarities were determined (Supplementary Table S2). The rates of methane production in 0.5 g/L amended coal treatments regardless of amendment type were significantly different from all 0.1 g/L amended coal and all GB treatments receiving the same amendment ($p < 0.032$). The 0.5 g/L cyanobacteria, yeast, and YE amended treatments did not have significantly different rates of methane production ($p > 0.413$). The maximum rate of the 0.5 g/L algae amended coal treatments was slightly lower and significantly different from the rates of the other 0.5 g/L amended treatments ($p < 0.032$). The maximum production rates of the coal only treatments were lower at 16.3 µg CH_4/g coal/day and were observed later, between days 68 and 83. The maximum methane production rate for 0.1 g/L amended GB treatments ranged from 2.7 to 5.1 µg CH_4/g GB/day. Algae and cyanobacteria amended GB treatments attained maximum methane production rates during the 68–83 day time period, whereas the yeast and YE amended treatments reached maximum methane production rates during the final 97–111 day time period. Furthermore, the 0.5 g/L amended GB treatments showed statistically significant differences in maximum methane production rates. The 0.5 g/L algae and cyanobacteria amended GB reached maximum rates during the 35–54 day time period and ranged from 1.6 to 2.2 µg CH_4/g GB/day, whereas yeast and YE amended treatments were later at 68–83 and 83–97 days, respectively, and with higher rates, 18.8 and 22.8 µg CH_4/g GB/day. ANOVA analysis confirmed higher rates for all amended coal treatments compared to the corresponding non-coal treatments ($p < 0.0005$) regardless of amendment type or concentration. In addition, the rate achieved between days 35 and 54 for amended coal treatments was significantly higher than the rates during any other period ($p < 0.0005$). The maximum methane production rate achieved by the 0.5 g/L amended coal treatments was statistically greater than the maximum rate achieved by the 0.1 g/L amended coal treatments.

3.2. Amendment concentration effect on coal-to-methane conversion

To evaluate the effect of amendment concentration, two amendment concentrations, 0.1 and 0.5 g/L, and unamended conditions were compared. While an increase in methane production was observed with

increasing amendment concentration for all 4 amendments (Fig. 1), the increase observed in amended coal treatments was not proportional to the amount of amendment added. The average total methane produced by the 0.5 g/L amended coal treatments was 2097 µg CH_4/g coal of which approximately 1421 µg CH_4/g coal was in excess of the production observed in unamended coal treatments. The average total methane produced in 0.1 g/L amended coal treatments was 1412 µg CH_4/g coal with approximately 736 µg CH_4/g coal in excess of the unamended coal treatment. The methane produced in excess of the unamended coal treatment can be attributed to the addition of amendment and includes both the methane due to enhanced coal-to-methane conversion and due to direct amendment-to-methane conversion. While the amendment concentration in the 0.5 g/L amendment treatments was five times greater than in the 0.1 g/L treatments, the increase in total methane production was only two times greater. Thus, it appears that increasing amendment concentration can lead to diminishing returns, and methane enhancement can be achieved while minimizing the amount of amendment needed. From this analysis, it appears that 0.1 g/L is advantageous for this purpose compared to 0.5 g/L.

3.3. Carbon source for increased methane production in amended coal treatments

The observed increase in methane production in amended coal treatments compared to unamended coal treatments could be attributed to the conversion of one or both of the major carbon sources present, coal or amendment. If the increased methane production is due to coal conversion, the effect of the amendment is enhancement. If the increased methane production is only due to direct conversion of the amendment to methane, the amendment's effect is only that of an alternative carbon source (feeding). The enhancement versus feeding effect of the amendments was analyzed in two ways.

First, it was observed for the duration of this study that the methane produced by amended coal treatments exceeded the sum of the methane produced by the corresponding amended GB treatments and the methane produced by the coal only treatments (Eq. (1)). The methane production of the unamended coal treatments was the 111-day methane potential of coal conversion alone while that of the amended GB treatments was the 111-day methane potential of conversion of the amendment alone. Methane produced by the corresponding amended coal treatments in excess of this sum was likely due to coal-to-methane conversion enhancement instead of amendment-to-methane conversion.

$$CH_{4(amended\ coal)} > CH_{4(unamended\ coal)} + CH_{4(amended\ GB)} \tag{1}$$

For example, on day 111, the sum of the methane produced by the unamended coal treatment and the 0.1 g/L algae amended GB was 787 µg CH_4/g coal or GB. The 0.1 g/L algae amended coal treatment produced 1371 µg CH_4/g coal. The 584 µg CH_4/g coal or GB difference is the amount of methane produced in these treatments due to enhancement of coal-to-methane conversion.

The total methane produced for all amended coal treatments, regardless of amendment type or amount, exceeded the sum of unamended coal and corresponding amended GB treatments for all sampling dates in this study. The difference was greatest on day 68 for all treatments. Thus, the results shown here clearly indicate an increase in methane production for amended coal treatments due to enhanced coal-to-methane conversion during the 111-day study.

A second analysis, based on a carbon balance, supports the hypothesis that complex organic amendments can enhance coal-to-methane conversion amounts and rates during the 111-day study. The total amount of carbon added as amendment was calculated for each treatment based on the elemental analysis of the amendments (Table 2). The total carbon moles (Cmol) of methane produced was calculated for each coal treatment using the methane produced for each sampling point

Table 2
Carbon added as amendment calculated from elemental analysis.

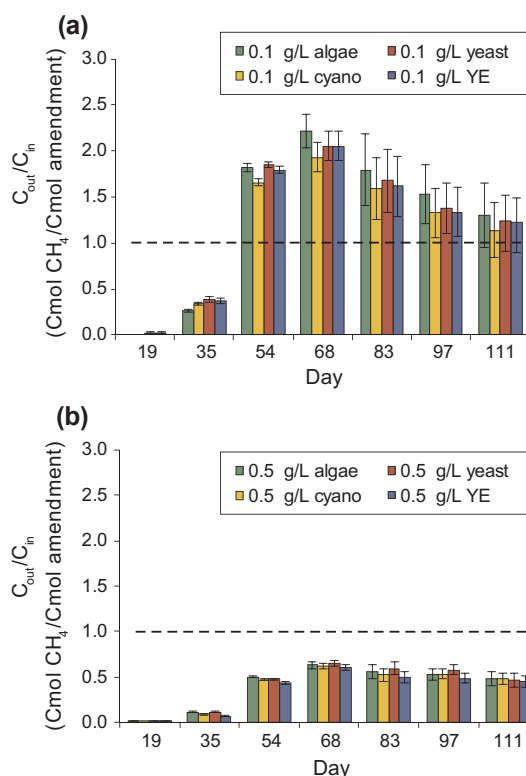| Amendment	Carbon added as Amendment (mg)		
	unamended	0.1 g/L amendment	0.5 g/L amendment
Algae	0	0.40	2.0
Cyanobacteria	0	0.47	2.4
Yeast	0	0.46	2.3
Yeast extract	0	0.48	2.4

Fig. 2. Amount of carbon detected as methane relative to the amount of carbon added as amendment for (a) 0.1 g/L amended systems and (b) 0.5 g/L amended systems. For the 0.1 g/L treatments, starting at 54 days, more carbon had been converted into methane than was added with the amendment, clearly indicating enhanced coal to methane conversion.

during the 111-day study (Fig. 1). Fig. 2 shows the ratio of Cmol of methane produced to Cmol of amendment added, or more simply, C_{out}/C_{in}, for the 0.1 g/L (Fig. 2a) and 0.5 g/L (Fig. 2b) amended coal treatments as calculated by Eq. (2):

$$\frac{C_{out}}{C_{in}} = \frac{Cmol\ CH_{4(amended\ coal)} - Cmol\ CH_{4(unamended\ coal)}}{Cmol_{(amendment)}} \tag{2}$$

The Cmol of methane produced by the unamended coal treatment was subtracted from the Cmol of methane produced by the amended coal treatments at each time point so that C_{out} reflects only the Cmol of methane produced up to that time point due to amendment, either by amendment conversion (feeding) or enhanced coal-to-methane conversion (enhancement).

For the 0.1 g/L coal treatments (Fig. 2a) C_{out}/C_{in} was greater than 1 for all amendments by day 54, demonstrating that the Cmol methane produced exceeded the Cmol added as amendment. Consequently, any produced methane resulting in a ratio greater than 1 is due to coal-to-methane conversion in excess of the unamended coal treatment and not just the conversion of the amendment itself to methane. Thus, it can be concluded that the increase in methane production in the presence of the 0.1 g/L amended coal treatments was due to enhanced coal-to-
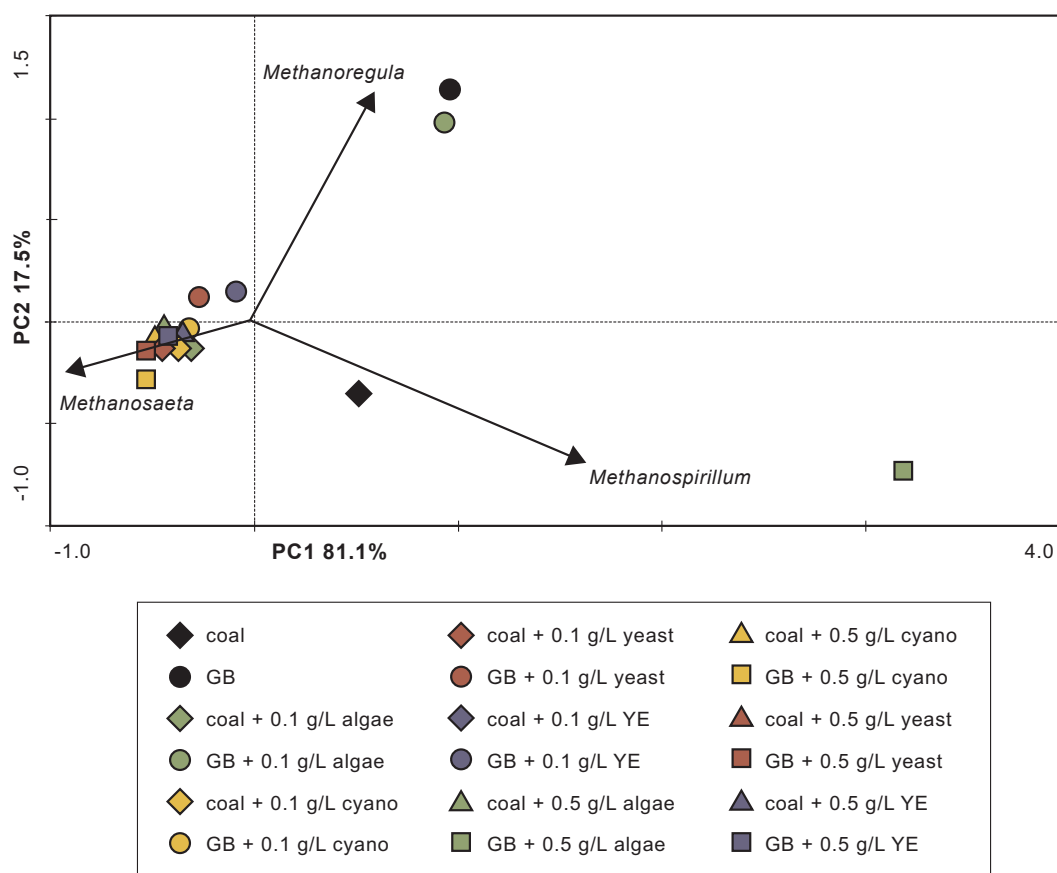
Fig. 3. Principal component analysis of non-transformed relative abundances of archaeal phylotypes (at the genus level) for all treatments on day 111.

methane conversion during the 111-day study. When similar calculations were made for the 0.5 g/L amended coal treatments (Fig. 2b), C_{out}/C_{in} was less than 1 for all 0.5 g/L amended coal treatments for the duration of the study. Therefore, for all time points, the Cmol added as amendment exceeded the Cmol produced in excess of the methane produced by unamended coal treatments for all 0.5 g/L amended coal treatments. Thus, unlike the 0.1 g/L amended coal treatments, it cannot be asserted with certainty that the increased methane production observed in the 0.5 g/L amended coal treatments was due to enhanced coal-to-methane conversion.

These calculations were made assuming all carbon added as amendment would have been converted to methane and none incorporated into biomass or converted to other carbon containing compounds such as CO_2. Thus, it is certain that any produced methane resulting in a C_{out}/C_{in} ratio greater than 1 was due to an enhancement of coal-to-methane conversion for that time point and not just due to the conversion of the amendment to methane. This difference between the 0.1 and 0.5 g/L amended treatments provides additional support that the lower amendment concentration may be advantageous for coal-to-methane conversion. While the production of CO_2 was not analyzed in these studies, future studies utilizing this type of carbon mass balance approach would benefit from the addition of CO_2 analysis resulting in a more accurate estimate of coal-to-methane conversion and reduce the underestimation effect of the assumption that all amendment carbon was converted to methane.

C_{out}/C_{in} was similar for all amendments of the same concentration at all time points and peaked at day 68 before declining due to the continued increase in methane production by the unamended coal treatment while the methane production of the amended coal treatments leveled out. It is clear from both of these described analyses that the amendments affect coal-to-methane conversion and certainly increase the rate at which coal-to-methane conversion occurs within the

duration of the 111-day study. However, it is not clear from this study what the mechanism is and whether the total amount of methane produced with amendment addition would be increased on a longer term. The greatest methane enhancement effect of the amendments during the 111-day study occurred by day 68, and a decline was observed for later time points for all amendments and concentrations with both analyses due to the continued methane production of the unamended treatments while the amended coal methane production leveled off. To acquire preliminary data to assess this effect, one of the unamended coal treatment replicates was observed until day 322 (data not shown). The methane production continued to increase until day 229 when it leveled out and was measured at 1330 μg CH_4/g coal, only slightly lower than the average 111-day methane production for the 0.1 g/L amended coal treatments of 1412 μg CH_4/g coal. While this was only one sample of the unamended coal treatment, this preliminary data suggests that both the 0.1 g/L amended coal treatments and the unamended coal treatments might produce similar amounts of methane in batch reactors, given sufficient time. Thus, the effect of the amendment is a definite increase in the coal-to-methane conversion rate but might not result in an increase in the long-term total methane production. While it will require further investigation to test this hypothesis, an increase in the rate of coal-to-methane conversion supports the use of these amendments as a potential strategy to extract more CBM in a shorter timeframe.

3.4. Microbial ecology differences due to the presence of coal or amendment

Principal component analysis (PCA) was used to identify differences in microbial community composition between the 18 treatments after 111 days. The bacterial and archaeal populations were examined separately.

3.4.1. Archaeal population diversity

The PCA of the archaeal communities showed little difference among the majority of the treatments except for unamended coal and GB treatments as well as algae amended GB treatments. Most treatments clustered together along the negative PC1 axis and correlated with a high relative abundance (> 0.75) of the genus *Methanosaeta* known for a preponderance of acetoclastic methanogens [30,31] (Fig. 3). A lower relative abundance of *Methanosaeta* was observed for the unamended coal (0.73), unamended GB (0.46), 0.1 g/L algae amended GB (0.51), and 0.5 g/L algae amended GB (0.17) treatments. The unamended coal treatments were still dominated by *Methanosaeta* but also had a higher relative abundance of the genus *Methanospirillum* (0.22), known for hydrogenotrophic members [32,33]. The unamended GB and the 0.1 g/L algae GB treatments were similar: lower relative abundances of *Methanosaeta* than the treatments clustered along the negative PC1 axis and higher relative abundances (0.3) of *Methanoregula* sequences, associated with hydrogenotrophic methanogenesis pathways [34,35]. The 0.5 g/L algae amended GB treatment was dominated by *Methanospirillum* (relative abundance 0.74). Overall, the PCA analysis indicates that the archaeal communities in the majority of the treatments were dominated by the genus *Methanosaeta* suggesting a high occurrence of acetate-to-methane conversion and that the amendment type did not significantly impact the archaeal community. The outlying treatments (unamended coal and unamended GB, 0.1 and 0.5 g/L algae GB), however, showed greater relative abundances of the genera *Methanoregula* and *Methanospirillum*, both known to contain hydrogenotrophic members.

3.4.2. Bacterial population diversity

The bacterial communities for all treatments were dominated by three major phyla: Firmicutes, Proteobacteria, and Bacteroidetes. These three phyla combined to a relative abundance of 0.76–0.94 for all treatments. Within these phyla, Clostridia and Bacilli were the dominant classes within the Firmicutes, and δ-Proteobacteria and β-Proteobacteria were the dominant classes within the Proteobacteria. All of these classes have been observed previously in coal environments and contain members that are obligate anaerobic or facultative organisms and associated with syntrophic complex carbon degradation [36,37,17]. While all treatments showed similar bacterial composition when compared at a phylum level, greater differences between treatments were observed when analyzed at a genus level.

The bacterial phylotypes were first examined using a PCA of all 18 treatments, resulting in no discernable trends. However, when the bacterial phylotypes were examined as two separate groups: unamended plus 0.1 g/L amended treatments (Fig. 4a) and 0.5 g/L amended treatments (Fig. 4b), trends were observed for the two separate groups. As shown in Fig. 4a, the unamended and 0.1 g/L amended treatments separated along the first principal component axis into coal (right) and GB (left) treatment groups. However, when examining the PCA for the 0.5 g/L amended treatments (Fig. 4b), a similar coal and GB separation was not clearly observed. For these 0.5 g/L amended treatments, the grouping pattern indicated an influence of amendment type instead of presence of coal with treatments loosely paired by amendment type. This observed difference in bacterial community relationships with increased amendment concentration suggests that the amendment concentration can cause shifts in the bacterial community structure and that higher amendment concentrations may have a greater influence than the presence or absence of coal. The shift could be associated with a decrease of the coal-to-methane conversion and an increase in the direct amendment to methane conversion. This is supported by the methane data indicating that higher concentrations of amendment may shift the carbon source for methane production from coal to the amendment itself.

4. Discussion

Yeast extract (YE), commonly added to undefined culture media as a complex nutrient source, has been investigated previously for its potential to enhance coal-to-methane conversion [14,38,36]. While some increase in methane production was observed in these studies, YE has costs associated with production and transport for *in situ* applications. This study investigated alternative complex nutrient sources and compared commercially available YE to three alternative amendments. Because all four amendments resulted in similar increases in methane production, algae, cyanobacteria, and yeast could be viable alternatives to YE for large-scale enhancement of microbial coal-to-methane conversion. Cost is always a large consideration, and each of these amendments would have varying costs associated with production, transport, and injection.

While CBM has promise as a domestic alternative to traditional coal energy, it is not without potential challenges. Because the rates of conventional CBM collection methods exceed natural rates of biogenic CBM formation, wells drilled in the PRB for CBM collection typically only have a 7–10 year lifespan [10]. During their active life, each well will produce up to 17,000 gallons of water per day, and this water must be treated before discharge or use for irrigation, resulting in thousands of holding ponds of low quality water in the PRB [39]. Barnhart et al. suggested the use of algal amendment for CBM enhancement for large-scale application utilizing production water ponds to grow algae that could be used for microbial CBM enhancement while also providing valuable byproducts, such as lipids for biofuels [21]. In addition, a microalgal strain isolated from a PRB CBM production water pond has been grown in formation water with limited nutrient addition in a laboratory setting. This alga can accumulate lipids for potential biofuel production when grown in CBM formation water [40]. Many species of cyanobacteria, including the species used in this study, can fix atmospheric nitrogen, potentially further reducing necessary nutrient inputs and thus costs. Phototrophic amendments grown in CBM production water holding ponds would make use of ponds, a by-product of CBM production, and reduce transport costs by producing the amendment on or very near the site of application while utilizing sunlight and CO_2 for growth.

Biogenic methane production was observed with all four amendments in the presence and absence of coal. The results presented here show that for a 0.1 g/L amendment concentration, the additional Cmol methane produced is greater than the Cmol added as amendment, clearly demonstrating that the amount of methane produced during the 111-day study duration exceeded the carbon input from the amendment. Thus, enhanced methane production with 0.1 g/L amendment addition is likely due to increased coal-to-methane conversion. However, while the 0.5 g/L amended coal treatments also produced more methane than the unamended coal treatments, the additional Cmol methane produced did not exceed the Cmol added through the amendment. Thus, the 0.1 g/L amendment concentration is more efficient for enhanced coal-to-methane conversion when considering the input-to-output ratio and potential costs.

While it is clear from these results that amended coal treatments produced more total methane at a higher rate than unamended coal treatments, no conclusions can be drawn regarding the longer-term effects of amendment addition on coal-to-methane conversion. It is clear that amendment addition increases the rate at which coal is converted to methane, but it appears from preliminary data that, given enough time, total methane produced from coal may be similar for both amended and unamended treatments. However, regardless of the total long-term methane production of unamended treatments, the increase in the rate of methane production with amendment addition suggests that biogenic CBM could be enhanced in the subsurface to result in the faster generation and retrieval of gases produced from coal conversion.

Bacterial community differences appeared to be influenced by the presence or absence of coal for unamended and 0.1 g/L amended
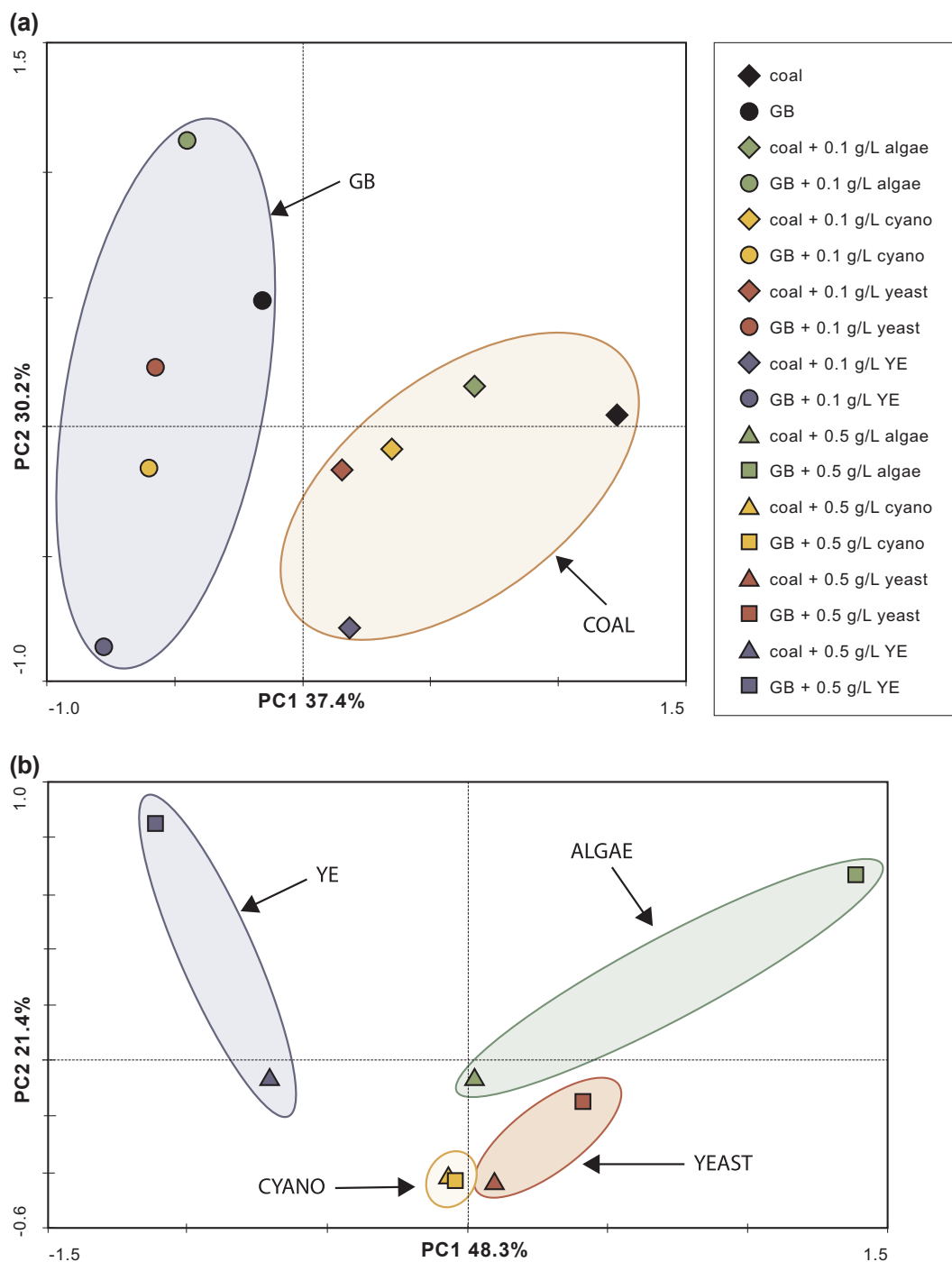
Fig. 4. Principal component analysis of non-transformed relative abundances of bacterial phylotypes (at the genus level) for (a) unamended and 0.1 g/L amended treatments and (b) 0.5 g/L amended treatments on day 111.

treatments, whereas the observed bacterial community differences for the 0.5 g/L amendment treatments appeared to be driven more by amendment type. These results coincide with the observed carbon input-to-output ratios for the 0.5 g/L treatments and indicate that too much amendment can affect the microbial community and shift the activity away from a coal-dependent system. All these observations together suggest that the lower amendment concentration provides a better return on investment and maintains coal-dependent conversions. The optimal type and amount of amendment for the most effective enhancement of coal-to methane conversion should be further investigated especially in the context of cost optimization and reducing microbial community shifts away from productive populations.

5. Conclusions

The research presented here shows (1) the importance of coal as a substrate in biogenic CBM production, as demonstrated in previous studies; (2) that algae, cyanobacteria, yeast and yeast extract amendments similarly increase rates of biogenic coal-to-methane conversion; (3) that increasing amendment concentrations increase the amount of methane formed but not proportionally to the amendment amount added; and (4) that microbial community composition appears to be dependent on the presence or absence of coal at low amendment concentrations but more dependent on amendment type at higher amendment concentrations. The results presented here also provide

evidence for the utility of photosynthetically produced biomass (algae and cyanobacteria) for increasing MeCBM production and lay a foundation for further investigation and scale-up of the microbially-enhanced coalbed methane production technology in the field.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fuel.2017.09.074.

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