ORGANIC AMENDMENTS FOR ENHANCING MICROBIAL COALBED METHANE PRODUCTION

by

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DEDICATION

This dissertation is dedicated to Dr. Warren Jones who always asked tough questions, inspired creative thinking, loved teaching Environmental Engineering, and was the most influential in starting me on the path leading to this work.

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ABSTRACT

Coalbed methane (CBM) is natural gas found in subsurface coal beds and supplies approximately 4-6% of the annual U.S. natural gas requirements. Many unmineable coal beds contain CBM produced by native microbial communities. Enhancing the microbial processes for coal-to-methane conversion can increase the rates of CBM production and the amount of extractable natural gas in these coal beds. Strategies for enhancing microbially-produced CBM must be logistically attainable and economically practical. The goal of this dissertation work was to determine a feasible methane enhancement strategy using organic amendments to increase microbial coal-to-methane conversion.

Four organic amendments were tested in coal-containing batch microcosms. Increased coal-to-methane conversion was demonstrated with small amounts of amendment addition, and all four tested amendments increased methane production similarly. Subsequent amendment addition produced smaller amounts of additional methane which appeared to be primarily due to amendment-to-methane conversion. ^{13}C-labeled algal and yeast amendments were used in coal systems for tracking carbon for methane production. It was shown that <22% of the amendment carbon was converted to methane. By tracking amendment carbon, it became clear that carbon sources besides coal and amendment are utilized for methane production; these carbon sources potentially include organic and inorganic carbon in the formation water and inoculum.

Amendment strategies tested in batch systems were scaled up and applied to column reactors. Methane production from coal increased with small amounts of ^{13}C-labeled algal amendment addition. However, unlike in batch experiments, methane production rates in the column flow reactors did not slow or cease after 60-90 days, and methane was still being produced after 176 days when the study was terminated.

The work presented here demonstrates that organic amendment addition is a viable methane enhancement strategy and all tested amendments were similarly effective. Algae can potentially be grown in CBM production water ponds near the amendment site and may thus offset costs associated with a CBM enhancement strategy based on the potential for producing value-added algal byproducts. These studies solidify the foundation for further studies targeting the scale-up of microbially enhanced CBM production in the field.

CHAPTER ONE

INTRODUCTION

Background

The research presented in this dissertation describes the potential of adding organic nutrient sources to coal environments to increase biogenic methane production from coal. Nutrient sources, such as microalgal biomass, provide a wide-range of nutrients for microbially-enhanced coalbed methane (MeCBM) production and a simpler "recipe" than synthetic nutrient mixtures. In addition, microalgal amendment has the potential to offset costs associated with amendment addition in the field because it can be grown in production water ponds near or onsite and can provide valuable byproducts for economic benefit. This dissertation investigates the applications of organic amendment additions with a focus on microalgal amendments to make advances towards scale-up and potential field applications.

This research has been sponsored by a U.S. Department of Energy (DOE) program, DE-FE0024068, "Increasing the rate and extent of microbial coal to methane conversion through optimization of microbial activity, thermodynamics, and reactive transport" and a Montana Research and Economic Development Initiative Contract # 51040-MUSRI2015-05. This research was conducted at the Center for Biofilm Engineering (CBE) at Montana State University (MSU) (Montana, USA). The research described here has been conducted concurrently and in coordination with other research efforts at the CBE focusing on microbial communities and processes involved in coal to

methane conversion and scale-up and field application design of MeCBM strategies. These efforts were supported by the National Science Foundation, #1322795, "Hydrodynamic controls on microbial community dynamics and carbon cycling in coal beds" and the U.S. Department of Energy, DE-FE0026155, "Optimization, Scale-up, and Design of Coal-Dependent Methanogenesis in Preparation for In-Situ Field Demonstration. Field site access and samples were provided by the USGS Energy Resource Program

Dissertation Overview

This dissertation provides a literature review of biogenic CBM research in relation to scale-up of laboratory MeCBM strategies for field applications in Chapter 2. Chapter 3 demonstrates the efficacy of four organic amendments for increasing the amounts and rates of biogenic methane production in coal systems. Chapter 4 discusses the potential for prolonging enhanced methane production by sequential re-amendment. Chapter 5 demonstrates a strategy for labeling amendments used to increase methane production from coal to better quantify the effect of an organic amendment on coal-to-methane conversion. Chapter 6 describes a column reactor system designed for anoxic studies and a scale-up study of enhanced coal-to-methane strategies presented in the previous Chapters. Chapter 7 outlines the conclusions of this work and describes recommendations for future research.

Chapter 2 is a review titled "Parameters to transfer *ex situ* studies of biogenic coal-to-methane to *in situ* coal bed applications: a review" and outlines some of the

potential issues with scale-up of laboratory CBM studies and parameters to consider for experimental design for field applications. The research presented in this dissertation seeks to address some of these issues to provide a more field-relevant base as pilot-scale studies are planned and implemented. This chapter is currently in preparation for submission to the peer-reviewed journal *International Journal of Coal Geology*.

In Chapter 3, four organic amendments at two concentrations were assessed for their potential to increase biogenic methane production in microcosms with and without coal. Increased amounts and rates of methane production were demonstrated for both concentrations of all amendments. Microbial communities were assessed, and shifts in microbial communities were observed with higher amendment concentrations. The results suggest that organic amendments are effective at enhancing methane production from coal but increasing amendment concentrations result in a decreased cost-to-benefit ratio and potentially detrimental microbial shifts. Chapter 3 was submitted for publication in the journal *Fuel* and is currently in review as "Type and amount of organic amendments affect enhanced biogenic methane production from coal and microbial community structure." The conclusions of Chapter 3 are utilized throughout the remainder of this dissertation work by supporting the use of algal amendments and showing that lower amendment concentrations are sufficient, if not ideal, for coal-to-methane conversion enhancement.

Chapter 4 details an investigation of the potential of increasing the methane production further through sequential amendment additions. Methane and inorganic carbon production were tracked and stoichiometric ratios of coal and amendment

conversion were determined. Sorption of methane and CO_2 to coal was discussed and recognized to be an important consideration in analyzing carbon conversion in coal systems. Chapter 4 has been prepared for submission to *Energy and Fuels* as a manuscript titled "Carbon conversion during enhanced microbial methane production from coal with repeated organic amendment."

Chapter 5 describes the use of ^{13}C-labeled amendments for quantifying methane production from coal and from amendments in coal systems. While carbon mass balances in Chapter 3 clearly showed coal-to-methane conversion in amended coal systems, the assumption that the amendment was fully converted to methane results in an underestimation of coal contributions. The isotopic labeling methods detailed in Chapter 5 allow the carbon source for methane production (coal or amendment) to be identified and result in more accurate estimates of coal conversion. These methods provide a useful tool for pilot-scale or field-scale demonstrations facilitating the transition to future commercial microbially-enhanced CBM endeavors. This chapter is titled "^{13}C-labeled amendments for enhanced biogenic methane production in coal systems indicate increased coal-to-methane conversion" and is currently in preparation for submission to *Nature*.

Chapter 6 details the development of a column reactor system for scale-up of laboratory batch studies of anoxic microbial processes under continuous flow conditions. The reactor system is designed to run at near atmospheric pressures and allows for capture of produced gases for analysis and quantification. Up to four reactor columns can be utilized from a single influent source and can be run as replicates or serve as separate

treatments to evaluate the effect of amendment addition at the reactor inlet. A scale-up experiment demonstrating the application of methods developed in Chapters 3, 4, and 5 is described and methane production demonstrated. This chapter has been prepared for submission to *International Journal of Coal Geology* and is titled "Development and pilot testing of column reactors for the study of anaerobic subsurface processes."

The appendices include other work completed during this PhD that did not effectively match the main themes but provided background or support for the main chapters of this dissertation. The abstract for a co-authored paper which introduced the use of organic amendments for enhancing CBM production and provided a foundation for the work in this dissertation is included as Appendix A. Investigations of coal surface area and buffering capacity are presented in Appendix B and C. The full amendment elemental analysis for amendments used in Chapters 3 and 4 are presented in Appendix D. The re-amendment of the microcosms presented in Chapter 3 provided preliminary data for the studies in Chapter 4, and results of re-amendment and an analysis of microbial community shifts are presented in Appendix E. Appendix F includes detailed descriptions of the deconvolution method used to analyze the GC-MS data for ^{13}C analysis used in Chapters 5 and 6. Data for the unlabeled treatments from Chapter 5 are presented in Appendix G. Preliminary column studies conducted during the iterative design process for the reactor systems used in Chapter 6 utilized a rapid-growing methanogen, *Methanosarcina acetivorans*, and are presented in Appendix H. A thermodynamic assessment of methanogenic pathways was performed and the results are presented in Appendix I.

CHAPTER TWO

TRANSITION OF BIOGENIC COAL-TO-METHANE CONVERSION FROM THE LABORATORY TO THE FIELD: A REVIEW OF IMPORTANT PARAMETERS AND STUDIES

Contribution of Authors and Co-Authors

Manuscript in Chapter 2

Author: Katherine J. Davis

Contributions: Developed topics, graphics, and table design. Wrote and revised manuscript.

Co-Author: Robin Gerlach

Contributions: Contributed to the writing, development and revisions of the manuscript with comments and feedback.

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Abstract

Coalbed methane (CBM) is an important unconventional natural gas resource in the U.S. and around the world. Many of the CBM containing coal formations contain microbial communities producing the gas by converting coal to methane. CBM produced biogenically provides an opportunity for developing technologies to enhance the microbial processes to increase the retrievable gas. In order to transfer strategies for biogenic CBM enhancement from small-scale laboratory studies to large-scale commercial applications in subsurface coal beds, there are several factors that should be considered to facilitate this transfer. Coal rank, structure, and chemistry, formation water chemistry, and microbial communities can vary widely between coal formations, and matching these components in laboratory studies to the coal bed of interest should be considered. More work needs to be performed to understand the effects of gas sorption, pressure, and water movement through coal formations on biogenic gas production. Additionally, methods for applying methane enhancement strategies *in situ* must be further investigated to develop commercial applications of enhanced microbial coalbed methane production.

Introduction

Most subsurface coal beds contain at least 2 types of fossil fuel energy: coal and natural gas. The natural gas, also known as coalbed methane (CBM), is an unconventional gas resource and has been extracted commercially in the United States since the late 1980s.[1] Due to the potential of microbial coal-to-methane conversion, it has

been suggested that technologies can be developed to enhance biological reactions producing CBM to increase the amount of natural gas available for extraction from coal beds.[2–4]

To advance the potential of microbial CBM enhancement *in situ*, laboratory experiments and field investigations have been published by researchers in many regions of the world. These studies have focused on the microbial communities responsible for the coal-to-methane conversion, coal bed hydrology and geochemistry, and methane enhancement strategies. While the general body of knowledge regarding microbial CBM production and potential enhancement strategies has greatly increased over the last 15 years, gaps still exist, inhibiting the transfer of bench-scale research to field-scale demonstrations and commercial *in situ* applications.

This review seeks to identify and discuss the parameters to be considered when transferring strategies for CBM enhancement developed in the laboratory to prospective *in situ* conditions. Gaps in the understanding of these parameters will be addressed, and suggestions will be made for the next steps necessary to apply enhancement strategies in the field.

Overview of Naturally Occurring Coalbed Methane

Coalbed Methane Extraction Techniques

Coalbed methane (CBM) is natural gas found in subsurface coal beds. Due to geological characteristics and recovery methods required for collection, CBM is categorized as an unconventional gas.[5] Conventional natural gas resources generally

require wells exceeding 5000 feet deep for extraction.[6] Most methane-producing coal beds are less than 2000 feet deep and many only a few hundred feet. This results in a much lower cost for drilling extraction wells for CBM compared to wells for conventional natural gas extraction [4]. While most of the CBM in subsurface coal beds is sorbed to the coal itself, the gas in conventional gas formations is typically found in the free state within the pores of the formation.[7]

It is generally accepted that CBM exists in three states in coal beds: (i) free state where CH_4 molecules exist as gas or dissolved within cleats (fractures) or macropores of the coal structure, (ii) adsorbed state where methane sorbs to the coal surface within coal micropores by physical (physisorption) or chemical (chemisorption) interactions, and (iii) an absorbed state where methane is held within the chemical structure of the coal itself.[7,8] It is believed that the majority of subsurface CBM is adsorbed, and the amount that can be adsorbed is dependent on the surface area of the pores rather than the pore volume.[9]

For commercial CBM extraction, wells are drilled into the coal beds of interest, and casings are screened in the methane-producing regions. To release the CBM adsorbed to the coal, water is pumped from the wells to reduce the hydrostatic pressure and allow the adsorbed gas to desorb.[10,11] While CBM wells are typically less expensive to drill than wells in conventional natural gas formations, most conventional natural gas wells produce more gas per well lifetime than CBM wells.[4]

Both conventional natural gas and CBM extraction generate large volumes of production water. However, due to the differences in how the gases are held in the formations, conventional gas initially produces low volumes of water and water volumes

increase as gas production decreases. In contrast, CBM extraction initially produces high volumes of water to release the adsorbed gas from the coal, and pumped water volumes decrease as gas production increases when the coal bed is dewatered (Figure 2.1).[7]

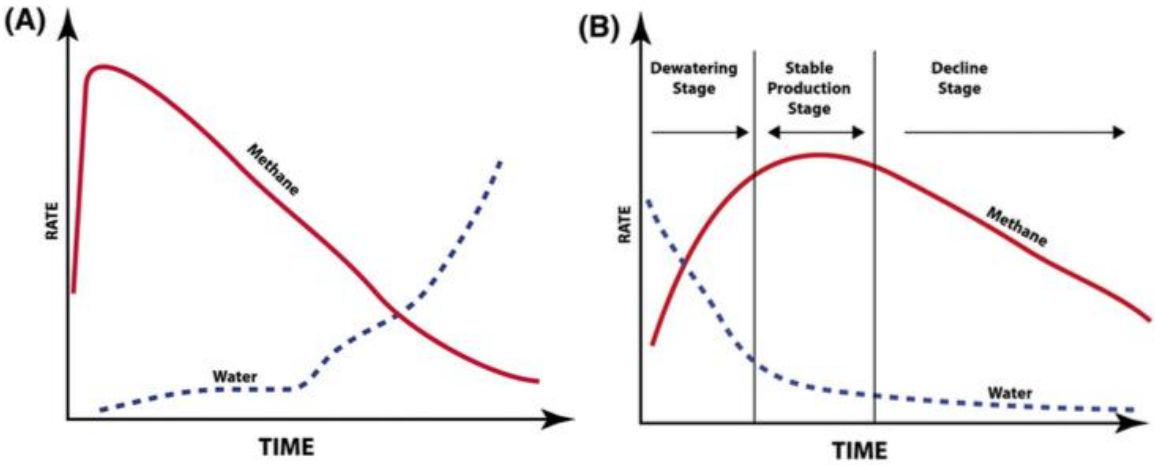

Figure 2.1: Schematic of water and gas production from (A) a typical conventional gas well in a clastic reservoir and (B) a typical CBM production profile showing the different phases of production. (Source: Moore, 2012)

The extraction of CBM produces large volumes of low quality water. The typical CBM well in the Powder River Basin (PRB) of Wyoming and Montana (U.S.A.) produces an average of approximately 17,000 gallons per day (64,300 L/day).[11] CBM production waters commonly have total dissolved solids (TDS) exceeding the 500 mg/L limit recommended for drinking water[12] and 1,000-2,000 mg/L maximum recommended for stock ponds or irrigation.[11] The main dissolved ions contributing to the elevated TDS in most CBM formations are sodium, bicarbonate, and chloride. While most CBM production waters are of better quality than water produced from conventional oil and gas wells, it is still often necessary to treat the production water prior to discharge or determine long-term storage solutions.[11]

In addition to the large quantities of water produced during CBM extraction, CBM wells typically have a short lifespan of only 7-10 years resulting in a need for new well development on shorter time intervals than with conventional gas wells.[10] Despite these issues with CBM extraction, there are also benefits to using CBM for electricity generation instead of the coal itself.

Natural gas is considered a cleaner energy source than coal, producing negligible amounts of mercury and sulfur compounds and approximately half the CO_2 per unit of energy generated. In addition to higher CO_2 emissions, combustion of coal results in 450% more NO_x, 500% more CO, and 400% more particulate emissions compared to the combustion of natural gas.[13,14] These advantages of natural gas for electricity generation relative to coal suggest similar advantages for CBM. CBM extraction instead of coal mining in coal beds producing gas would allow for the utilization of a potential energy source while reducing many harmful emissions and the environmental impact caused by mining coal.

Table 2.1: Summary of U.S. estimated coal reserves in 2015 (in million short tons).[14]

	Demonstrated Reserve Base	**Estimated Recoverable Reserves**	**Recoverable Reserves at Producing Mines**
Underground – Mineable Coal	327,791	146,785	7,120
Surface – Mineable Coal	149,287	108,111	11,207
Total	477,078	254,896	18,327

Coal reserves contain approximately an order of magnitude more energy than natural gas reserves in the U.S. (Table 2.1).[13,14] However, only ~4% of the demonstrated coal reserve base is estimated to be recoverable with current infrastructure and active

mines. For underground coal reserves, the amount of recoverable coal is only ~2% of the reserve base. The vast unmineable underground coal reserves are likely a source of CBM and could represent an ideal environment for methane enhancement technologies.

Origins of CBM

CBM is formed in two ways. (i) Thermogenic methane is formed through abiotic processes requiring heat, pressure, and geological time scales; it is formed during the coal aging process and results from the thermally-induced conversion of larger coal molecules.[7,15] (ii) The second pathway for CBM formation is the conversion of coal to methane by microbial processes and is continually occurring in many coal formations.[3,4] The multi-step processes of biogenic coal-to-methane conversion involves diverse microbial consortia containing both bacterial and archaeal members. It is generally accepted that bacteria sequentially break down the complex carbon in coal to intermediate and simple byproducts.[2,16,17] Some of the simplest byproducts of the bacterial biodegradation of coal are the substrates required by methanogenic archaea to produce methane gas (Figure 2.2).

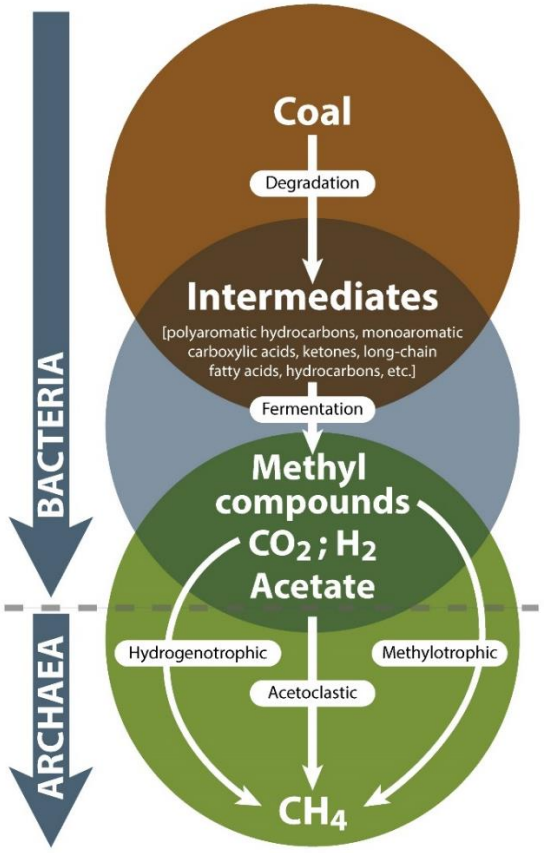

Figure 2.2: Schematic of the sequential microbial degradation of coal to produce coalbed methane.[1,7,17]

The three primary pathways for archaeal methane production are hydrogenotrophic (Eq.1), acetoclastic (Eq. 2), and methylotrophic (Eq. 3) reactions. The H_2, acetate, and methanol (or methyl-group containing molecules) are the byproducts of the bacterial degradation of coal and the substrates for methanogenic archaea.[1–3]

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad \text{(Eq.1)}$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad \text{(Eq. 2)}$$

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \quad \text{(Eq. 3)}$$

The gases found in coal beds can be purely thermogenic, biogenic, or of mixed origin, and stable isotope analysis of produced gases can be used to determine gas origins. Plots of δ^{13}C-CH_4 versus δ^{2}H-CH_4 for CBM, as shown in Figure 2.3, can suggest the origins of produced gases and differentiate between the dominant biogenic pathways.[18]

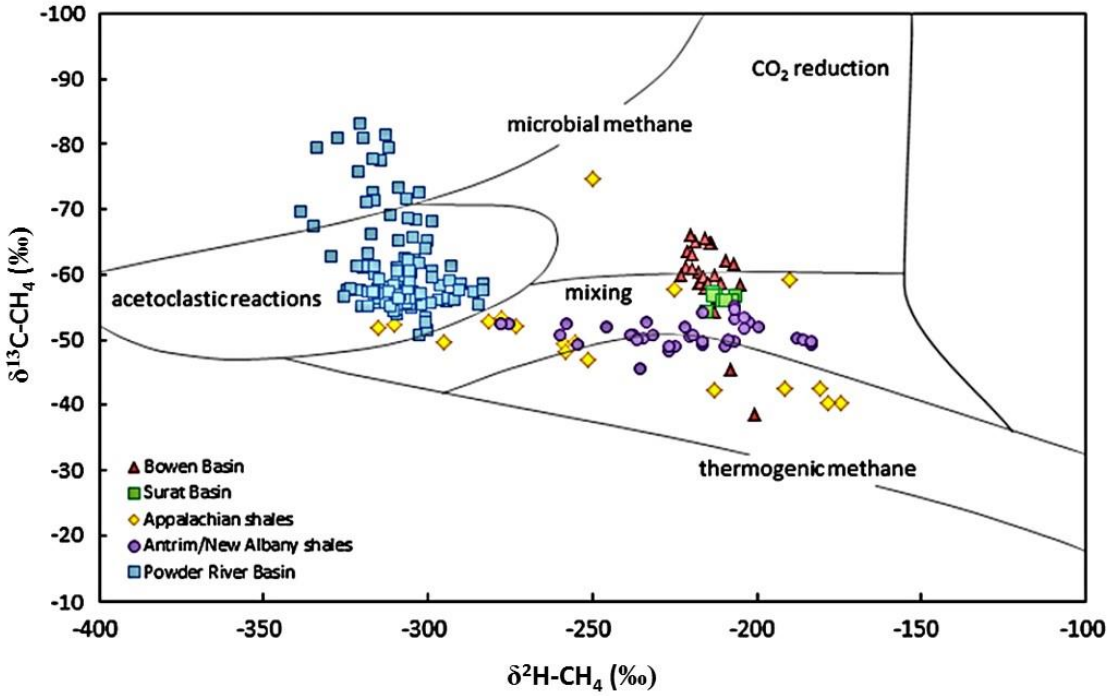

Figure 2.3: Methane δ^{13}C versus δ^{2}H for several CBM and shale gas formations. The relationship between the two indicate varying origin modes for different basins (Source: Golding et al., 2013).

Stable isotope analyses have been published for several CBM producing formations around the world. The Bowen and Surat Basins of Australia have isotopic signatures indicating that CBM is likely of mixed origins.[19,20] The Forest City and Powder River Basins of the U.S were found to have signatures indicative of almost exclusively biogenic CBM formation.[21,22] Much of the methane found in Illinois Basin

coal and shale formations have a thermogenic or mixed origin isotopic signature. Several studies have shown that CBM gases produced along the eastern margins of the basin are primarily of biogenic origin and are associated with meteoric groundwater recharge associated with glacial melting.[23–26] An Indonesian coal bed in the South Sumatra Basin has an isotopic signature suggesting mixed origin gas. Microbial community analysis from this coal formation are indicative of the communities found in known biogenic CBM producing formations, and laboratory microcosms using formation water from this Indonesian coal bed produced biogenic methane.[27]

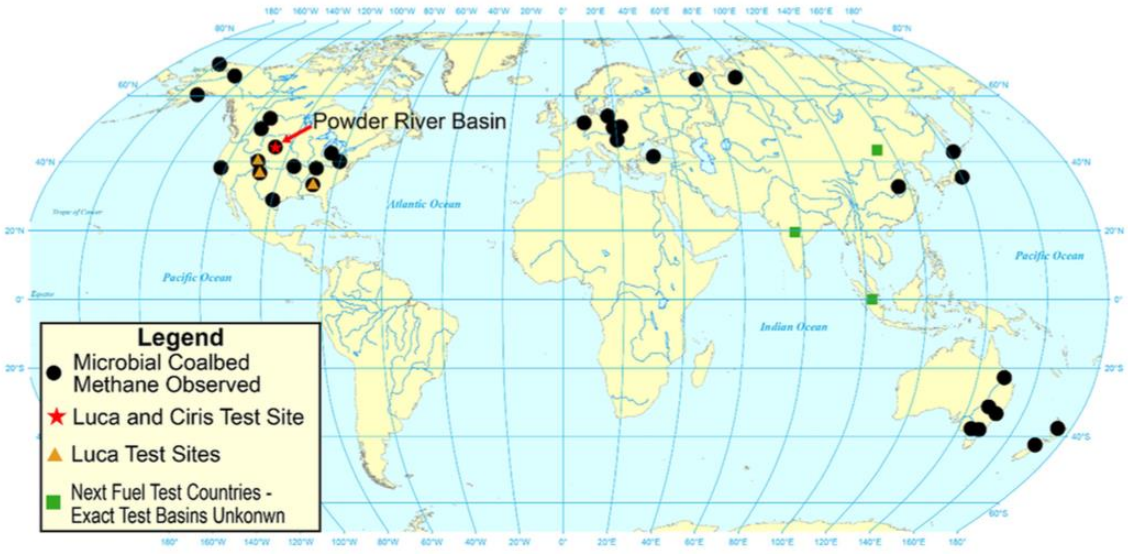

Figure 2.4: Map showing locations of coal beds with microbially produced CBM and locations where pilot tests have been performed to attempt enhancement of the coal-to-methane conversion. (Source: Ritter et al., 2015)

CBM has been found in many locations around the world. While some CBM formations contain primarily thermogenic gases, many coal beds contain mixed origin methane, and the CBM of the Powder River Basin is thought to be solely of biogenic origin (Figure 2.4).[4] While CBM can be extracted from coal beds regardless of gas origin,

biogenic production of CBM provides the potential to enhance the microbial communities to increase the rates of coal-to-methane conversion, increase the extractable methane, and thus extend the life of in-place wells.

Microbially Enhanced Coalbed Methane (MeCBM)

Three recently published reviews summarized the most studied methods for enhancing microbial CBM.[2–4] Laboratory studies to enhance microbial CBM fall into three categories: bioaugmentation, coal treatment to increase bioavailability, and biostimulation.

Bioaugmentation is the addition of a coal degrading, methanogenic microbial consortium to the coal environment and has been implemented in laboratory studies where biogenic coal-to-methane was studied with non-coal sourced microbial consortia.[28–32] While this strategy would allow the possibility of enhancing biogenic CBM in coal beds without current biogenic production, obtaining permits from regulatory agencies is likely difficult.[3,4]

Coal treatment to increase its bioavailability for bacterial degradation is a second proposed strategy for enhancing biogenic CBM production. Hydraulic fracturing is a commonly used method for increasing surface area and releasing gases during shale gas extraction.[2] Similar techniques could be applied in coal beds to release CBM and increase the surface area accessible for microbial degradation. However, fluids used for hydraulic fracturing could be detrimental to CBM-producing microbes or contaminate nearby drinking water aquifers. Strategies for pre-treating the coal have been studied in the laboratory; treatments include the use of strong oxidants such as hydrogen peroxide or

solvents to increase coal bioavailability, but these may also be harmful to the microbial populations or contaminate drinking water aquifers.

Biosurfactants and chemical surfactants can reduce surface and interfacial tensions between coal molecules to increase solubility.[2] Biosurfactants produced by a strain of *Pseudomonas stutzeri*, isolated from an Indian coal bed, increased coal solubility in two ways: 1) improving contact between hydrophilic enzymes and hydrophobic coal surfaces and 2) increasing the solubility of coal humates by binding metals involved in ionic linkages.[33] Increased biogenic methane production from coal was demonstrated for Surat Basin coals with the addition of the chemical surfactant Zonyl FSN.[34]

The most extensively investigated CBM enhancement strategy, however, is biostimulation via nutrient addition. To apply biostimulation methods *in situ* or *ex situ*, a viable microbial consortium capable of coal-degradation and methanogenesis must be present. Some biostimulation studies have added methanogenic substrates (e.g. formate, acetate, H_2) as nutrient amendments.[35,36] These amendments were shown to increase methane production, but it is likely these additions only provided methanogenic substrates and did not significantly increase the desired coal-to-methane conversion. Therefore, when choosing nutrients for addition, the target for stimulation should likely be the coal-degrading members of the community instead of the methanogens themselves.

A few companies have made commercial attempts to enhance biogenic CBM production. Luca Technologies, Inc. applied its nutrient addition strategies at U.S. sites in the PRB (WY), Uinta Basin (UT), San Juan Basin (NM), and Black Warrior Basin (Ala.).

The nutrient mixture includes synthetic vitamins and minerals, complex nutrients like yeast extract and soy proteins, glycerol, and weak organic acids. Next Fuel, Inc. utilized nutrient additions containing synthetic, non-carbonaceous nutrients, trace metals, and vitamins at several sites in China, India and Indonesia. Cirus Energy attempted *in situ* biostimulation in the coal beds of the PRB using synthetic nutrients and yeast extract. [4]

While biostimulation strategies have been tested extensively in the laboratory and some commercial applications have been applied *in situ*, transferring these methods to large-scale field applications faces several challenges.

Increasing Field-Relevance of Laboratory Biogenic CBM Enhancement Strategies

Before applying laboratory-investigated microbially enhanced coalbed methane (MeCBM) strategies *in situ*, several aspects should be considered. Bench-scale studies are greatly simplified representations of the subsurface coal environment, providing greater control of conditions, thus allowing for a better understanding of each step of coal-to-methane conversion. However, to scale-up methane production enhancement strategies developed in the laboratory for application in the field, it is important to consider the design of *ex situ* experiments and how to account for differences between the laboratory and environmental conditions.

The subsurface coal environment can vary between coal beds in several ways. Formation water chemistry, coal rank, native microbial consortium composition, pressure, and seepage velocity are some of the *in situ* variations to be considered when designing *ex situ* laboratory experiments. While many bench-scale studies have shown

methane enhancement, it is necessary to consider which laboratory conditions are representative of the coal environment of interest and which require further investigation.

Formation Water Chemistry

Most subsurface coal aquifers contain elevated total dissolved solids concentrations. Sodium, chloride and bicarbonate are the dominant species in CBM waters while concentrations of calcium, magnesium and sulfate are generally relatively low (Table 2.2). Compared to municipal drinking water, CBM formation waters generally contain up to two orders of magnitude more sodium, chloride, and bicarbonate[37] but can vary widely in concentration between formations. Sodium concentrations have been reported to range from 4.8-300 mmol/L; chloride from 0.1-100 mmol/L; and bicarbonate from 6.9-300 mmol/L.[38,39]

Most previously published bench-top studies of biogenic methane production have used synthetic media designed to imitate formation water. The same medium described by Tanner (2007)[40] has, for instance, been used in laboratory studies of coals from the Surat Basin in Australia,[34] several coal beds from the Powder River Basin in Wyoming and Montana (U.S.A.),[36,41,42] and the South Sumatra and Kutai Basins in Indonesia.[43] While the use of synthetic media can remove some variability and can increase reproducibility, using the same medium recipe on coals originating in formations with different aquifer chemistries may not well represent the different *in situ* conditions, because of the wide variation in formation water composition observed (Table 2.2). While synthetic media can provide greater compositional control, formation water may contain (or lack) unknown essential or inhibitory trace compounds not added to the

synthetic medium, or the medium may be more nutrient-rich than the *in situ* formation water. These differences can cause an over- or under-estimate of the *in situ* coal-to-methane conversion potential. Thus, it is important to consider variations in methane production in a laboratory setting due to the medium or formation water used in studies.

To address this concern and provide more *in situ* relevance to *ex situ* experiments, some CBM studies have used formation water in lieu of synthetic media in laboratory enrichments.[44–47] By using formation water sourced from the coal bed of interest, concerns of chemistry differences between laboratory coal microcosms and the *in situ* conditions can be reduced.

Table 2.2: Formation water composition of 8 CBM producing coal beds and one municipal drinking water treatment effluent for comparison. All concentrations are given in mmol/L.[37–39]

	Bowen Basin, Australia[38]				**U.S.A.**				
	Durham Ranch	**Fairview**	**Upper seam**	**Lower seam**	**Black Warrior Basin**	**San Juan Basin**[38]	**Uinta Basin**	**Powder River Basin**	**Municipal Drinking Water**
Calcium	0.35	0.015	1.25	0.75	0.65	0.7	1.35	0.1-1.7	0.548
Magnesium	0.4	0.04	1	0.65	0.5	0.6	1.3	0.1-1.9	1.323
Sodium	100	11	100	100	70	300	160	4.8-33.9	0.259
Chloride	70	6	80	80	55	60	100	0.1-1.8	0.169
Sulfate	0.85	0.05	0.005	0.01	5	0.075	0.05	<0.2	0.033
Bicarbonate	12	10	10	20	10	300	70	6.9-38.0	1.33

Microbial Inocula for Laboratory Studies

Microbial populations involved in coal degradation and methane production vary between coal bed locations. Studies of the bacterial populations in the Powder River and Illinois Basins in the U.S. and basins in Australia, Canada, and Japan have shown high relative abundance of the bacterial phyla Firmicutes and Proteobacteria.[2,35,42,48–50] However, other dominant bacterial groups varied with PRB studies showing high abundances of Actinobacteria and Spirochaetes[35,42] while the bacterial communities of the basins in Australia, Canada, and Japan showed high abundance of Bacteroidetes.[48–50] While there is bacterial similarity between methane producing coal beds at the phylum level, differences in individual genera and species within these phyla may contribute more variability between coal beds than is apparent from phylum level comparisons.

Archaeal methanogenic communities may also show differences between coal bed locations. While the most commonly found archaeal orders are Methanobacteriales, Methanomicrobiales, and Methanosarcinales, the dominant members are not consistent between locations. It was found in two PRB studies that members of the Methanosarcinales dominated the archaeal populations.[35,42] However, in an Indian coal bed, Methanomicrobiales and Methanobacteriales members dominated.[46] These differences suggest that the dominant methanogenic pathways may be different as Methanosarcinales have been described to be largely acetoclastic methanogens[51] while Methanomicrobiales and Methanobacteriales appear to represent strictly hydrogenotrophic members.[52,53] These variations in the apparent preferential methanogenic pathway suggest the presence of different metabolic pathways upstream

during the coal degradation process generating different methanogenic substrates and may suggest dissimilarities in bacterial populations as well.

In laboratory studies, the source of the coal-degrading, methanogenic microbial consortium can significantly affect the *ex situ* experimental relevance to the *in situ* condition. Many CBM-related studies have used microbial consortia derived from non-coal sources such as wetlands, animal dung, anaerobic digester fluids, termite guts, and lake sediments.[28,32,54] Jones et al. (2008) developed a bioassay to test methane potential of coal using the WBC-2 consortium that was derived from wetland sediments.[30] Huang et al. (2013a, 2013b) used a *Pseudomonas putida* F1 strain to develop a bioassay to test coal bioavailability changes resulting from pretreatment with nitric acid (HNO_3), sodium hydroxide (NaOH), potassium permanganate ($KMnO_4$), and catalyzed hydrogen peroxide (H_2O_2).[55,56] Other studies have used microbial consortia from coal cores [36], pumped formation water,[34,47,49] or a specialized microbial sampler.[35] Microbial samples obtained with this sampler were shown to have higher cell counts than formation water alone.[35] Selection of microbial consortia for laboratory coal-to-methane studies is an important consideration when transferring *ex situ* developed technologies to possible *in situ* applications.

Coal Source and Treatment

The source and treatment of coal used in bench-top experiments can also have implications for the transferability of laboratory results to *in situ* applications. The importance of formation water and microbial consortium sources has been discussed, and similar reasoning applies to the coal used in laboratory experiments. To make *ex situ*

experiments most representative of the subsurface coal environment, the coal used is ideally sourced from the same coal bed as the formation water and microbial consortium. At the time of this review, few published studies are available, which use coal, microbes, and formation water from the same coal bed.[46,47] One PRB study used coal and formation water from the Fort Union Formation coal bed and used microbes enriched from the formation water as inoculum to assess microbial metabolites.[47] Singh et al. (2012) presented microcosm studies using coal and formation water from the Jharia coal field in eastern India to assess the methane-enhancement potential of the consortium in the formation water.[46] The work presented by Davis (2017) used coal and formation water from the Flowers-Goodale coal bed in the PRB and microbial consortia from the same coal bed obtained using a microbial sampler similar to the one described by Barnhart et al. (2013).[57] To build on previous studies and transfer to field experiments, transitional studies should ideally be performed using coal, formation water and microbes from the coal bed of interest.

In addition to coal source, handling and treatment of coal samples for laboratory studies should also be considered. For studies focusing on microbial community composition, it is important to eliminate possible contaminants. Thus, some studies used autoclaved coal in microcosm studies to ensure that the inoculum was the only microbial source.[46,58,43,59] However, the autoclave process could result in chemical or physical changes in the coal structure[60] potentially causing changes in coal bioavailability. Thus, laboratory experiment using autoclaved coal, even when all the components are from the

same coal bed, must consider the probable differences in methanogenic potential of the autoclaved coal when transferring to field applications in the same coal formation.

Some of the methane enhancement strategies tested in the laboratory include coal treatments for enhanced bioavailability. Horizontal drilling and hydraulic fracturing have been used to release the gases from unconventional shale gas formations. These technologies increase the permeability of the formation and allow gases to be more easily recovered.[61] Many coal formations have low permeability and a dense matrix. Hydraulic fracturing has been shown to increase the permeability of subsurface coal beds by increasing the number of fractures in the coal matrix and improving coal dewatering for CBM recovery.[62,63] It has been proposed that similar technologies could potentially increase the bioavailable surface area in coal beds to increase microbial CBM production.[2]

To test the hypothesis that increasing bioavailable coal surface area would increase biogenic methane production, a few laboratory studies have sieved crushed coal and tested the biogenic methane production of each size fraction in microcosm studies. Green et al. (2008) used three separate coal sizes and found that the treatments with the smallest particles (105-177 μm) produced the most methane.[42] Papendick et al. (2011) also demonstrated the highest methane production with the smallest size fraction tested (<300 μm) and similar methane production with coal sized at 300-600 μm and 600-850 μm.[34] Gupta and Gupta (2014) tested four coal fractions and found that the 250-595 μm particle size produced the most methane. However, this fraction was the second largest particle size tested and produced only slightly more than the 0.595-1.3 mm fraction but

significantly more than the treatments with smaller coal particles. [2828] A similar study also found that an intermediate coal particle size (0.6-1.18 mm) produced slightly more methane than other coal sizes tested.[57] Thus, the published research on the effects of increasing coal surface area as a method for increasing biogenic CBM production have not produced consistent results. Until more studies have been performed, it cannot be concluded that hydraulic fracturing in coal beds, and thus presumably increased bioavailable surface area, would enhance biogenic methane production.

Most coal used in laboratory studies is dried and crushed before use in microcosm studies. Exposure to atmospheric oxygen may oxidize the coal and potentially change the bioavailability of the coal organic matter. Reduced biogenic methane production was demonstrated when coal was oxidized by exposure to atmospheric oxygen.[41] Another study showed increased methane production using the WBC-2 assay in microcosms containing potentially oxidized coal from dewatered coal beds when compared to coal from non-dewatered coal formations. This same study used a strong oxidant, hydrogen peroxide, to pretreat coal from three sources and showed increased methane production potential after peroxide oxidation.[64] Huang et al. (2013a, 2013b) pretreated coal with another oxidant, potassium permanganate, and demonstrated increased methane production and higher dissolved organics compared to untreated coal.[55,56]

Thus, the type of coal used in experiments can affect the comparability and applicability of the laboratory studies to the coal subsurface environment. In addition, the pre-treatment of the coal in the laboratory can affect the methane production potential which could in turn affect the comparability of microcosm studies with *in situ* conditions.

However, coal pre-treatment strategies proven to increase biogenic coal-to-methane conversion in the laboratory and suitable for field application may represent a strategy for enhancing *in situ* CBM production.

Sorption of Methane and CO_2 to Coal

When biogenic coal-to-methane conversion is studied in the laboratory, methane production is almost always measured by sampling the microcosm headspace and measuring the concentration. This measurement does not take into account methane that is dissolved in the liquid or sorbed to the coal. The dissolved methane concentration can be estimated using Henry's Law assuming equilibrium conditions, but sorption of methane to coal is more difficult to discern.

Sorption studies have shown preferential sorption of CO_2 to coal with a sorption capacity 2-4 times greater than for methane.[65,66] Harpalani et al. (2006) state that the preferential sorption of CO_2 is influenced by the higher atmospheric boiling point of CO_2 which results in higher sorption strength.[66] Milewska-Duda et al. (2000) demonstrated that absorption accounted for only a small fraction of the total methane sorption while it accounted for nearly half of the total sorption of CO_2.[9]

Most coal sorption studies have used dry coal to measure sorption and desorption isotherms. It has been shown that the rates of sorption are higher with dry coal and moisture in the coal reduces overall gas sorption capacity.[8,67] While some studies have addressed the potential effects of sorption on CBM production, it is likely that the sorption capacity, rates, and preferentially sorbed gases vary between coal sources. Thus, it can be difficult to determine how much total methane is produced in coal microcosms

or in field studies because sorption rates and the amounts of sorbed methane and CO_2 are challenging to estimate.

While there is still more work to be done to reliably quantify the amount of methane and CO_2 sorbed to coal, the observed preferential sorption of CO_2 has been proposed for enhanced CBM recovery while sequestering CO_2 in coal beds.[68–70] When carbon dioxide is injected into CBM containing coal beds, the CO_2 will sorb to the coal and displace some of the previously sorbed CBM. Field applications of CO_2 injection into coal beds have demonstrated enhanced CBM recovery by CO_2 displacement of sorbed methane.[71,72]

Pressure

Pressure is another environmental parameter to be considered and can vary significantly between coal beds due to depth and hydrostatic pressure. For example, two coal beds at a PRB field site [73] have estimated pressures between 100 and 200 psi for wells screened at approximately 350 and 530 feet below the surface (unpublished data). Increased hydrostatic pressures have been shown to decrease microbial growth in the deep-sea environments.[74,75] While the pressures observed in most coal beds are less than in the deep-sea environment, it can be hypothesized that decreased hydrostatic pressures in laboratory studies may alter microbial activity and community structure and thus the biogenic conversion of coal to methane. Perhaps of greater consequence, pressures can impact the sorption and solubility of methane and CO_2 in the subsurface coal beds and consequently affect the efficiency of produced methane quantification.[65,67] Methods for

measuring produced gases in laboratory experiments at atmospheric pressures do not adequately measure *in situ* gas production.

The most commonly used batch reactors for CBM studies are glass serum bottles which are set up at atmospheric pressure. Produced gases may increase the system pressure, but pressures in the serum bottles are still substantially less than in subsurface coal beds and therefore not representative of the *in situ* condition. Thus, it might be important to design laboratory systems that can withstand the higher pressures observed in the subsurface. Performing studies of microbial CBM production at increased pressure will provide useful information to improve the design of field applications.

Transitional Laboratory Systems

Most previously published studies have investigated biogenic coal-to-methane conversion in batch reactors. While these systems allow for greater control of the initial conditions, are easier to reproduce, and are relatively inexpensive, batch systems can be limited due to substrate depletion and/or by-product accumulation which can result in a cessation of microbial processes.[76] These potential issues limit the transfer of methane enhancement strategies developed in batch systems to *in situ* applications.

Maintaining an oxygen-free environment is one of the greatest challenges to running microbial coal-to-methane studies in flow systems in the laboratory. Because many members of coal-degrading, methanogenic microbial consortia are typically considered to be strict anaerobes, oxygen infiltration into the experimental system could change the community and its metabolic pathways, potentially reducing the coal-to-methane conversion potential. In addition, it might be more difficult to achieve good

reproducibility in flow systems than in batch systems as there can be variability in the packing of the columns potentially resulting in preferential flow paths through the coal media as well as differences in flow rates due to pump variations.

Saturated subsurface coal formations have continuous flow with formation-specific (and possibly season-specific) seepage velocities.[73] In order to transfer benchtop batch system strategies to the field, it might be important to investigate stimulation methods in flow reactors. While flow reactors are more expensive to run, they do not have the same issues of substrate depletion or by-product inhibition characteristic of batch reactors. A core flooding study investigated biogenic methane production in a coal system under pressure with continuous flow.[77] This study used synthetic media and ground the coal to a particle size of less than 150 μm. The study was performed under a continuous flow of 0.006 mL/min (seepage velocity could be estimated if reactor dimensions were known) and pressures ranging from 250-500 psi. Biogenic methane production was observed under both flow and higher than atmospheric pressure conditions.

Mesle et al. (unpublished data) have run flow column studies using a pulse flow strategy at a pressure of approximately 80 psi using formation water, coal, and inoculum from PRB sources and produced biogenic methane in these coal systems. Davis (2017) designed an upflow column reactor that can separate produced gases for ease of measurement. This system was filled with 2-4 mm sized coal from the Flowers-Goodale formation in the PRB. The formation water and microbial inoculum were also from the same coal formation. Methods for enhancing biogenic coal-to-methane conversion with

algae amendment, previously tested in batch systems, were applied and resulted in increased methane production.[57]

There is indeed evidence that flow systems might represent an important transitional step in the scale-up of field applicable MeCBM strategies. Because field applications can be costly and opportunities are limited, using laboratory-scale flow reactors might be an important step for determining the best practices to apply coal-to-methane enhancement strategies in subsurface coal beds.

Enhancement of Biogenic CBM with Amendment Additions

Microbial conversion of coal to methane can be enhanced with nutrient additions. Some studies have demonstrated increased methane production with the addition of methanogenic substrates: acetate, formate, methanol, methylamine, and H_2 gas.[35,36,42,78] While biogenic methane production was increased in these studies, the carbon sources for methane production were likely the amendments themselves and not the coal.

For MeCBM biostimulation strategies to be economically viable, the addition of amendment should target the coal degraders to increase the rate of coal degradation and production of byproducts that become the substrates for methanogenesis.[2,4] Many of the published studies have provided macronutrients (ammonium, phosphate) and micronutrients (trace minerals, vitamins) in defined form in coal microcosms. Jones et al. (2010) used a nutrient-free medium, adding non-carbonaceous nutrients to only some treatments, and showed increased methane production with nutrient addition.[31] Another study using non-carbonaceous nutrients to enhance biogenic coal-to-methane conversion

showed a two- to four-fold increase in methane production from lignite sourced from a coal bed without detectable *in situ* biogenic CBM.[44]

The type and amounts of nutrients necessary for optimized methane production might be different for different coal beds. The process of determining the necessary nutrients and optimized amounts can be difficult, time consuming, and costly. To address these concerns, the use of "multi-nutrients" has been introduced. Luca Technologies, Inc. used yeast extract, brewer's yeast, soy protein, and peptones in its proprietary nutrient mix for enhancing biogenic CBM *in situ.*[4] In a recent publication, yeast extract and its common components, peptone, glutamate, and vitamins, were investigated for their effects on biogenic coal-to-methane conversion.[79] Yeast extract had a greater enhancement effect than any of the individual components. These results support the use of complex nutrient sources for increasing biogenic methane production instead of investing the time and resources into determining the optimal nutrient composition needed for each coal bed. In addition to yeast extract, Barnhart et al. (2017) tested the effects of lipid-extracted algal biomass as a methane enhancing amendment. The algae-amended treatments showed increased methane production amounts and rates similar to the enhanced levels observed for yeast extract-amended treatments.[79]

In a separate study, Davis (2017) showed that algae, cyanobacteria, and yeast biomass, as well as commercial yeast extract, can all be used to enhance biogenic coal-to-methane conversion to a similar extent.[57] Phototrophic microalgae and cyanobacteria have the potential to be grown in production water holding ponds on or near the site where CBM enhancing amendments are to be applied *in situ*. In addition to providing a

nutrient amendment for CBM enhancement, microalgae can produce lipids for biofuels and both algae and cyanobacteria can produce other high value chemicals or can be used as biofertilzer or aquaculture feedstock,[80] and these other products could help offset the cost investment for CBM enhancement.

A microalga, *Neospongiococcum* sp., was isolated from a CBM production water pond and has been shown to produce lipids when grown in production water with limited nutrient addition.[81] Thus, it has been shown that algae and cyanobacteria can be used to enhance biogenic coal-to-methane conversion and at least one native strain of lipid-producing microalgae can be grown in CBM production water with limited nutrient addition. Figure 2.5 shows a conceptual model of how the use of algae or cyanobacteria as a CBM enhancing amendment might be implemented. In addition, some of the costs associated with CBM enhancement could be reduced with this strategy by decreased amendment transport costs when the algae can be grown on site as well as off-set costs by producing other valuable and marketable algae-based products.

Strategies for delivery of subsurface amendments to enhance biogenic CBM production are still being researched. One strategy is to inject the algae (or other amendments) in an injection well while pumping water and harvesting CBM from a second well (Figure 2.5).[79] A second strategy would be to use a “push-pull” method where the amendment is injected into the subsurface where it can enhance CBM production. After a certain amount of incubation time, water is pumped from the same well to extract CBM. To implement either strategy, the amount of amendment to be added must be considered based upon desired volume of coal to be impacted.

Considerations must also be made to reduce the likelihood of clogging of the cleats and pores of the coal matrix by the added amendment or resulting microbial growth.

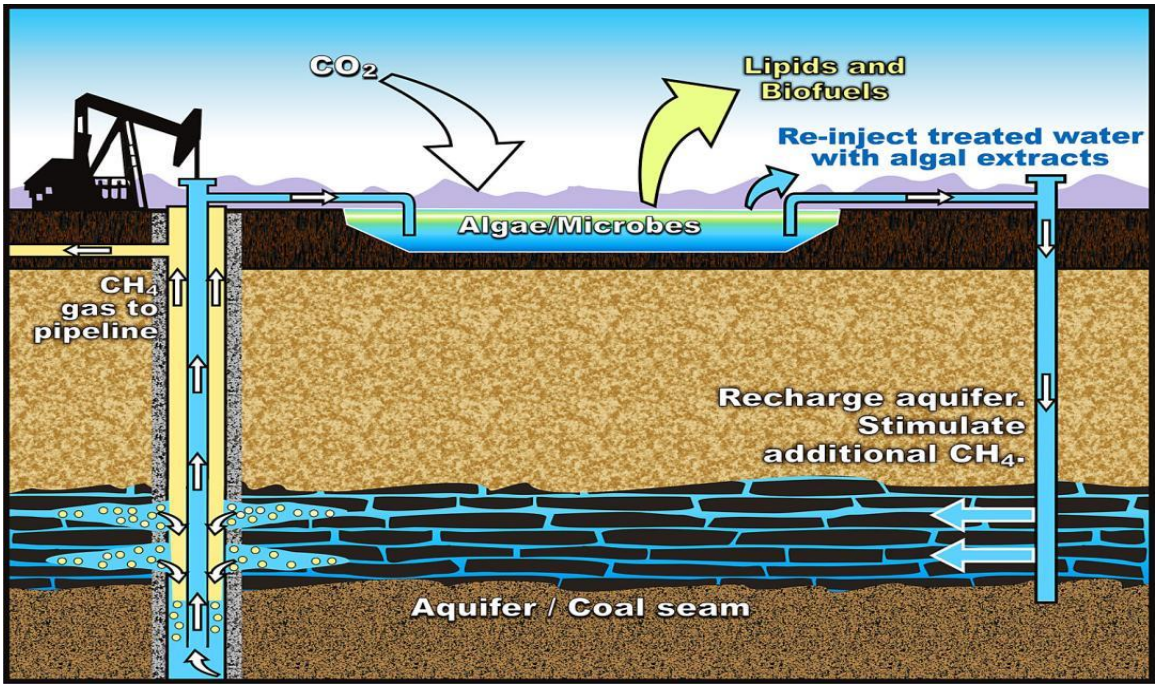

Figure 2.5: Conceptual model of coalbed methane enhancement using algae. Algae fix atmospheric CO2 when grown in the production water ponds. Lipids and other valuable products are harvested, and the residual biomass is used to enhance biogenic CBM production. (Source: Barnhart et al., 2017)

Most of the flow through a subsurface coal bed is through cleats (fractures) that generally have apertures of less than 100 µm,[82] but small amounts of liquid transport can also occur through the coal matrix itself. The porous coal matrix contains macropores (> 50 nm), mesopores (2-50 nm), and micropores (≤ 2 nm) that are assumed to be too small for microbial access.[83] A typical cell size for *Chlorella vulgaris*, a much studied green microalga, ranges from 2-10 µm.[84] If the algae cells are not disrupted prior to amendment injection in the subsurface coal, it is certain the only flow path available to the nutrient addition would be through the cleats. In addition, whole algal cells could block cleats that

are smaller in diameter. Thus, processing of the algal amendment to break up the cells might be advantageous and result in smaller particle sizes and would also likely increase the bioavailability of the nutrients to the microbial communities.

Conclusions

Laboratory experiments investigating coal-to-methane conversion have produced a reasonable body of knowledge, and as a result, microbial communities and geochemistry in subsurface coal beds are better understood. Strategies for enhancing the microbial processes converting coal to methane have been developed in the laboratory and applied *in situ* on a small scale from several commercial ventures. However, due to the costs associated with applying CBM enhancement strategies *in situ*, it is important to consider the relatability of the laboratory studies to subsurface applications. To make *ex situ* studies as relevant to the subsurface condition as possible, the formation water, coal, and microbial consortium should be sourced from the coal formation of particular interest. This will ensure the best simulation of the subsurface environment. In addition, it is important to consider how the coal is processed for laboratory studies to minimize changes to the coal chemistry and potential bioavailability. Pressure differences between *in situ* coal conditions and laboratory studies should be considered for effects on both microbial processes and sorption of methane and CO_2. In addition to pressure effects on methane and CO_2 sorption, it is necessary to increase the understanding of competitive sorption and desorption of gases and differences in sorption characteristics between different coal beds. Flow cannot be discounted, and strategies for coal-to-methane

conversion should be tested in flow reactors prior to application *in situ*. Lastly, methods for applying amendments for enhancing biogenic CBM production in the subsurface will require further development to ensure maximum enhancement with minimal costs associated with injection or long-term effects to water flow through the coal formation.

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CHAPTER THREE

TYPE AND AMOUNT OF ORGANIC AMENDMENTS AFFECT ENHANCED BIOGENIC METHANE PRODUCTION FROM COAL AND MICROBIAL COMMUNITY STRUCTURE

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

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Contributions: Performed extractions and sequencing for community analysis.

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Abstract

Slow rates of coal-to-methane conversion limit biogenic methane production from coal beds. The studies presented here show 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 exceeded the amount of carbon added in the form of amendment, indicating 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 much, 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 *in situ* coal-to-methane production is to be promoted.

Introduction

Coalbed methane (CBM), formed in subsurface coal seams by thermogenic and biogenic processes, is an unconventional natural gas resource. In 2015, CBM production contributed approximately 3.9% of the total natural gas produced in the United States.

The total proven CBM reserves were 12,517 billion cubic feet, enough to supply over 42,000 homes for the next 50 years.[5,13] Biogenic CBM is the result of coal-to-methane conversion by a diverse, natural microbial community.[1,2,17] 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 with interactive metabolic strategies for sequential fermentative processes to degrade coal to simpler fermentation byproducts.[1,2,17] Biogenic methane is produced continuously in active coal basins, and methods have been proposed for increasing the rate and volume of microbially produced methane (i.e. Ritter et al. (2015)).[4]

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.[5,13] Several studies have shown that the CBM produced in the PRB is primarily or completely of biogenic origin,[1,18,21] but rates of 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 environment for microbially-enhanced CBM (MeCBM) methods to increase the lifespan of current and future wells by increasing the rate and volume of CBM production.

Ritter et. al. (2015) summarized the commercial *in situ* applications of MeCBM techniques using various nutrient and amendment methods.[4] Several studies have investigated MeCBM strategies through stimulation methods[1,4,42,47] or coal pre-treatment to enhance bioavailability.[41,56,64] While many previous CBM stimulation studies have used simple carbon substrates, such as acetate[35] or formate,[36] or inorganic nutrients[31,42] to enhance methane production, a few have successfully enhanced CBM production utilizing yeast extract with or without other inorganic nutrients or simple carbon substrates.[35,47] While adding simple carbon substrates alone will 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.

A review of microbial life under extreme energy limitations suggested that microbial communities in subsurface environments can obtain necessary nutrients through biomass turnover.[85] Using this principle, additions of biomass as a nutrient source to the coal environment could provide the nutrients necessary to encourage microbial growth and enhance coal degradation. Barnhart et al. (2017) used yeast and algal extracts to stimulate biogenic methanogenesis from coal and demonstrated a production increase with both amendments.[79] Bioenhancement with yeast extract or other complex nutrient sources could provide limiting nutrients needed for both the bacterial and archaeal population and result in increased coal-to-methane conversion with potentially reduced cost and need to determine 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 complex nutrient amendments to enhance methane production from coal (algae, cyanobacteria, yeast cells, and granulated yeast extract), (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, (3) determine whether amendment addition causes significant shifts in the microbial community involved in coal-dependent methanogenesis.

Materials and Methods

Site and Sample Collection

The sampling site, located near Birney (Montana, USA) in the Powder River Basin, was thoroughly described by Barnhart et al.[73] Water from the Flowers-Goodale (FG) coal bed 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 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 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. 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.[35] Slurry 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.

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[86] in tube photobioreactors using methods previously described.[87] *Anabaena cylindrica* strain UTEX 1611, a nitrogen-fixing cyanobacterium was cultured using methods similar to SLA-04 cultivation using Blue-Green Medium (BG-11)[88] 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, $6.0x10^7$ and $4.0x10^7$ cells/mL respectively. A yeast, *Saccharomyces cerevisiae* strain EtOH-Red, was cultured in 100 mL of Yeast Extract Peptone Dextrose (YPD) medium[89] in 250 mL flasks at 37°C and shaken at 100 rpm to keep cells in suspension. Optical density (OD) at 600nm was measured daily. Five mL of the yeast culture was collected in 26 mL Balch tubes, and OD was measured with Unico 1100RS tube spectrophotometer (Dayton, NJ, USA). OD increase from an initial OD of 0.77 to 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 S3.1).

Microcosm set up

All microcosms were set up anoxically in 26 mL Balch tubes with butyl rubber stoppers and aluminum crimp seals. 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 (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 glass beads. The formation water was sparged for 5 hours 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 cells, and granulated yeast extract) were ground to a fine powder with a ceramic mortar and pestle. Two concentrations of each amendment were prepared at 10X 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 1mL 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[73] and adjusted with 1M HCl as necessary. All inoculated treatments received 1 mL of the inoculum described above; 3 mL of the inoculum slurry were stored at -80°C for microbial community analysis. All microcosms were incubated at room temperature in the dark for 111 days, and headspace gas was sampled and analyzed approximately every 2 weeks.

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 x 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 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.

DNA Extraction and Microbial Community Analysis

On day 111, one replicate of each treatment was destructively sampled for DNA analysis. The coal or glass beads (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 minutes. 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 minutes 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, 200µL the centrifuged liquid fraction and 200µL of the pre-treated coal/SDS fraction were used. Times for homogenizing, binding matrix, and air drying after the SEWS/ethanol wash 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 with thirty cycles of (DreamTaq PCR Master Mix, Thermo Fisher Scientific) with the annealing temperature of 55 ºC for 30 seconds using the universal prokaryotic primers Pro341F (5'-CCTACGGGNBGCASCAG-3') and Pro805R (5'-GACTACNVGGGTATCTAATCC-3'), which amplify the V4 region of the 16S rRNA gene of bacteria and archaea.[90] 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.[91] In brief, paired reads were firstly 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 RDP training sets with cutoff value of 80. Chloroplast-, Mitochondria- and Eukaryota-like

sequences were removed from the analysis. Sequences were binned into phylotypes according to taxonomic classification and a relative abundance plot for each library was obtained. Amplicon sequences from this study were uploaded to NCBI's Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra) under accession numbers SRR5342596 to SRR5342613.

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.

Results

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 3.11). All coal treatments produced a detectable amount of methane by day 19 except for the unamended coal and the 0.1 g/L algae and cyanobacteria amended coal treatments (Figure 3.1). 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. 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 (Figure 3.1).

Table 3.1: Treatment conditions, final amount of methane produced per g of coal, and maximum methane production rates. Variation is expressed as 1 standard deviation for three replicates of each treatment

Treatment	**Solid Substrate** (1g)	**Amendment**	**Amendment concentration** (g/L)	**Methane produced in 111 days** (μg CH_4/g coal)	**Maximum methane production rate** (μg CH_4/g coal/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

The total methane after 111 days was 676μg CH_4/g coal for the coal only treatment. For coal treatments with the same amendment concentration, final amounts of methane were not statistically 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 1370 to 1456 μg CH_4/g coal while the 0.5 g/L amended coal treatments ranged from 1959 to 2185 μg CH_4/g coal. All four 0.1 g/L amended GB treatments produced methane ranging from 111-147 μg CH_4/g glass beads (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 609μg CH_4/g glass beads, respectively, and significantly more than the 0.5 g/L algae or cyanobacteria amended GB treatments at 64 and 91μg CH_4/g glass beads (p<0.001). Statistically significant groupings for the total methane produced for all treatments can be found in the Supplementary Information (Table S3.2).

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.[47,79] 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 might be slightly more bioavailable for the methane-producing microbial consortia.

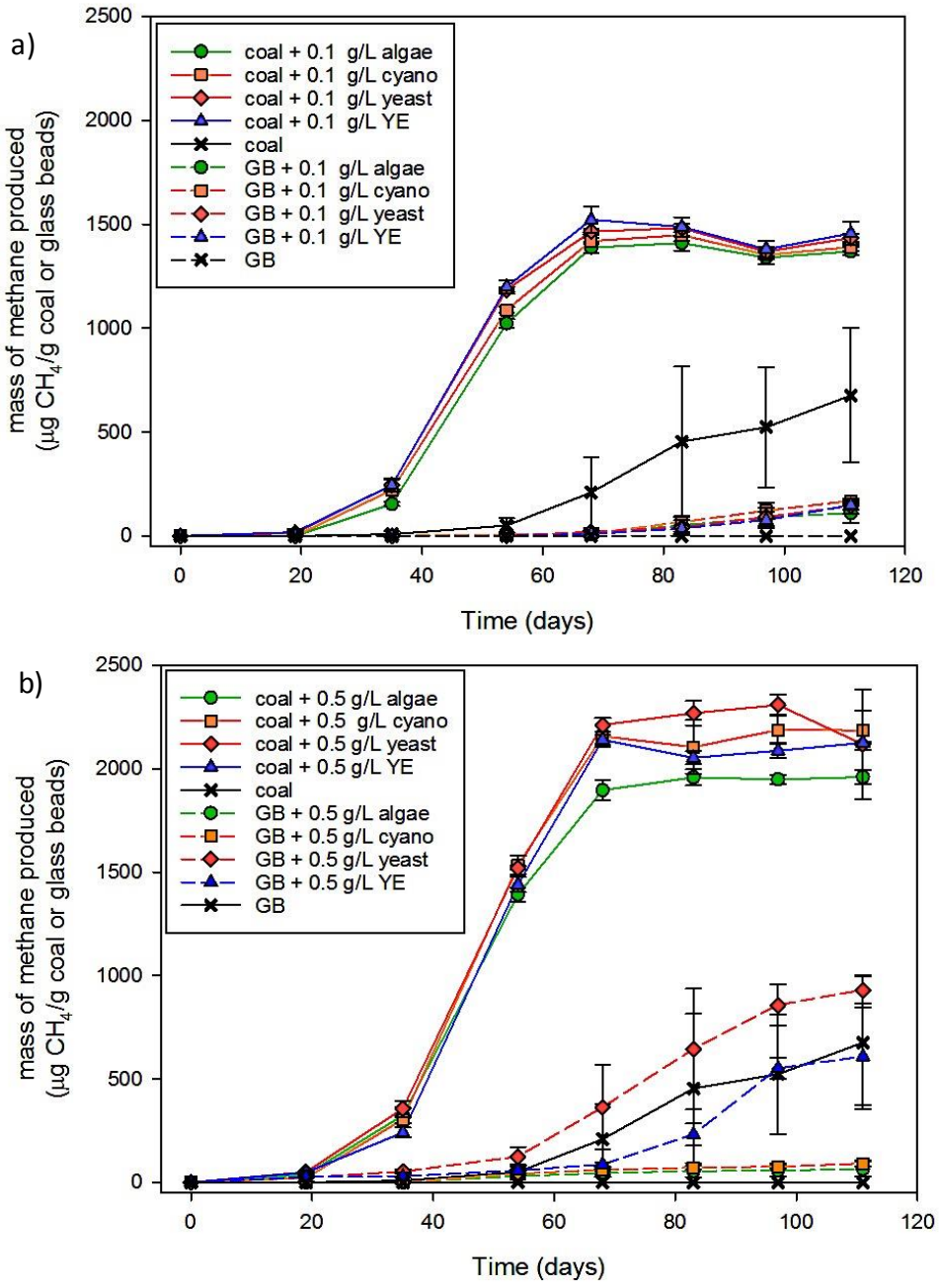

Figure 3.1: Methane production over time for (a) unamended and 0.1 g/L amended treatments and (b) unamended and 0.5 g/L amended treatments showing the cumulative methane produced at each sampling day. The methane produced is the sum of methane measured in the headspace and what can be assumed to be dissolved according to Henry's law. It does not include methane that may be sorbed to the coal or glass beads. Error bars represent 1 standard deviation for triplicates of each treatment.

The average rate of methane production was calculated for each treatment for every time-period between samplings (Supplementary Table S3.3). Maximum production rates are shown in Table 3.1. 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 56.2-63.1 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 S3.2). All 0.5 g/L amended coal treatments except for the algae-amended treatment did not have significantly different rates of methane production (p>0.413) but were significantly different from all other treatments (p<0.032). The 0.5 g/L algae amended coal treatment maximum rate was slightly lower than the rates of other 0.5 g/L amended treatments and statistically different from all other treatments (p<0.032). The maximum production rate of the coal only treatment was lower at only 16.3 μg CH_4/g coal/day and was observed later, between days 68 and 83. The maximum methane production rate for 0.1 g/L amended GB treatments ranged from 2.7-5.1 μg CH_4/g coal/day. Algae and cyanobacteria amended GB treatments attained maximum methane production rates during the 68-83 day time period while 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 variations for maximum methane production rates when compared to algae and cyanobacteria amended treatments. The 0.5 g/L algae and cyanobacteria amended GB reached their maximum rates during the 35-54 day time period and ranged from 1.6-2.2 μg CH_4/g coal/day while 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 coal/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.

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 (Figure 3.1), 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 1430 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 amendment concentration in the 0.5 g/L amendment treatments was 5 times greater than in the 0.1 g/L treatments but the increase in total methane production was only 1.9 times. 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.

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 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 glass bead treatment and the methane produced by the coal only treatment (Eq. 1). The methane production of the unamended coal treatment was the methane potential of the conversion of coal alone. That of the amended glass bead treatments was the methane potential of conversion of the amendment alone. Methane produced by the corresponding amended coal treatment in excess of this sum was likely due to enhancement instead of amendment to methane conversion.

$$CH_{4(amended\ coal)} > CH_{4(unamended\ coal)} + CH_{4(amended\ GB)} \quad \text{(Eq. 1)}$$

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. The 0.1 g/L algae amended coal treatment produced 1370 μg CH_4/g coal. The 583 μg CH_4/g coal 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.

Table 3.2: Carbon added as amendment calculated from elemental analysis

	Carbon added as Amendment (mg)		
Amendment	**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

The second analysis supporting the hypothesis that complex organic amendments can enhance coal-to-methane conversion is based on a carbon balance. The total amount of carbon added as amendment was calculated for each treatment based on the elemental analysis of the amendments (Table 3.2). The total carbon moles (Cmol) of methane produced was calculated for each coal treatment using the methane produced for each sampling point during the 111-day study (Figure 3.1). Figure 3.2 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 3.2a) and 0.5 g/L (Figure 3.2b) amended coal treatments as calculated by equation (2):

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

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

For the 0.1 g/L coal treatments (Figure 3.2a) 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 an enhancement of coal-to-methane conversion and not just due to 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-methane conversion. When similar calculations were made for the 0.5 g/L amended coal treatments (Figure 3.2b), 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.

C_{out}/C_{in} was similar for all amendments of the same concentration at all time points and peaked at day 68 before declining. This observed decline of the C_{out}/C_{in} ratio is due to the continued increase in methane production by the unamended coal treatment while the methane production of the amended coal treatments leveled out.

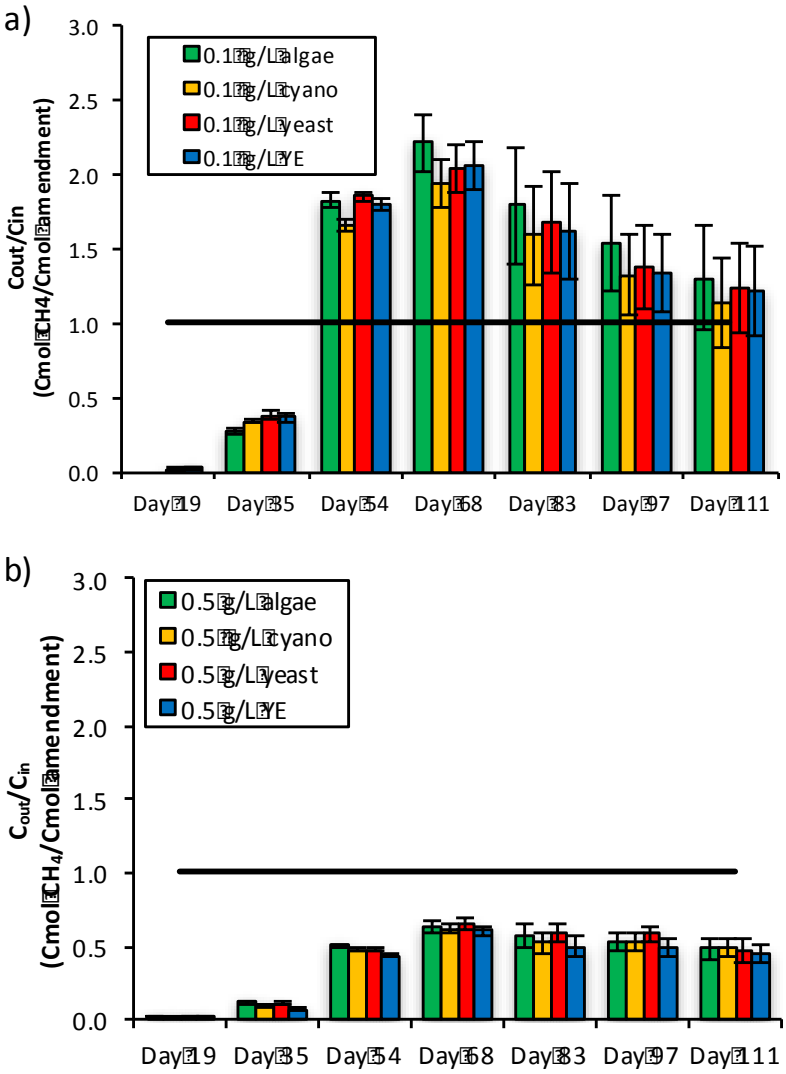

Figure 3.2: Amount of carbon detected as methane relative to the amount of carbon added as amendment for (a) 0. g/L amended systems and (b) 0.5 g/L amended systems. For the 0.1 g/L treatments, starting at 54 days, more carbon had been converted into methane than was added with the amendment, clearly indicating enhanced coal to methane conversion.

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 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 provide more advantageous enhancement.

While it is clear from both of these described analyses that the amendments affect coal-to-methane conversion, it is not clear from this study what the mechanism is and whether longer-term methane production would be increased with amendment addition or just the rate at which the methane is produced. The greatest methane enhancement effect of the amendments 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. This decrease occurred because the unamended coal continued to produce methane for the duration of the study while the amended coal methane production leveled off after day 68. 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, comparable to 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 replicate of the unamended coal treatment, it appeared that the total methane production in batch reactors, given enough time, would be similar in both the 0.1 g/L amended coal treatments and the unamended coal treatments. Thus, the effect of the amendment may be to increase the coal-to-methane conversion rate and 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.

Microbial ecology differences due to the presence of coal or amendment

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

Archaeal population diversity**.** The PCA of the archaeal communities showed little difference among the majority of the treatments except for unamended controls and alga amended GB treatments. Most treatments clustered together along the negative PC1 axis and correlated with a higher relative abundance (0.76-0.94) of the genus *Methanosaeta* known for a preponderance of acetoclastic methanogens[92,93] (Figure 3.3). A decrease in the relative abundance of *Methanosaeta* was observed for the unamended coal, unamended GB, and both the 0.1 g/L and 0.5 g/L algae amended GB treatments. The unamended coal treatments still exhibited a moderately high relative abundance of *Methanosaeta* (0.73) but also had a higher relative abundance of the genus *Methanospirillum* (0.22), known for hydrogenotrophic members.[94,95]

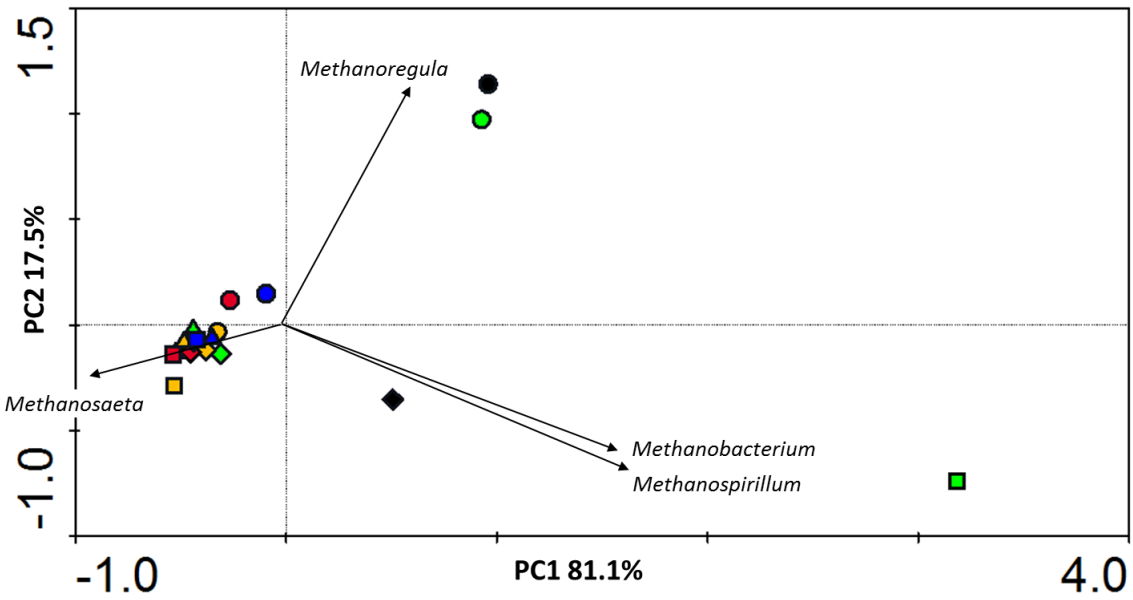

Figure 3.3: Principal component analysis of non-transformed relative abundances of archaeal phylotypes (at the genus level) for all treatments on day 111.

The unamended GB and the 0.1 g/L algae GB treatments were similar and had relative *Methanosaeta* abundances of 0.46 and 0.51 respectively, significantly lower than the treatments clustered along the negative PC1 axis. Sequences indicative of *Methanoregula*, previously shown to utilize hydrogenotrophic methanogenesis pathways,[53,96] had a higher relative abundance (0.3) for both the unamended GB and the 0.1 g/L algae amended treatments. The 0.5 g/L algae amended GB treatment had the lowest relative *Methanosaeta* abundance of 0.17 and was dominated by *Methanospirillum* (relative abundance 0.74). While hydrogenotrophic-associated *Methanobacterium* [52] had a relative abundance <0.02 in all other treatments, the relative abundance of *Methanobacterium* in the 0.5 g/L algae amended GB treatments was 0.06. 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 GB, 0.1 and 0.5 g/L algae GB), however, showed a larger relative abundance of the genera *Methanoregula, Methanospirillum,* and *Methanobacterium* which are known to contain hydrogenotrophic members.

Bacterial population diversity**.** 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 (Figure 3.4a) and 0.5 g/L amended treatments (Figure 3.4b), trends were observed for the two separate groups. As shown in Figure 3.4a, 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 (Figure 3.4b), 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 pairing 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.

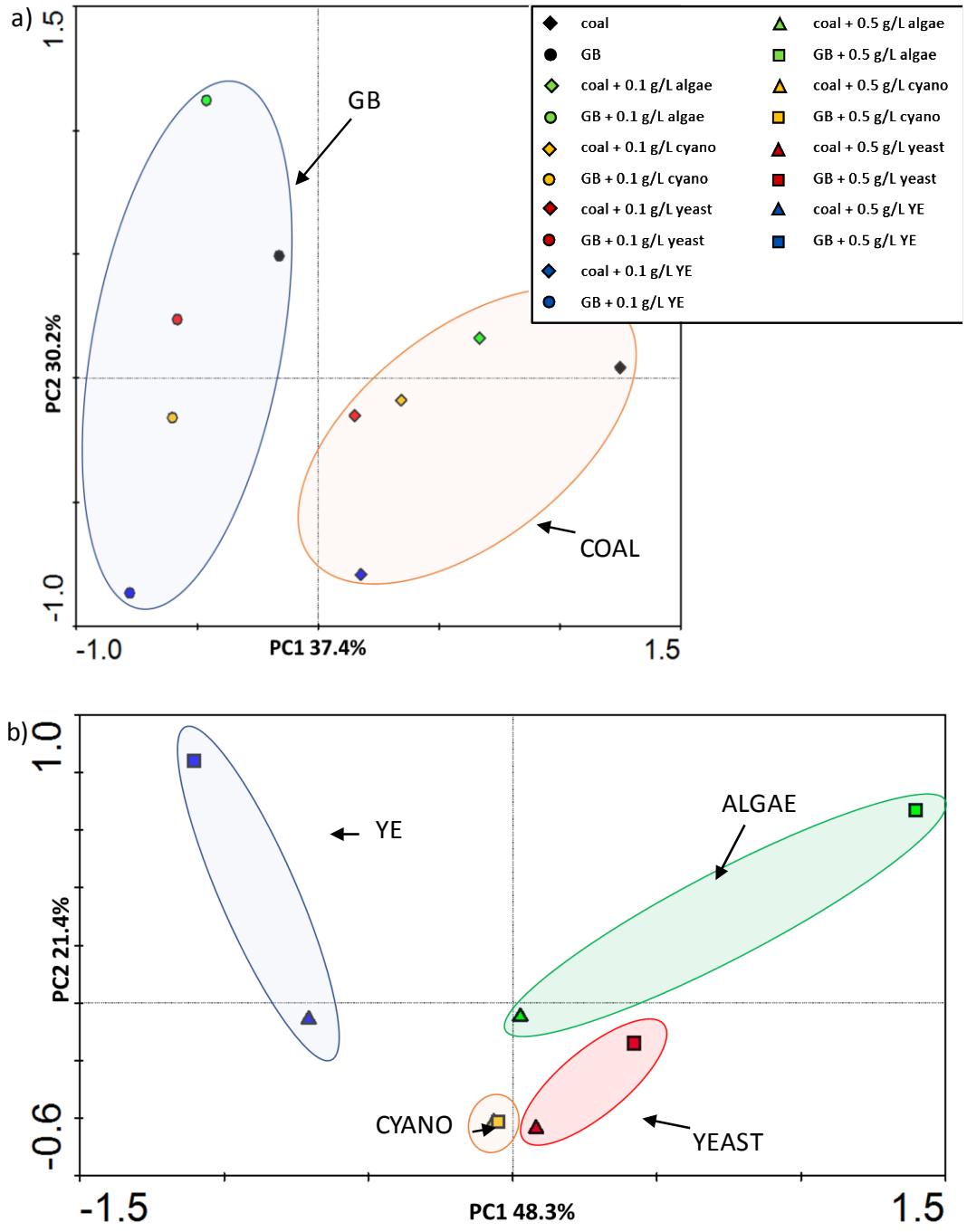

Figure 3.4: Principal component analysis of non-transformed relative abundances of bacterial phylotypes (at the genus level) for (a) unamended and 0.1 g/L amended treatments and (b) 0.5 g/L amended treatments on day 111.

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. [46,59,31]

Discussion and Conclusions

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. [42,97,46] 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.[11] Barnhart et al. suggested the use of algal amendment for CBM enhancement for large scale application utilizing production water ponds to grow algae which could be used for microbial CBM enhancement while also providing valuable byproducts, such as lipids for biofuels.[79] 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. [81] 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 already 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 Cmol methane produced is greater than the Cmol added as

amendment, clearly demonstrating that the amount of methane produced 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.

Bacterial community differences appeared to be influenced by the presence or absence of coal for unamended and 0.1 g/L amended treatments while 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. 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.

In summary, the research presented here shows 1) the importance of coal as a substrate in biogenic CBM production; 2) that algae, cyanobacteria, yeast, and yeast extract amendments similarly increase biogenic coal-to-methane conversion; 3) that

increasing amendment concentrations increase the amount of methane formed but not proportionally to the amendment amount added; 4) microbial community composition appear to be dependent on the presence or absence of coal at low amendment concentration 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 increased MeCBM production and lay a foundation for further investigation and scale-up of the microbially enhance coal bed methane production technology in the field.

Acknowledgments

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Chapter Specific Supporting Information

Table S3.1: Elemental analysis results for the 4 biostimulants used for CBM enhancement

Biostimulant	**Carbon** (g/g)	**Nitrogen** (g/g)	**Phosphorus** (g/g)
algae	0.40	0.11	0.012
cyanobacteria	0.47	0.10	0.013
yeast	0.46	0.08	0.022
yeast extract	0.48	0.11	0.020

Table S3.2: Grouping information using the Tukey Method and 95% confidence intervals for the final methane production on day 111 and the methane production rate between 35 and 54 days when the rate was at its maximum for most of the coal treatments

	Grouping	
	Methane production (day 111)	**Methane production rate (days 35-54)**
coal + 0.5 g/L cyano	A	W
coal + 0.5 g/L YE	A	W
coal + 0.5 g/L yeast	A	W
coal + 0.5 g/L algae	A	X
coal + 0.1 g/L cyano	B	Y
coal + 0.1 g/L YE	B	Y
coal + 0.1 g/L yeast	B	Y
coal + 0.1 g/L algae	B	Y
GB + 0.5 g/L YE	C	Z
GB + 0.5 g/L yeast	C	Z
GB + 0.1 g/L algae	D	Z
GB + 0.1 g/L cyano	D	Z
GB + 0.1 g/L YE	D	Z
GB + 0.1 g/L yeast	D	Z
GB + 0.5 g/L algae	D	Z
GB + 0.5 g/L cyano	D	Z

Table S3.3: Average methane production rates calculated for each time period between sampling events

	Average Rate (μgCH_4/g coal/day)							
Day range	**0-19**	**19-35**	**35-54**	**54-68**	**68-83**	**83-97**	**97-111**	**max**
coal only	0.0	0.6	2.2	11.3	16.3	5.0	10.9	16.3
GB only	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
coal + 0.1 g/L algae	0.0	9.6	45.8	26.1	1.5	-5.0	2.1	45.8
GB + 0.1 g/L algae	0.0	0.0	0.0	0.7	2.7	2.7	1.6	2.7
coal + 0.1 g/L cyano	0.0	13.8	45.7	23.6	1.9	-6.8	2.6	45.7
GB + 0.1 g/L cyano	0.0	0.0	0.4	0.3	3.7	3.9	3.4	3.9
coal + 0.1 g/L yeast	0.8	14.4	49.5	20.0	1.1	-8.1	4.8	49.5
GB + 0.1 g/L yeast	0.0	0.0	0.0	1.4	1.5	3.6	3.8	3.8
coal + 0.1 g/L YE	0.9	14.1	50.2	23.1	-2.3	-7.7	5.4	50.2
GB + 0.1 g/L YE	0.0	0.0	0.0	0.6	1.8	2.9	5.1	5.1
coal + 0.5 g/L algae	2.0	17.8	56.2	36.0	4.1	-0.8	0.9	56.2
GB + 0.5 g/L algae	0.0	0.0	1.6	1.1	0.6	0.3	0.4	1.6
coal + 0.5 g/L cyano	1.2	17.7	64.7	44.5	-3.5	5.9	-0.1	64.7
GB + 0.5 g/L cyano	0.0	0.0	2.2	1.4	0.7	0.3	1.1	2.2
coal + 0.5 g/L yeast	2.6	19.1	61.3	49.3	3.8	2.8	-13.6	61.3
GB + 0.5 g/L yeast	1.2	1.8	3.8	17.1	18.8	15.2	5.2	18.8
coal + 0.5 g/L YE	2.5	12.1	63.1	50.0	-5.8	2.5	2.7	63.1

CHAPTER FOUR

FATE OF CARBON DURING ENHANCED MICROBIAL METHANE PRODUCTION FROM COAL WITH REPEATED ORGANIC AMENDMENT

Contribution of Authors and Co-Authors

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Manuscript Information Page

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Abstract

Understanding the effect of repeated amendment additions on the rate and extent of enhanced coal-dependent methane production and the influences of repeated nutrient additions on subsurface microbial populations has become increasingly important with the growing interest in enhancing coalbed methane (CBM) production. Addition of organic amendments to laboratory coal systems has been shown to increase the rates and total production of biogenic methane for 60-90 days. Algal biomass, as an organic amendment, was added at a concentration of 0.1 g/L to microcosms with and without coal on days 0, 76, and 117, and methane and inorganic carbon production were measured every 2-4 weeks. Coal treatments amended three times produced successively decreasing amounts of methane with each amendment; at least 113% of carbon added during the first amendment were recovered as methane, 39% of carbon added with the second and 32% of the carbon with the third amendment. Additionally, amended coal treatments produced 180% more methane than amended coal-free treatments after one amendment; a second amendment addition resulted in only a 20% increase in methane production for coal versus non-coal treatments and a third amendment addition resulted in similar methane production in both coal and non-coal treatments. These results suggest a shift from coal-to-methane conversion to amendment-to-methane conversion as the primary methane source with repeated amendment additions.

Introduction

Natural gas found in many of the world's coal reserves is commonly referred to as coalbed methane (CBM). CBM can be formed by thermogenic or biogenic processes and is commercially produced in some regions. Unlike thermogenic CBM, formed by heat and pressure on geologic timescales, biogenic CBM is formed by microbial processes occurring continuously on shorter timescales, thus providing an opportunity for enhancement strategies for increased production.[7]

The Powder River Basin (PRB) in Montana and Wyoming is the largest known U.S. coal reserve and a site of commercial CBM collection. Previous studies have shown that the methane collected in the PRB is almost exclusively of biogenic origin.[1,18,21] Commercial collection of biogenic CBM often exceeds the rate of microbial methane production, resulting in short well lifespans.[10] When wells no longer produce enough gas to justify continued pumping, they are often abandoned, leaving valuable infrastructure in place. In the Montana PRB alone, there were 1046 shut-in or abandoned wells and an additional 597 with expired permits in 2015.[98] Thus, the PRB is potentially lucrative environment for application of strategies to increase the rates and amounts of microbially-produced methane by utilizing the already in place infrastructure for collection.

Previous studies have tested methods for enhancing biogenic coal-to-methane conversion in the laboratory. Enhancement of biogenic methane production from coal has been demonstrated on the laboratory-scale using inorganic and organic nutrient additions[1,42,47] and coal oxidation pre-treatment.[41,56,64] However, most of these tested

methods could be too costly to implement *in situ* due to the investment required for production, transport, and application of the amendment. The use of yeast extract which contains a broad spectrum of nutrients has been implemented as an alternative methane-enhancing amendment strategy to reduce the necessity of determining the exact "recipe" of nutrients needed.[4]

In an effort to address potential costs associated with enhanced CBM production by amendment addition, Barnhart et al. (2017) introduced the use of algal extract as an alternative amendment to yeast extract or synthetic amendments for increasing microbial coal-to-methane conversion.[79] Microalgae grow in CBM production water ponds, and a *Neospongiococcum* spp. isolated from a production water pond has been shown to accumulate lipids.[81] In addition to lipids for potential biofuel production, microalgae have also been grown commercially for production of nutritional supplements, food additives, fertilizer, aquaculture feedstock, and other high-value chemicals.[80] Potential amendment transportation costs could be reduced by growing algae on or near the CBM collection site, while providing alternative economic benefits in the form of biofuels or other value-added products. Thus, the costs associated with CBM enhancement could be reduced by using microalgal amendments.

Work presented in Chapter 3 demonstrated the methane enhancement effect of four organic amendments at two concentrations. The studies presented here focus on microalgal amendment at the lower amendment concentration of 0.1 g/L to minimize the observed shifts in microbial community structure at higher amendment concentrations.

Most CBM enhancement studies have been performed using batch systems. In most of these studies, methane production rates and amounts increase for a period of 60-90 days before methane production slows down or ceases completely. The purpose of this study was to determine whether methane production can be increased repeatedly when systems are re-amended with algae after enhanced methane production has slowed down. For a long-term application of enhanced CBM strategies *in situ*, it might be necessary to ascertain the potential benefits or problems arising from the repeated amendment of coal systems for increasing coal-to-methane conversion. The results of this study contribute to the overall objective of designing a field-scale application of amendment-enhanced microbial coalbed methane production.

Materials and Methods

Site and Sample Collection

The sampling site, located near Birney, Montana in the Powder River Basin (PRB), was thoroughly described by Barnhart et al.[73] Water from the Flowers-Goodale (FG) coal bed was pumped and retrieved in May 2016 from the FGP-13 well. Two well volumes were pumped prior to formation water collection. Plastic jugs (6-gallon volume) were rinsed twice with formation water before being filled and stored at 4°C prior to microcosm set up. Coal cores were collected during the July 2013 drilling of the Flowers-Goodale monitoring wells (FGM-13 and FGP-13). Twelve-inch sections of the 2-inch diameter cores were placed in PVC tubes which were filled completely with formation water pumped from the FG-11 well and sealed with flexible rubber caps to allow room

for gas desorption. Microbial cultures were collected from the FGM-13 well in September 2015 using microbial samplers similar to those described by Barnhart et al.[35] Liquid from the FGM-13 sampler was added to 3 serum bottles prepared with 5 g FG coal and 45 mL anoxic FG formation water before being incubated at room temperature in the dark. The three serum bottles were combined prior to use as inoculum for the studies described here.

Amendment Growth and Preparation

A *Chlorella* microalga species, strain SLA-04, was grown in photobioreactors for biomass accumulation as previously described in Chapter 3. The biomass was lyophilized and stored at -20°C before being used in microcosm studies. The algal amendment was ground to a fine powder with a ceramic mortar and pestle. A 1g/L stock solution (dry weight/v) was prepared in 0.2 µm filtered and degassed FG formation water and sealed in oxygen-free serum bottles using anoxic methods.

Microcosm Set Up and Re-Amendment

Microcosms were set up anoxically in 26 mL Balch tubes with butyl rubber stoppers and aluminum crimp seals. The FG coal core (depth 374-376') was opened in an anaerobic glove bag where it was dried, crushed, and sieved to an effective size range of 0.85-2.0 mm. The prepared coal was stored in oxygen-free glass bottles until microcosms were set up. Borosilicate glass beads (1 mm) were autoclaved and used in lieu of coal to provide a carbon-free solid substrate as appropriate controls. Each Balch tube received 1 g of prepared coal or glass beads (GB). The formation water was filtered with 0.2 µm

bottle top filters and sparged for 5 hours with an oxygen-free gas mixture of 5% CO_2/95% N_2. Sodium sulfide (1 mM as $Na_2S{\cdot}9H_2O$) was used as an oxygen scavenger to ensure low redox conditions. All amended treatments received 1mL of the prepared amendment concentrate. The initial pH of the degassed and reduced formation water was 7.6, similar to what has been observed in the FG formation water.[73] All inoculated treatments received 1 mL of inoculum from the combined serum bottles of the previously enriched FG microbial consortium. The initial total liquid volume of all microcosms was 10 mL. Inoculum slurry was frozen and lyophilized for carbon analysis (5 mL). All microcosms were incubated at room temperature in the dark, and headspace gas was sampled and analyzed approximately every 2 weeks.

Table 4.1: Experimental treatments for each amendment time period. Amendment addition is indicated for each amendment time as "+" (addition of algal amendment in FG water) or "-" (addition of FG water only).

Days 0-76	Days 76-117	Days 117-159
Coal -	Coal - -	Coal - - -
	Coal - +	Coal - + -
		Coal - + +
GB +	GB + -	GB + - -
	GB + +	GB + + -
		GB + + +
GB -	GB - -	GB - - -
Coal +	Coal + -	Coal + - -
	Coal + +	Coal + + -
		Coal + + +

After 76 and 117 days, appropriate treatments were re-amended as shown in Table 4.1. The amendment was prepared as for initial microcosm set up and 1 mL of prepared amendment was added to each of the re-amended treatments. Treatments not re-

amended with additional algae amendment received 1 mL of degassed FG formation water to account for volume changes and potential nutrient addition from the formation water added with the algal amendment.

Gas Analysis

Methane and carbon dioxide were monitored using an SRI Instruments (Torrance, CA, USA) Model 8601C GC equipped with a thermal conductivity detector (TCD) interfaced with PeakSimple Chromatography software. A Supelco HayeSep-D packed stainless-steel column (6 feet x 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 8 psi. Gas (1 mL) was sampled from the microcosm headspace for GC injection. To prevent creating a negative pressure in the tubes, 1 mL of anoxic 5% CO_2/95% N_2 gas was injected to replace the sample volume removed. After the initial 20-day incubation period, reactors were sampled every 12-16 days for gas analysis for the duration of the 159-day study.

Carbon Analysis

Dissolved inorganic carbon (DIC) and non-purgeable organic carbon (NPOC) were measured using a Formacs HT/TN (Skalar, Inc., Buford, GA, USA). Samples were centrifuged for 10 minutes at 4700 rpm and 4°C and filtered through 0.7 μm GD/X filters. NPOC samples were acidified with 3N HCl to decrease the pH below 2 and purged with oxygen gas for 180 seconds prior to analysis. DIC and NPOC measurements were taken every 4 weeks and on each re-amendment day. Because these analyses

required destructive sampling of reactors, only one reactor per treatment was sampled for each sampling date except at the end of the experiment on day 176 when DIC and NPOC measurements were taken by destructively sampling the three remaining samples of each treatment. Carbon, nitrogen, hydrogen, and sulfur analysis were performed on the dry algal biomass and lyophilized inoculum using a Thermo Scientific (Waltham, MA, USA) CE Elantech Flash 2000 CHNS-O Analyzer.

Results

Methane Production

Experimental treatments were sampled for gas analysis approximately every 2 weeks. All treatments, regardless of solid substrate (coal or glass beads (GB)) or amendment regimen, produced methane during the 159-day study (Figure 4.1a-c), and the largest amount of methane was produced during the first amendment period (days 0-76). During the second and third amendment periods, less methane was produced than during the initial amendment period, but more methane was produced by treatments that were re-amended on days 76 and/or 117 than treatments that were not re-amended (Figure 4.1d-f). After the initial amendment period, treatments that were not re-amended leveled off and little additional methane production was observed.

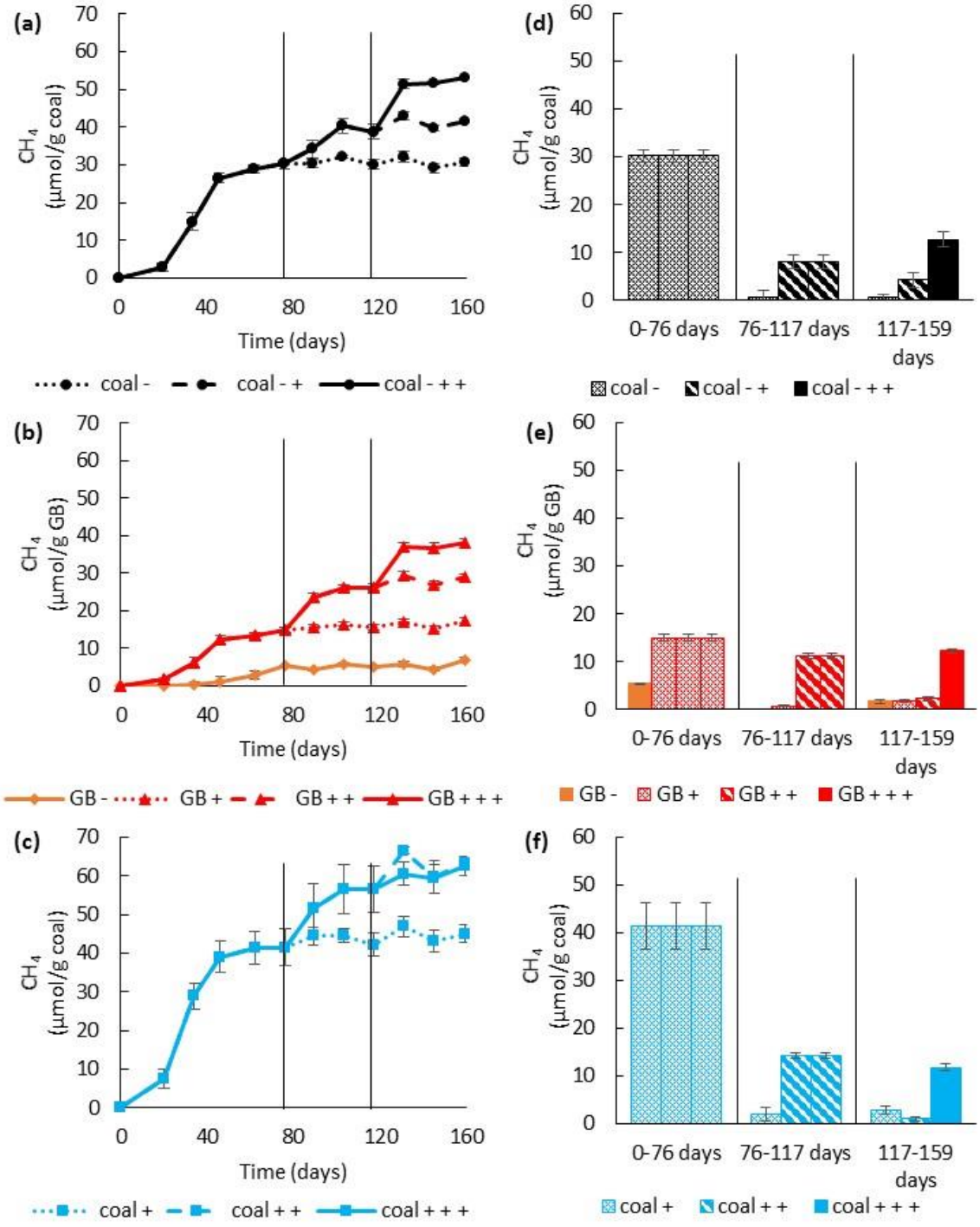

Figure 4.1: Methane production shown as a time series (a-c) and as production per amendment period (d-f) for initially unamended coal (a, d), glass bead (b, e), and initially amended coal (c, f) treatments. Error bars represent one standard deviation. Vertical lines indicate re-amendment period change.

Initially Unamended Coal Treatments. During the first amendment period (days 0-76), the unamended coal treatments (coal -) produced 30.2 ± 1.3 µmol CH_4/g coal (n=9) (Figure 4.1d). The methane production during the second amendment period (days 76-117) for unamended coal treatments (coal - -) was 0.7 ± 1.5 µmol CH_4/g coal (n=3). Initially unamended coal treatments that were amended on day 76 (coal - +) produced an additional 8.2 ± 1.4 µmol CH_4/g coal during the second amendment period (n=6). During the third and final amendment period from days 117-159, the unamended coal treatments (coal - - -) produced 0.6 ± 0.6 µmol CH_4/g coal (n=3); coal - + - treatments that were amended on day 76 but not re-amended on day 117 produced an additional 4.3 ± 1.6 µmol CH_4/g coal (n=3). Coal treatments amended on both days 76 and 117 (coal - + +) produced 12.7 ± 1.5 µmol CH_4/g coal during amendment period 3 (n=3).

Glass Bead Treatments. During the first amendment period (days 0-76), the unamended GB treatment (GB -) produced 5.4 ± 0.2 µmol CH_4/g GB (n=3), and the initially amended GB treatments (GB +) produced 15.0 ± 0.7 µmol CH_4/g GB (n=9) (Figure 4.1e). On day 76, six of the original GB + treatments were re-amended. Between days 76 and 117, unamended GB treatments produced -0.2 ± 0.1 µmol CH_4/g GB (GB - -) and 0.7 ± 0.2 µmol CH_4/g GB (GB + -) (n=3, both). The six re-amended GB treatments (GB + +) produced 11.2 ± 0.5 µmol CH_4/g GB. During the third amendment period (days 117-159), GB amendments not re-amended on day 117 produced 1.7 ± 0.4 µmol CH_4/g GB (GB - - -), 1.8 ± 0.3 µmol CH_4/g GB (GB + - -), and 2.2 ± 0.4 µmol CH_4/g GB (GB + + -) (n=3, each). GB + + + treatments were re-amended on day 117 and produced an additional 12.3 ± 0.4 µmol CH_4/g GB (n=3).

Initially Amended Coal Treatments. During the first 76-day amendment period, the initially amended coal treatments (coal +) (Figure 4.1f) produced 41.5 ± 4.9 µmol CH_4/g coal (n=9). Between days 76 and 117, coal + - treatments (n=3), which were not amended on day 76, produced an additional 2.0 ± 1.4 µmol CH_4/g coal. Coal + + treatments (re-amended on day 76) produced 14.3 ± 0.6 µmol CH_4/g coal (n=6) during the second amendment period. During the third amendment period (days 117-156), treatments not re-amended on day 117 produced 2.9 ± 0.9 µmol CH_4/g coal (coal + - -) and 1.1 ± 0.5 µmol CH_4/g (coal + + -) (n=3, both). Coal treatments (coal + + +), re-amended on both day 76 and 117, produced 11.8 ± 0.6 µmol CH_4/g coal during the third amendment period.

Comparisons of Amendment Regimens. During the first amendment period, the amended coal (coal +) treatments produced 1.4X more methane than the unamended coal (coal -) treatments and 2.8X more methane than the amended glass bead (GB +) treatments. However, during the second amendment period, the coal + + produced 1.7X and 1.3X the amount of methane produced by the coal - + and GB + + treatments. During the last amendment period, the coal + + + produced a similar amount of methane as the coal - + + and GB + + + treatments, 0.93X and 0.96X respectively. When comparing the coal treatments amended on day 0 only (coal + - -) to coal treatments never amended (coal - - -), coal + - - produced more methane than coal - - -, 1.4X during amendment period 1, 2.9X during amendment period 2, and 3.0X during amendment period 3.

Methane Production Rates

Maximum rates of methane production for all treatments during each amendment period are shown in Table 4.2.

Table 4.2: Rates of methane production were calculated for each treatment as an average for each time period between gas analyses, and maximum methane production rates for the 10 inoculated treatments for each amendment period are shown. The error is one standard deviation of three replicates.

	Maximum methane production rate (µmol CH_4/g coal/day)		
Treatment	**Days 0-76**	**Days 76-117**	**Days 117-159**
coal - - -	0.97 ± 0.21	0.12 ± 0.12	0.15 ± 0.05
coal - + -		0.44 ± 0.08	0.40 ± 0.12
coal - + +			0.80 ± 0.14
GB + - -	0.50 ± 0.07	0.06 ± 0.02	0.16 ± 0.01
GB + + -		0.66 ± 0.03	0.20 ± 0.03
GB + + +			0.79 ± 0.01
GB - - -	0.18 ± 0.06	0.10 ± 0.01	0.05 ± 0.02
coal + - -	1.52 ± 0.13	0.32 ± 0.02	0.33 ± 0.06
coal + + -		0.73 ± 0.10	0.31 ± 0.06
coal + + +			0.70 ± 0.08

Initially Unamended Coal Treatments. The maximum rate of methane production observed during the first amendment period for unamended coal treatments (coal -) was 0.97 ± 0.21 µmol CH_4/g coal/day and occurred between days 34 and 46. During the second amendment period, both coal - - and coal - + treatments had lower maximum methane production rates compared to the first amendment period. Coal - - was not amended on day 76 and had a methane production rate of 0.12 ± 0.12 µmol CH_4/g coal/day. Coal - + treatments were amended at the beginning of the second amendment period and had maximum methane production rates for this period of 0.44 ± 0.08 µmol CH_4/g coal/day. During the third amendment period (days 117-156), treatments not re-

amended on day 117 had maximum methane production rates of 0.15 ± 0.05 and 0.40 ± 0.12 µmol CH_4/g coal/day for coal - - - and coal - + -, respectively. Coal - + + treatments which were re-amended on day 117 had a maximum methane production rate of 0.80 ± 0.14 µmol CH_4/g coal/day for the third amendment period. The maximum methane production rates were highest during the first amendment period for all initially unamended coal treatments. With amendment addition on days 76 and/or 117, maximum methane production rates were higher for coal - + than coal - - and higher for coal - + + than both coal - - - and coal - + -.

Glass Bead Treatments. The maximum rate for the GB - treatments during the first amendment period was 0.18 ± 0.06 µmol CH_4/g GB/day and occurred between days 62 and 76. The maximum rate for amended GB treatments occurred between days 34 and 46 and averaged 0.50 ± 0.07 µmol CH_4/g GB/day for all nine samples (GB +). For the GB treatments during the second amendment period, the maximum rates for GB treatments not re-amended during the second amendment period were 0.10 ± 0.01 µmol CH_4/g GB/day (GB - -) and 0.06 ± 0.02 µmol CH_4/g GB/day (GB + -). The maximum rate for GB treatments amended initially and re-amended on day 76 (GB + +) was 0.66 ± 0.03 µmol CH_4/g GB/day, an increase from the 0.50 ± 0.07 µmol CH_4/g GB/day rate observed for initially amended GB treatments during the first period. Maximum methane production rates observed during the third amendment period for GB treatments not amended on day 117 were 0.05 ± 0.02 µmol CH_4/g GB/day for GB - - -, 0.16 ± 0.01 µmol CH_4/g GB/day for GB + - -, and 0.20 ± 0.03 µmol CH_4/g GB/day for GB + + -. Only the GB + + + treatments were re-amended on day 117 and had a maximum rate of

methane production of 0.79 ± 0.01 μmol CH_4/g GB/day, an increase over both of the previous amendment periods.

Initially Amended Coal Treatments. During the first amendment period, the highest maximum rate for all treatments was observed in the amended coal treatments (coal +) and occurred between days 20 and 34. This maximum rate averaged across all nine samples was 1.52 ± 0.13 μmol CH_4/g coal/day. During the second amendment period (days 76-117), treatments not amended on day 76 (coal + -) had a maximum methane production rate of 0.32 ± 0.02 μmol CH_4/g coal/day. The maximum rate for coal treatments amended initially and re-amended on day 76 (coal + +) was 0.73 ± 0.10 μmol CH_4/g coal/day (n=6). These rates were lower than the maximum rate observed for the coal + treatments during the first amendment period but higher than the rates for coal + -. During the third amendment period (days 117-156), the maximum methane production rates of treatments not amended on day 117 were 0.33 ± 0.06 and 0.31 ± 0.06 μmol CH_4/g coal/day for coal + - - and coal + + -. On day 117, only the coal + + + treatments were re-amended and had a maximum methane production rate of 0.70 ± 0.08 μmol CH_4/g coal/day, similar to the rate observed for coal + + treatments during the second amendment period.

Inorganic carbon (IC) production

Methanogenic pathways (Eq. 1-3) can produce or utilize carbon dioxide (CO_2). Hydrogenotrophic methanogenesis (Eq. 1) consumes carbon dioxide while acetoclastic (Eq. 2) and methylotrophic (Eq. 3) methanogenesis produce CO_2. In addition to methanogenesis, coal degradation reactions can produce or utilize CO_2.[16,17]

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad \text{(Eq. 1)}$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad \text{(Eq. 2)}$$

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \quad \text{(Eq. 3)}$$

Due to speciation of inorganic carbon (IC), CO_2 is not the only form of IC in these systems and measuring CO_2 in the headspace is not sufficient to track IC changes. Bicarbonate and carbonate are other IC species, and the concentrations of each IC species is dependent on pH and partial pressure of gaseous CO_2. Furthermore, CO_2 sorption to coal can occur and affect the ability to properly track IC in the systems described here.

Dissolved Inorganic Carbon (DIC) and CO_2 Concentrations. Dissolved Inorganic Carbon (DIC) was measured every 2-4 weeks during the 159-day study. The initial DIC for the prepared formation water used to set up all microcosms was 284 ± 0.8 µmol C/g coal or GB and was the same for all treatments. Because destructive sampling was required for measurement, only one sample for each treatment was analyzed for each time point except for the final reading on day 156 when triplicate samples were analyzed. Therefore, the variability between equivalent treatments could only be assessed for the final measurement. When the DIC was measured on day 7, the GB treatments exhibited a potentially small increase to 287 µmol C/g GB while coal treatments saw a decrease in DIC to 224 µmol C/g coal for unamended coal treatments and 229 µmol C/g coal for amended coal treatments (Figure 4.2a). All GB treatments had higher DIC concentrations than all coal treatments for the duration of the study. This initial decrease in DIC in coal treatments but not in GB treatments could be the result of CO_2 sorption to the coal but not

to GB. After the initial decrease in DIC, all treatments saw a general trend toward increasing DIC for the rest of the 159-day study.

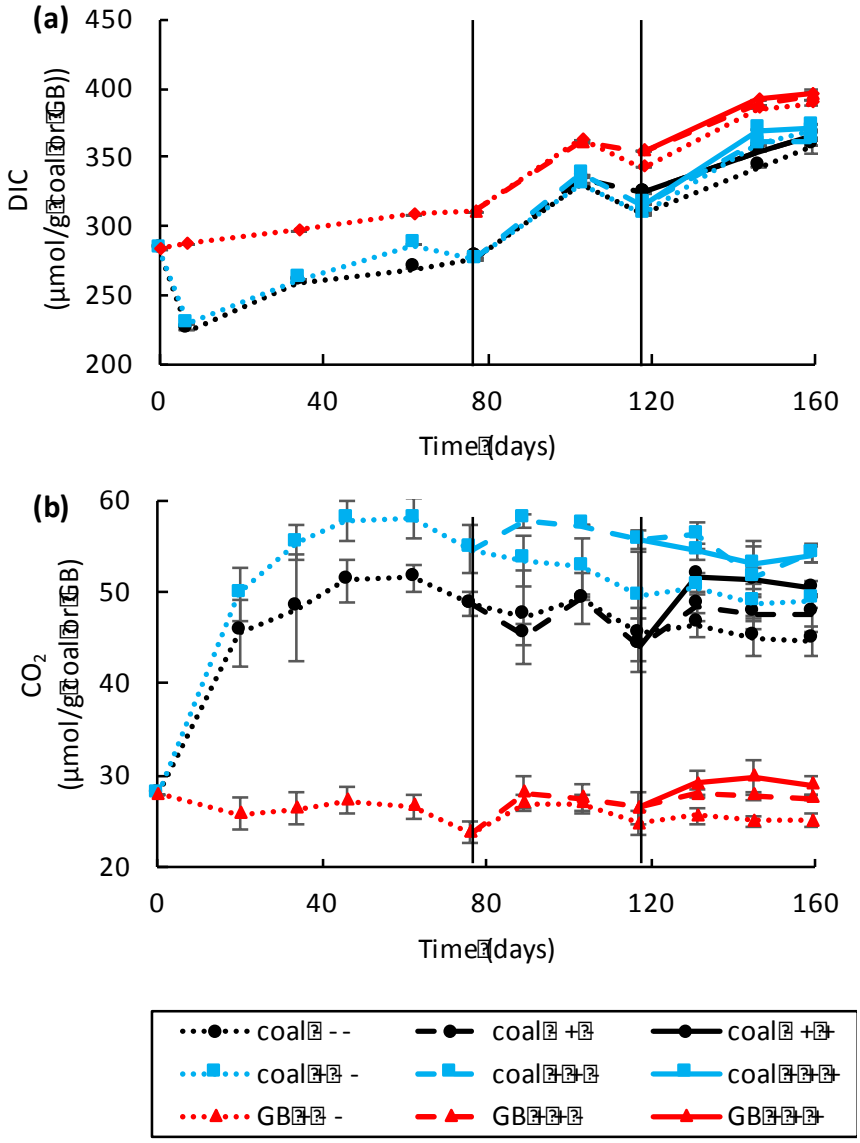

Figure 4.2: (a) Dissolved inorganic carbon (DIC) and (b) headspace CO_2 concentrations in treatments. Re-amendment (where appropriate) is indicated by vertical lines.

Headspace CO_2 concentrations were measured for all treatments approximately every 2 weeks during the 159-day study (Figure 4.2b). The initial CO_2 in the headspace

of all treatments was 28.0 ± 0.0 µmol/g coal or GB. By the first sampling on day 20, unamended coal treatments (coal -) had a headspace CO_2 concentration of 45.5 ± 3.6 µmol/g coal; amended GB treatments (GB +) had a CO_2 concentration of 25.8 ± 1.8 µmol/g GB; and amended coal treatments (coal +) had a headspace CO_2 concentration of 49.7 ± 3.0 µmol/g coal. From day 20 to the end of the study, headspace CO_2 concentrations changed little for all treatments, and, in contrast to the DIC concentrations, CO_2 concentrations were higher for the coal treatments than for the GB treatments.

Total System Inorganic Carbon. System IC was calculated from headspace CO_2 and dissolved inorganic carbon (DIC) concentrations. For this analysis, the sorption of IC to coal was assumed to have no effect on the measureable IC in the system. Produced amounts of IC for all systems are shown in Figure 4.3. DIC for unamended GB treatments (GB -) was not measured during the study because destructive sampling required more replicates than initially set up. IC changes are reported here as "production", and a negative value for production would indicate a potential utilization of IC. However, because it was possible to only measure IC for one sample for each treatment for all time points except the final measurement on day 156, some of the negative "production" values may be due to naturally occurring differences between samples. All systems had a cumulative IC production for the 176-day study (Figure 4.3 a-c).

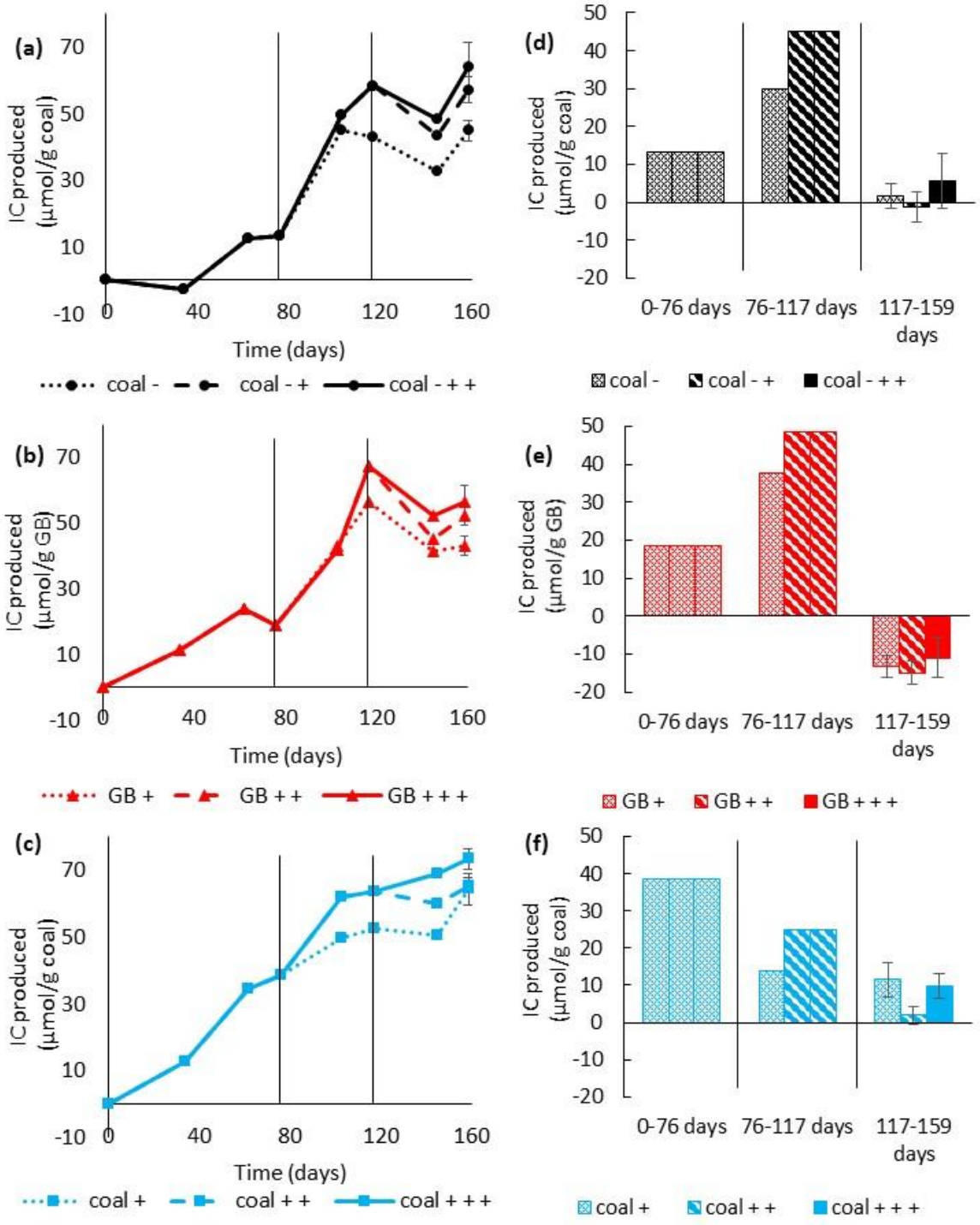

Figure 4.3: Inorganic carbon (IC) produced as time series (a-c) and as production per amendment period (d-f) for initially unamended coal (a, d), glass beads (b, e), and initially amended coal (c, f) treatments. Error bars on day 159 represent one standard deviation for 3 samples. Vertical lines designate amendment addition days.

During the first amendment period (days 0-76), unamended coal treatments (coal -) produced 13.3 µmol IC/g coal. Amended GB treatments (GB +) produced 18.7 µmol IC/g GB while amended coal treatments (coal +) produced 38.6 µmol IC/g coal. During the second amendment period, coal - - treatments produced 29.9 µmol IC/g coal while coal - + treatments amended on day 76 produced 45.1 µmol IC/g coal. GB + - treatments produced 37.6 µmol IC/g GB, and GB + + treatments produced 48.6 µmol IC/g GB during amendment period 2. Initially amended coal treatments produced 14.0 µmol IC/g coal for treatments not amended on day 76 (coal + -) and 24.8 µmol IC/g coal for amended treatments (coal + +).

During the third amendment period, coal treatments that were never amended (coal - - -) produced 1.7 ± 3.2 µmol IC/g coal (n=3). IC production by coal - + - treatments was -1.2 ± 3.9 µmol IC/g coal. Coal - + + treatments amended on day 76 and day 117 produced 5.8 ± 7.3 µmol IC/g coal. Glass bead treatments, during the third amendment period, produced -13.2 ± 2.8 µmol IC/g GB for GB + - - treatments, -15.0 ± 3.1 µmol IC/g GB for GB + + - treatments, and -10.9 ± 5.2 µmol IC/g GB for GB + + + treatments. IC produced during the third amendment period by initially amended coal treatments was 11.6 ± 4.6 µmol IC/g coal for coal + - - treatments, 1.9 ± 2.3 µmol IC/g coal for coal + + -, and 9.8 µmol IC/g coal for coal + + +.

Dissolved Non-Purgeable Organic Carbon

Non-Purgeable Organic Carbon (NPOC) was also measured every 2-4 weeks during the study. The initial NPOC for the prepared formation water used to set up microcosms was 57.9 ± 0.8 µmol C/g coal or GB. The initial spike in NPOC observed on

day 7 (Figure 4.4) to 187, 162, and 228 µmol C/g coal or GB for unamended coal (coal -), amended GB (GB +), and amended coal (coal +), respectively, can potentially be attributed to NPOC carryover from the inoculum, dissolved organics in the algae amendment, and/or dissolution of coal organics after microcosm set up. After this initial spike, NPOC decreased to 35, 14, and 26 mg C/L for coal -, GB +, and coal + treatments, respectively, on day 35. After this time, the NPOC decreased to less than 20 µmol C/g coal or GB for all treatments and remained below 20 µmol C/g coal or GB for all subsequent time points.

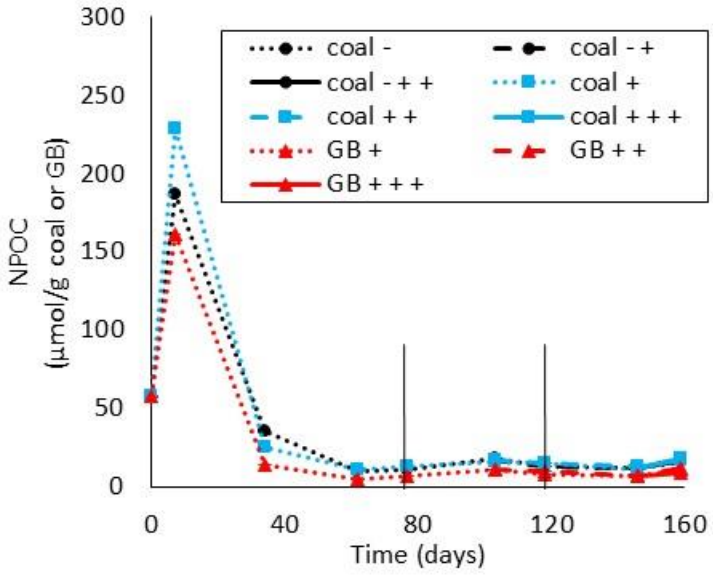

Figure 4.4: Non-purgeable organic carbon (NPOC) for treatments. The day 0 reading is the NPOC measured in the degassed formation water at microcosm set up. Days of amendment addition, where appropriate, are indicated by vertical lines.

IC/CH_4 Ratios

To estimate contributions of produced CH_4 and CO_2 by algae and coal conversions, ratios of produced IC to produced methane (IC/CH_4) were calculated for all

treatments (Table 4.3). These ratios indicate relationships between the produced CH_4 and IC. During the first amendment period (0-76 days), unamended coal treatments (coal -) have an IC/CH_4 ratio of 0.4 which implies that for every mole of methane produced, 0.4 moles of IC were produced (Eq. 4). Amended GB treatments (GB +), which contained algae but no coal, exhibited an IC/CH_4 ratio of 1.2, indicating that 1.2 moles of IC are produced for every mole of CH_4 produced (Eq. 5). The coal + treatments contain coal and algae which both contribute to produced methane and CO_2 by microbial conversion. The IC/CH_4 ratio for coal + treatments is 0.9, implying 0.9 moles of IC are produced for every mole of CH_4 in these systems (Eq. 6).

$$coal \rightarrow CH_4 + 0.4CO_2 \qquad \text{(Eq. 4)}$$

$$algae \rightarrow CH_4 + 1.2CO_2 \qquad \text{(Eq. 5)}$$

$$coal + algae \rightarrow CH_4 + 0.9CO_2 \qquad \text{(Eq. 6)}$$

Assuming these reactions are an accurate representation of the methane and CO_2 produced for each component alone and together, contributions of coal and algae conversion to the total methane and CO_2 produced by the amended coal treatments (coal +) can be estimated at 40% coal conversion and 60% algae conversion during the initial amendment period (days 0-76).

IC/CH_4 ratios of the total amounts of IC and methane produced during the 156-day study are higher for treatments not re-amended on days 76 and 117 than for treatments re-amended on days 76 and 117. For coal - - - treatments, the total IC/CH_4 ratio was 1.5 ± 0.1 compared to 1.2 ± 0.0 for the coal - + + treatments. The total IC/CH_4

ratio for unamended GB treatments (GB - - -) was 5.6 ± 0.6 compared to 2.5 ± 0.1 for the GB + - - treatments and 1.5 ± 0.0 for the GB + + + treatments. For initially amended coal treatments, coal + - - treatments had a total IC/CH_4 ratio of 1.4 ± 0.1 compared to 1.2 ± 0.0 for the coal + + + treatments.

Table 4.3: Ratio of IC produced to CH_4 produced. Values indicate the moles of carbon produced as IC for every mole of carbon produced as CH_4. Negative values reflect periods where IC consumption appeared to be greater than IC production.

	IC/CH_4 (Cmol/Cmol)			
	0-76 days	**76-117 days**	**117-159 days**	**TOTAL**
coal -	0.4 ± 0.0	10.9 ± 0.0	-2.5 ± 6.1	1.5 ± 0.1
coal - +		5.7 ± 1.1	-0.3 ± 0.1	1.4 ± 0.0
coal - + +			0.5 ± 0.1	1.2 ± 0.0
GB +	1.2 ± 0.1	60.8 ± 17.8	-7.5 ± 1.1	2.5 ± 0.1
GB + +		4.3 ± 0.2	-7.0 ± 1.1	1.0 ± 0.1
GB + + +			-0.9 ± 0.0	1.5 ± 0.0
GB - - -				5.6 ± 0.6
coal +	0.9 ± 0.1	4.8 ± 0.2	4.5 ± 1.3	1.4 ± 0.1
coal + +		2.2 ± 0.4	28.6 ± 3.8	1.8 ± 0.0
coal + + +			0.8 ± 0.0	1.2 ± 0.0

Carbon Produced per Carbon Added

The total amount of carbon produced as methane and IC was compared to the total amount of carbon added as algae amendment (C_{out}/C_{in}) during the 159-day study (Figure 4.5). C_{out}/C_{in} ratios greater than 1 indicate that the amount of carbon converted to CH_4 and IC exceeded the amount of carbon added with the algal amendment.

Comparing treatments of the same initial condition (coal -, GB +, and coal +), regardless of solid substrate, C_{out}/C_{in} ratios decreased with each subsequent amendment (i.e. coal + - - > coal + + - > coal + + +). Unamended coal treatments (coal - - -) were not amended during the study (C_{in} = 0) and therefore cannot be analyzed with this method.

However, coal - + - treatments had a C_{out}/C_{in} ratio of 2.7 ± 0.1 while coal - + + treatments had a lower ratio of 1.6 ± 0.1. For initially amended GB treatments, GB + - - treatments had a C_{out}/C_{in} ratio of 1.6 ± 0.1 while re-amendment on day 76 resulted in a ratio of 1.1 ± 0.0 for GB + + - treatments and re-amendment on both day 76 and 117 resulted in a ratio of 0.9 ± 0.1 for GB + + + treatments. The C_{out}/C_{in} ratio for initially amended coal treatments was 3.0 ± 0.1 for coal + - - treatments, 1.7 ± 0.1 for coal + + - treatments, and 1.2 ± 0.0 for coal + + + treatments. The observation of decreasing C_{out}/C_{in} ratios with increasing re-amendment suggests that re-amendment results in a greater portion of produced methane being derived from the conversion of the amendment itself and less from other carbon sources including the conversion of coal to methane.

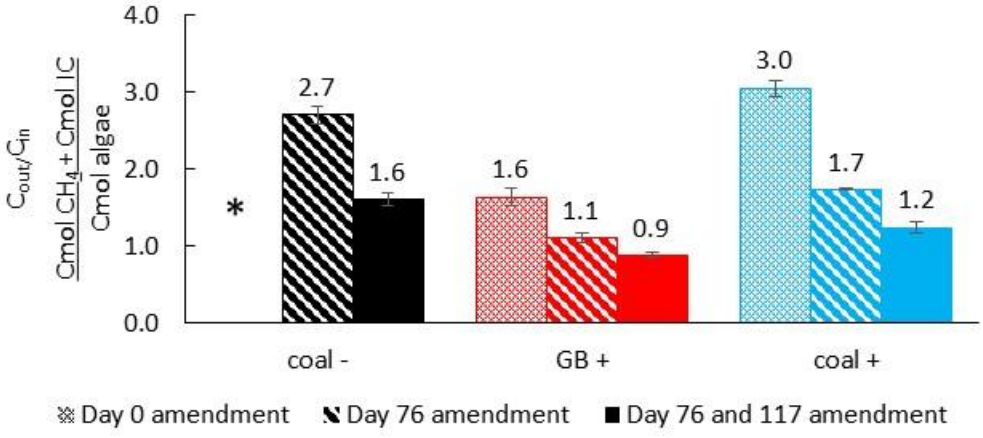

Figure 4.5: Ratio of produced methane and IC to algae added (normalized to carbon) for the entire 159-day study. Ratios greater than 1 indicate carbon production (as methane and IC) in excess of the carbon added as algae amendment. The initial amendment condition is labeled below each group. *Coal - - - treatments have no data for this comparison because they were never amended.

All amended coal and GB treatments except the GB + + + treatments had a C_{out}/C_{in} ratio greater than 1. Therefore, all treatments except for the GB + + + treatments produced more carbon as methane and IC than was added as algal amendment. Coal is indeed a significant carbon source for methane production in treatments containing coal. However, a C_{out}/C_{in} ratio greater than 1 was also observed in the GB + - - and GB + + - treatments which did not have coal as an alternative carbon source. Other sources of carbon in all systems potentially contributing to methane and IC production include initial dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), carbon carried over with the inoculum, or biomass turnover of the initial microbial members.

Discussion

During the initial amendment period, coal treatments produced more methane than the corresponding GB treatments for both amended and unamended treatments. This indicates that coal is an important substrate for methane production, which has been demonstrated in Chapter 3 and other previous studies.[79] However, while the coal + treatments produced 2.8 times more methane than the GB + treatments during the first amendment period, during the second amendment period coal + + only produced 1.3 times more methane than GB + +. During the third amendment period, the coal + + + treatments produced approximately the same amount of methane as the GB + + + treatments. Additionally, while the coal + treatment had a higher methane production rate in the first amendment period, the methane production rate for coal + + treatments was the same as the GB + + during the second amendment period. During the third

amendment period, the GB + + + treatments produced methane at a maximum rate greater than the coal + + + treatments. These results support the hypothesis that re-amendment of batch coal systems possibly results in a shift in the utilization of carbon sources for methane production from coal to the amendment itself. These potential substrate shifts are likely to result in shifts in the microbial community as demonstrated previously (Chapter 3).

Previous research indicates coal-to-methane conversion in batch studies levels off after an initial production period.[36,42,47,64] This can likely be attributed to common limitations of batch systems such as substrate depletion or byproduct inhibition.[76] In batch systems containing a limited amount of coal, the most bioavailable coal will be degraded first to produce CH_4, IC, and other byproducts. Once this easily degraded coal fraction has been utilized, the rate of methane production appears to decrease as the microbial community begins to degrade the more recalcitrant coal matrix. In addition to possible coal substrate depletion, the accumulation of degradation byproducts could become inhibitory for some microbial processes, resulting in the cessation of methane production from coal.

Re-amended treatments appear to produce similar amounts of methane, regardless of the solid substrate present (coal or glass beads). This observation suggests amendment-to-methane conversion is the primary source for methane production in all re-amended systems. Amendment addition after methane production has slowed facilitates more methane production, and thus re-amended systems which are no longer substrate-limited

due to re-amendment do not appear to be inhibited by byproduct accumulation for amendment utilization during this study.

All treatments were inoculated with 1 mL of the FG microbial consortium. Each mL of inoculum contained a measured 39.3 ± 0.0 µmol C as biomass, DOC, DIC, and coal microparticles. It is unlikely that all of this carbon was converted to CH_4 or IC, but it cannot be completely ruled out as a potential carbon source for methane or IC production. As shown in Figure 4.4, the measured NPOC, after an initial spike on day 7, decreased; a comparison of NPOC measurements between day 0 and day 159 indicate a net loss of NPOC during the experiment: 41 µmol C for coal treatments and 47 µmol C for GB treatments. This net loss could be attributed to microbial processes resulting in methane or IC production. The decrease in NPOC could also be due to consumption of organic carbon by microbes for biomass growth or sorption of organic carbon to coal which would not contribute to CH_4 or IC production.

All estimates of methane and IC production made in this manuscript assume the effects of sorption of CH_4 and CO_2 to coal are either negligible or affect all systems equally. However, it is possible that neglecting sorption of both CH_4 and CO_2 results in an underestimation of the amount of CH_4 and/or IC present in the system due to the inability to measure the amounts of sorbed CH_4 and CO_2 in these systems.

Coal sorption studies have shown preferential sorption of CO_2 over CH_4, and estimates for the relative amount of CO_2 sorbed to coal compared to CH_4 sorbed to coal vary from 2:1 to 10:1.[66] It is hypothesized that this preferential sorption can be attributed to several differences: the higher boiling point of CO_2 and variations in physicochemical

sorption processes result in increased adsorption energy of CO_2, and CO_2 is smaller than CH_4 and thus can diffuse into smaller pores not accessible to CH_4.[66] Many coal sorption studies have used dry coal. However, studies considering the effect of moisture on CH_4 and CO_2 sorption have shown that moisture reduces the sorption capacity of coal though CO_2 is still the preferentially sorbed compound.[8,67]

Busch et al. (2003) performed adsorption and desorption studies on dry Wyodak coal from the Powder River Basin. This subbituminous coal is from the Tongue River Member of the Fort Union formation as is the Flowers-Goodale coal used in this study and is thus more relevant to the potential sorption effects in this study.[65] The ratio of CO_2 sorption relative to CH_4 was estimated at 2.69 for Wyodak coal. Additionally, the adsorption and desorption isotherms indicated that even at atmospheric pressure, not all CH_4 and CO_2 can be desorbed from the Wyodak coal. This suggests that the systems presented here, at near atmospheric pressure, are likely to have some amount of sorbed methane and CO_2.

A decrease in DIC from day 0 to day 7 was observed in all coal treatments: -59.9 and -55.0 µmol carbon/g coal for unamended and amended coal treatments, respectively. Over the same time period, amended GB treatments had a possibly small increase in DIC of +3.4 µmol carbon. During the rest of the study, all samples had an overall trend of increasing DIC. While a few small decreases in DIC were observed later in the study, none was as large as the decrease observed in the first 7 days in the coal treatments. This initial decrease in DIC observed in coal treatments could be attributed to the sorption of CO_2 to coal but not to glass beads. After day 7, the rate of IC production likely exceeded

the sorption rate or produced methane sorption displaced some sorbed CO_2 resulting in an increase in the DIC.

With an estimated CO_2 to CH_4 sorption ratio between 2:1 and 4:1,[66] it is likely that CH_4 sorption to coal is also a factor in these systems. While sorption was not measured, the study described here provides preliminary observations to support the necessity for future field studies to be better able to account for actual methane produced per volume of *in situ* coal.

Summary and Conclusions

The results presented here indicate that the rate and amount of biogenic CH_4 from coal can be increased by the addition of organic amendments that stimulate indigenous microbial populations. However, in batch systems, after 60-90 days, CH_4 production slows down or ceases completely as demonstrated in Chapter 3. For potential long term commercial applications, it is necessary to determine the potential benefits, concerns and feasibility of adding additional amendment to coal systems to prolong the enhanced CH_4 production and increase the total recoverable CH_4.

Amendment addition after initial CH_4 production slowed resulted in a temporary increase in CH_4 production rates and additional produced CH_4 before CH_4 production slowed down again. During the initial amendment period, amended coal treatments (coal +) produced approximately 38% more CH_4 than unamended coal treatments (coal -), and the total amount of carbon produced as CH_4 was approximately 113% the amount of carbon added as amendment. After a repeated addition of amendment in these coal

systems, the amount of carbon produced as additional CH_4 was approximately 40% of the carbon added with the second amendment and 32% with the third amendment. In addition, when compared to similarly amended GB treatments, coal + treatments produced 180% more CH_4 than GB + treatments during the first amendment period, but coal + + treatments only produced approximately 20% more additional CH_4 than GB + + treatments after a second amendment addition. After a third amendment addition, coal + + + and GB + + + treatments produced approximately the same amount of additional CH_4. These results suggest a greater contribution of amendment-to-methane conversion in coal systems with each subsequent amendment addition. The apparent shift from coal-to-methane conversion to amendment-to-methane conversion with repeated amendment addition results in diminishing returns on investment and is similar to the previous assessment in Chapter 3 that high amounts of amendment result in greater amendment-to-methane conversion instead of the desired coal-dependent methanogenesis.

To more accurately quantify the fate of carbon in these systems, the fraction of sorbed methane and inorganic carbon should be quantified, but the time necessary for these studies did not permit another repetition as part of this doctoral thesis.

CHAPTER FIVE

^{13}C-LABELED AMENDMENTS FOR ENHANCED BIOGENIC METHANE PRODUCTION IN COAL SYSTEMS INDICATE INCREASED COAL-TO-METHANE CONVERSION

Contribution of Authors and Co-Authors

Manuscript in Chapter 5

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Contributions: Designed and performed experimental work, analyzed data. Wrote and revised manuscript.

Co-Author: Matthew W. Fields

Contributions: Contributed to experimental design. Contributed to writing and revision of the manuscript with comments and feedback.

Co-Author: Robin Gerlach

Contributions: Contributed to experimental design and data analysis. Contributed to writing and revision of the manuscript with comments and feedback.

Manuscript Information Page

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Nature

Abstract

Strategies for enhancing biogenic coal-to-methane conversion utilizing algae and yeast amendments have proven to increase the total amount and rate of methane (CH_4) production in laboratory batch systems. To more accurately quantify new CH_4 production for scale-up of these strategies, ^{13}C-labeled microalgae and yeast preparations were used to track the fate of carbon during stimulated biological coal-to-methane conversion. Systems amended with 0.1 g/L ^{13}C-labeled (~95% ^{13}C) amendments produced 10-13% of the total CH_4 as $^{13}CH_4$ in coal treatments and 31-35% as $^{13}CH_4$ in non-coal treatments. Amendment with 0.5 g/L resulted in an increase to 38-39% and 53-56% of $^{13}CH_4$ for coal treatments and non-coal treatments, respectively. In non-coal treatments, the 44-47% CH_4 produced as $^{12}CH_4$ indicates the utilization of alternate carbon sources. Microcosms using 0.2 µm filtered formation water showed a reduction in approximately two-thirds of the $^{12}CH_4$ produced in non-coal treatments. The remaining $^{12}CH_4$ is likely accounted for by conversion of dissolved organic carbon in the formation water or dissolved or particulate organic matter in the inoculum. Using these insights into the fate of carbon sources for CH_4 production, the increase in coal-to-methane conversion in treatments with small amounts of algal amendment can be quantified and was approximately 2.5 times the coal-to-methane conversion in unamended coal treatments.

Introduction

Coalbed methane (CBM) is a form of natural gas found in many subsurface coal formations and is an unconventional gas commercially extracted around the world.[2,5]

While some CBM is formed by thermogenic processes involving heat and pressure over geologic timescales, biogenic CBM is the result of coal-to-methane conversion by diverse, *in situ* microbial communities on shorter timescales.[1,2,17] The microbial consortium converting coal to methane includes bacterial members with diverse metabolic strategies for sequential fermentative processes to degrade coal to simpler byproducts that ultimately become the substrates for methanogens to produce biogenic methane.[1,2,17] Methods for increasing biogenic methane production in coal beds have been investigated, and the current state of commercial CBM enhancement strategies were recently described by Ritter et al. (2015).[4]

Several studies have shown that methane in the coal formations of the Powder River Basin (PRB) in Montana and Wyoming (U.S.A.) is primarily or completely of biogenic origin.[1,18,21] In 2015, 16.3% of the CBM commercially extracted in the U.S was biogenically produced gas from the PRB.[5,13] Rates of biogenic CBM collection often exceed the rates of microbial production, causing a reduction in extractable gas and short lifespans for CBM production wells.[10] This has increased the cost-to-revenue ratio for CBM retrieval, and many wells have been abandoned. The existing infrastructure in the PRB creates a potential opportunity for applications of methods to increase the rate and volume of biogenic CBM production thus increasing the lifespan of current and future wells.

Methane enhancement strategies to stimulate microbial processes have been investigated and shown to increase the amounts and rates of methane production.[1,4,42,47] Previous CBM stimulation studies have used methanogenic substrates, such as acetate[35]

or formate,[36] or inorganic nutrients with or without additional carbon substrates[31,42]. Other studies have demonstrated enhanced biogenic methane production with complex organic nutrient additions like yeast extract[35,47] and microalgae extract.[79] While most of these amendments increased methane production, it is unclear whether these additions enhanced the coal-to-methane conversion or merely supplied a more easily metabolized substrate for bacteria and/or methanogens. Although some studies performed controls for the CH_4 generated from coal or nutrient amendment alone, it is still difficult to ascertain the exact contributions of each when coal is incubated with different amendments.

The investigation of four organic amendments for biogenic coal-to-methane enhancement presented by Davis et al. (in review) (Chapter 3 of this thesis) used carbon mass balance calculations to show that the addition of small amounts of amendment to coal systems indeed increased the coal-to-methane conversion. However, these methods assumed the carbon added as amendment was completely converted to methane, likely resulting in an underestimation of the coal-to-methane conversion. To better quantify enhanced coal conversion to methane with amendment addition, methods for differentiating and quantifying methane from coal and amendment conversion must be developed.

Carbon occurs almost completely in two stable isotope forms, ^{12}C and ^{13}C, which comprise approximately 98.9% and 1.1% of the total carbon. Stable isotopes do not decay like the rarer radioisotope ^{14}C and thus shifts in ^{13}C concentrations can be used to understand carbon cycling.[99] A previous study used ^{13}C-labeled methanogenic substrates to investigate substrate utilization and carbon incorporation into coal-degrading microbial

consortia,[100] but to date, there are no published studies using ^{13}C-labeled amendments to enhance biogenic methane production from coal.

The study presented here used algal and yeast biomass amendments previously shown to enhance biogenic coal-to-methane conversion to assess the source of produced CH_4 via ^{13}C-labeling of the amendment biomass. The goals of the studies described here are (1) to develop a method to identify methane produced by amendment conversion, (2) quantify methane produced by coal-to-methane conversion to assess the amount of methane enhancement due to amendment addition, and (3) assess the contribution of carbon sources alternative to coal and amendment, such as dissolved and suspended organic carbon, dissolved inorganic carbon, and carbon transferred with the inoculum, to methane production.

Materials and Methods

Site and Sample Collection

The sampling site, located near Birney, Montana in the Powder River Basin, was thoroughly described by Barnhart et al.[73] Water from the Flowers-Goodale (FG) coal bed was collected from the FG-09 well in September 2015 (study 1) and from the FGP-13 well May 2016 (study 2). Plastic storage jugs (6-gallon volume) were rinsed twice with formation water before being filled, transported to the laboratory, and stored at 4°C prior to 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 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. For study 1, microbial cultures were collected from the FG-09 well in September 2015 using the diffusive microbial samplers (DMS) described by Barnhart et al.[35] Slurry from the DMS was added to two 120 mL serum bottles prepared with 5 g FG coal and 45 mL anoxic FG formation water and incubated at room temperature in the dark for 4 months prior to being used to inoculate the studies described here. For study 2, microbial cultures were collected from three FG wells in April 2016 (FG-09, FG-11, FGM-13) and from the FGP-13 well in May 2016 using the DMS samplers described. The samples were processed and incubated as described above.

Amendment Growth

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[86] with 2X nitrate in tube photobioreactors as previously described[87] with the following modifications. Five photobioreactors, each containing 1.2 L of sterile media, were sparged (20%:80% O_2:N_2) at a rate of 0.4 mL/min to reduces atmospheric CO_2 dissolution. Sodium bicarbonate ($NaHCO_3$) was added at an initial concentration of 10mM as the sole inorganic carbon source. During the 14-hour light period, pH was measured within the first hour of the light phase and then every 4 hours until the beginning of the dark phase. pH was maintained above 8.5 to reduce IC loss through CO_2 off gassing and adjusted with 2M HCl or KOH as needed to maintain a pH range between 8.5 and 10. DIC was measured twice daily, and $NaHCO_3$ was added as needed to

maintain DIC between 5 and 10 mM. The photobioreactors were inoculated with $5x10^5$ cells/mL, and daily cell counts were used to determine stationary phase when the cell counts were highest, $1.2x10^8$ cells/mL. This procedure was followed once with standard $NaHCO_3$ with assumed environmental ^{13}C concentration approximately 1.1%. The procedure was then repeated with labeled $NaH^{13}CO_3$.

Red Star® Active Dry bread yeast, *Saccharomyces cerevisiae,* was cultured at 30°C for biomass accumulation in two tube reactors, each with 1.2 L of Yeast Nitrogen Base w/o Amino Acids glucose added for a concentration of 10 g/L. The tube reactors were sparged with atmospheric air at a rate of 0.4 L per minute. Standard D-glucose with assumed environmental ^{13}C concentration of approximately 1.1% was used to grow unlabeled yeast. Fully ^{13}C-labeled D-glucose was used to grow labeled yeast. Optical density (OD) at 600nm was measured daily using a Unico 1100RS tube spectrophotometer (Dayton, NJ, USA). Yeast culture (5 mL) was collected in 26 mL Balch tubes. The unlabeled yeast was inoculated with 0.5 g/L *S. cerevisiae*, and OD(600) increased from 1.150 to 1.940 at stationary phase. The labeled yeast was inoculated with 0.25 g/L *S. cerevisiae,* and OD(600) increased from 0.767 to 1.954 at stationary phase.

The biomass from each of the amendment cultures (unlabeled and ^{13}C-labeled of both algae and yeast) was concentrated by centrifugation, dried by lyophilization, and stored at -20°C until use in coal microcosms.

Carbon analysis

The four amendments and the coal used in the experiments were sent to the Stable Isotope Facility at the University of California (Davis, CA) for carbon-13 analysis by

elemental analyzer-isotope ration mass spectrometer (EA-IRMS). Dissolved inorganic carbon (DIC) was measured during algal culturing using a Formacs HT/TN (Skalar, Inc., Buford, GA, USA). Samples were prepared by filtering with 0.2 µm syringe filter. Carbon, nitrogen, hydrogen, and sulfur analysis were performed on the dry algal and yeast biomasses, lyophilized inoculum (study 2 only), and coal using a Thermo Scientific (Waltham, MA, USA) CE Elantech Flash 2000 CHNS-O Analyzer. The results of these analyses are summarized in Table 5.1.

Table 5.1: Summary of carbon analyses for microcosm components.

Sample ID	**%C (dry weight)**	**%^{13}C (of total C)**	**δ^{13}C (‰)**
^{12}C-algae	48.5	1.1%	-2.1
^{13}C-algae	46.8	94.8%	1,621,359
^{12}C-yeast	45.9	1.1%	-8.9
^{13}C-yeast	45.2	97.1%	2,978,635
coal	58.1	1.1%	-24.1
Inoculum (study 2)	20.5	n.m.	n.m.

Microcosm set up

All microcosms were set up in triplicate using anoxic techniques in 26 mL Balch tubes (study 1) or 120 mL serum bottles (study 2) sealed with butyl rubber stoppers and aluminum crimp seals. The FG coal core (depth 384-385') was opened in an anaerobic glove bag where it was dried, crushed, and sieved to an effective size range of 0.85-2.0 mm. The prepared coal was stored in oxygen-free glass bottles until microcosm set up. Borosilicate glass beads (1 mm diameter) were autoclaved and used in lieu of coal to provide a carbon-free solid substrate. Each Balch tube received 1 g of prepared coal or glass beads, and each serum bottle received 5 g. The formation water (unfiltered in study

1 and filtered with a 0.2 µm bottle top filter for study 2) was sparged overnight with anoxic 5% CO_2 balance of nitrogen gas and reduced with sulfide (1 mM as $Na_2S \cdot 9H_2O$). The amendments (^{12}C-algae, ^{13}C-algae, ^{12}C-yeast, ^{13}C-yeast) were ground to a fine powder with a ceramic mortar and pestle. Two concentrations of each amendment were prepared at 10X desired concentration (0.1 and 0.5 g/L final concentration) in degassed FG formation water and sealed anoxically in serum bottles. For study 2, only one concentration for ^{12}C-algae and ^{13}C-algae (0.1 g/L final concentration) were made. All amended treatments received 1mL (study 1) and 5 mL (study 2) of this prepared amendment concentrate as appropriate. pH was tested to ensure a range of 7.5-8.5 as observed in the FG formation water[73] and adjusted with 1M HCl or KOH as necessary. Prior to inoculation, the liquid fraction from two (study 1) and five (study 2) FG DMS samples were combined. All inoculated treatments received 1 mL (study 1) or 5 mL (study 2) of the enrichment of previously collected FG microbial consortium. The final culture volume was 10 mL in the Balch tubes for study 1 and 50 mL in the serum bottles for study 2. Three mL of the inoculum slurry was stored at -80°C for microbial community analysis for both studies, and an additional 5 mL were frozen and lyophilized for carbon analysis in study 2. All microcosms were stored at room temperature in the dark for 133 days for study 1 and 126 days for study 2. Headspace gas was sampled and analyzed approximately every 2 weeks.

Gas analysis

Methane production analyzed using an SRI Instruments (Torrance, CA, USA) Model 8601C GC equipped with a thermal conductivity detector (TCD) and interfaced

with PeakSimple Chromatography software. In the first study, a Supelco Molecular Sieve 13X packed stainless steel column (6 feet x 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 microcosm headspace for GC injection. One mL of anoxic 5% CO_2 in N_2 gas was injected to replace the sample volume removed.

In the second study, headspace gases (CH_4 and CO_2) were analyzed 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 HayaSep-D packed stainless steel column (6 feet x 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, carrier gas pressure 8 psi, and 1 mL injections. An Agilent 6890 GC 5973 electron impact ionization mass selective detector (Agilent Technologies, Palo Alto, CA, USA) interfaced with Agilent Enhanced ChemStation software and operated in scan mode was used to measure isotope ratios of $^{13}CH_4$:$^{12}CH_4$ and $^{13}CO_2$:$^{12}CO_2$. A GS-Carbonplot column (60 m × 0.320 mm i.d. × 1.50 µm film thickness) was used for analysis. The following parameters were used: 500 µL manual split ratio 30:1 injection, constant flow at 1 mL/min, injector temperature of 185 °C, interface 60 °C, and scan range m/z 2-100. Ultra-high purity helium was the carrier gas. Both headspace gas samples were taken at the same time and 1.5 mL of anoxic 5% CO_2 was injected to replace the sample volume. The GC-MS deconvolution is described in Appendix F of this thesis.

Results and Discussion

Study 1

Total methane production. In the first study, two amendments (algae and yeast) were investigated for enhancement of biogenic methane production in coal systems. ^{13}C-labeled and unlabeled amendments were tested at two different concentrations (0.1 and 0.5 g/L) for each amendment. By the end of the 133-day study, similar total methane production was observed in treatments with the same amendment type, concentration, and solid substrate, regardless of amendment label (Figure 5.1). For 0.1 g/L algae with coal, treatments amended with unlabeled algae produced 57.3 ± 3.6 µmol CH_4/g coal, and ^{13}C-labeled treatments produced 58.4 ± 1.8 µmol CH_4/g coal. For glass bead (GB) treatments with 0.1 g/L algae, unlabeled treatments produced 23.5 ± 1.2 µmol CH_4/g GB and ^{13}C-labeled produced 20.9 ± 2.7 µmol CH_4/g GB (Figure 5.1a). For coal treatments amended 0.1 g/L yeast, unlabeled yeast treatments produced 58.7 ± 4.7 µmol CH_4/g coal, and ^{13}C-labeled treatments produced 65.0 ± 16.2 µmol CH_4/g coal. GB treatments with 0.1 g/L yeast produced 25.9 ± 1.4 µmol CH_4/g GB with unlabeled yeast amendment and 25.9 ± 1.6 µmol CH_4/g GB with ^{13}C-labeled yeast treatments (Figure 5.1b). At a concentration of 0.1 g/L, all coal treatments produced a similar amount of total methane, regardless of amendment type (yeast or algae). Similarly, all GB treatments with 0.1 g/L amendment produced similar amounts of methane.

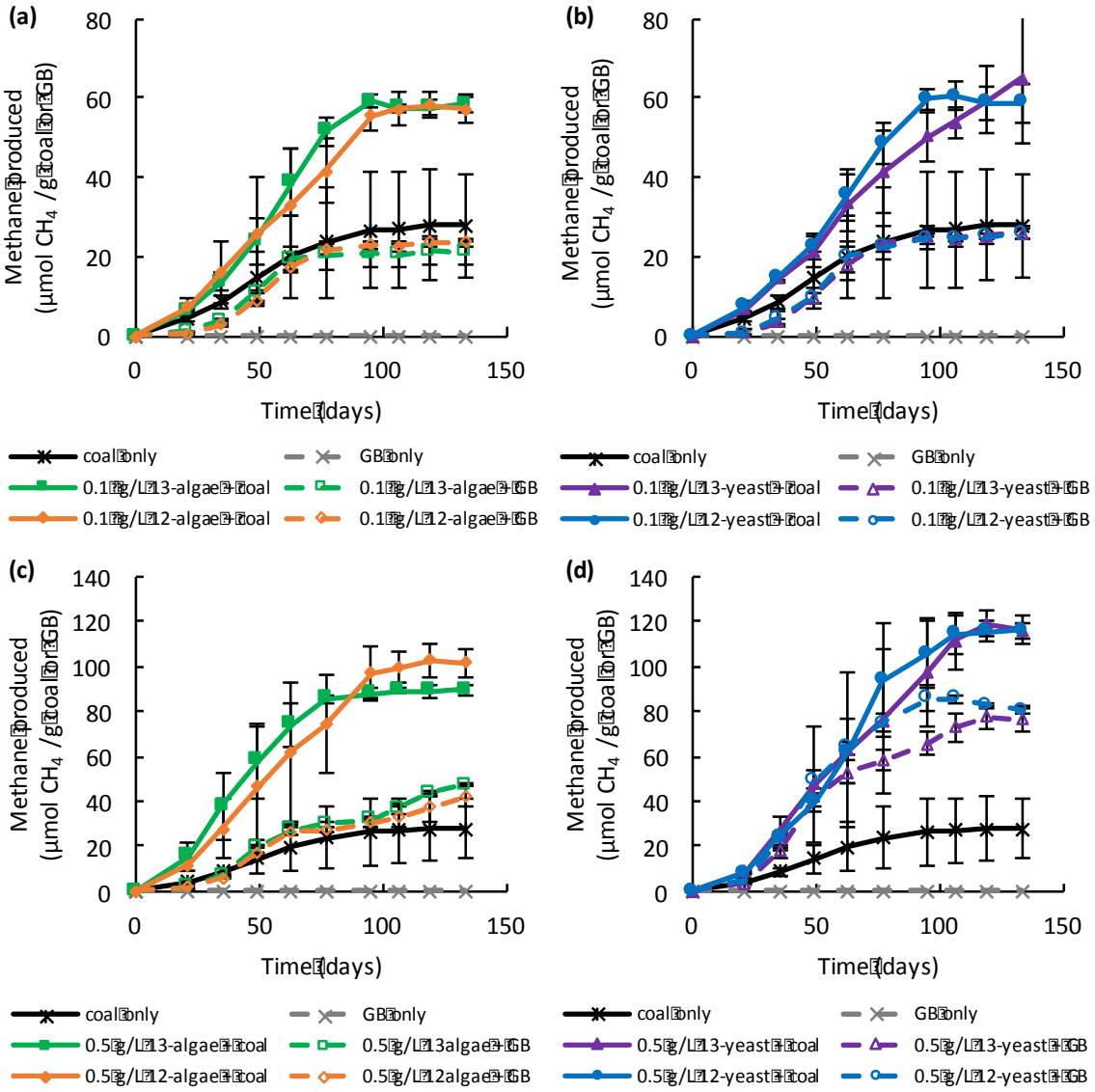

Figure 5.1: Methane production over time for unlabeled and ^{13}C-labeled amended treatments for a) 0.1 g/L algae, b) 0.1 g/L yeast, c) 0.5 g/L algae, and d) 0.5 g/L yeast treatments.

Coal treatments with 0.5 g/L algae produced 101.4 ± 6.2 µmol CH_4/g coal for unlabeled algae treatments and 89.4 ± 1.9 µmol CH_4/g coal for ^{13}C-labeled algae treatments. For GB treatments with 0.5 g/L algae, unlabeled algae treatments produced 42.2 ± 4.5 µmol CH_4/g GB and ^{13}C-labeled algae treatments produced 47.1 ± 0.6 µmol

CH_4/g GB (Figure 5.1c). Coal treatments with 0.5 g/L yeast produced 115.7 ± 3.4 µmol CH_4/g coal for unlabeled yeast treatments and 115.9 ± 6.4 µmol CH_4/g coal for ^{13}C-labeled yeast treatments. For GB treatments with 0.5 g/L yeast, unlabeled yeast treatments produced 80.3 ± 1.5 µmol CH_4/g GB and ^{13}C-labeled yeast treatments produced 76.5 ± 5.8 µmol CH_4/g GB (Figure 5.1d). Unlike 0.1 g/L amended treatments, 0.5 g/L treatments had higher methane production for yeast amended treatments than for algae amended treatments for both coal and GB treatments. This observation is similar to what was previously observed by Davis et al. (2017) (Chapter 3 of this thesis).

In addition to the amended treatments, unamended coal and GB controls were also analyzed. The unamended GB treatments produced no methane during the 133-day study. The unamended coal treatments produced a total of 27.8 ± 13.1 µmol CH_4/g coal during the study. These controls are shown in all graphs in Figure 5.1 to facilitate comparison to amended treatments. The unamended coal produced less methane than all amended coal treatments and 0.5 g/L amended GB treatments but produced similar amounts of methane as the 0.1 g/L amended GB treatments.

Analysis of carbon sources for methane production. By day 133, the total amount of methane produced in all amended coal treatments exceeded the amount of methane produced by the unamended coal plus the amount of methane produced by the corresponding amended GB treatments. This observation suggests an enhancement of coal-to-methane conversion due to amendment of coal treatments based on two assumptions: 1) the methane produced by the unamended coal treatments is representative of the amount of methane potential of coal without amendment, and 2) the

methane produced by the amended GB treatment is representative of the methane potential of amendment alone. The details of this analysis were previously described by Davis et al. (in review) (Chapter 3 of this thesis).

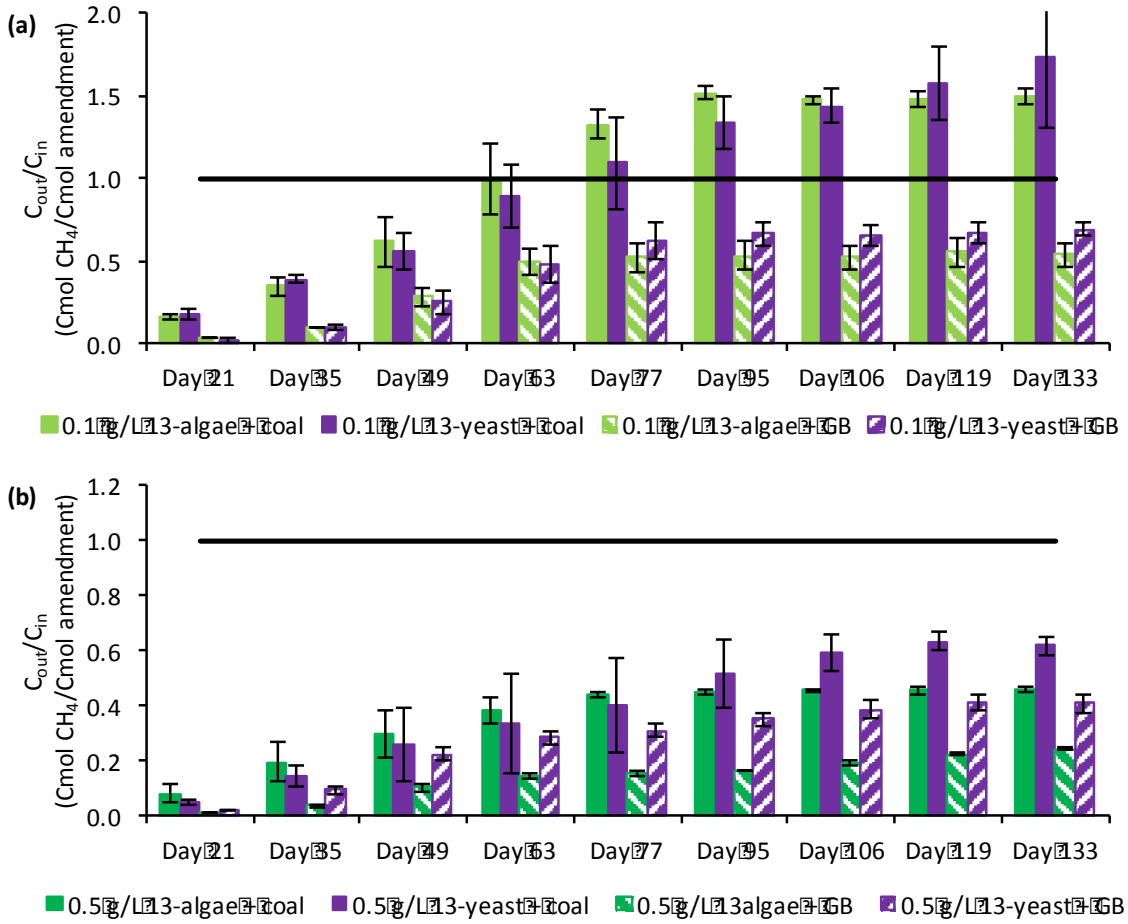

Figure 5.2: Comparison of total carbon produced as methane to carbon added as amendment for a) 0.1 g/L and b) 0.5 g/L ^{13}C-label-amended treatments. Error bars represent one standard deviation. A value greater than 1 indicates more methane production than could be accounted for by complete amendment conversion to methane.

To assess the coal-to-methane conversion, the total carbon moles (Cmol) of produced methane is compared to the Cmol added as amendment (C_{out}/C_{in}). When the C_{out}/C_{in} ratio is greater than 1, more methane was produced than can be accounted for by

complete conversion of amendment to methane and is indicated by the horizontal lines in Figure 5.2. For coal treatments with 0.1 g/L amendment, C_{out}/C_{in} was greater than 1 by day 77 through the end of the study on day 133 (Figure 5.2a). This indicates that the Cmol methane produced is greater than the Cmol added as amendment, and thus, it can be definitively concluded that the methane produced in 0.1g/L amended coal treatments by day 77 is due in some part to coal-to-methane conversion and not merely amendment-to-methane conversion.

For 0.5 g/L amended coal treatments (Figure 5.2b) and all GB treatments, C_{out}/C_{in} was less than 1 for the entire duration of this study. This observation indicates not all of the amendment was converted to methane but offers no conclusion on the contribution of coal-to-methane conversion in 0.5 g/L amended coal treatments. The unlabeled amended treatments were analyzed with this method (results shown in Appendix G), and C_{out}/C_{in} values were similar between treatments with the same amendment type, concentration, and solid substrate. While this analysis can give a conclusive indication of coal-to-methane conversion, such as with the 0.1 g/L amended coal treatments in this study, the assumption that all of the amendment carbon is fully converted to methane only likely results in an underestimation of the amount of biogenic methane produced from coal. In Chapter 4 of this dissertation, it was shown that conversion of coal or amendment to methane also results in similar amounts of inorganic carbon production.

13C amendments and methane. The natural abundance of ^{13}C is approximately 1.1%.[99] Unamended coal and all unlabeled amended treatments produced methane that consisted of 0.9-1.1% $^{13}CH_4$ (Table 5.2 and Appendix G). This is similar to the natural

abundance of ^{13}C and was expected for treatments that were not enriched by amending with ^{13}C-labeled amendments.

$^{13}CH_4$ production. All treatments that were amended with ^{13}C-labeled amendments produced methane with higher than 1.1% $^{13}CH_4$ (Table 5.2). This observation indicates that the microbial consortium is converting at least some amendment to methane. Methane produced by coal treatments with 0.1 g/L amendment was 10.8% $^{13}CH_4$ for algae (9765‰ $\delta^{13}C$-CH_4) and 12.5% for yeast (11748‰ $\delta^{13}C$-CH_4). In GB treatments with 0.1 g/L amendment, the percentage of methane produced as $^{13}CH_4$ was higher, 30.9% (38703‰ $\delta^{13}C$-CH_4) and 34.7% (46290‰ $\delta^{13}C$-CH_4) for algae and yeast, respectively. Coal treatments with 0.5 g/L amendment produced 38.0% (53488‰ $\delta^{13}C$-CH_4) and 38.6% (54929‰ $\delta^{13}C$-CH_4) of total methane as $^{13}CH_4$ for algae and yeast, respectively. Methane produced by 0.5 g/L amended GB treatments was 53.1% $^{13}CH_4$ (99664‰ $\delta^{13}C$-CH_4) for algae and 55.8% (111445‰ $\delta^{13}C$-CH_4) for yeast.

Table 5.2: Summary of produced $^{13}CH_4$ and labeled amendment conversion.

Treatment	**$^{13}CH_4$ (% of total methane)**	**$\delta^{13}C$-CH_4 (‰)**	**% ^{13}C amendment as $^{13}CH_4$**
unamended coal	0.9 ± 0.1	-180	n.a.
0.1 g/L 13-algae + coal	10.8 ± 0.9	9765	15.3 ± 0.7
0.1 g/L 13-algae + GB	30.9 ± 1.2	38703	15.7 ± 1.6
0.1 g/L 13-yeast + coal	12.5 ± 3.0	11748	17.8 ± 1.4
0.1 g/L 13-yeast + GB	34.7 ± 0.8	46290	21.9 ± 0.8
0.5 g/L 13-algae + coal	38.0 ± 1.2	53488	15.4 ± 0.8
0.5 g/L 13-algae + GB	53.1 ± 0.5	99664	11.5 ± 0.0
0.5 g/L 13-yeast + coal	38.6 ± 1.2	54929	21.7 ± 0.5
0.5 g/L 13-yeast + GB	55.8 ± 1.3	111445	21.2 ± 1.2

All ^{13}C-amended GB treatments had a higher percentage of total methane produced as $^{13}CH_4$ than the corresponding coal treatments. This observation would be expected because the primary carbon source in the GB treatments is the amendment itself. The treatments with 0.5 g/L amendment produced a greater fraction of methane as $^{13}CH_4$ than the 0.1 g/L amended treatments with the same solid substrate indicating a greater amount of amendment-to-methane conversion in the treatments at higher amendment concentration.

Quantifying amendment conversion. Comparing the amount of $^{13}CH_4$ produced to the amount of ^{13}C added as amendment gives the percentage of ^{13}C from the amendment that was converted to methane (Table 5.2). For all ^{13}C-amended treatments, regardless of solid substrate, less than 22% of the amendment ^{13}C was converted to $^{13}CH_4$. 0.1 g/L algae amended treatments converted 15.3% and 15.7% of the ^{13}C-amendment to $^{13}CH_4$ for coal and GB treatments, respectively. Coal and GB treatments amended with 0.5 g/L algae converted 15.4% and 11.5%, respectively, of the ^{13}C from the amendment to $^{13}CH_4$. 0.1 g/L yeast amended treatments converted 17.8% and 21.9% of the ^{13}C-amendment to $^{13}CH_4$ for coal and GB treatments, respectively. Coal and GB treatments amended with 0.5 g/L yeast converted 21.7% and 21.2%, respectively, of ^{13}C-amendment to $^{13}CH_4$. From this analysis, it appears that a smaller percentage of algae is converted to methane than for yeast, and these results suggest that less of the algae extract is used directly by methanogens and/or more is used by primary- and secondary degraders compared to yeast extract. For treatments with the same amendment type, similar fractions of the

added amendment were converted to $^{13}CH_4$ regardless of solid substrate or initial amendment concentration.

Though ^{13}C from the amendment that was not converted to methane (approximately 78% or greater) could remain unutilized by the microbial community and therefore unchanged, it is also possible that it could be converted to other byproducts besides methane. It is likely that some of the ^{13}C from the amendment was converted to $^{13}CO_2$ and slightly elevated $^{13}CO_2/^{12}CO_2$ ratios were observed (Appendix G). Other potential fates of the ^{13}C from the amendment addition are intermediate byproducts, such as acetate, and incorporation into biomass.

Other carbon sources for methane production. Glass bead treatments amended with ^{13}C-labeled algae or ^{13}C-labeled yeast at both concentrations investigated never exceeded 56% of the total amount of methane produced as $^{13}CH_4$. If the ^{13}C-labeled amendment was the only available carbon source in these treatments, it could be assumed that the percentage of total methane produced as $^{13}CH_4$ should be equivalent to the ^{13}C percentage of total amendment carbon: approximately 95% $^{13}CH_4$ for ^{13}C-algae amended treatments and 97% $^{13}CH_4$ for ^{13}C-yeast amended treatments. Because the GB treatments with ^{13}C-amendment produced significantly less $^{13}CH_4$ than expected, it is hypothesized that there are other carbon substrates for biogenic methane production available in these treatments.

One of the other potential carbon sources for methane production is carbon in the formation water used in the microcosms. This water contains dissolved organic and inorganic carbon (DOC and DIC) and, because the formation water was unfiltered,

particulate organics also. A second potential carbon source for methane production is the carbon transferred with the inoculum. Each batch reactor was inoculated with slurry from a Flowers-Goodale derived enrichment that was 10% of the total reactor volume. The inoculum slurry also contains DOC and DIC that could potentially be used for methane production. In addition, the inoculum was a coal enrichment and may contain small coal particles that were transferred from the inoculum enrichment to the GB microcosms. Lastly, the microorganisms may themselves be a carbon source for methane production during biomass turn over. To assess these potential carbon sources for methane production, a second study was performed using filtered formation water and measuring initial DOC (measured as NPOC – non-purgeable organic carbon), DIC, and inoculum carbon content.

Study 2

To further investigate the conversion of coal and amendment to methane, a second study was performed using only algae amendment at a concentration of 0.1 g/L. This study included unamended coal, 0.1 g/L unlabeled algae amended coal and GB treatments, and 0.1 g/L ^{13}C-labeled algae amended coal and GB treatments. To assess other carbon sources besides coal and amendment for biogenic methane production, the initial DIC and DOC concentrations were measured. All treatments initially contained 257 µmol DIC/g coal (or GB) and 6 µmol DOC/g coal (or GB). The inoculum used contributed an additional 55 µmol C/g coal (or GB). It was assumed that the added DIC, DOC, and inoculum carbon contained of ^{13}C at a natural isotope abundance of

approximately 1.1%. Conversions of any of these carbon sources to methane are assumed to produce unenriched methane with 1.1% or less as $^{13}CH_4$.

Total Methane Production. All 5 treatments in the second study produced methane during the 126-day study. Coal treatments amended with 0.1 g/L algae produced similar amounts of total methane: 49.3 ± 0.9 µmol CH_4/g coal for treatments amended with unlabeled algae and 45.6 ± 1.0 µmol CH_4/g coal for treatments amended with ^{13}C-labeled algae. For GB treatments amended with 0.1 g/L algae, treatments amended with unlabeled algae produced a total of 13.0 ± 0.5 µmol CH_4/g GB and treatments amended with ^{13}C-labeled algae produced 12.7 ± 0.1 µmol CH_4/g GB. Unamended coal treatments produced 19.3 ± 13.5 µmol CH_4/g coal by the end of the study (Figure 5.3a).

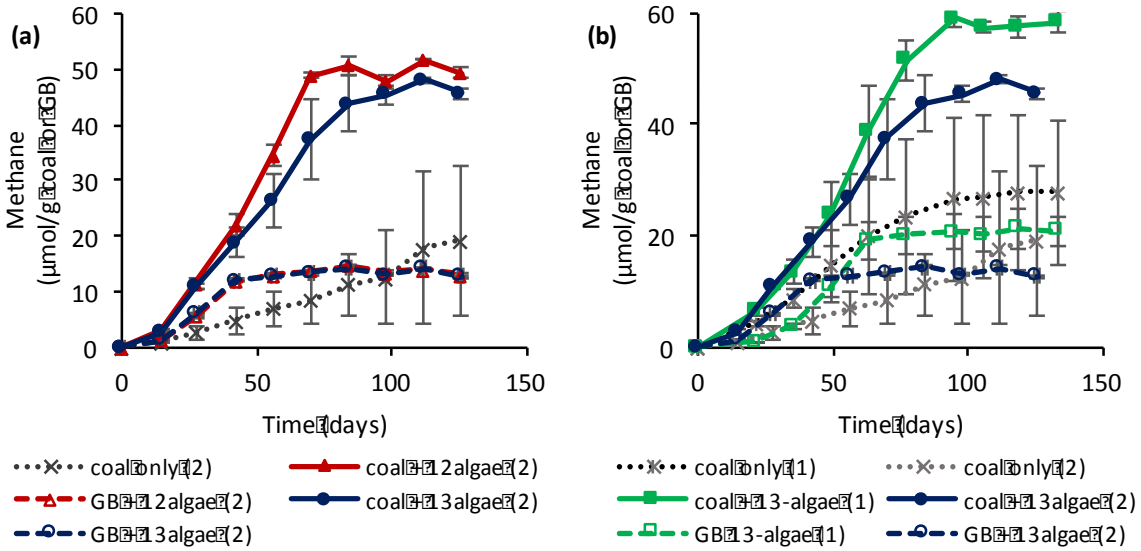

Figure 5.3: (a) Total methane production versus time for all treatments from experiment 2 and (b) a comparison of methane production of similar unamended coal and 0.1 g/L ^{13}C-labeled algae amended treatments for both studies (experiment 1 and 2).

When the total methane production is compared for similar treatments in study 1 and study 2 (Figure 5.3b), it is observed that all treatments in study 1 produced more methane than the same treatment in study 2: 12.7 ± 2.1 µmol CH_4/g coal more for ^{13}C-algae amended coal treatments, 8.2 ± 2.7 µmol CH_4/g GB more for ^{13}C-algae amended GB treatments, 8.5± 18.8 µmol CH_4/g coal more for unamended coal treatments.

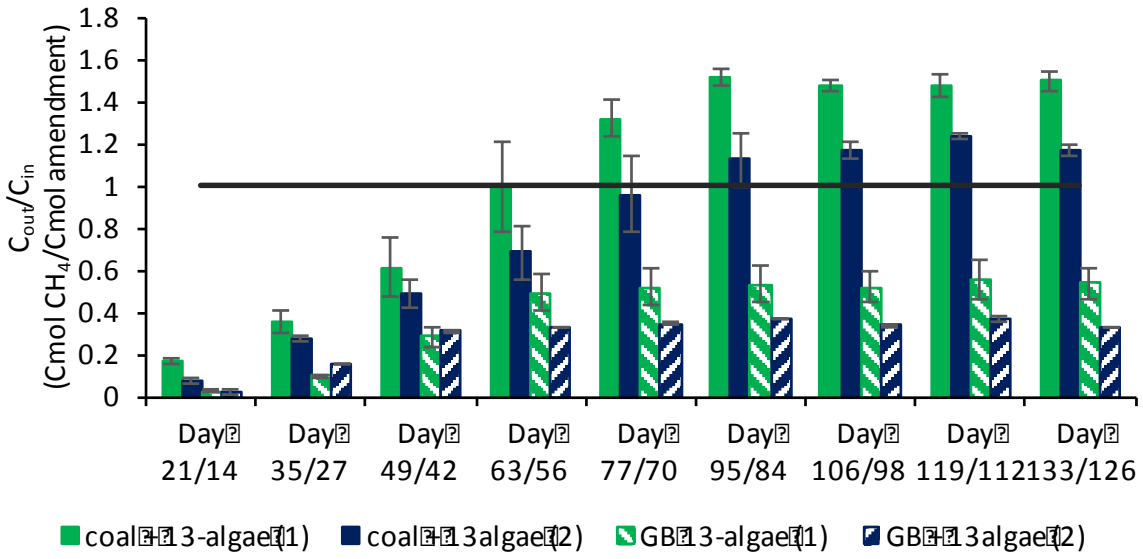

Figure 5.4: Comparison of total carbon produced as methane to carbon added as amendment for 0.1 g/L ^{13}C-algae amended coal and GB treatments from both studies. Error bars represent one standard deviation. A value greater than 1 indicates more methane production that could be attributed to complete amendment conversion to methane. Days are presented as "Day study 1/study 2".

When the amounts of carbon produced as methane are compared to the carbon added as algae amendment (C_{out}/C_{in}) as previously described, the ^{13}C-algae amended coal treatments from the second study have a C_{out}/C_{in} ratio greater than 1 by day 84 indicating definite coal-to-methane conversion (Figure 5.4). The ^{13}C-algae amended GB treatments from study 2 do not have a C_{out}/C_{in} ratio greater than 1 at any time during this study. These observations are similar to what was observed for the same treatments in study 1.

However, the treatments in study 1 have a higher C_{out}/C_{in} ratio than the corresponding treatment in study 2 for both coal and GB treatments due to the higher amounts of methane produced.

The primary difference between the initial conditions of each study was that in study 1, unfiltered formation water was used and, in study 2, 0.2 µm filtered formation water was used. This difference in total methane production between the 2 studies suggests that a carbon source for methane production in study 1 was particulate organic matter in the formation water > 0.2 µm in diameter.

^{13}C Amendments and Methane Production. The methane produced by unamended coal treatments from the second study was approximately 0.9% $^{13}CH_4$, similar to the natural abundance of ^{13}C and the amount produced by the unamended coal treatments in study 1. The ^{13}C-algae amended coal treatments produced 4.9 ± 0.2 µmol $^{13}CH_4$/g coal which was approximately 10.7% of the total amount of methane produced. The ^{13}C-algae amended GB treatments produced 6.9 ± 0.0 µmol $^{13}CH_4$/g coal which was approximately 54.2% of the total amount of methane produced (Table 5.3).

Table 5.3: Percentages of $^{13}CH_4$ produced and amounts of $^{13}CH_4$ and $^{12}CH_4$ produced in both studies.

Treatment	**% Total Methane as $^{13}CH_4$**		**Total $^{13}CH_4$ (µmol/g coal or GB)**		**Total $^{12}CH_4$ (µmol/g coal or GB)**	
	Study 1	**Study 2**	**Study 1**	**Study 2**	**Study 1**	**Study 2**
coal only	0.9 ± 0.1	0.9 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	27.5 ± 12.9	19.1 ± 13.3
13-algae + coal	10.8 ± 0.0	10.7 ± 0.2	6.3 ± 0.0	4.9 ± 0.2	52.1 ± 3.6	40.8 ± 0.9
13-algae + GB	30.9 ± 0.0	54.2 ± 0.5	6.5 ± 0.0	6.9 ± 0.0	14.4 ± 1.2	5.5 ± 0.1

Effects of filtered formation water. The use of filtered CBM formation water in the second study resulted in a decrease in overall methane production in all treatments

when compared to similar treatments from the first study, regardless of amendment addition or presence or absence of coal. For all ^{13}C-amended treatments, comparable amounts of $^{13}CH_4$ were produced by similar treatments in both studies, and the observed decrease in total methane production was almost entirely a decrease in the amount of produced $^{12}CH_4$ (Table 5.3). This observation indicates that the carbon source for the additional methane production observed in study 1 was not the amendment itself. Thus, it is hypothesized that the higher amounts of methane produced in treatments during study 1 versus comparable treatments during study 2 was due to the presence of an additional easily converted carbon source in the unfiltered formation water that was removed during filtration for the second study. The methane potential of this particulate carbon (>0.2 µm) in the unfiltered production water is assumed to be equivalent to the differences observed in $^{12}CH_4$ production between the 2 studies and is estimated to be between 8.4 and 11.3 µmol CH_4/g coal.

In the second study with filtered formation water, the ^{13}C-algae amended GB treatments produced a greater percentage of the total amount of produced methane as $^{13}CH_4$: 54.2% versus 30.9% in the first study. However, the total amount of $^{13}CH_4$ produced was similar in both experiments: 6.5 ± 0.0 µmol $^{13}CH_4$/g GB for study 1 versus 6.9 ± 0.0 µmol $^{13}CH_4$/g GB for study 2. The primary methane production difference between the study 1 and study 2 0.1 g/L ^{13}C-algae amended glass bead treatments was the amount of $^{12}CH_4$ produced: 14.4 ± 1.2 µmol $^{12}CH_4$/g GB in study 1 and 5.5 ± 0.1 µmol $^{12}CH_4$/g GB in study 2. While the amended GB treatments in study 2 indeed produced less $^{12}CH_4$ than in study 1 (likely due to the removal of particulate organic matter by

filtration of the formation water used for microcosm set up), it is clear that there is still another carbon source in these treatments that has approximately natural abundance of ^{13}C.

Other carbon sources for methane production. The alternate carbon sources in the formation water might be as previously stated, DIC and DOC, carbon transferred with the inoculum slurry, or biomass turnover of the microbial consortium itself. The initial DIC in the systems was 257 µmol carbon/g GB. In the studies presented in Chapter 4, it was shown that total system inorganic carbon increased during incubation in similar treatments, and it is thought that CO_2 (inorganic carbon) is another byproduct of coal and amendment degradation. Thus, it is hypothesized that while DIC could be converted to CH_4 by hydrogenotrophic methanogens, it is unlikely that this would cause a net decrease in DIC.

The initial DOC in treatments in study 2 was 6 µmol carbon/g GB. Dissolved organic compounds commonly found in CBM production water include phenols, heterocyclic compounds, and polyaromatic hydrocarbons.[101] It is likely that microbial degradation of coal and algal amendment produces additional dissolved organic compounds that can be utilized by other microorganisms and, ultimately, methanogens. DOC is likely a more bioavailable nutrient source for microbial processes than the coal and possibly even intact algae cells and could thus be a carbon source for methane production. If all of the initial 6 µmol C/g coal or GB DOC was converted to methane, this would account for the 5.5 µmol $^{12}CH_4$/g GB produced in the ^{13}C-algae amended GB treatments in study 2. However, as previously discussed in Chapter 4, conversion of

organic compound to methane also produces some amount of CO_2. Thus, it is likely that even full conversion of the initial DOC would account for only part of the 5.5 µmol $^{12}CH_4$/g GB produced in the ^{13}C-algae amended GB treatments in study 2.

The third potential source of carbon for methane production is the inoculum slurry which contributed 55 µmol carbon/g GB to the system. The inoculum slurry was transferred with a 23-gauge needle (inner diameter ~337 µm). Because the inoculum was a microbial consortium from a coal-containing batch reactor, it is likely that some DOC and coal particles <337 µm in diameter were also added to the experimental treatments. These additional carbon sources contain environmental concentrations of ^{13}C, approximately 1.1%, and could be a substrate for methane production. In addition, it has been shown that the turnover of microbial cells for metabolic processes does occur[85] and thus, methane could also be produced from the breakdown of dead biomass. Thus, from this discussion of alternative carbon sources for methane conversion, it is likely that some of the $^{12}CH_4$ produced in coal-containing microcosms that has been attributed to coal-to-methane conversion is actually due to the conversion of the alternative carbon sources discussed in the context of the GB treatments.

Conclusions

The work presented suggest that ^{13}C-labeled organic amendments for enhancement of coal-to-methane conversion are as effective as unlabeled amendments. It appears that the effect of microbial preference for the ^{12}C isotope is undetectable with the accuracy of the methods used. As shown in Chapter 3, the treatments amended with 0.1

g/L algae or yeast cells produced more methane than can be accounted for by complete conversion of carbon from amendment addition while 0.5 g/L amended treatments did not. Treatments that were unamended or amended with unlabeled amendment produced unenriched methane that contained near natural abundance of ^{13}C. All treatments containing ^{13}C-labeled amendments produced methane that was significantly enriched in $^{13}CH_4$. Treatments without coal, containing glass beads instead, and with equivalent amounts of ^{13}C-labeled amendments produced methane that was more highly enriched in $^{13}CH_4$ than treatments containing coal. Also, treatments amended with 0.5 g/L ^{13}C-labeled amendment produced more ^{13}C-enriched methane than treatments amended with 0.1 g/L with the same solid substrate.

Formation water from a CBM producing aquifer appears to contain particulate organic matter, which can function as a carbon source for methane production. This became obvious when the use of 0.2 µm pore filtered formation water for microcosm studies resulted in a decrease in the total amount of methane produced by approximately 8-12 µmol CH_4/g coal or GB.

However, even in filtered formation water, non-coal treatments still produced approximately 40% of the total methane as unlabeled $^{12}CH_4$ indicating another carbon source with natural abundance of ^{13}C besides particulate organic matter in unfiltered CBM formation water. Potential contributors to unlabeled methane production in the non-coal treatments are initial DOC and DIC in the filtered formation water, dissolved or nanoparticulate organic carbon as well as organic carbon or biomass contained in the inoculum.

Using the methane carbon source insights obtained by the used of ^{13}C-labeled amendments, the methane produced from direct coal conversion was estimated at approximately 35 µmol CH_4/g coal for 0.1 g/L ^{13}C-algae amended coal treatments and 14 µmol CH_4/g coal for unamended coal treatments. Thus, the use of ^{13}C-labeled amendments not only confirmed that amendment addition does indeed increase coal-to-methane conversion, it provided the data to quantify the coal-to-methane conversion increase of approximately 250%.

CHAPTER SIX

DEVELOPMENT AND PILOT TESTING OF COLUMN REACTORS FOR THE STUDY OF ANAEROBIC SUBSURFACE PROCESS

Contribution of Authors and Co-Authors

Manuscript in Chapter 6

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Contributions: Designed and performed experimental work, analyzed data. Wrote and revised manuscript.

Co-Author: George A. Platt

Contributions: Contributed to reactor design and experimental work.

Co-Author: Randy Hiebert

Contributions: Contributed to reactor design and implementation.

Co-Author: Robert Hyatt

Contributions: Contributed to reactor design and manufacture.

Co-Author: Matthew W. Fields

Contributions: Contributed to experimental design and writing and revision of the manuscript with comments and feedback

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Contributions: Contributed to experimental design and writing and revision of the manuscript with comments and feedback

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Abstract

A bench-scale column reactor system was constructed for investigations of subsurface anaerobic microbial processes. The system described can maintain an oxygen-free environment with continuous or pulse flow through a porous media packed column to mimic the subsurface environment. Sampling flexibility for both gas and liquid is ensured with septum sampling/injection ports in multiple locations, and produced gases can be separated and collected in the "gas trap" for analysis. Studies utilizing this reactor system can provide important information for implementing the scale-up of methods developed in batch microcosms systems. Additionally, strategies can be devised for *in situ* applications by providing a surrogate subsurface environment requiring less time and economic investment for investigations. To demonstrate the effectiveness of the described reactor system, methods for enhancing microbial coal-to-methane conversion developed in previous batch system experiments were implemented with the described system. Four reactor columns were packed with coal and inoculated with a microbial consortium from the same Flowers-Goodale coal bed. Two of the reactors were amended with ^{13}C-labeled algal biomass on day 0, and two were left unamended. On day 61, one previously amended and one previously unamended reactor were re-amended. Produced gases were captured in the gas trap, and CH_4 and CO_2 were quantified. Amendment was shown to increase the rate of coal-to-methane conversion and total gas production under flow regimes.

Introduction

Transition from batch to flow laboratory experiments

Laboratory studies of subsurface microbial processes are generally limited to small scale, batch reactors that cannot account for the effects of groundwater flow on abiotic and biotic processes. Opportunities for field studies of the subsurface environment are limited, work-intensive, and costly and thus require significant planning and preparation to maximize success. To facilitate the transition from small, batch scale experiments to field experiments and technology development, it is important to scale-up laboratory studies to better mimic the *in situ* environmental conditions. Experiments using flow reactors can scale-up the reactor volume to investigate effects of flow and transport on the processes of interest to expand understanding for the development of strategies for field-scale experiments and technology application.

Many subsurface microbial processes require oxygen-free environments and only occur at low redox potential. Batch reactors are a simple and useful tool for studying subsurface microbial processes that require a strict oxygen-free environments and have been used to investigate various microbial processes such as environmental contaminant degradation[102–104], corrosion with sulfate-reducing bacteria[105,106], and microbial coalbed methane (CBM) production[31,35,107]. While these systems are simple to use, they are limited in scope due to potential microbial inhibition resulting from substrate depletion, gas accumulation, or byproduct inhibition. While many studies have been performed in the laboratory using batch reactors assembled using anaerobic techniques, very few

studies have been published that utilize flow reactor systems that can maintain an oxygen-free environment for studying subsurface processes with a continuous flow. In addition to creating more field-relevant conditions, flow systems can also reduce the effects of substrate depletion and byproduct accumulation.

Continuous or pulse flow reactors have been developed to study the scale-up of various microbial processes such as sulfate reduction[108–110], reductive dechlorination[111,112], and biomineralization[113,114]. However, studying anaerobic microbial processes in flow systems comes with a unique set of challenges. Challenges to any flow reactor system include choosing an appropriate flow rate to mimic observed *in situ* flow rates, reproducibility between systems, and potentially higher operating costs. Microbial processes can be affected by flow rates, nutrient loading, gas accumulation, substrate utilization, and byproduct accumulation.[76] Adding the requirement of an oxygen-free environment results in additional challenges. Some of these challenges are purging oxygen from the system prior to adding oxygen-free media and anaerobic cultures, preventing oxygen infiltration, and keeping media reduced to the appropriate oxidation-reduction potential (ORP) for the duration of the study. These challenges for anaerobic systems can be overcome by constructing reactors and running experiments in an anaerobic chamber. However, the difficulty of working within an anaerobic chamber can make this option less appealing.

The upflow column reactor system discussed here is designed for laboratory bench-top use and can maintain an oxygen-free environment for all components upstream of the reactor column, the reactor column itself, and the gas collector column to ensure

the desired low ORP. The system can be run at varying flow rates. Each reactor system has sampling ports at the top and bottom for injection or sampling and a gas trap where the produced gases are separated and collected for sampling and analysis. The system can run up to four separate reactor systems from the same influent liquid source to allow up to four replicates of the same treatment or different treatments of each reactor while each has the same influent source.

Microbially Enhanced Coalbed Methane (MeCBM)

Biogenic coalbed methane (CBM) is produced by microbial consortia that convert coal to methane in subsurface coal seams and is considered an unconventional gas resource.[2,4] CBM has been extracted in many coal basins around the world, but commercial extraction rates often exceed rates of microbial coal-to-methane conversion, resulting in short well lifespans. To enhance coal-to-methane conversion, methods have been investigated for biostimulation using various organic and/or inorganic nutrient amendments[2,4,79] and Chapter 3. Other studies have investigated methods for increasing coal bioavailability through oxidation[41,64] or chemical treatment[56] or by increasing surface area.[28,34,42] Research to develop methods for increasing total methane and rates of microbial coal-to-methane conversion to produce more CBM faster has mostly utilized batch systems. While batch studies are useful and fairly inexpensive systems for experimental purposes, their meaningfulness can be limited and since they neglect to account for important *in situ* conditions. In order to apply the methane-enhancing strategies developed in batch systems *in situ*, it is necessary to scale-up these strategies to intermediate systems that incorporate more field-relevant parameters such as flow. To

date, only one flow system study of microbial coal-to-methane conversion has been published.[77] The reactor system described here was designed with scale-up of microbial CBM processes in mind.

Motivation for investigation

The purpose of this paper is to describe the upflow column reactor system for studies of gas-producing microbial processes requiring an oxygen-free environment. To demonstrate the potential of this system, an experiment was conducted under oxygen-free conditions to implement methods for increasing the microbial coal-to-methane conversion that were previously investigated in batch systems. Implementation of these methods in flow reactors is an important intermediate step to implementing these strategies in a future field trial.

Materials & Methods

Reactor Design and Construction

The upflow column reactor system was designed to maintain an oxygen-free environment under flow conditions to capture produced gases for studies of anaerobic microbial processes. This reactor system can be assembled on a laboratory benchtop and does not require an oxygen-free environment outside of the reactor to maintain the conditions necessary for studies of anaerobic processes. A schematic of the system described is shown in Figure 6.1.

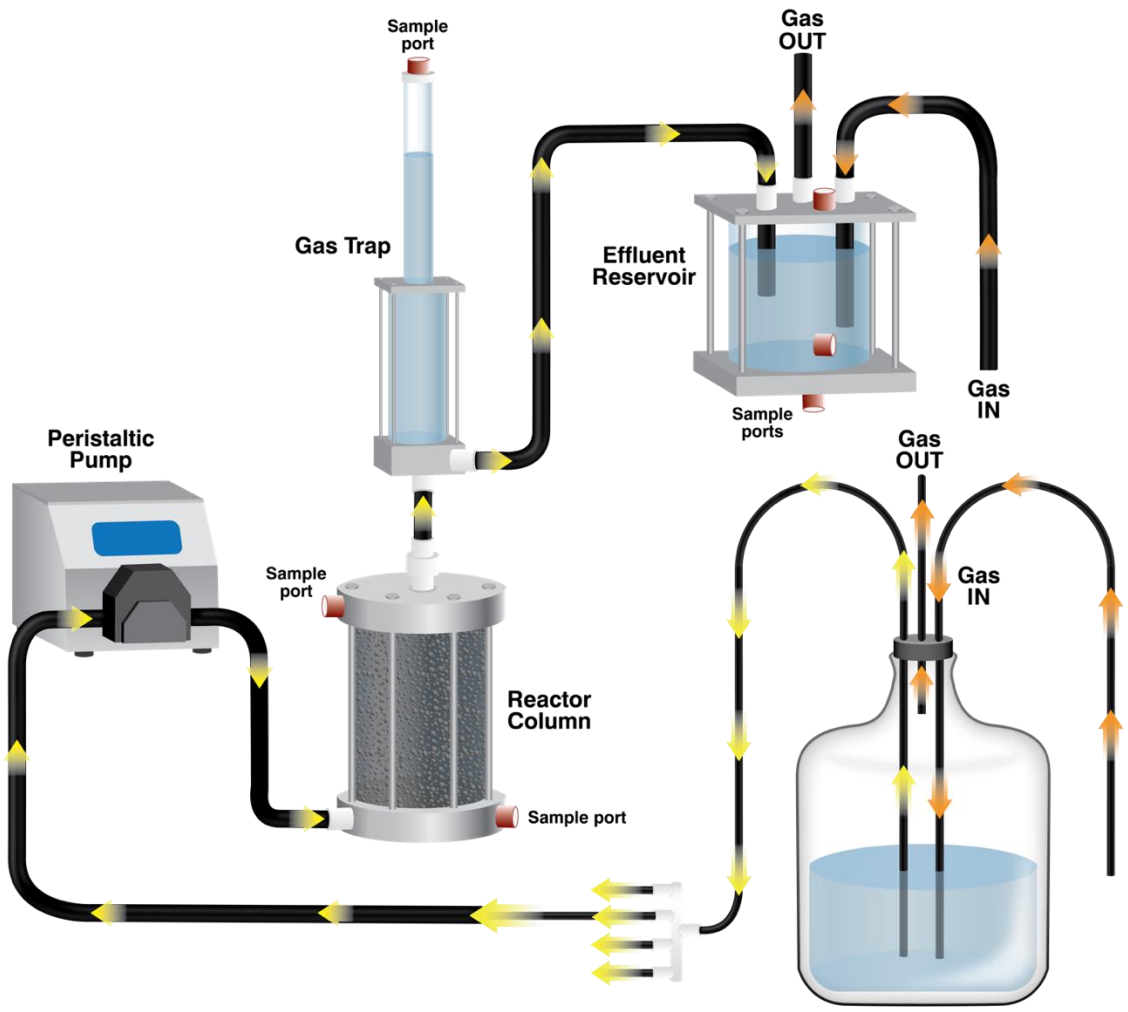

Figure 6.1: A schematic of the reactor system showing all components of a single reactor system and designating the split from the carboy supplying the influent to three other identical setups.

The reactor column was constructed from type 316/316L stainless steel threaded pipe with inner diameter of 2.067 inches, wall thickness of 0.154 inches, and length of 3 inches. The threaded caps were 316 stainless steel rated to a pressure of 150 psi. All components for the reactor body were purchased from McMaster-Carr (Elmhurst, IL, USA). Holes were drilled in both caps and 1/8 inch NPT threads were cut for fittings. The bottom reactor cap (influent side) had two threaded holes on the side of the cap, 90

degrees apart. One was fitted with a 1/16-inch barbed fitting for influent flow and attached to size 13 Masterflex® Norprene tubing. The other was fitted with a septum port fitting for sampling and injection. The top reactor cap had two fitting holes, one on the side and one on the top. The side hole was fitted with a septum port fitting for reactor sampling. The top hole was fitted with a ¼-inch barbed fitting for effluent flow and attached to size 25 Masterflex® Norprene tubing. The total sealed reactor volume is 300 mL. The reactor body can be packed with any porous media for a planned experiment, such as sand, soil, or coal.

The gas collection tube ("gas trap") was made of polycarbonate tubing. Two sizes were made to accommodate experiments with varying amounts of expected gas production. The smaller gas trap has a total volume of 55 mL while the larger has a total volume of 125 mL. Each gas trap was fitted on the top with ¼-inch NPT septum port for gas collection and sampling. The gas trap was designed such that the effluent from the reactor (gas-trap influent) enters at least 3 inches above where the gas-trap effluent leaves the gas trap to go to the effluent reservoir. This allows for gas/liquid separation and results in the gas being captured in the gas trap where it can be sampled for analysis while the liquid effluent continues to flow to the effluent reservoir. The effluent fitting is a ¼-inch barbed NPT attached to size 25 Masterflex® Norprene tubing.

The effluent reservoir is a 4.5-inch long, 3.5-inch inner diameter polycarbonate tube. The tube is capped with polycarbonate plates which were drilled for fitting attachment. The base has one ¼-inch NPT hole for a septum fitting. The lid had 4 holes drilled: one for ¼-inch NPT barbed fitting connected to Masterflex® Norprene tubing for

the reactor effluent flow from the gas trap, one for a septum sampling fitting, and two for ¼-inch NPT barbed fittings for periodic sparging and venting the reservoir with oxygen-free gas.

The liquid media reservoir is a 15-L glass carboy. For long-term experiments with low flows requiring low ORP influents, a secondary containment was included inside the glass influent carboy. An 8L gas impermeable Tedlar® bag was place inside the carboy and attached to the tubing leading out of the carboy. This bag is loaded with the desired influent media to provide 2 layers of protection against oxygen infiltration. The Tedlar® bag is fitted with a 1/8-inch barbed fitting and attached to the reactor system plumbing. The bag is loaded with the desired liquid media. The carboy is sealed with a butyl rubber stopper. The rubber stopper was drilled to install a piece of glass tubing which is attached to Masterflex® Norprene tubing and the oxygen-free gas purge. A second hole was drilled for 1/8-inch stainless steel tubing attached inside the carboy to the Tedlar® bag holding the media and outside the carboy to the first 3-way ball valve leading to the reactor systems. This 3-way valve is connected to a second 3-way ball valve which is connected to 1/8-inch stainless steel tubing leading to the reactor systems. The two 3-way ball valves in series allow the reactor systems to be isolated from the media carboy so that the systems can be purged with oxygen-free gas prior to loading and inoculation from the effluent reservoir to the top of the carboy where the second 3-way valve serves as a vent. The first 3-way valve can isolate the carboy from the reactor systems so that media can be prepared outside of the system and then loaded into the influent Tedlar® bag reservoir.

From the top of the influent carboy to the peristaltic pumps, the system was plumbed with 1/8-inch stainless steel tubing with a valve system designed so that 1-4 reactors can be run simultaneously and the number of active reactors can be changed by opening or closing valves instead of re-working the plumbing. The stainless-steel tubing and fittings were all fabricated by Swagelok (Solon, OH). Two Masterflex® L/S Standard Digital Drive peristaltic pumps with two-channel Easy-Load II pump heads (Cole-Instrument Company, Vernon Hills, IL, USA) were used for the 4 reactor systems with each pump feeding two reactor columns. The valve and tubing layout are shown in schematic in Figure 6.2.

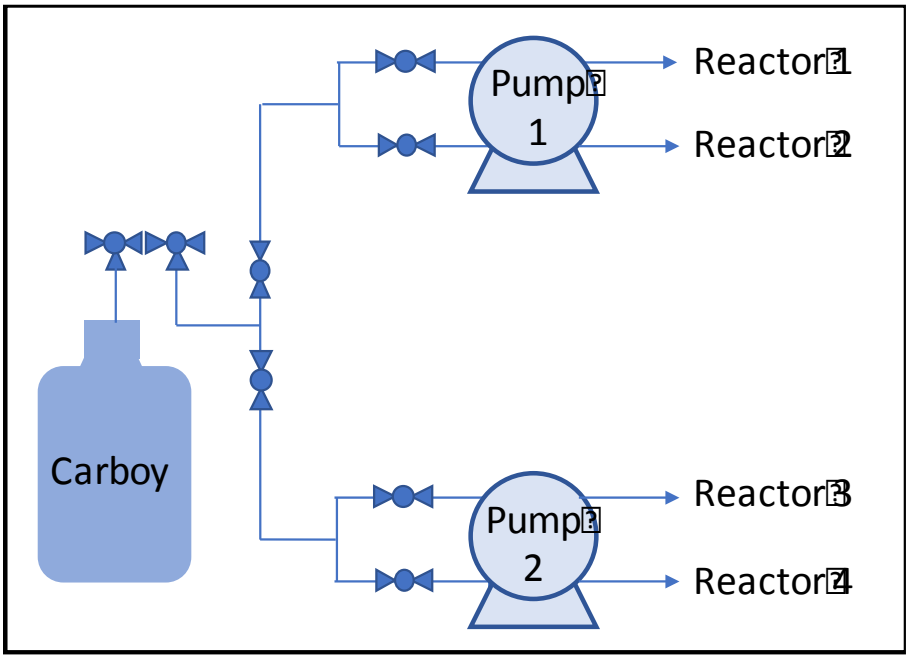

Figure 6.2: Schematic showing the valves and plumbing set up of the reactor system containing 4 individual reactor columns.

Sampling protocols

The reactor system was designed with flexibility in sampling protocols in mind. Liquid samples can be taken via septum sampling ports from the bottom and top of the reactor body and the effluent reservoir and via a luer-lock fitting on the first 3-way valve to sample the influent reservoir. Additions to the system can also be made at any of these locations. Gas can be sampled from the top of the gas trap and the top of the effluent reservoir. Sampling port locations are designated in Figure 6.1. Destructive sampling at the end of the experiment allows access for sampling the porous media within the reactor body.

Reactor flow variation

This system can be rearranged to switch from a single-pass flow regime to a recirculating flow. To do this, the effluent reservoir of the single-pass system becomes both the influent and the effluent reservoir of the recirculating system. The septum sampling port in the base of the effluent reservoir can be replaced with a barbed fitting and connected to the Norprene tubing going through the pump.

Experimental Design

Enhancement methods for the microbial conversion of coal to methane described in previous chapters were scaled-up from batch reactors and tested in the reactor system described here.

Material sources and preparation

Coal cores from the USGS field site near Birney, MT were collected in 2013 from the Flowers-Goodale (FG) coal bed when three new wells were drilled and completed in this coal bed.[73] The coal cores were stored in FG formation water in PVC tubes at room temperature until use for this study. The FG core was dried in the anaerobic glove bag, crushed, and sized to 2-4 mm. This size was chosen to reduce the coal fines that could cause blockage of reactor plumbing. Coal density was estimated at 1.25g/cm^3 by the water displacement method. The volume of a known mass of coal was determined by measuring the amount of water it displaced. This measured density was slightly lower than the reported range of 1.44-1.54 for Flowers-Goodale coals.[73]

The formation water used in this study was collected in May 2016 from the Birney field site FGM-13 well. The plastic storage jugs were rinsed twice with formation water before being filled and stored at 4°C until use in this study. The microbial consortium used to inoculate the reactor system was collected 2 months prior to the start of the study from the USGS field site FG-09 well and treated as previously described in Chapter 3. The algal amendment used to enhance the coal-to-methane conversion was ^{13}C-labeled algal biomass grown and processed as previously described in Chapter 5 with a total carbon content of 46.8 % (w/w) and 95% labeled with ^{13}C. Seventy-five mg of ground algae was added to 5mL of degassed, reduced FG formation water in an oxygen-free serum bottle. One mL of this prepared amendment was added to designated amended reactors.

Reactor preparation

Prior to the experimental run of the reactor systems, all Norprene tubing and sampling port septa were replaced. The system was chemically disinfected by the following protocol. Two liters of each disinfection solution was prepared for: 1) 1% v/v bleach, 2) 1% w/v sodium chloride, 3) 0.25% w/v sodium thiosulfate, 4) 70% v/v ethanol. Each solution was pumped first into the influent bag and then into the reactor system. The entire volume of each solution was pumped into the reactor systems before the next was loaded into the influent bag. After all disinfectant solutions were pumped through the reactor system, three liters of 0.2 μm filtered FG formation water was loaded into the influent bag and distributed through the reactor systems to remove any residual disinfection solutions. All fluids were drained from the reactors, gas traps, and effluent reservoirs. The disinfected reactor columns were removed from the system and moved to a microbiological safety cabinet. One hundred ninety grams of prepared coal was loaded into each reactor column, and columns were replaced in the system.

Eight liters of formation water were filtered with 0.2 μm bottle top filters. Resazurin (1 mg/ L) was added as an oxygen indicator, and the water was sparged with 5% CO_2 balance of N_2 for 18 hours to reduce dissolved oxygen concentration. The formation water was reduced with sulfide (1 mM as $Na_2S{\cdot}9H_2O$) which acts as an oxygen scavenger. The prepared FG formation water was pumped into the Tedlar® bag inside the influent carboy. The FG water was left in the influent Tedlar® bag overnight to ensure the water maintained the desired low ORP as indicated by a lack of Resazurin color change.

The reactor system was purged of atmospheric air, specifically targeting the removal of O_2 by purging the effluent reservoir with 5% CO_2/95% N_2 and venting first from the top of the gas trap and then at the valve on top of the influent carboy. The reactor system was filled (all 4 reactors and gas traps) from the prepared FG formation water in the influent carboy by pumping at 6mL/minute. All four reactor systems were filled until the water just reached the effluent reservoir with gas displaced by formation water from all components except the effluent reservoir.

Both pumps to the reactors were run at the experimental flow rate of 0.005 mL/min for 48 hours to ensure all gas was removed and redox potential was maintained as indicated by no change of color due to Resazurin. All four reactors were inoculated from the bottom sampling port with 15 mL of microbial consortium (~10% v:v) collected from the FG-09 well. Reactors #1 and #2 were amended with 1 mL of the prepared algae amendment, resulting in 15 mg per reactor and 0.1 g/L amendment concentration. Two mL of the microbial consortium inoculum was stored at -80ºC for DNA extraction and community analysis.

Reactor breakdown and sampling

On day 172, reactor 1 was destructively sampled. The reactor column was isolated and removed from the rest of the system. The liquid contents were pushed out of the column into a Tedlar® bag by purging with nitrogen gas from the top of the column. The liquid contents were placed on a rocker table for 24 hours to allow gas dissolution and gas was analyzed. The inlet to the column was plugged and a second Tedlar® bag was attached to the top of the column for 7 hours to collect desorbed gases. Two vacuum

desorption tests were run using an ISCO D1000 pump (1000 mL) (Teledyne, Nebraska, USA). The first test applied a vacuum of -7 psi on the reactor column. The column was isolated under vacuum and the gases collected in the pump were collected in a Tedlar® gas sampling bag. A second vacuum of -10 psi was drawn on the column and maintained for 18 hours. These gases were again collected. All gases were analyzed analogously to the gas samples collected from the gas trap.

Gas analysis

Gas samples were collected from the gas trap for quantification and analysis using a gas tight syringe when at least 2 mL gas had accumulated. Methane and carbon dioxide were quantified 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 HayeSep-D packed stainless steel column (6 feet x 1/8" O.D.) was used with ultra-high purity helium carrier gas for separation using the following conditions: 1 mL manual injection, oven temperature 40°C, TCD temperature 150°C, and carrier gas pressure 8 psi. An Agilent 6890 GC 5973 electron impact ionization mass selective detector (Agilent Technologies, Palo Alto, CA, USA) interfaced with Agilent Enhanced ChemStation software and operated in scan mode was used to measure isotope ratios of $^{13}CH_4$:$^{12}CH_4$ and $^{13}CO_2$:$^{12}CO_2$. A GS-Carbonplot column (60 m × 0.320 mm i.d. × 1.50 µm film thickness) was used for analysis. The following parameters were used: 500 µL manual split ratio 30:1 injection, constant flow at 1 mL/min, injector temperature of 185 °C, interface 60 °C, and scan range m/z 2-100.

Ultra-high purity helium was the carrier gas. The % $^{13}CH_4$ and $^{13}CO_2$ were determined as described in Appendix F of this dissertation.

Results and Discussion

The pore volume for all reactors was calculated using the measured coal density and total reactor volume. With 190g of coal with a density of 1.25 g/cm^3 and 300 mL total reactor volume, the pore volume was calculated at 148 mL and effective porosity 0.49. At the flow rate of 0.005 mL/minute used in this study, each reactor had an average hydraulic residence time of 20.5 days. The reactor system was run for 172 days, or 8.4 retention times, before destructively sampling reactor 2 for desorption studies.

The seepage velocity in the FG formation at the USGS field site is estimated to be approximately 0.00634 ft/day.[73] The lowest accurate flow rate for the reactor system was determined to be 0.005 mL/min, which results in a seepage velocity of 0.022 ft/day in the reactors. Due to the lower flowrate limits (regarding accurate flow rates) of the peristaltic pumps used in this work, the seepage velocity of the reactors was about 3.5 times that observed in the FG coal bed. However, other coal beds have seepage velocities similar or exceeding the seepage velocity of the reactor systems.[73]

Gas Production

The amendment schedule and observation of first produced gas are summarized in Table 6.1. On day 0, reactors 1 (R1) and 2 (R2) were amended with 1 mL of the prepared ^{13}C-labeled algae amendment. The mass of amendment added per reactor was 15 mg resulting in an approximate concentration of 0.1 g/L, the concentration previously shown

by Davis et al. (2017) to enhance biogenic coal-to-methane conversion while minimizing microbial community changes. Reactors 3 (R3) and 4 (R4) were initially unamended. Gas was first observed in the gas trap of R1 on day 27 and R2 on day 33. On day 61, R1 was re-amended with 1 mL of the prepared algae amendment. R3, which was initially unamended and had not yet produced any measureable gas, was also amended with 1 mL of the prepared algae amendment. Continued gas production was observed for both R1 and R2 (initially amended reactors) for the duration of the study. Gas production was first observed in R3 on day 142, 81 days after algae amendment addition. R4, which was never amended, produced no measurable gas at any point during the 172-day study.

Table 6.1: Summary of amendment strategy and first observed gas for all four reactors.

Reactor	**Initial algae amendment (mg)**	**Day 61 amendment (mg)**	**First gas observed (day)**
R1	15	15	27
R2	15	0	33
R3	0	15	142
R4	0	0	n.a.

The methane and carbon dioxide production curves shown for all reactors in Figure 6.3 reflect measurements of gases collected from the gas trap. These production curves do not account for dissolved methane and CO_2, dissolved inorganic carbon (DIC), or gases sorbed to the coal. During the 172-day study, R1 produced a total of 1712.6 µmol of CH_4 and 128.6 µmol of gaseous CO_2; ^{13}C-label was measured at 2.3% $^{13}CH_4$ and 1.1% $^{13}CO_2$. R2 produced a total of 1485.8 µmol of CH_4 and 113.0 µmol of CO_2. Of these total produced gases 1.3% was measured as $^{13}CH_4$ and 1.0% as $^{13}CO_2$. R3 produced 278.9 µmol of CH_4 and 25.1 µmol of gaseous CO_2 with initial gas production observed

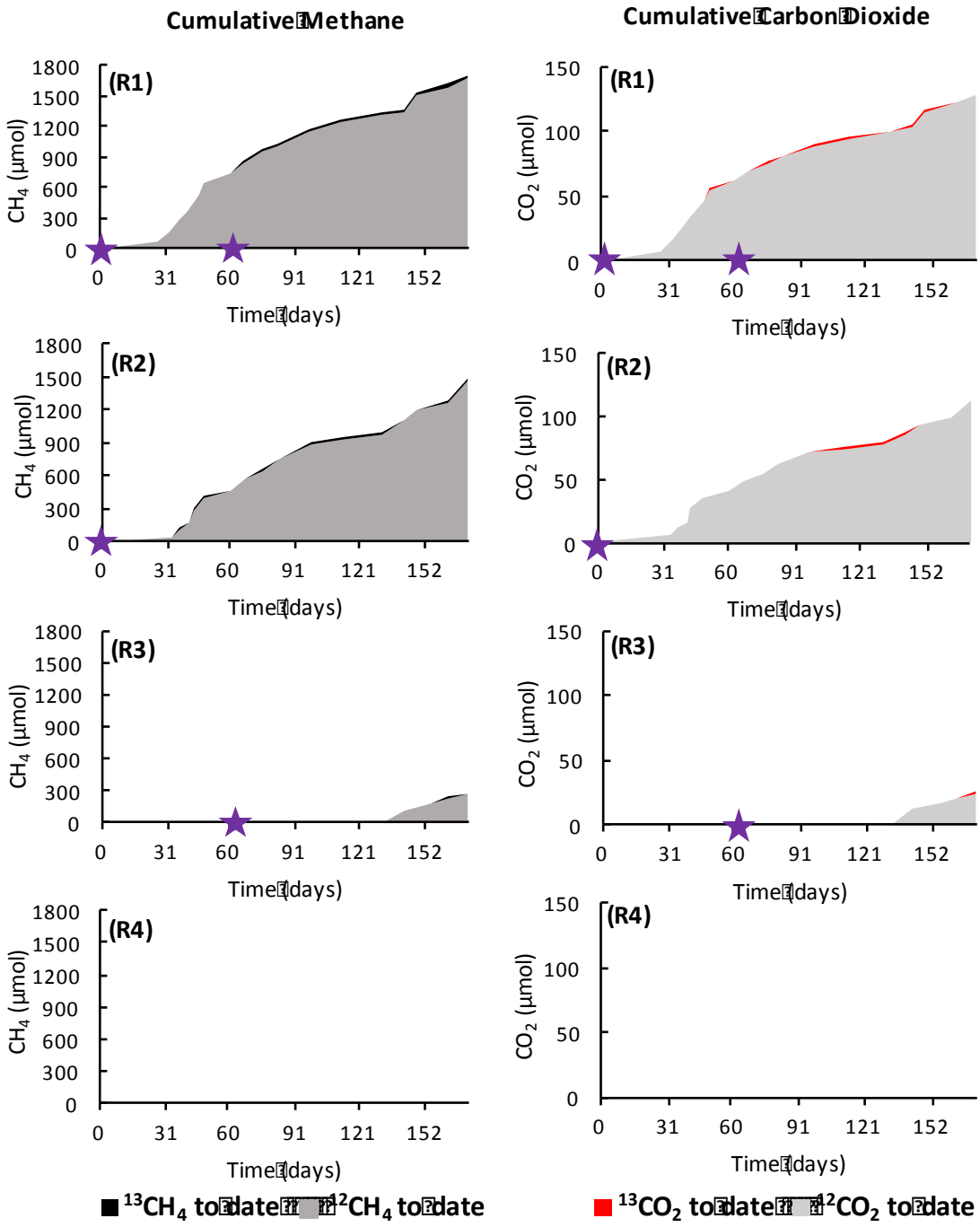

Figure 6.3: Methane and CO_2 production over time for all four reactors showing the ^{13}C-labeled contribution of each. Time of algae amendment is designated by stars (★).

on day 142; 1.9% of the methane was $^{13}CH_4$ and 1.0% of the CO_2 was $^{13}CO_2$. R4 produced no gases during the 172-day study period.

During the first 61 days, R1 and R2 were amended replicates while R3 and R4 were unamended replicates. Neither of the unamended reactors (R3 and R4) produced measurable gases by day 61. R1 produced a total of 740.3 µmol CH_4 and 62.0 µmol of CO_2; R2 produced 463.5 µmol of CH_4 and 41.2 µmol of CO_2. Both of these amended reactors produced 1.3% of total methane as labeled $^{13}CH_4$. The $^{13}CO_2$ fraction detected in both reactors appeared to be below the natural abundance of ^{13}C (1.1%). The differences in gas production between the amended reactors could be due to differences between the columns, such as preferential flow paths, minor variation in flow rates between pumps (though not observed to an appreciable amount over the duration of the study), or possible small leaks. A potential biotic difference between reactor systems is variation in microbial metabolic rates due to differences in microbial community structure. By day 83, R2 had produced a total of 760.5 µmol of CH_4 and 63.1 µmol of gaseous CO_2, reaching the 61-day totals for R1.

From day 61, when R1 was re-amended with a second algae addition and R3 was amended with a first algae addition, to the end of the 172-day study, the 4 reactor systems were all different treatments and no replicates existed. Comparing gas production for the 111 days remaining in the study, R1, amended initially and again on day 61, produced an additional 972.3 µmol of CH_4 and 66.6 µmol of CO_2. Of these produced gases, methane was 2.3% as $^{13}CH_4$ and carbon dioxide was 1.1% as $^{13}CO_2$. R2, initially amended but not re-amended on day 61, produced an additional 1022.3 µmol of CH_4 and 71.8 µmol of

CO_2. Of these produced gases, methane was 1.3% as $^{13}CH_4$ and carbon dioxide was 1.0% as $^{13}CO_2$. R3, which was unamended initially but amended on day 61, produced measureable gas 81 days after amendment on day 61. R3 produced 278.9 µmol of CH_4 and 25.1 µmol of CO_2. Of each of these total gases produced, 1.9% was $^{13}CH_4$ and 1.0% was $^{13}CO_2$. R4, the column that did not receive amendment, did not produce any measureable gas throughout the 172-day study.

While R1 produced the greatest sum of CH_4 and CO_2 gases of all the reactors, R2 produced 50 µmol more CH_4 than R1 during the last 111 days of the study, even though R1 received twice the amendment of R2. This observation suggests that the amendment serves to "jump-start" the microbial processes resulting in coal-to-methane conversion. Additional amendment, at least at the time-scale used in this study, may be unnecessary for increased coal-to-methane conversion once the "jump-start" of processes has occurred.

On a moles-produced basis, R3 produced 11.1x more CH_4 than CO_2, R1 produced 13.3x, and R2 produce 18.7x more CH_4 than CO_2. This observation, combined with the higher fraction of ^{13}C in the produced gases in R1, suggests that the utilization of the algal amendment itself results in a smaller CH_4 to CO_2 ratio than coal-to-methane conversion processes.

Analysis of Substrates for Gas Production

The total amount of carbon produced as CH_4 and CO_2 produced (C_{out}) was compared to the total amount of carbon added to the system in the form of algal amendment (C_{in}). Table 6.2a summarizes the comparison of C_{out}/C_{in} for the first 61 days

and the total study duration (Day 172). A C_{out}/C_{in} ratio of greater than 1 indicates conversion of coal to CH_4 and CO_2. The method of this comparison is explained in greater detail in Davis et. al. (in review) (Chapter 3).

Table 6.2: Comparison of (a) total carbon produced as CH_4 and CO_2 gases as a fraction of the carbon content of the algae amendment and (b) 13-carbon produced as $^{13}CH_4$ and $^{13}CO_2$ gases as a fraction of the 13-carbon content of the algae amendment.

(a)	**Day 61**				**Day 172**			
Reactor	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
CH_4 (µmol C)	740	464	0	0	1713	1486	279	0
CO_2 (µmol C)	62	41	0	0	129	113	25	0
Algae (µmol C)	585	585	0	0	1170	585	585	0
C_{out}/C_{in}	1.37	0.86	n.a.	n.a.	1.57	2.73	0.52	n.a.

(b)	**Day 61**				**Day 172**			
Reactor	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
CH_4 (µmol C)	9.4	6.0	0	0	38.7	19.0	5.4	0
CO_2 (µmol C)	0.5	0.4	0	0	1.5	1.1	0.3	0
Algae (µmol C)	556	556	0	0	1112	556	556	0
$^{13}C_{out}/^{13}C_{in}$	0.018	0.012	n.a.	n.a.	0.036	0.036	0.010	n.a.

In this study, R3, which was amended on day 61 and did not begin gas production until day 132, never produced enough CH_4 and CO_2 gases to exceed the amendment potential. However, the C_{out}/C_{in} ratio for R1 was greater than 1 on both day 61 and day 172. Therefore, in both the first 61 days after the first amendment and by the end of the study after a second amendment, R1 had produced more carbon in the form of methane and CO_2 than could have been attributed to amendment conversion alone. Thus, coal-to-methane conversion in this column indisputably occurred. The C_{out}/C_{in} for R2 was less than 1 after 61 days of the experiment and therefore it cannot be concluded with certainty that coal-to-methane conversion occurred in this reactor. However, on day 172, C_{out}/C_{in} for R2 was 2.73, the highest of all three gas producing reactors for both time

points assessed. This supports coal-to-methane conversion in R2 and indicates that almost 2/3 of the gases produced were certainly from the degradation of coal and not from amendment conversion.

While a direct comparison of C_{out}/C_{in} is useful for assessing the possible carbon source for gas production, this method assumes complete conversion of the algae amendment to CH_4 or CO_2. It should be noted that because it is likely that not all of the algae amendment was converted to CH_4 and CO_2, this method underestimates the contribution of coal conversion for gas production.

A second method was used to analyze the substrates used for gas production comparing the amount of ^{13}C produced as $^{13}CH_4$ and $^{13}CO_2$ produced relative to the ^{13}C added as amendment. These comparisons are summarized in Table 6.2b where $^{13}C_{out}/^{13}C_{in} << 1$ indicating that only a small amount of amendment was converted to measurable gas.

Thus, it can be concluded that some of the produced gases result from the conversion of the amendment itself and can be estimated by measuring the ^{13}C-labeled gasses. However, due to the low $^{13}C_{out}/^{13}C_{in}$ ratios, it is apparent that the majority of gas production in these systems is due to coal conversion. The microbial conversion of ^{13}C-labeled amendment to methane and CO_2 is measurable, and low amounts of $^{13}CH_4$ and $^{13}CO_2$ show that coal, not algal amendment, is the primary carbon source for gas production in these coal reactor systems.

Desorption Analysis

On day 172, column R1 was taken off-line for gas desorption analysis. Figure 6.4 shows the amounts and % ^{13}C-labeled of CH_4 and CO_2 recovered from the column R1

from (i) the gas trap during the 172-day experiment, (ii) the gases recovered from the reactor fluids, and (iii) from 3 different desorption steps. R1 produced 1712.6 µmol of CH_4 during the 172-day study which was 74.2% or the total methane recovered from the system. An additional 78.5 µmol of CH_4 was detected in the reactor fluid at the end of the experiment. The initial desorption lasting for 6.5 hours without any vacuum applied resulted in a recovery of 9.6 µmol of CH_4 which is equivalent to about 0.4% of the total methane recovered. The first subsequent vacuum desorption step (-7 psi) desorbed 304.4 µmol of CH_4 while the second vacuum desorption (-10 psi for 18 hours) desorbed an additional 201.5 µmol of CH_4 or 13.2 and 8.7%, respectively, of the total methane recovered (Figure 6.4a). The $^{13}CH_4$ contribution of each fraction is shown in Figure 6.4a as a percent label for each.

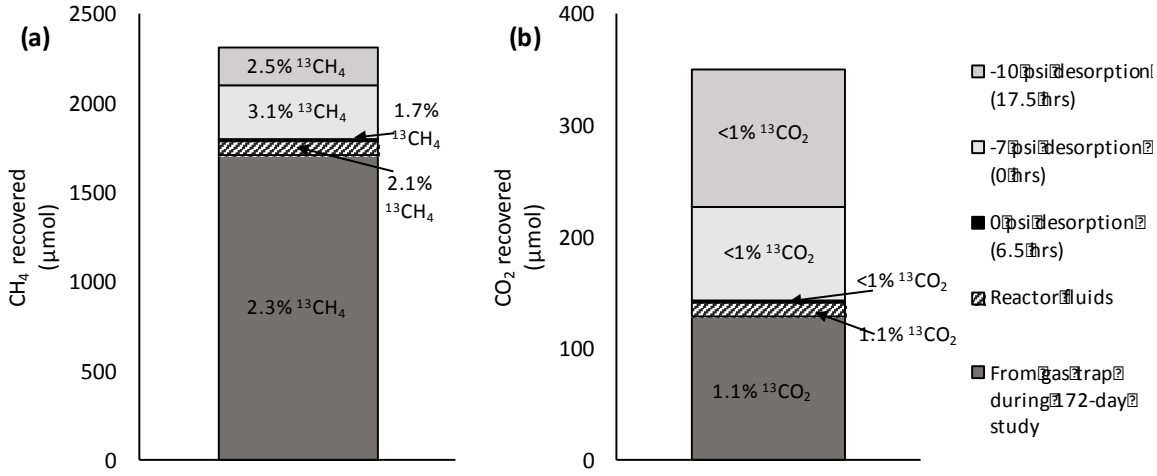

Figure 6.4: (a) Methane and (b) carbon dioxide produced by R1 during the experiment and during desorption. Percentages of the total gas measured as ^{13}C labeled gases are shown for each phase.

Column R1 produced 128.6 µmol of CO_2 during the 172-day study which was 36.7% of the total CO_2 recovered. An additional 12.3 µmol of CO_2 (or 3.5% of the

total CO_2) gas were detected in the reactor fluids at the end of the experiment. The 6.5-hour, no vacuum desorption resulted in 1.81 µmol of CO_2 which was only 0.5% of the total CO_2. During the -7 psi and -10 psi desorption phases, 83.9 and 123.4 µmol of CO_2 were recovered, respectively. These amounts were 24 and 35.3% of the total CO_2 retrieved in this study. Figure 6.4b shows these values and the percentage of each that was $^{13}CO_2$. All retrieved CO_2 fractions were near natural abundance ^{13}C which is similar to what was observed in all reactors during the course of the 172-day study.

More CH_4 was retrieved on a molar basis than CO_2; most of the CH_4 was produced and removed while fluids flowed through the reactors (~75%) while just over a third of the total CO_2 was detected in the gas traps. Almost 60% of the total CO_2 retrieved from the system was the result of the 2 vacuum desorption phases. While the amount of methane desorbed decreased during the two sequential vacuum desorption phases, the amount of CO_2 desorbed increased. It has been shown previously that CO_2 has a higher affinity for coal adsorption than CH_4 and methods for enhanced CBM recovery by injection CO_2 have been previously utilized.[69,70]

The amounts of produced CH_4 and CO_2 reported for this study reflect only what could be collected as gas. Some of the produced methane and CO_2 was dissolved in the reactor fluids and not captured or quantified in this study. Due to the speciation of inorganic carbon (CO_2, H_2CO_3, HCO_3^-, CO_3^{2-}), the production of CO_2 during microbial processes also cause shifts in the concentrations of the other dissolved inorganic carbon (DIC) species. DIC was not measured in this study, and therefore, the amounts of CO_2

reported do not represent the production or utilization of the total inorganic carbon present in the system.

Busch et al. observed that not all methane and CO_2 could be desorbed from Wyodak coal during adsorption and desorption studies.[65] Subbituminous Wyodak coal is part of the Tongue River Member of the Fort Union Formation in the Powder River Basin like the Flowers-Goodale coal used in this study. Thus, it is likely that in the reactor system described here some methane and CO_2 remained sorbed to the coal even after vacuum desorption and therefore was not quantifiable.

Summary & Conclusions

The reactor system described here has a total reactor volume of 300 mL, significantly greater than the typical ≤50mL used in batch reactors, and can be packed with any desired porous medium for studies of anaerobic subsurface processes. The single influent source for the four reactor systems allows for treating all systems similarly. Systems can also be run under different flow conditions or amendment strategies to increase understanding of treatments in the subsurface. By providing an increased volume relative to batch experiments, introducing flow, and allowing multiple reactors to be run in parallel, the system described here is a useful tool for testing strategies developed in batch systems before more expensive meso-scale or pilot-scale demonstrations. This strategy of scale-up of small laboratory experiments to larger systems prior to field trials has proven to be a successful strategy to decrease costs while facilitating successful implementation of a biomineralization sealing, developed in the

laboratory before transferring these methods into the field to seal a leaking gas well.[113,115,116]

The preliminary study discussed here examining strategies for enhancing microbial coal-to-methane conversion applied methods previously developed in batch studies to a more field-relevant continuous flow system. Amendment strategies developed in batch systems were applied to enhance the coal-to-methane conversion (Chapter 3). Re-amendment strategies were scaled up for further investigation and to determine applicability in future field studies (Chapter 4). Additionally, ^{13}C-labeled algae amendment, previously used to amend batch systems to trace the origins of enhanced methane production (Chapter 5), was utilized to determine the viability of this method in flow systems, potentially useful in the development of analyses for use in *in situ* applications. While amendment of coal systems in previous batch studies increased the rates of production and total methane produced in the initial 60-80 days, little methane production was observed after this point. The lack of methane production after approximately 80 days in these systems could have many causes, but inhibition due to substrate depletion or accumulation of byproducts are common causes of growth cessation in batch systems.[76] By amending flow reactor systems, these issues were eliminated and methane production did not cease during the 176-day study. This study and potential future studies utilizing the flow reactor system described here are useful in designing a potential field-scale application of enhanced coal-to-methane conversion in subsurface coal beds.

CHAPTER SEVEN

CONCLUSIONS AND FUTURE WORK

Conclusions

Coalbed methane (CBM) is a significant contributor to the overall annual natural gas production in the U.S. (5-6% for 2013-2016),[13] and biogenic production can potentially be increased. Enhancing naturally occurring microbial processes that convert coal to methane gas would increase the rates of biogenic methane production and therefore increase the amounts of CBM and rates at which CBM can be extracted.

In summary, this dissertation demonstrates that biogenic coal-to-methane conversion can be enhanced with the addition of small amounts of organic amendments but that larger amounts of amendment or repeated amendment additions appear to have limited effects on coal-to-methane conversion (at least in batch systems). A method for tracking amendment carbon using ^{13}C-labeled amendments was developed that provided insights into carbon sources for methane production allowing quantification of the amendment enhancement effect on coal-to-methane conversion. ^{13}C-labeled amendments were finally used in a column reactor system to provide a proof-of-principle for the ability of using organic amendments to enhance coal-to-methane conversion under more coal bed-like continuous flow conditions.

Specifically, the work presented in Chapter 3 demonstrates the potential of four organic amendments (algae, cyanobacteria, and yeast cells, and granulated yeast extract) for increasing biogenic CBM production. In coal systems, each amendment studied

increased methane production similarly at equal amendment concentrations. Higher concentrations of amendment addition resulted in higher methane production, but the amount of methane produced was not proportional to the amount of amendment added. In addition, higher concentrations of amendment appeared to cause shifts in the microbial community. A mass balance revealed that even if the amendment had been fully converted to methane, it can be concluded with certainty that the amended coal treatments receiving the lower amendment concentration produced a greater amount of coal-derived methane than unamended coal treatments. These results supported the use of algae or other organic amendments at the low concentrations (0.1 g/L tested here) for use in subsequent experiments.

Algal amendments at a concentration of 0.1 g/L were used to investigate the potential of repeated amendment addition to coal systems to extend increased methane production (Chapter 4). Additional methane was produced with repeated amendment additions, but the amount of additional methane produced decreased with subsequent algal amendment additions. Coal containing and coal-free treatments produced similar amounts of additional methane after repeated amendment additions suggesting that in both systems, the additional methane produced was derived from the conversion of the amendment to methane and not from coal-to-methane conversion. Based on methane and inorganic carbon (IC) measurements, it was shown that in all treatments, including amended non-coal treatments, more carbon was produced as methane and IC than can be accounted for by amendment conversion alone. In coal treatments, this observation supports the conversion of coal to methane while for amended non-coal treatments higher

than predicted production of methane and IC from amendment conversion suggests alternative carbon sources, such as initial dissolved organic or inorganic carbon or carbon transferred with the inoculum (cells, particulate carbon, dissolved carbon).

In Chapter 5, work is described in which algae and yeast containing 95% ^{13}C-labeled carbon were used to amend treatments with and without coal. All ^{13}C-amended treatments produced ^{13}C-enriched methane. Non-coal treatments produced a smaller amount of total methane, but the produced methane was more highly enriched in $^{13}CH_4$ than for coal treatments amended with the same amount of amendment. These observations support the occurrence of coal-to-methane conversion in coal-containing treatments. However, the ^{13}C-amended non-coal containing treatments produced at least 50% of the total methane as $^{12}CH_4$ indicating other sources of methane production besides coal and amendment (also shown in Chapter 4). Filtration (0.2 µm pore size) of the formation water used in the microcosms resulted in two-thirds less unlabeled $^{12}CH_4$ produced by ^{13}C-amended non-coal treatments than produced in similar treatments with unfiltered formation water. This indicates that there is particulate organic matter in the unfiltered formation water contributing to methane production.

Chapter 6 finally focuses on the use of a continuous flow column reactor system which was designed to scale-up and demonstrate the feasibility of using the organic amendment strategies developed in Chapters 3, 4, and 5 in flow-through systems. The reactors were amended with ^{13}C-labeled algae at a concentration of 0.1 g/L, and one reactor was re-amended after 60 days. While the liquid to solid ratio in the batch systems was 10:1, the formation water to coal ratio in the reactor systems was approximately 1:1,

resulting in less amendment added per gram of coal. In the reactors systems, less than 4% of the added algal amendment was converted to measurable methane or inorganic carbon. The amount of methane produced in the reactor systems on a per g of coal basis was approximately 25% of that produced in batch systems during a similar time frame. However, while methane production rates decreased in batch systems after 60-90 days, methane production in the reactor systems continued at a steady rate until the end of the 172-day study. Thus, the reactor system described provides a useful tool for transitioning strategies for enhancing microbial coal-to-methane conversion from small batch systems to meso-scale and pilot-scale systems.

Future Work

The results presented in this dissertation demonstrate the potential use of organic nutrient sources for enhancing microbial coal-to-methane conversion, investigated the carbon conversion of coal and amendments to produce methane, and presented an initial scale-up of the strategies previously examined in microcosms. However, to transition these amendment strategies into the field, more research should be performed, both to enhance fundamental understanding of the microbial coal-to-methane conversion and to address potential issues associated with the scale-up of this technology.

Fundamental Work

- Methane production from alternative carbons sources: Further investigation of carbon sources for methane production other than coal and amendment (such as

dissolved and particulate organics) are needed to increase the accuracy of quantification of coal-to-methane conversion.

- Inorganic carbon production in amended coal systems: The work in Chapter 4 addressed net inorganic carbon production in batch systems but was limited due to sample size and challenges related to the sorption of methane and CO_2 to coal. Additional work to specifically investigate the role of inorganic carbon in methane producing systems would improve carbon mass balance calculations for these coal systems.
- Sorption of methane and carbon dioxide: Sorption of CH_4 and CO_2 to coal is difficult to measure in the three-phase systems used here (solid-, liquid-, and gas-phase) and can vary depending on pressure, temperature, gas concentration, coal rank, and likely other parameters.[65–67] Increasing the understanding of the role of sorption will allow better quantification of produced gases and provide insights into the influence that inorganic carbon sorption may have on the amount of methane that can be sorbed.

Addressing Scale-up Questions

- Amendment injection strategies: To apply algal amendment to subsurface coal beds, an injection strategy must be developed that considers the area impacted by the amendment, the time of incubation before CBM extraction, and infrastructure needed. Injection strategies that could be used include injecting amendment and extracting CBM from the same well ("push/pull") and injecting into one well and extracting from a second well.

- Processing algae amendment: Efforts must be made to determine the best processing of the algal amendment to reduce any effects of coal pore or cleat clogging due to either the amendment itself or the increased biomass due to amendment addition.
- Algae sources: More work must be performed to determine how to effectively grow algae in CBM production water ponds for use as amendment. Which algae or algal consortia and be grown in CBM production water and what the nutrient requirements might be for maximizing algal growth will have to be determined. It will also be of interest to assess whether algal high-value products, such as lipids, can be produced, separated, processed, and sold to offset the costs associated with CBM enhancement strategies.
- Untapped vs. "depleted" coal beds: CBM has been extracted from many coal beds until the amount of extractable methane was not economical anymore resulting in many "depleted" coal beds. Other methane-containing coal beds have not yet been tapped for CBM extraction. Before broad commercial applications of amendment-enhanced coal-to-methane conversion strategies can be attempted, it is necessary to determine whether these strategies are effective in both of these types of coal beds.

APPENDICES

APPENDIX A

ADDITIONAL RESEARCH: ENHANCED COAL-DEPENDENT METHANOGENESIS COUPLED WITH ALGALE BIOFUESL: POTENTIAL WATER RECYCLE AND CARBON CAPTURE

Abstract

Many coal beds contain microbial communities that can convert coal to natural gas (coalbed methane). Native microorganisms were obtained from Powder River Basin (PRB) coal seams with a diffusive microbial sampler placed downhole and used as an inoculum for enrichments with different nutrients to investigate microbially-enhanced coalbed methane production (MECoM). Coal-dependent methanogenesis more than doubled when yeast extract (YE) and several less complex components (proteins and amino acids) were added to the laboratory microcosms. Stimulated coal-dependent methanogenesis with peptone was 86% of that with YE while glutamate- stimulated activity was 65% of that with YE, and a vitamin mix had only 33% of the YE stimulated activity. For field application of MECoM, there is interest in identifying cost-effective alternatives to YE and other expensive nutrients. In laboratory studies, adding algal extract (AE) with lipids removed stimulated coal-dependent methanogenesis and the activity was 60% of that with YE at 27 d and almost 90% of YE activity at 1406 d. Analysis of British Thermal Unit (BTU) content of coal (a measure of potential energy yield) from long-term incubations indicated N99.5% of BTU content remained after coalbed methane (CBM) stimulation with either AE or YE. Thus, the coal resource remains largely unchanged following stimulated microbial methane production. Algal CBM stimulation could lead to technologies that utilize coupled biological systems (photosynthesis and methane production) that sustainably enhance CBM production and generate algal biofuels while also sequestering carbon dioxide (CO_2).

Citation

Barnhart, E.P.; Davis, K.J.; Varonka, M.; Orem, W.; Cunningham, A.B.; Ramsay, B.D.; Fields, M.W., Enhance coal-dependent methanogenesis coupled with algal biofuel: Potential water recycle and carbon capture. *International Journal of Coal Geology* 2017, 171, 69-75

APPENDIX B

SURFACE AREA EFFECTS ON COAL-DEPENDENT METHANOGENESIS

Experimental Justification

To understand methods for microbially enhanced coalbed methane (MeCBM), it is important to assess coal bioavailability. The surface area accessible to the microbes can affect how much carbon is available for coal-to-methane conversion. This experiment was designed to determine whether coal particle size, and therefore assumed bioavailable surface area, can have an effect on total biogenic methane produced.

Materials and Methods

Cores from the Flowers-Goodale (FG) coal bed collected in 2013 and described in Barnhart et al.[73] were removed from the collection tubes and dried at room temperature in atmospheric air. The coal was crushed and sieved to 4 separate size fractions (Table B.1 and Figure B.1) and stored in autoclaved glass jars prior to reactor set up.

Table B.1: Treatment size fraction and standard sieve sizes used

Treatment	**Size fraction (mm)**	**Sieve sizes**	**Total Surface Area (cm^2)**
S	0.106-0.3	140, 50	247.4
M	0.6-1.18	30, 16	82.8
L	3.35-4.75	6, 4	34.3
XL	6.3-9.52	¼”, 3/8”	14.9

The XL size was chosen to ensure the coal pieces could fit through the 13 mm Balch tube opening. Each treatment size was chosen such that there was a size gap between treatments to ensure that each treatment was separate with no size overlap. Formation water was collected from the FG-09 well in July 2014 and stored in plastic

jugs at 4°C until reactor set up. The microbial consortium used to inoculate the reactors was an enrichment from the HWC-01 well collected in 2010 and transferred to fresh CBM formation water twice previously.

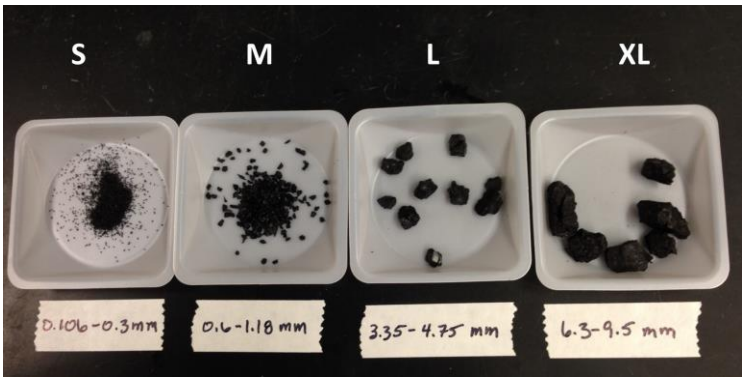

Figure B.1: Coal particles by size fraction.

Batch reactors were set up using anaerobic methods in 26 mL Balch tubes with butyl rubber stoppers and aluminum crimp seals. One gram of crushed and sized coal was added to each tube. Degassed FG formation water was added anoxically. The tubes were sealed with a 5% CO_2 headspace. Resazurin was used as a redox indicator, and sodium sulfide was added to each tube as an oxygen scavenger. Yeast extract was added to amended treatments at a concentration of 0.1 g/L. Reactors were inoculated with 1 mL of the HWC-01-originating consortium. The total reactor volume was 10 mL. Each treatment had duplicates of both unamended and 0.1 g/L yeast extract amended conditions. Additionally, uninoculated controls for each treatment were set up and sampled to account for any methane desorption or abiotic formation.

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 x 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. One mL of 5% CO_2 in N_2 gas was added to the reactors prior to sampling to ensure a positive pressure in the reactor to reduce the risk of oxygen being introduced into the system.

Results

No abiotic methane formation or desorption was observed in the uninoculated controls. Thus, it can be assumed that all methane produced and measured was produced biogenically during the duration of the experiment.

More methane production was observed for all YE amended treatments compared to the corresponding unamended treatment for all time points. Methane production was first observed on sampling day 49 for YE amended treatments of all coal size fractions (Figure B.2a). Total methane production continued to increase until day 228. Between day 228 and the last sampling point on day 301, total methane production remained within the sample standard deviation for all treatments except M+YE treatments which saw a small increase.

Methane production was not observed for any of the unamended treatments until day 228 when methane production was observed in both M replicates and one L replicate. On day 301, methane production was observed in one S replicate, both M replicates, and

one L replicate. No methane was observed for the XL treatment at any point during the duration of the experiment (FigureB.2b).

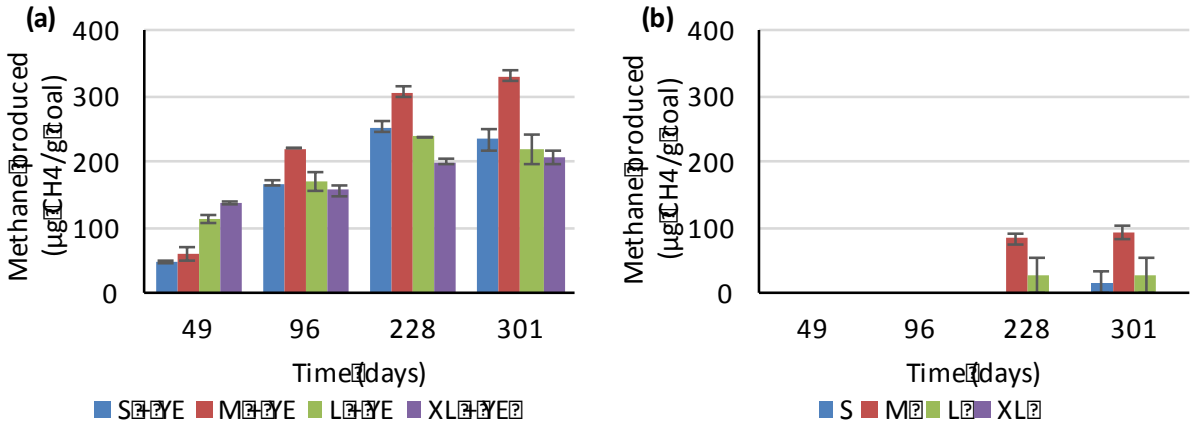

Figure B.2: Methane production for all treatments at four time point over the duration of the experiment for (a) yeast extract (YE) amended and (b) unamended treatments. Error bars represent one standard deviation of duplicates

Discussion and Conclusions

The results of this experiment showed no conclusive correlation between increased surface area and increased methane production. The S, L, and XL treatments produced a similar amount of methane with YE amendment for the duration of the experiment. By day 96, the M + YE treatment had produced more methane than the other treatments. This trend remained for the rest of the experiment.

Previous studies on the effect of coal particle size on biogenic methane production are also inconclusive with one study showing increased methane production with decreasing particle size[42] while another showed increased methane production with increasing particle size.[28] Thus, the results shown here along with the results from these

two previous studies suggest that, while coal particle size may have an effect on methane production, it appears that there are other influences that can override this effect.

These results were used to determine coal particle size for future experiments. All future batch experiments used coal sized between 0.8 and 2.0 mm. Using size fractioned coal increases the homogeneity of particle size within experimental treatments and accuracy of weighing the coal for small batch reactors.

APPENDIX C

ACID-BASE TITRATION OF FLOWERS-GOODALE COAL TO ASSESS COAL BUFFERING CAPACITY

Experimental Justification

Coal is complex, heterogeneous, and high in carbon content. The effects coal has on the aqueous environment are not well understood. This study was designed to investigate the capacity of coal to buffer pH to provide insights into the geochemistry of the coal environment and to assist in setting up later studies.

Materials and Methods

Flowers-Goodale (FG) coal from the FGM-13 cores was dried at room temperature in atmospheric conditions. The coal was crushed and sieved to 1.19-2.0 mm particle size. Two-liter glass bottles were set up as shown in Table C.1. Each bottle received 1L of deionized water (DI). Treatments 2 and 4 received 100 g of crushed and sized coal, i.e. the same 10:1 liquid to solid ratio used in microbial CBM batch reactor studies.

Table C.1: Experimental treatment design.

Treatment	Coal (g)	DI water (mL)	Experiment
1	0	1000	A
2	100	1000	A & B
3	0	1000	B
4	100	1000	B

Prepared bottles were allowed to equilibrate with the atmosphere for 72 hours loosely covered with foil and continuously stirred. Two experiments were run. Treatments 1 and 2 were used in experiment A, and treatments 2, 3, and 4 were used in experiment B. Experiment A examined the buffering capacity of coal during addition of a

strong acid, HCl. Experiment B examined the buffering capacity during addition of a strong base, NaOH. Experiment A had only a DI water control and an untreated coal and DI water treatment, and Experiment B had the previously acid-treated coal and DI water treatment in addition to those in Experiment A.

For both experiments, 3 days of titrations were performed: 1) 72 hours, 2) 120 hours, and 3) 168 hours after bottle assembly. Between titrations, bottles were loosely covered with foil and left on a stir plate to ensure adequate mixing and equilibrium attainment. On the first sample day, 0.1M HCl or NaOH was added by pipette in 10-200 μL amounts. pH was monitored with a calibrated pH probe. On the second and third sample days, 1M HCl or NaOH was added in increasing amounts from 10 to 100 μL amounts for HCl and 10 to 500μL amounts for NaOH.

Results

The titration curves for the DI controls are shown in Figure C.1. These curves show a typical titration curve for water as shown in the inset graph where acid titration is considered a removal of base. The base titration resulted in an overall increase in pH of 7.94 from a starting pH of 4.12 to and ending pH of 12.06. The acid titration resulted in an overall decrease in pH of -2.97 from a starting pH of 5.1 and ending pH of 2.13.

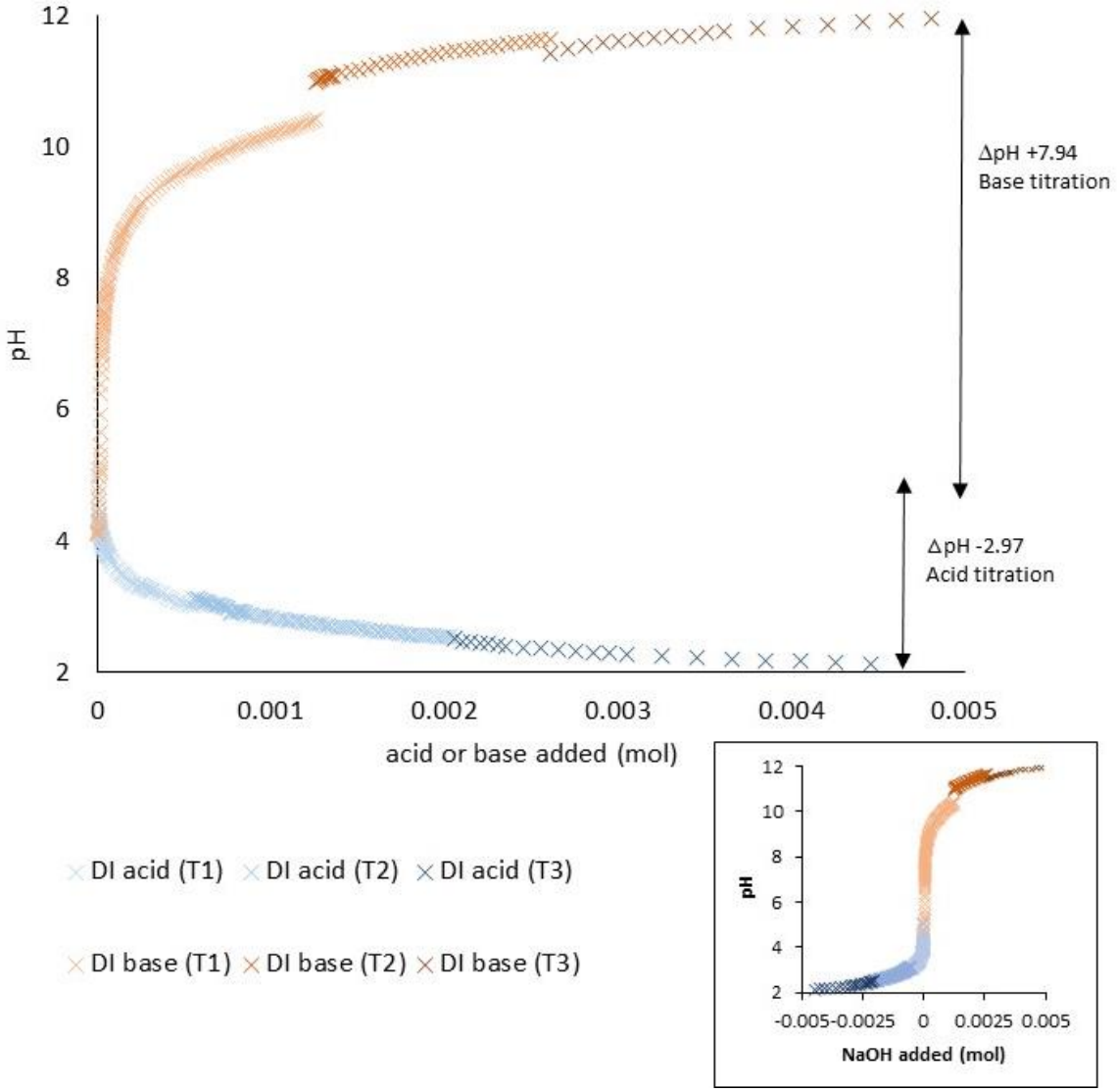

Figure C.1: Titration curves for DI water with no coal. The inset figure is the same data but shown as a more typical titration curve for a base titration. In this case, the acid titration was considered a removal of base.

The titration of DI with coal is shown in Figure C.2. The top set of lines (grey with a positive slope) show the results of the base titration. The titration on day1 started at a pH of 8.1 and increased to 9.12. Forty-eight hours later for the second titration, the pH had decreased 0.79 pH points to 8.33. The second titration resulted in an increased pH of 9.51. The pH again decreased over the next 48 hours, and the third titration started at a

pH of 8.6. This titration resulted in an increased pH of 10.1. The lower lines (blue with a negative slope) show the results of the acid titration. The first titration started at a pH of 7.43 and ended with a pH of 6.72. The pH increased by 0.52 units in the 48 hours between the first and second titration. The second titration started with a pH of 7.24 and ended at 5.86. The pH again increased during the 48 hours between the second and third titrations to 6.18. The final pH for the third titration was 5.09.

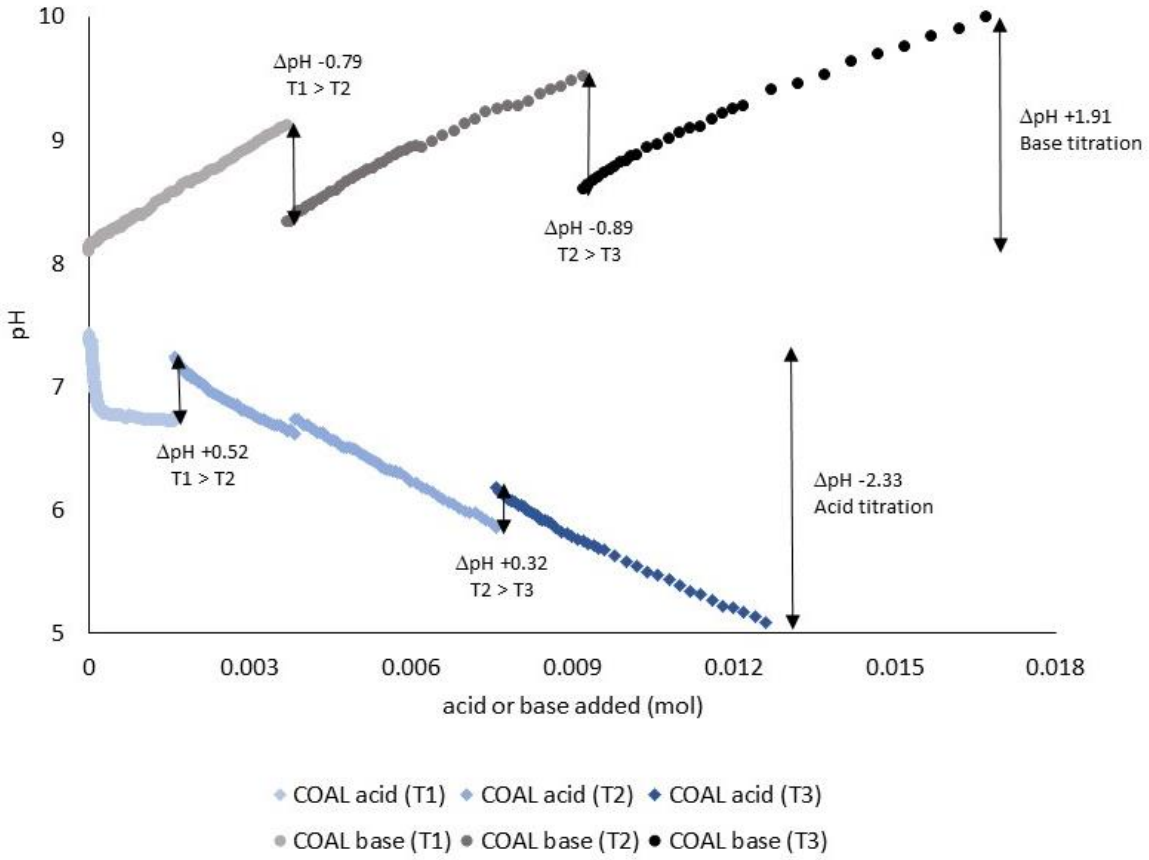

Figure C.2: Titration curves for the base (grey) and acid (blue) titrations with coal.

Figure C.3 shows the titrations of the same coal/DI sample. The acid titration was performed first and previously described (above). The final pH change for the acid titration was -2.34. The same coal/DI sample was used for a base titration. Between

titrations, the pH of the sample increased from 5.09 to 5.57. The first base titration resulted in an increase in pH to 6.67. During the 48 hours between the first and second base titration, the pH decreased -0.43. The pH for the second titration started at pH 6.24 and increased to pH 8. The pH decreased to 7.19 during the 48 hours between titration 2 and 3. The final pH after the third base titration was 8.37.

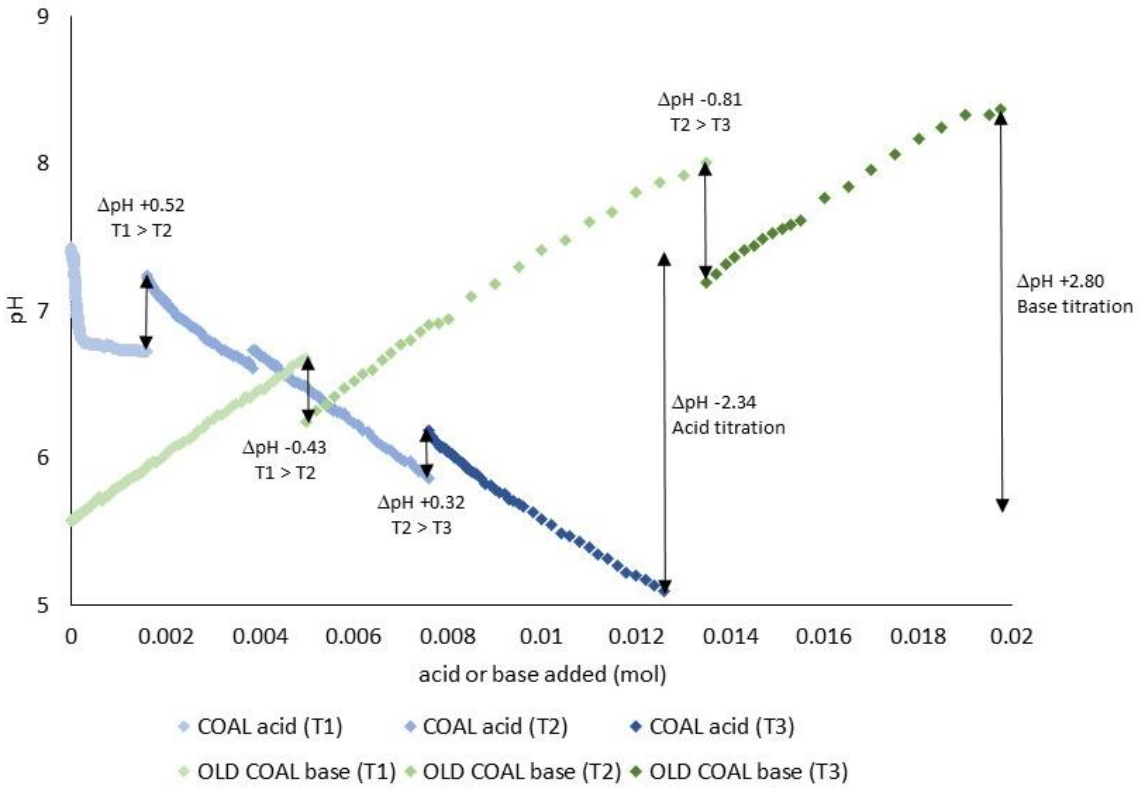

Figure C.3: Titration curves for coal in DI. Acid titrations (blue) followed by a base titration (green).

Discussion and Conclusions

The results of this study showed that coal itself acts to buffer the pH of the aqueous system. While titrations with a strong acid or a strong base do result in an overall

pH change, the coal was able to offset much of this change. The coal/DI sample was treated with 3.1 times as much base as the DI only sample, but the overall pH increase was only +1.91 compared to the increase of +7.94 for the DI only sample. The coal/DI sample was treated with 2.8 times as much acid as the DI only sample, resulting in a pH decrease of -2.34 versus -2.88 for the DI only.

The observation of the apparent buffering capacity of coal was useful in planning later experiments. Because the coal systems have such a large pH buffer, it is less necessary to get the starting formation water to an exact pH as the coal will tend to dictate the system pH. However, it is important to note that in systems without coal (such as glass beads in lieu of coal), the buffering capacity is much reduced and attention to starting system pH is necessary for these systems.

APPENDIX D

ELEMENTAL ANALYSIS OF FOUR AMENDMENTS FROM CHAPTER 3

The complete elemental analysis is shown for the four amendments used in Chapter 3: algae (SLA-04), cyanobacteria (Anabaena), yeast cells (EtOH-Red), and yeast extract (YE). The alga was also the amendment used in Chapter 4. This analysis was performed at the Soil and Plant Analysis Laboratory at Iowa State University.

Table D.1: Complete elemental analysis of amendments used in Chapters 3 and 4.

(a)	**Total C (%)**	**Total N (%)**
SLA-04	40.1	10.9
Anabaena	46.7	9.9
EtOH-Red	45.5	7.7
YE	47.9	10.6

(b)	**P (mg/kg)**	**K (mg/kg)**	**Na (mg/kg)**	**Mg (mg/kg)**	**Ca (mg/kg)**
SLA-04	12360	55980	1360	343.4	2340
Anabaena	12720	7718	2082	732	916.6
EtOH-Red	21620	17220	2910	9380	7936
YE	20260	14500	718.8	6192	5898

(c)	**Mn (mg/kg)**	**Fe (mg/kg)**	**Cu (mg/kg)**	**Zn (mg/kg)**	**Al (mg/kg)**	**S (mg/kg)**
SLA-04	3.2	33.72	0.58	85.94	26.62	6266
Anabaena	14.42	59.6	1.22	113.2	19.08	3820
EtOH-Red	182.6	3068	266.8	410.4	74.5	5870
YE	948	3534	30.28	167.4	28.26	7002

(d)	**Ni (ppm)**	**Co (ppm)**	**Mo (ppm)**	**B (ppm)**	**Se (ppm)**	**W (ppm)**
SLA-04	14.48	0.62	10.98	3.46	76.46	BDL
Anabaena	14.98	2.42	11.52	7.82	54.2	BDL
EtOH-Red	13.94	1.48	8.02	55.58	108.2	BDL
YE	13.24	0.6	9.18	67.28	178.6	BDL

APPENDIX E

RE-AMENDMENT OF BATCH TREATMENTS USING FOUR ORGANIC AMENDMENTS AT 2 CONCENTRATIONS

Experimental Justification

The initial study in Chapter 3 examined the methane enhancement potential of 4 amendments (algae, cyanobacteria (cyano), yeast, and yeast extract (YE)) at 2 different concentrations. Significant coal-to-methane enhancement was observed with all 4 amendment additions. After one replicate of each treatment was destructively sampled for microbial community analysis, two replicates of each treatment remained. To assess the potential for achieving repeated methane enhancement with repeated amendment addition, these remaining treatments were re-amended with the same type and amount of amendment as the initial condition.

Materials and Methods

The initial experimental preparation and design was previously described in Chapter 3. One replicate of each treatment was destructively sampled for microbial community analysis on days 111, 172, and 322. After these samplings, the remaining replicates were re-amended. Only one replicate for each treatment was sampled for the entire 322-day study, and only one replicate per treatment was sampled for community analysis at each time point.

To re-amend the reactors, one mL of amendment prepared as previously described was added to each batch system. Each reactor was re-amended with the same amount of the same amendment as the initial condition. To account for volume changes and potential nutrient addition from the formation water itself, one mL of degassed Flowers-Goodale formation water was added to all unamended treatment.

Headspace methane concentrations were monitored by gas-chromatography. Samples for microbial community analysis were processed and analyzed as previously described using FastSpin soil extraction kits and Illumina MiSeq sequencing platform. Sequences were compared between treatments and times using similarity percentage (SIMPER) analysis in the PAST3 software.[117] OTUs contributing at least 1% dissimilarity between treatments of interest were evaluated.

Results

Methane Production

Methane production was observed on all time periods for all treatments except for the glass bead (GB) only treatment which produced no methane for the entire 322-day study. Figure E.1 shows the amount of methane produced by each treatment for each amendment period: 1) 0-111, 2) 111-172, and 3) 172-322 days. The amount of methane produced by all coal treatments was greatest in the first amendment period of 111 days while methane production declined in subsequent amendment periods. In contrast, the amended GB treatments produced increasing amounts of methane for each amendment period. In the first amendment period, the coal treatments produced more methane than the corresponding GB treatment regardless of amendment type or amount. However, in the second and third amendment periods, the corresponding coal and GB treatments produced similar amounts of methane for all treatments except for unamended and 0.5 g/L amended algae treatments. For both of these amendment regimens, the coal

treatments produced more methane than the corresponding GB treatments on all three amendment periods.

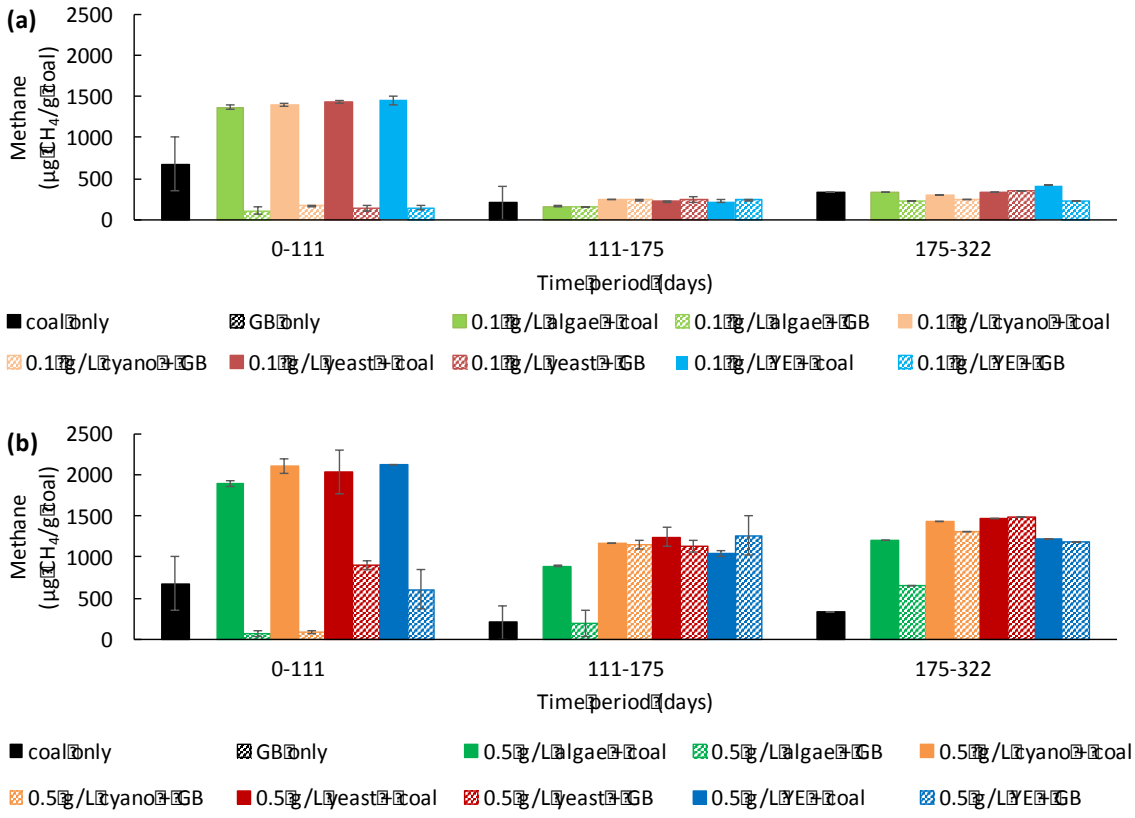

Figure E.1: Methane produced on each amendment time period for (a) unamended and 0.1 g/L amended and (b) 0.5 g/L amended treatments. Error bars represent one standard deviation for triplicate (0-111 days) and duplicate (111-175 days). Only one replicate remained dor days 175-322.

As previously discussed in Chapter 3 and shown in Figure E.1, it was observed during the first 111 days that more methane was produced with 0.5 g/L amendment than with 0.1 g/L amendment concentration, but the increase in methane production was not proportional to the amount of amendment added. The 0.5 g/L amended coal treatments produced an average of 1.4 times the methane of the 0.1 g/L amended treatments (Table

E.1). However, a difference was observed in the amended GB treatments during this period. The 0.5 g/L algae and cyanobacteria amended treatments produced less methane than the corresponding 0.1 g/L amended treatments while the yeast and YE GB treatments produced 6.3 and 4.1 times more, respectively, than the corresponding 0.1 g/L treatments.

Table E.1: A comparison of the methane produced with 0.5 g/L amendment to 0.1 g/L amendment by treatment. The unitless value is the factor by which the 0.5 g/L amended treatment exceeds the 0.1 g/L amended treatment (0.5 g/L: 0.1 g/L with same solid substrate, amendment type, and concentration).

Time period (days) **Treatment**	**0-111**	**111-172**	**172-322**	**TOTAL 0-322**
coal + algae	1.4	5.4	3.6	2.1
coal + cyanobacteria	1.5	4.7	4.7	2.4
coal + yeast	1.4	5.7	4.4	2.4
coal + YE	1.5	4.6	3.0	2.1
GB + algae	0.6	1.2	2.8	1.8
GB + cyanobacteria	0.5	4.8	5.3	3.9
GB + yeast	6.3	4.5	4.3	4.8
GB + YE	4.1	5.3	5.3	5.0
coal average	1.4	5.1	3.9	2.3
GB average	2.9	3.9	4.4	3.9

However, during the second amendment period, the amount of methane produced in the 0.5 g/L amended coal treatments averaged 5.1 times greater than in the corresponding 0.1 g/L amended coal treatments. The 0.5 g/L amended GB treatments for this amendment period averaged 3.9 times greater than 0.1 g/L amended GB treatments, but the algae amended GB treatments still seemed to lag and only produced 1.2 times the methane with the higher amendment concentration.

During the third amendment period, the coal treatments produced an average of 3.9 times more methane with the 0.5 g/L amendment concentration than with the 0.1 g/L

amendment concentration. The GB treatments for this period varied with the 0.5 g/L amendment to 0.1 g/L amendment ratio lower for the algae amended GB treatments at 2.8 while the cyanobacteria, yeast, and YE GB treatments varied from 4.3 to 5.3.

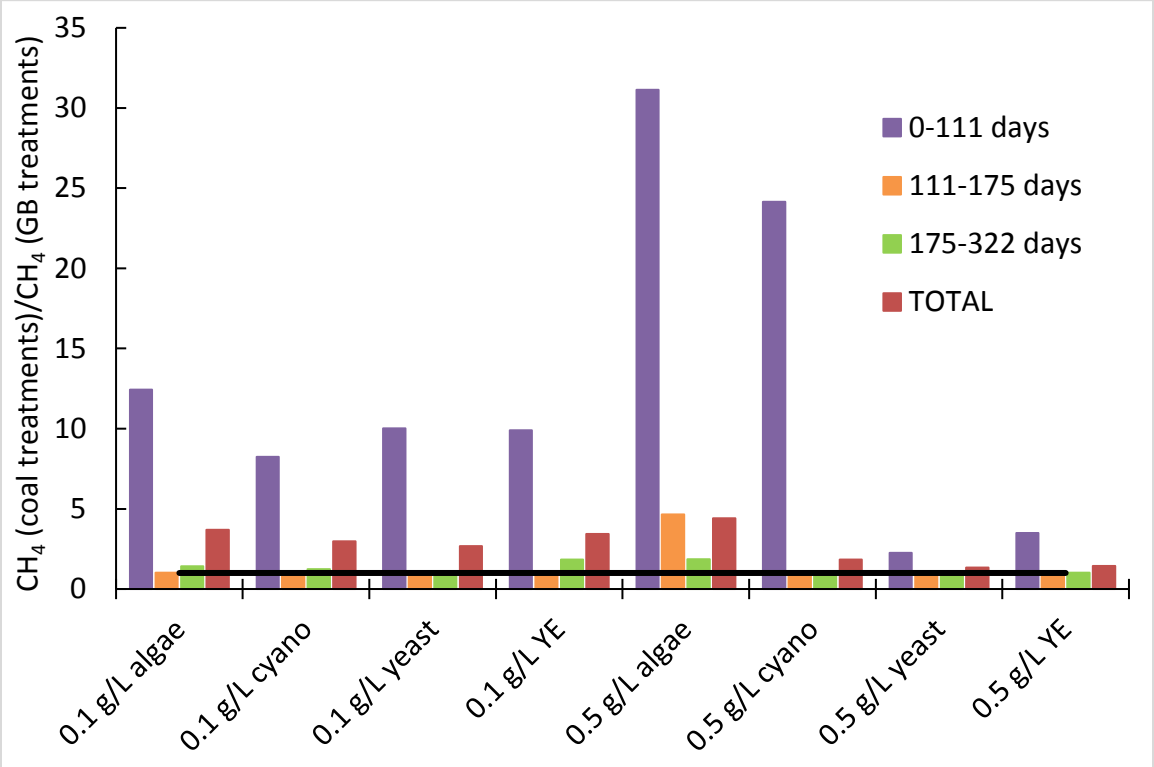

Figure E.2: Ratio showing the amount of methane produced in the coal treatments as a multiple of the methane produced by the corresponding GB treatment. The black line indicates the ratio at which methane production in coal and GB treatments are equal. Bars that extend above this line show conditions where coal treatments produced more methane than corresponding GB treatments for that amendment time period.

Figure E.2 shows the ratio of coal:GB methane productions with the same amendment type and concentration. When coal treatments produce more methane than the corresponding GB treatment, this ratio is >1. When the GB treatments produce more methane than the corresponding coal treatment, this ratio is <1. While coal treatments produced more methane than the corresponding GB treatments in the first amendment

period, subsequent amendment periods resulted in similar methane production in coal and GB treatments of same amendment and concentration. The only large discrepancy from this observation was for the 0.5 g/L algae amended treatments where the coal treatment produced more methane than the corresponding GB treatment for all three amendment periods. However, the enhanced methane observed in the 0.5 g/L amended algae appeared to shift toward greater amendment-to-methane conversion and less enhanced coal-to-methane conversion due to the diminishing coal to GB methane ratio.

Methane Production Rates

The maximum methane production rates for all 18 treatments on each of the three amendment periods are shown in Table E.2. The maximum rate during the first amendment period for all amended coal treatments and 0.5 g/L algae and cyanobacteria amended GB occurred between days 35 and 54 after amendment. However, the maximum rate for unamended coal and most amended GB treatments occurred later after day 68. During the second amendment period, the maximum methane production rate occurred within 24 days of re-amendment with the exception of the unamended coal (days 34-44 after amendment) and 0.5 g/L amended cyanobacteria (days 24-34 after amendment). During the third amendment period, the maximum methane production rate occurred in the first 13 days after amendment for all treatments except both 0.1 and 0.5 g/L yeast amended coal treatments (days 13-23 after amendment) and unamended coal and 0.5 g/L algae amended GB (days 23-33). From this observation, it appears that the maximum methane production rate occurs earlier with each additional amendment, as also observed in Chapter 5.

Table E.2: Maximum methane production rate for each treatment for each amendment period. Error are one standard deviation where the first time period has 3 replicates, the second two, and the third only 1.

Treatment	**Max. Rate of CH_4 Production (µg CH_4/g coal)**		
	Day 0-111	**Day 111-175**	**Day 175-322**
coal only	17.0 ± 13.2	25.3 ± 15.0	14.5
GB only	0.0 ± 0.0	0.0 ± 0.0	0.0
0.1 g/L algae + coal	45.8 ± 0.7	10.0 ± 0.5	10.8
0.1 g/L algae + GB	3.0 ± 0.5	7.7 ± 0.5	10.0
0.1 g/L cyano + coal	45.7 ± 0.5	14.5 ± 1.4	12.9
0.1 g/L cyano + GB	4.2 ± 1.0	9.8 ± 0.3	14.3
0.1 g/L yeast + coal	49.5 ± 0.9	13.5 ± 4.1	13.1
0.1 g/L yeast + GB	3.9 ± 0.6	9.5 ± 0.9	12.5
0.1 g/L YE + coal	50.3 ± 1.5	14.5 ± 1.1	13.8
0.1 g/L YE + GB	4.7 ± 1.7	9.5 ± 0.3	15.5
0.5 g/L algae + coal	56.3 ± 0.4	32.4 ± 4.7	42.1
0.5 g/L algae + GB	1.7 ± 0.3	8.2 ± 0.3	12.6
0.5 g/L cyano + coal	64.7 ± 2.8	52.1 ± 1.5	56.8
0.5 g/L cyano + GB	2.3 ± 0.2	48.1 ± 0.7	49.3
0.5 g/L yeast + coal	61.3 ± 1.7	48.9 ± 7.2	54.1
0.5 g/L yeast + GB	19.7 ± 13.7	46.6 ± 4.1	50.4
0.5 g/L YE + coal	63.1 ± 2.7	47.6 ± 7.2	55.4
0.5 g/L YE + GB	23.1 ± 1.5	60.5 ± 0.7	61.2

For all amended coal treatments, the maximum rate of methane production was highest during the first amendment period and decreased in the second and third amendment periods. The maximum rate was lowest during the first amendment period for all amended GB treatments and increased during the subsequent amendment periods. During the first amendment period, the amended coal treatments had higher methane production rates than the corresponding GB treatments for all amendments and concentrations. In the second and third amendment periods, the differences between maximum rates observed for corresponding amended coal and GB treatments were smaller.

Microbial Community Analyses

Archaeal and bacterial sequencing data were analyzed as two separate groups using SIMPER analysis. Archaea were compared at a genus level while bacteria were compared on an order level. SIMPER analysis indicates dissimilarity between samples based on the relative abundance of OTUs from sequencing analysis.

Table E.3: Comparison of the same treatment condition between the 3 amendment periods for (a) archaeal and (b) bacterial communities using SIMPER analysis.

(a)	Average Dissimilarity		
	T1:T2	T2:T3	T1:T3
coal	19.4	7.7	19.6
GB	15.1	25.1	14.0
0.1 algae coal	8.0	3.8	4.3
0.1 algae GB	43.1	26.5	36.6
0.1 cyano coal	7.0	6.7	1.2
0.1 cyano GB	6.4	13.3	7.4
0.1 yeast coal	5.1	2.4	2.9
0.1 yeast GB	4.2	9.8	9.3
0.1 YE coal	3.9	4.7	1.3
0.1 YE GB	11.5	6.8	11.8
0.5 algae coal	5.7	6.6	6.8
0.5 algae GB	21.8	84.3	71.3
0.5 cyano coal	3.6	17.6	16.1
0.5 cyano GB	11.5	12.0	11.5
0.5 yeast coal	5.2	15.8	5.2
0.5 yeast GB	8.1	8.8	8.1
0.5 YE coal	7.0	6.7	7.0
0.5 YE GB	3.4	5.5	3.4

(b)	Average Dissimilarity		
	T1:T2	T2:T3	T1:T3
coal	25.5	30.4	36.2
GB	28.8	22.6	24.0
0.1 algae coal	21.2	12.6	26.9
0.1 algae GB	29.1	29.1	49.3
0.1 cyano coal	23.9	37.1	31.5
0.1 cyano GB	39.1	23.4	40.7
0.1 yeast coal	23.5	21.3	29.7
0.1 yeast GB	20.4	30.7	29.2
0.1 YE coal	30.5	21.2	37.1
0.1 YE GB	38.1	20.7	35.0
0.5 algae coal	43.1	36.9	37.9
0.5 algae GB	77.8	73.3	69.6
0.5 cyano coal	28.8	31.4	43.7
0.5 cyano GB	70.2	33.8	67.2
0.5 yeast coal	22.5	25.8	38.8
0.5 yeast GB	30.9	18.0	37.8
0.5 YE coal	29.4	34.0	36.8
0.5 YE GB	45.9	46.5	40.1

A broad analysis of dissimilarity was performed for both groups on all treatment comparing dissimilarity over time, between coal and GB treatments with the same amendment regimen, and between unamended and amended treatments with the same solid substrate. Dissimilarity generally was greater between amendment periods for GB treatments than for coal treatments for both archaeal and bacterial communities (Table E.3). When comparing amended coal treatments to the corresponding GB treatments,

bacterial community dissimilarity was greater than archaeal dissimilarity except for the 0.5 g/L algae amended treatments on the first two amendment periods. For both archaeal and bacterial communities, the unamended treatments showed more dissimilarity than the amended treatments (Table E.4). When comparing amended and unamended treatments, the general trend observed for both archaeal and bacterial communities was that GB treatments had greater dissimilarity than the similarly amended coal treatments for almost all time points (Table E.5).

Table E.4: Comparison of the coal and glass bead (GB) treatments with the same amendment regimen for each of the 3 amendment periods for (a) archaeal and (b) bacterial communities using SIMPER analysis.

(a)	Average Dissimilarity			(b)	Average Dissimilarity		
	T1	T2	T3		T1	T2	T3
coal vs GB	44.9	35.5	49.4	**coal vs GB**	70.8	79.4	60.3
0.1 algae coal vs 0.1 algae GB	42.3	4.1	27.5	**0.1 algae coal vs 0.1 algae GB**	59.3	41.3	31.7
0.1 cyano coal vs 0.1 cyano GB	6.6	4.0	14.2	**0.1 cyano coal vs 0.1 cyano GB**	53.2	36.2	47.1
0.1 yeast coal vs 0.1 yeast GB	9.7	10.2	13.8	**0.1 yeast coal vs 0.1 yeast GB**	39.6	38.2	33.1
0.1 YE coal vs 0.1 YE GB	12.3	6.8	7.1	**0.1 YE coal vs 0.1 YE GB**	45.7	52.7	51.9
0.5 algae coal vs 0.5 algae GB	81.0	94.1	7.1	**0.5 algae coal vs 0.5 algae GB**	55.3	73.1	68.4
0.5 cyano coal vs 0.5 cyano GB	5.7	13.3	19.9	**0.5 cyano coal vs 0.5 cyano GB**	29.7	63.9	60.4
0.5 yeast coal vs 0.5 yeast GB	2.7	8.7	14.0	**0.5 yeast coal vs 0.5 yeast GB**	31.1	26.7	23.3
0.5 YE coal vs 0.5 YE GB	6.1	7.1	6.9	**0.5 YE coal vs 0.5 YE GB**	45.8	37.5	46.2

From these comparisons across all treatments, it appears that bacterial communities have more dissimilarity between treatments than archaeal communities. In addition, greater dissimilarity was observed between GB treatments when compared to coal treatments suggesting that the presence or absence of coal can have a significant impact on community structure for both domains. Because algal amendment has become the preferred amendment for future work, only the unamended and algae amended treatments were analyzed in greater detail.

Table E.5: Comparison of the unamended and amended treatments with the same solid substrate for each of the 3 amendment periods for (a) archaeal and (b) bacterial communities using SIMPER analysis.

(a)	Average Dissimilarity		
	T1	T2	T3
coal vs 0.1 algae coal	22.1	6.2	7.5
GB vs 0.1 algae GB	13.0	33.8	55.4
coal vs 0.1 cyano coal	21.7	6.4	7.5
GB vs 0.1 cyano GB	41.5	32.8	48.1
coal vs 0.1 yeast coal	22.0	11.3	8.0
GB vs 0.1 yeast GB	39.0	27.9	50.2
coal vs 0.1 YE coal	22.0	8.8	5.0
GB vs 0.1 YE GB	34.4	30.7	53.1
coal vs 0.5 algae coal	22.3	5.9	5.6
GB vs 0.5 algae GB	73.9	92.5	57.4
coal vs 0.5 cyano coal	22.1	6.4	13.5
GB vs 0.5 cyano GB	48.0	31.5	56.3
coal vs 0.5 yeast coal	22.7	9.2	16.9
GB vs 0.5 yeast GB	47.1	30.4	51.2
coal vs 0.5 YE coal	21.6	7.2	4.5
GB vs 0.5 YE GB	43.9	28.6	52.2

(b)	Average Dissimilarity		
	T1	T2	T3
coal vs 0.1 algae coal	38.7	54.4	42.7
GB vs 0.1 algae GB	64.1	70.2	63.8
coal vs 0.1 cyano coal	43.2	48.2	38.8
GB vs 0.1 cyano GB	57.8	72.1	64.9
coal vs 0.1 yeast coal	48.1	59.1	44.7
GB vs 0.1 yeast GB	63.4	71.2	64.3
coal vs 0.1 YE coal	54.6	38.0	31.5
GB vs 0.1 YE GB	65.4	83.5	77.4
coal vs 0.5 algae coal	56.0	69.0	52.8
GB vs 0.5 algae GB	79.5	79.3	84.1
coal vs 0.5 cyano coal	49.8	62.6	53.9
GB vs 0.5 cyano GB	69.1	86.9	87.0
coal vs 0.5 yeast coal	62.1	60.4	56.1
GB vs 0.5 yeast GB	75.7	75.9	66.0
coal vs 0.5 YE coal	58.6	60.0	54.0
GB vs 0.5 YE GB	73.2	83.3	81.9

Unamended and Algae Amended Archaeal SIMPER Analysis. When comparing the same treatment between amendment periods, the smallest amount of archaeal community dissimilarity was observed for the 0.1 g/L algae amended coal treatments on all periods. From this observation, it can be inferred that these treatments had the smallest archaeal community shifts (Table E.6). *Methanosaeta* sequences significantly contributed to dissimilarity between time points for all treatments analyzed and was the largest contributor for most treatments. *Methanospirillum* sequences contributed greater than 1% dissimilarity in all treatments (except 0.1 g/L algae coal) for all time comparisons (except GB treatments no T2:T3 and T1:T3). Other significant players contributing to dissimilarity in both coal and GB and both unamended and amended treatments were *Methanoregula* and an unclassified *Methanobacteriaceae* OTU. Contributions to

dissimilarity by assumed methylotrophic *Methanolobus*[118] occurred in all GB treatments regardless of amendment and the 0.5 g/L algae amended coal.

Table E.6: Comparisons of the archaeal communities from the same treatment condition between the 3 amendment periods for (a) unamended, (b) 0.1 g/L algae amended, and (c) 0.5 g/L algae amended treatments using SIMPER analysis.

(a)	Average Dissimilarity					
	coal only			GB only		
	T1:T2	T2:T3	T1:T3	T1:T2	T2:T3	T1:T3
Methanosaeta	9.7	2.0	7.7	7.5	11.1	3.6
Methanospirillum	7.9	1.8	9.7	1.0		
Methanoregula	1.7	3.8	2.1	5.0	11.8	6.9
Methanobacteriaceae_unclassified						1.4
Methanolobus						1.0
Contribution of genera w/ > 1% dissimilarity	19.3	7.6	19.5	13.5	22.9	12.9
TOTAL av. dissim.	19.4	7.7	19.6	15.1	25.1	14.0

(b)	Average Dissimilarity					
	0.1 algae coal			0.1 algae GB		
	T1:T2	T2:T3	T1:T3	T1:T2	T2:T3	T1:T3
Methanosaeta	4.0	1.9	2.1	21.5	12.8	8.7
Methanospirillum				3.4	12.9	9.6
Methanoregula				16.1		16.3
Methanobacteriaceae_unclassified	2.4	1.1	1.3			
Methanolobus				1.3		1.5
Contribution of genera w/ > 1% dissimilarity	6.4	3.0	3.3	42.2	25.8	36.0
TOTAL av. dissim.	8.0	3.8	4.3	43.1	26.5	36.6

(c)	Average Dissimilarity					
	0.5 algae coal			0.5 algae GB		
	T1:T2	T2:T3	T1:T3	T1:T2	T2:T3	T1:T3
Methanosaeta	21.5	12.8	8.7	6.5	42.1	35.6
Methanospirillum	3.4	12.9	9.6	10.8	42.0	31.2
Methanoregula	16.1		16.3			
Methanolobus	1.3		1.5	1.4		1.3
Methanobacterium				2.8		2.8
Contribution of genera w/ > 1% dissimilarity	42.2	25.8	36.0	21.4	84.1	71.0
TOTAL av. dissim.	43.1	26.5	36.6	21.8	84.3	71.3

When comparing dissimilarity between coal and GB treatments with the same amendment regimen, the greatest archaeal dissimilarities were observed for the 0.5 g/L

algae amended treatments for the first and second amendment periods (Table E.7). The main contributors to the dissimilarity comparison of unamended coal and GB were *Methanoregula* and *Methanosaeta* with smaller contributions from *Methanospirillum*, *Methanolobus*, and an unclassified *Methanobacteriaceae*. For the 0.1 g/L algae amended treatments, *Methanoregula* was a major contributor to overall dissimilarity only during the first amendment period, and *Methanosaeta* contributed to dissimilarity in the first and third amendment periods. *Methanospirillum*, *Methnolobus*, and an unclassified *Methanobacteriacece* also made contributions to the dissimilarity between 0.1 g/L algae amended coal and GB. For 0.5 g/L algae amended coal and GB dissimilarity, *Methanosaeta* and *Methanospirillum* contributed more dissimilarity while *Methanoregula*, *Methnolobus*, *Methanobacterium*, and an unclassified *Methanobacteriacece* made smaller contributions to dissimilarity but still >1%.

Table E.7: Comparisons of the archaeal communities for coal and GB treatments with similar amendment regimen between the 3 amendment periods using SIMPER analysis.

	Average Dissimilarity								
	coal vs GB			**0.1 algae coal vs GB**			**0.5 algae coal vs GB**		
	T1	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**
Methanoregula	13.8	10.6	18.6	15.5			3.7	1.3	1.4
Methanosaeta	13.5	15.6	24.7	17.2		10.6	36.2	45.0	
Methanospirillum	9.0	2.1		4.0	1.3	13.5	36.5	47.0	3.2
Methanolobus	5.7	5.5	4.7	1.6			1.3		
Methanobacteriaceae_unclassified	2.1	1.2		3.8	1.6	2.7			1.5
Methanobacterium							2.7		
Contribution of genera w/ > 1% dissimilarity	44.0	35.1	48.0	26.6	2.9	26.8	76.7	92.0	4.7
TOTAL av. dissim.	44.9	35.5	49.4	42.3	4.1	27.5	81.0	94.1	7.1

Archaeal community dissimilarity between unamended and amended treatments with the same solid substrate were compared (Table E.8), and coal treatment had less

dissimilarity than the corresponding GB treatments for almost all amendment periods. The average dissimilarity between unamended and amended coal, regardless of amendment amount, showed decreasing dissimilarity with each subsequent amendment period. The first and second amendment periods of coal comparisons had dissimilarity dominated by *Methanospirillum* and *Methanosaeta*. During the third amendment period, the coal treatments saw a greater contribution of *Methanoregula* and an unclassified *Methanobacteriaceae*. GB treatment comparisons saw more genera contributing to dissimilarity than coal treatment comparisons. The largest contributors to dissimilarity were *Methanosaeta*, *Methanospirillum*, and *Methanoregula*. Both GB comparisons had dissimilarity due to methylotrophic *Methanolobus*, and the 0.5 g/L algae amended comparison also showed in influence of *Methobacterium* in the dissimilarity in the first and second amendment periods.

Table E.8: Comparisons of archaeal communities for unamended and amended treatments with the same solid substrate for each of the 3 amendment periods using SIMPER analysis.

	Average Dissimilarity											
	coal vs 0.1 algae coal			GB vs 0.1 algae GB			coal vs 0.5 algae coal			GB vs 0.5 algae GB		
	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3
Methanospirillum	10.4	3.1		2.6		12.9	11.1	2.9		34.4	46.2	3.8
Methanosaeta	6.3			2.6	16.6	14.8	8.3	1.0		14.4	28.3	24.8
Methanobacteriaceae_unclassified	4.0	1.6	2.7	1.9	1.3				1.5			
Methanoregula			3.1		10.1	22.2	1.9	1.2	2.6	15.6	10.7	22.6
Methanolobus				4.1	5.1	4.5				4.4	5.6	4.7
Methanobacterium										2.6	1.3	
Contribution of genera w/ > 1% dissimilarity	20.6	4.7	5.9	11.1	33.1	54.4	21.4	5.1	4.0	71.3	92.0	56.0
TOTAL av. dissim.	22.1	6.2	7.5	13.0	33.8	55.4	22.3	5.9	5.6	73.9	92.5	57.4

Unamended and Algae Amended Bacterial SIMPER Analysis. Unamended and 0.1 and 0.5 g/L algae amended treatment bacterial communities were compared for dissimilarity (Table E.9). For all amendment levels, the GB treatments had more diversity in orders contributing >1% dissimilarity.

Unamended coal treatment dissimilarity was primarily due to Clostridiales and Desulfuromonadales over all time comparisons. Unamended GB treatment dissimilarity resulted from more orders that had smaller contributions to dissimilarity over all: Campylobacterales, Desulfovibrionales, Syntrophabacterales, Clostridiales, and others. Clostridiales and unclassified Bacteriodetes were the only contributors >1% dissimilarity in 0.1 g/L amended coal for all time comparisons. For 0.1 g/L GB treatments, Clostridiales, unclassified Bacteroidetes, unclassified Bacteria, and Bacillales were the largest contributors to dissimilarity. Other contributors for the 0.1 g/L GB dissimilarity include Anaerolineales, Desulfovibrionales, and Desulfobacterales.

For the 0.5 g/L algae amended coal treatments, Clostridiales and Bacteroidales were the largest contributors to dissimilarity across all time comparisons. These were also the largest contributors to dissimilarity for 0.5 g/L algae amended GB treatments along with Bacillales. The 0.5 g/L GB treatments also had contributions to dissimilarity between amendment periods from Enterobacteriales, Rhodocyclales, Desulfovibrionales, Desulfuromonadales, and Syntrophobacterales.

Table E.9: Comparisons of the bacterial communities from the same treatment condition between the 3 amendment periods for (a) unamended, (b) 0.1 g/L algae amended, and (c) 0.5 g/L algae amended treatments using SIMPER analysis.

(a)	Average Dissimilarity					
	coal only			GB only		
	T1:T2	T2:T3	T1:T3	T1:T2	T2:T3	T1:T3
Clostridiales	14.6	7.9	16.3		2.4	2.6
Desulfuromonadales	4.0	11.6	7.9			
Desulfovibrionales	2.2		1.8	2.1	1.9	1.4
Coriobacteriales		2.2	1.9		1.4	
Anaerolineales		1.4	1.2	1.6		2.0
Desulfobacterales		1.3	1.4	2.1		2.0
Campylobacterales				10.9	6.4	4.4
Syntrophobacterales				2.2		2.3
Pseudomonadales				1.9	2.1	
Bacteria_unclassified					2.2	1.5
Bacteroidetes_unclassified					1.1	
Burkholderiales						1.0
Contribution of orders w/ > 1% dissimilarity	20.8	24.5	30.6	20.8	17.5	17.3
TOTAL av. dissim.	25.5	30.4	36.2	28.8	22.6	24.0

(b)	Average Dissimilarity					
	0.1 algae coal			0.1 algae GB		
	T1:T2	T2:T3	T1:T3	T1:T2	T2:T3	T1:T3
Clostridiales	8.0	4.9	11.1	8.0	8.3	12.4
Bacteroidetes_unclassified	6.1	1.8	7.9	3.5	3.1	6.6
Bacteria_unclassified				6.4		5.9
Bacillales				2.5	8.1	10.5
Anaerolineales				1.8	1.3	3.1
Desulfovibrionales				1.1	2.4	3.3
Desulfobacterales				1.1		1.2
Contribution of orders w/ > 1% dissimilarity	14.1	6.7	19.1	24.3	23.3	42.9
TOTAL av. dissim.	21.2	12.6	26.9	29.1	29.1	49.3

(c)	Average Dissimilarity					
	0.5 algae coal			0.5 algae GB		
	T1:T2	T2:T3	T1:T3	T1:T2	T2:T3	T1:T3
Clostridiales	27.5	17.4	15.0	25.2	24.1	12.9
Bacteroidales	4.3		5.3	5.5	22.1	17.2
Bacteria_unclassified	3.1		1.2	4.4		4.0
Bacteroidetes_unclassified	2.3		5.2	3.7	3.4	7.1
Flavobacteriales	1.3		1.2			
Anaerolineales			2.8			
Bacillales			1.1	17.5		18.2
Enterobacteriales				6.5	9.2	3.1
Rhodocyclales				3.4	3.1	
Desulfovibrionales				4.2	2.0	2.2
Desulfuromonadales				1.3	1.3	
Syntrophobacterales					1.2	1.2
Contribution of orders w/ > 1% dissimilarity	38.4	17.4	31.9	71.5	66.3	65.9
TOTAL av. dissim.	43.1	36.9	37.9	77.8	73.3	69.6

Table E.10: Comparisons of the bacterial communities for coal and GB treatments with similar amendment regimen between the 3 amendment periods using SIMPER analysis.

	Average Dissimilarity								
	coal vs GB			0.1 algae coal vs GB			0.5 algae coal vs GB		
	T1	T2	T3	T1	T2	T3	T1	T2	T3
Clostridiales	18.4	20.0	19.4	23.0	14.7	13.9	22.4	39.2	25.2
Desulfuromonadales	17.2	21.2	9.7	5.2	4.6	5.1	2.0		1.4
Syntrophobacterales	6.2	3.8	3.2						2.3
Desulfobacterales	5.7	3.7	3.6	1.3					
Anaerolineales	4.5	3.0	1.3		1.6	2.5	1.1	1.2	4.0
Pseudomonadales	3.4	5.5	3.4						
Desulfovibrionales	3.1	2.3	3.9	3.6	2.7			4.4	2.2
Burkholderiales	2.1	1.8	1.4						
Betaproteobacteria_ unclassified	1.1								
Campylobacterales		10.9	4.4						
Bacteria_unclassified			1.8	4.9	1.2	1.2		1.4	2.8
Bacillales				12.6	9.3	1.9	15.7	1.8	1.4
Flavobacteriales				1.3			1.5		
Coriobacteriales				1.1					
Bacteroidetes_ unclassified				1.0	1.6		2.0	3.4	3.9
Bacteroidales							5.2	4.5	17.0
Enterobacteriales								6.7	3.0
Rhodocyclales								3.4	
Contribution of orders w/ > 1% dissimilarity	61.7	72.1	52.1	53.9	35.7	24.6	49.8	66.0	63.1
TOTAL av. dissim.	70.8	79.4	60.3	59.3	41.3	31.7	55.3	73.1	68.4

For bacterial community comparisons of dissimilarity between amended coal treatments and the corresponding GB treatments (Table E.10), Clostridiales contributed the greatest dissimilarity for all comparisons over all time points. For unamended comparisons, Desulfuromonadales was also a large contributor to dissimilarity. Several other bacterial orders contributed >1% dissimilarity but a lesser amount than those named above. For the 0.1 g/L algae amended treatment comparison, Bacillales were a large contributor to dissimilarity and several others contributed >1%. For 0.5 g/L algae amended treatments, Bacillales were a high contributor to dissimilarity in the first

amendment period while unclassified Bacteriodetes was a great contributor in the third amendment period.

Table E.11: Comparisons of bacterial communities for unamended and amended treatments with the same solid substrate for each of the 3 amendment periods using SIMPER analysis.

	Average Dissimilarity											
	coal vs 0.1 algae coal			GB vs 0.1 algae GB			coal vs 0.5 algae coal			GB vs 0.5 algae GB		
	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3
Desulfuromonadales	12.7	17.1	4.9				16.1	17.1	8.8			
Clostridiales	11.1	17.7	14.2	5.1	13.0	14.0	16.2	17.7	19.7	5.1	23.3	17.0
Bacteroidetes_ unclassified	2.5	9.1	10.2	2.8	7.1	3.8	7.3	9.1	1.9	2.8	1.7	2.8
Bacillales	2.3	3.3	2.6	14.9	12.6	4.5	2.7	3.3	1.6	14.9		
Desulfovibrionales	2.2			4.5	1.5	9.1	2.3			4.5	2.8	2.0
Flavobacteriales	1.3						1.5					
Bacteria_ unclassified	1.0	1.1		5.1		2.2	2.9	1.1	1.3	5.1	1.6	3.3
Anaerolineales		1.3		3.5		1.7		1.3	2.5	3.5	3.0	3.0
Coriobacteriales			2.2	1.0		2.0			2.7	1.0		2.1
Desulfobacterales			1.2	5.3	3.8	3.8			1.6	5.3	3.8	3.9
Syntrophobacterales				5.9	4.0	3.3			1.0	3.4	5.3	2.8
Pseudomonadales				3.4	5.4	3.3				5.9	4.3	5.4
Burkholderiales				2.0	1.9	1.7				2.0	1.5	1.6
Betaproteobacteria_ unclassified				1.1						1.1		
Campylobacterales					10.9	4.5					10.9	4.5
Bacteroidales									5.4			22.1
Enterobacteriales											6.7	3.3
Rhodocyclales											3.4	
Contribution of orders w/ > 1% dissimilarity	33.2	49.6	35.4	54.7	60.2	53.9	48.9	49.6	46.5	54.7	68.2	73.8
TOTAL av. dissim.	38.7	54.4	42.7	64.1	70.2	63.8	56.0	69.0	52.8	79.5	79.3	84.1

For comparisons between bacterial communities in amended and unamended treatments, Clostridiales and Desulfuromonadales were the largest contributors to dissimilarity in all coal treatment comparisons for all time periods (Table E.11). Unclassified Bacteroidetes and Bacillales were other larger dissimilarity contributors with several other less significant but >1% dissimilar members. The GB comparisons had

more bacterial orders contributing to dissimilarity than the coal comparisons. For the 0.1 g/L algae amended and unamended GB comparison, Clostridiales and Bacillales were the 2 largest contributors to dissimilarity on all time points. Thirteen total bacterial orders contributed >1% dissimilarity on at least one time points while Desulfobacterales Syntrophobacterales, and Campylobacterales were larger contributors on some time points. For the 0.5 g/L algae amended GB versus unamended GB comparison, Clostridiales was the largest contributor to dissimilarity. Desulfovibrionales were a large contributor during amendment period 1, Campylobacterales were a large contributor during the second amendment period, and Bacteroidales were a large contributor during the third amendment period.

Discussion and Conclusions

Methane production in coal treatments was highest in the first amendment period and decreased after subsequent re-amendments while GB treatments produced similar or more methane after subsequent re-amendments. Methane production during the first amendment period was higher for coal treatments than for the corresponding GB treatments. During the second and third amendment periods, corresponding coal and GB treatments produced similar amounts of methane. The maximum methane production rate for coal treatments was highest during the first amendment period and decreased with re-amendment while GB treatments observed an increased maximum methane production rate with each amendment period. By the third amendment period, the corresponding coal and GB treatments had a similar maximum methane production rate for most of the

treatments. Additionally, the methane produced in 0.1 and 0.5 g/L treatments was not proportional to the amount of amendment added during the first amendment period. During the second and third amendment periods, the methane produced was roughly proportional to the amount of amendment added. All of these observations together support a shift of the carbon source for methane production from a higher coal-to-methane conversion in the first amendment period to greater influence of direct amendment-to-methane conversion with re-amendment in the second and third amendment periods.

For SIMPER comparisons of all treatments, there was generally more dissimilarity in GB archaeal communities than in the corresponding coal treatments over time while bacterial communities showed similar dissimilarity between coal and GB treatments. Unamended treatments showed greater dissimilarity for both archaeal and bacterial communities for all corresponding coal and GB comparisons except for the archaeal comparisons for 0.5 g/L algae amended treatments. In addition, when comparing amended and unamended treatments with the same solid substrate, GB comparisons showed more dissimilarity for both archaeal and bacterial communities for all conditions when compared to the same treatment with coal. These observations support the conclusions that the presence or absence of coal can have a significant impact on microbial community structure and that the addition of amendment can cause community shifts especially without the influence of coal.

The major archaeal contributors to dissimilarity in all comparisons of unamended and algae amended treatments were *Methanosaeta*, *Methanoregula*, and

Methanospirillum which correlates with the principal component analysis (PCA) in the original study presented in Chapter 3 that showed these genera as the most influential in the differences observed in archaeal population. *Methanolobus*, described as capable of methylotrophic methanogenesis [50,118], was observed to contribute to dissimilarity only in comparisons with GB treatments. This observation could suggest that methylotrophic methanogenesis may not be a big contributor in coal-to-methane conversion but may play a part in the conversion of amendment itself.

Similar to the SIMPER comparisons with archaeal communities, bacterial comparisons had more contributors of more than 1% dissimilarity for GB treatments than coal treatments. The bacterial community dissimilarities for nearly all comparisons had a large influence of Clostridiales, Bacillales, Bacteroidetes, and Desulfuromonadales. All of these groups have been previously identified in coal microbial communities and are thought to contain members contributing to the degradation of organic matter.[2,42,49,59,119]

Coriobacteriales, an order in the phylum Actinobacteria, was found to be a bacterial member contributing in several comparisons but more strongly correlating to GB treatments than coal treatments. Members of Coriobacteriales have been associated with biosurfactant production.[120] In addition, some members have been found to be facultative or even microaerophilic, needing low levels of oxygen for respiration but possibly being inhibited by high levels.[121] Two other groups associated with microaerophilicity, Burkholderiales[122,123] and Campylobacterales[124], were also found to contribute to dissimilarity comparisons primarily in comparison including treatments with GB. The higher influence of microaerophilic-associated OTUs in the GB treatments

may suggest that there is some characteristic of coal that reduces the oxygen concentration in the system either by abiotic or biotic processes that is not supported in GB treatments.

Several orders associated with sulfur cycling were also found with SIMPER analysis to be >1% contributors to dissimilarity in many comparisons and most often in GB treatments. The Campylobacterales contain members associated with sulfur-oxidation[124] and contributed to dissimilarity in all comparisons to unamended GB treatments. Desulfovibrionales, Desulfobacterales, Desulfuromonadales, and Syntrophobacterales were all found to contribute to dissimilarity and contain members associated with sulfate reduction.[125–129] Most of these had higher association with dissimilarity comparisons including GB treatments than with coal treatments, except for Desulfuromonadales which had high association with coal comparison dissimilarities. The genus *Geobacter* is a member of the Desulfuromonadales and has been previously associated with methane-producing coal enviroments.[2,31,130] This could explain the higher association with coal treatments instead of GB treatments for the Desulfuromonadales dissimilarity contributions.

While SIMPER analysis cannot expose or explain all microbial community differences observed in these treatments, the observations made with this method suggest that there may be two groups-of-interest contributing to significant differences between coal and GB treatments: groups that are aero-tolerant or microaerophilic and groups associated with sulfur cycling. This supports previous observations that coal is an important component of CBM production, not only as a substrate for eventual

methanogenesis, but also for its influences on limiting microbial community shifts away from coal-degrading capabilities.

While the results of this study suggest general trends in methane production and microbial community changes with repeated amendment of coal and non-coal treatments, only one replicate of each treatment was sampled for the entire duration of the 322-day study, and only one replicate was sequenced for community analysis. In addition, on re-amendment days 111 and 172, all replicates remaining were re-amended. Thus, there were no non-re-amended treatment controls to compare. Regardless, this experiment laid the foundation and provided preliminary data for the study presented in Chapter 4 where a similar study was performed with more replicates and controls for the unamended and 0.1 g/L amended algae subset of the treatments presented here.

APPENDIX F

DECONVOLUTION OF GC-MS DATA TO DETERMINE THE ^{13}C CONTENT OF PRODUCED GASES: METHANE AND CARBON DIOXIDE

Gas Chromatography-Mass Spectrometry Analytical Parameters

To evaluate the ^{13}C content of produced CH_4 and CO_2 gases, a 500 µL gas sample was manually injected on an Agilent 6890 GC 5973 electron impact ionization mass selective detector (Agilent Technologies, Palo Alto, CA, USA) interfaced with Agilent Enhanced ChemStation software and operated in scan mode. A GS-Carbonplot column (60 m × 0.320 mm i.d. × 1.50 µm film thickness) was used for analysis. The following parameters were used for analysis: 500 µL manual injection with 30:1 split ratio, constant flow at 1 mL/min, injector temperature 185ºC, constant interface temperature 60ºC, and scan range m/z 2-100. The carrier gas was ultra-high purity helium.

The areas obtained from extracted ion chromatograms were used for analysis. For methane, m/z = 13, 14, 15, 16, and 17 were used. These m/z peaks represented all the measureable ion fragments of methane. For CO_2, m/z = 44 and 45 were used for deconvolution. These m/z areas were the largest fraction of the CO_2 ion fragments and $^{12}CO_2$ and $^{13}CO_2$ fragmented similarly. Standards were run and analyzed to deconvolute this data for the determination of the fraction of ^{13}C in CH_4 and CO_2.

Methane

Methane standards were analyzed on the GC-MS. The natural abundance CH_4 standard was assumed to be 1.1% $^{13}CH_4$. The $^{13}CH_4$ standard was 99% purity $^{13}CH_4$. Extracted ion chromatograms for each of these standards were used to obtain areas of each mass-to-charge ratio's (m/z) peak and fractional abundance values were calculated for 13, 14, 15, 16, and 17 shown in Table F.1.

Table F.1: Areas and fractional abundance of the 10% methane m/z values used for deconvolution analysis. The CH_4 standard is assumed to have approximately 1.1% $^{13}CH_4$ reflecting the natural abundance of ^{13}C.

	CH_4		**$^{13}CH_4$**	
m/z	**Area**	**Abundance**	**Area**	**Abundance**
13	801365	0.040	211634	0.007
14	626461	0.032	1200410	0.041
15	8273527	0.417	1245327	0.042
16	10011016	0.505	12256870	0.416
17	113538	0.006	14565605	0.494

To calculate the percentage of $^{13}CH_4$ in the sample analyzed, all m/z peak areas were compared to the m/z = 17 peak (Table F.2). It was assumed that any ions with a m/z of 17 must be from $^{13}CH_4$. Thus, in a fully labeled methane standard, the ratios of any m/z value (for 13, 14, 15, 16) to the m/z 17 will represent an assumed consistent ion fragmentation pattern for all $^{13}CH_4$.

Table F.2: Ratios of the m/z areas for 10% $^{13}CH_4$ methane standard.

m/z ratio	**^{13}STD ratios**
13/17	0.0145
14/17	0.0824
15/17	0.0855
16/17	0.8415
17/17	1

The ratios shown in Table F.2 were used to calculate the contribution of $^{13}CH_4$ to each m/z peak in samples. The area attributed to $^{13}CH_4$ by this method were subtracted from the observed peak area for measured m/z area of samples to calculate the $^{12}CH_4$ as shown in Table F.3. These calculated individual m/z areas for $^{12}CH_4$ and $^{13}CH_4$ were used to calculate the $\%^{13}CH_4$ of the total methane for each sample.

Table F.3: Outline of calculations made to determine $\%^{13}CH_4$ of the total sample methane.

m/z	Sample Area	$12CH_4$	$13CH_4$
13	$sample_{13}$	$sample_{13} - {}^{13}STD_{13/17} * sample_{17}$	${}^{13}STD_{13/17} * sample_{17}$
14	$sample_{14}$	$sample_{14} - {}^{13}STD_{14/17} * sample_{17}$	${}^{13}STD_{14/17} * sample_{17}$
15	$sample_{15}$	$sample_{15} - {}^{13}STD_{15/17} * sample_{17}$	${}^{13}STD_{15/17} * sample_{17}$
16	$sample_{16}$	$sample_{16} - {}^{13}STD_{16/17} * sample_{17}$	${}^{13}STD_{16/17} * sample_{17}$
17	$sample_{17}$		$sample_{17}$
SUM	$sample_{total}$	$12C_{total}$	$13C_{total}$
%		$12C_{total}/ sample_{total}$	$13C_{total}/ sample_{total}$

To test this deconvolution method, 3 samples were sent to the UC Davis Stable Isotope Facility where they were analyzed using an isotope ratio mass spectrometer (IRMS). A comparison of the $\%^{13}CH_4$ using the deconvolution method and the amount measured at UC Davis is shown in Table F.4. The percent difference of the $\%^{13}CH_4$ from the deconvolution method described here compared to the IRMS results varied from 0.15-2.40%. Thus, the deconvolution method presented here was considered a sufficiently accurate calculation to estimate the $\%^{13}CH_4$ with less than 3% difference to established IRMS methods.

Table F.4: Comparison of 3 samples for $\%^{13}CH_4$ using the deconvolution method presented here and the result of the UC Davis IRMS analysis; percent differences between the methods were also calculated.

Sample ID	IRMS -UC Davis	Deconvolution	% difference
POC5-1'	40.86%	40.92%	0.15%
POC5-2'	41.19%	40.68%	-1.23%
POC5-3'	42.54%	41.52%	-2.40%

Carbon Dioxide

CO_2 standards were also analyzed on the GC-MS. The areas and fractional abundances of the four most abundant m/z fractions are shown in Table F.5. The CO_2

standard contains natural abundance of $^{13}CO_2$, assumed to equal 1.1%. Because both $^{12}CO_2$ and $^{13}CO_2$ fragment similarly during GC-MS analysis, the sum of the m/z 44 and m/z 45 areas is representative of the sum of both$^{12}CO_2$ and $^{13}CO_2$. Using this assumption and summing the m/z 44 and m/z 45 abundance (representing the $^{12}CO_2$ and $^{13}CO_2$, respectively) in the environmental concentration CO_2 standard the abundance of the CO_2 ion is 0.919 and equal to the abundance of the m/z 45 in the $^{13}CO_2$ standard.

Table F.5: Areas and fractional abundance of the 100% CO_2 m/z values for analysis. The CO_2 standard is assumed to have approximately 1.1% $^{13}CO_2$ reflection the accepted environmental concentrations.

	99.9% CO_2		**99.9% $^{13}CO_2$**	
m/z	**Area**	**Abundance**	**Area**	**Abundance**
28	40138712	0.081	438840	0.001
29	312598	0.001	48078232	0.080
44	449702494	0.909	83.4	0.000
45	4831525	0.010	551233324	0.919

For making calculation of $\%^{13}CO_2$, the sample m/z 44 and 45 were used as in Equation 7.

$$\%^{13}CO_2 = \frac{Area\left(\frac{m}{z}=45\right)_{sample}}{Area\left(\frac{m}{z}=44\right)_{sample}+Area\left(\frac{m}{z}=45\right)_{sample}} \quad \text{(Eq. 7)}$$

When Eq. 7 is applied to the natural abundance CO_2 standard (Table F.5), an abundance of 1.01% $^{13}CO_2$ is calculated which is similar to the natural abundance of 1.1% and confirms this analysis.

Produced $^{13}CH_4$ Can Be Used as a Surrogate for Amendment Conversion

The amount of methane produced from coal-to-methane conversion versus amendment-to-methane conversion can be assessed using the amount of ^{13}C-label in the amendment and coal, the amount of $^{12}CH_4$ produced, and the amount of $^{13}CH_4$ produced. It is assumed that the preferential uptake of ^{12}C over ^{13}C by the microbial consortium is insignificant for this analysis. Therefore, methane produced from coal conversion will have the same $^{12}C/^{13}C$ ratio as the coal itself and methane produced from amendment conversion will have the same $^{12}C/^{13}C$ ratio as the amendment itself. With these assumptions, the amount of methane produced from coal and amendment can be estimated according to Eq. 1 and 2.

$$[^{12}CH_4] = 0.989[CH_{4_{coal}}] + \left(1 - \%^{13}C_{amendment}\right) * [CH_{4_{amendment}}] \quad \text{(Eq.1)}$$

$$[^{13}CH_4] = 0.011[CH_{4_{coal}}] + \left(\%^{13}C_{amendment}\right) * [CH_{4_{amendment}}] \quad \text{(Eq. 2)}$$

From these equations, the amount of methane produced from amendment and coal conversion were calculated and are shown in Table F.6, along with the measured $^{13}CH_4$ and $^{12}CH_4$ produced amounts. Coal treatments amended with 0.1 g/L ^{13}C-algae produced 6.0 ± 0.3 µmol CH_4/g coal from algae and 52.3 ± 2.1 µmol CH_4/g coal from coal. These calculated values were similar to the measured amounts of produced $^{13}CH_4$ and $^{12}CH_4$, 6.3 ± 0.3 µmol $^{13}CH_4$/g coal and 52.1 ± 2.0 µmol $^{12}CH_4$/g coal. The calculated methane produced from amendment and coal in 0.1 g/L ^{13}C-yeast amended coal treatments was

6.4 ± 0.6 and 58.5 ± 16.4 µmol CH_4/g coal, respectively. The measured values for produced $^{13}CH_4$ and $^{12}CH_4$, 6.9 ± 0.5 µmol $^{13}CH_4$/g coal and 58.1 ± 16.2 µmol $^{12}CH_4$/g coal, were similar to the calculated contributions from amendment and coal.

Table F.6: Methane production due to coal or amendment conversion.

Treatment	**CH_4 from amendment (µmol/g coal)**	**CH_4 from coal (µmol/g coal)**	**$^{13}CH_4$ (µmol/g coal)**	**$^{12}CH_4$ (µmol/g coal)**
0.1 g/L 13-algae + coal	6.0 ± 0.3	52.3 ± 2.1	6.3 ± 0.3	52.1 ± 2.0
0.1 g/L 13-yeast + coal	6.4 ± 0.6	58.5 ± 16.4	6.9 ± 0.5	58.1 ± 16.2
0.5 g/L 13-algae + coal	32.3 ± 1.6	56.7 ± 0.7	31.6 ± 1.6	57.8 ± 0.7
0.5 g/L 13-yeast + coal	42.0 ± 0.9	73.5 ± 5.4	42.0 ± 1.0	73.9 ± 5.4

For 0.5 g/L amended coal treatments, calculated amounts of methane produced from amendment and coal were also similar to the measured amounts of $^{13}CH_4$ and $^{12}CH_4$. For the 0.5 g/L ^{13}C-algae amended coal treatments, calculated methane amounts from amendment and coal were 32.3 ± 1.6 and 56.7 ± 0.7 µmol CH_4/g coal, respectively. The measured amounts of $^{13}CH_4$ and $^{12}CH_4$ were 31.6 ± 1.6 µmol $^{13}CH_4$/g coal and 57.8 ± 0.7 µmol $^{12}CH_4$/g coal. For the 0.5 g/L ^{13}C-yeast amended coal treatments, calculated methane amounts from amendment and coal were 42.0 ± 0.9 and 73.5 ± 5.4 µmol CH_4/g coal, respectively. The measured amounts of $^{13}CH_4$ and $^{12}CH_4$ were 42.0 ± 1.0 µmol $^{13}CH_4$/g coal and 73.9 ± 5.4 µmol $^{12}CH_4$/g coal.

The calculated methane produced from coal is less than 2% different from the measured $^{12}CH_4$, and the calculated methane produced from amendment is less than 5% different from the measured $^{13}CH_4$. These errors also approximately correspond with the ^{13}C abundance in coal (1.1%) and the ^{12}C abundance in the amendments (5% for algae and 3% for yeast). Thus, these results suggest the measured amount of $^{13}CH_4$ produced in

coal systems amended with ^{13}C-labeled amendment provides a good approximation for the contribution of amendment-to-methane conversion to methane production and the amount of $^{12}CH_4$ for the contribution of coal-to-methane conversion.

APPENDIX G

SUPPLEMENTARY FIGURES FOR CHAPTER 5

Supplementary figures for Chapter 5

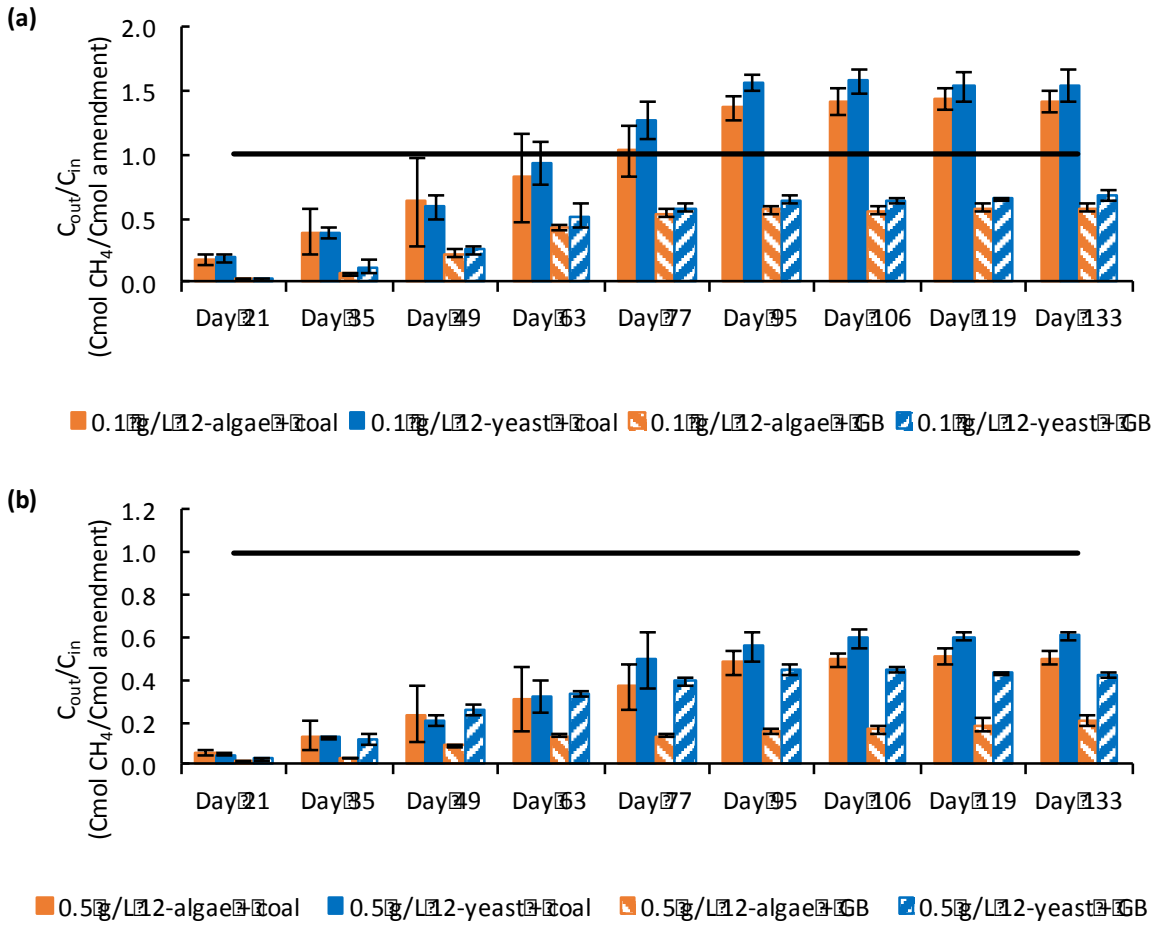

Figure G.1: Comparison of total carbon produced as methane to carbon added as amendment for a) 0.1 g/L and b) 0.5 g/L unlabeled amendment treatments in study 1 from Chapter 5. Error bars represent one standard deviation. A value greater than 1 indicates more methane production than could be accounted for by complete amendment conversion to methane.

Table G.1: Percentage of the total produced as $^{13}CH_4$ and $^{13}CO_2$ for all treatments in study 1 presented in Chapter 5.

Treatment	**$^{13}CH_4$ (% of total methane)**	**$^{13}CO_2$ (% of total CO_2)**
coal only	0.9 ± 0.1	0.9 ± 0.0
GB only	NA	0.9 ± 0.0
0.1 g/L 13-algae + coal	10.8 ± 0.7	3.4 ± 0.3
0.1 g/L 13-algae + GB	31.0 ± 1.0	4.6 ± 0.1
0.1 g/L 12-algae + coal	1.0 ± 0.0	0.9 ± 0.0
0.1 g/L 12-algae + GB	0.9 ± 0.0	0.9 ± 0.0
0.1 g/L 13-yeast + coal	11.2 ± 2.5	4.9 ± 0.4
0.1 g/L 13-yeast + GB	32.8 ± 0.8	5.6 ± 0.1
0.1 g/L 12-yeast + coal	1.0 ± 0.0	0.9 ± 0.0
0.1 g/L 12-yeast + GB	0.9 ± 0.0	0.9 ± 0.0
0.5 g/L 13-algae + coal	35.4 ± 1.0	16.6 ± 0.2
0.5 g/L 13algae + GB	50.2 ± 0.7	15.2 ± 0.4
0.5 g/L 12-algae + coal	1.0 ± 0.0	1.0 ± 0.1
0.5 g/L 12algae + GB	1.0 ± 0.0	0.9 ± 0.0
0.5 g/L 13-yeast + coal	36.3 ± 1.2	20.8 ± 0.5
0.5 g/L 13-yeast + GB	53.8 ± 1.6	22.4 ± 0.3
0.5 g/L 12-yeast + coal	1.1 ± 0.0	1.5 ± 0.1
0.5 g/L 12-yeast + GB	1.0 ± 0.0	1.4 ± 0.0

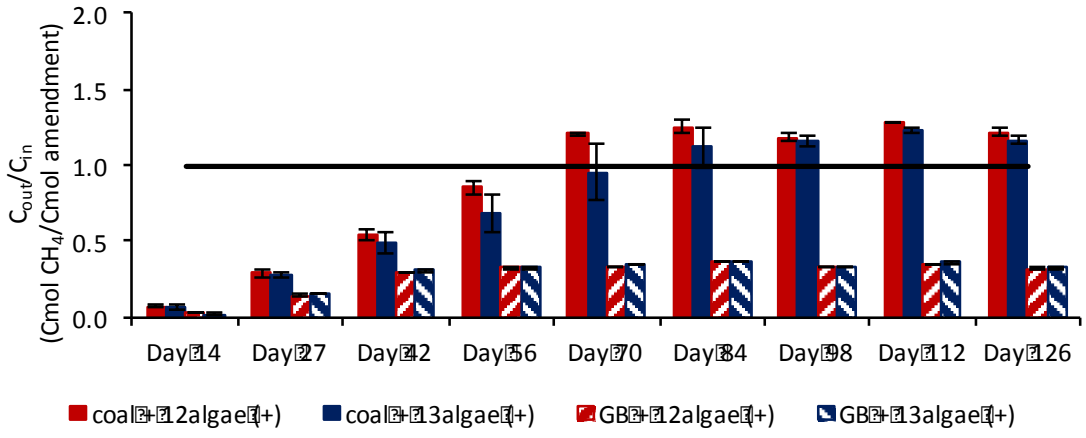

Figure G.2: Comparison of total carbon produced as methane to carbon added as amendment for treatments in study 2 from Chapter 5. Error bars represent one standard deviation. A value greater than 1 indicates more methane production than could be accounted for by complete amendment conversion to methane.

Table G.2: Percentage of the total produced as $^{13}CH_4$ and $^{13}CO_2$ for all treatments in study 2 presented in Chapter 5.

Treatment	**$^{13}CH_4$ (% of total methane)**	**$^{13}CO_2$ (% of total CO_2)**
coal only	0.8 ± 0.0	0.9 ± 0.0
0.1 g/L 12-algae + coal	1.0 ± 0.0	0.9 ± 0.0
0.1 g/L 12-algae + GB	0.8 ± 0.0	0.8 ± 0.0
0.1 g/L 13-algae + coal	10.8 ± 0.0	2.0 ± 0.0
0.1 g/L 13-algae + GB	54.1 ± 0.0	5.3 ± 0.0

APPENDIX H

DEMONSTRATION OF REACTOR DESIGN FOR GAS PRODUCTION AND CAPTURE USING *METHANOSARCINA ACETIVORANS* AS A MODEL METHANOGEN

Experimental Justification

A reactor system was developed for scale-up of the enhanced coal-to-methane conversion experiments performed previously in batch systems. The reactor system described and utilized in Chapter 6 required multiple iterations and testing with a model methanogen to optimize performance. The original design had a recirculating flow and in-line pH and oxidation-reduction potential (ORP) monitoring probes. The final design was for a single-pass flow with the capability of up to 4 simultaneous reactors from a single influent source. *Methanosarcina acetivorans* is a fast-growing methanogen that can utilize both acetate and methanol to produce methane.[51,131] This organism was used to test the reactor system's ability to maintain an oxygen-free environment and to separate and collect gases as well as the adaptability and accessibility for benchtop usage.

Study 1: Recirculating Flow

The original recirculating reactor system was designed to reduce the locations of potential oxygen infiltration when testing methane enhancement methods for coal systems and is shown in Figure H.1. The entire system, except the inline pH and ORP probes, was autoclaved prior to set up. The pH and ORP probes were cleaned with 100% methanol and reinstalled in the autoclaved system. The reactor column was taken to the microbiological safety cabinet and filled with 2-4 mm effective size Flowers-Goodale coal. The DSMZ 141c *Methanosarcina* sp. medium was made with the modifications recommended for DSM strain 2834.[132] The media was autoclaved, then cooled to room temperature under 20% CO_2/ 80% N_2 anoxic gas sparge. The autoclaved reactor system

was purged with the same gas mixture to remove atmospheric oxygen, and 400 mL of the cooled medium was pumped into the media reservoir.

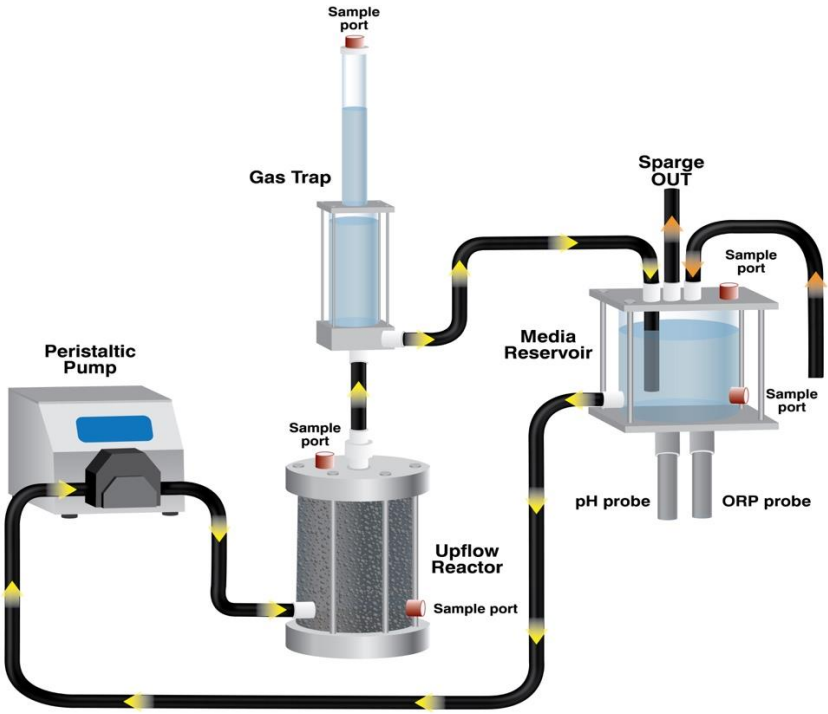

Figure H.1: The original recirculating flow for th upflow column reactor system with in-line pH and ORP probes.

The medium was circulated through the reactor system at 1 mL/min for 48 hours. The pumping flow rate was reduced to 0.055 mL/min. The pH was measured at 6.9 and the ORP at -178mV. The reactor column was inoculated at the influent sampling port with 25 mL of active *M. acetivorans* monoculture. The system was monitored for gas production. Once gas was detected in the gas trap, the system was sampled approximately every 12 hours. A one mL sample was taken from the gas trap. The remainder of the gas was removed from the gas trap and the volume was measured to quantify the total gas produced. A one mL sample was taken from the headspace of the media reservoir. The

media reservoir was then sparged for one minute with 20% CO_2/80% N_2 anoxic gas to remove any collected gases.

In the first study of one reactor with recirculating flow, the first measureable gas collected in the gas trap was at 36 hours after inoculation (Figure H.2a). At this time, 17.2 mL of gas was collected from the gas trap with a total of 0.23 mmol of CH_4. No methane was detected in the media reservoir.

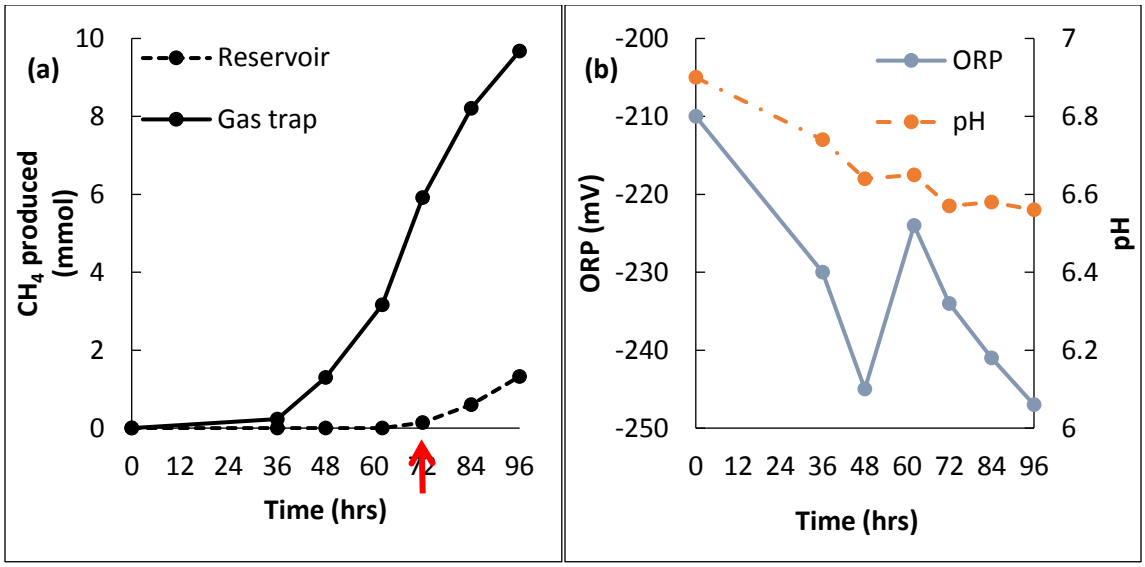

Figure H.2: (a) Methane captured and quantified from the gas trap and reservoir of the recirculating reactor design. The first quantifiable methane in the reservoir was measured after 72 hours and is designated with the arrow. (b) ORP and pH measured with inline probes during recirculating system study.

The first detectable methane sampled in the media reservoir was at 72 hours. At this point 0.15 mmol CH_4 was removed from the headspace of the media reservoir. The last sample time was at 96 hours. The total gas collected in the gas trap for the entire study duration was 479.2 mL and 9.68 mmol CH_4. The total methane measured in the reservoir was 1.32 mmol CH_4.

pH and ORP measurements were taken at each sampling time with the inline probes and shown in Figure H.2b. Both saw small decreases during the 96-hour study. The ORP decreased from -210 to -247 mV, and the pH decreased from 6.9 to 6.56. With longer usage, the pH and ORP probes experienced biofouling and reduced accuracy. With inline installation, it was not possible to remove these propes for cleaning and recalibration during longer studies. With the small changes in pH and ORP observed in this study, it was decided that these inline probes were not absolutely necessary and thus were eliminated in later iterations of the reactor design.

Study 2: Single-pass Flow

The recirculating reactor system was adapted to a single-pass flow to reduce the accumulation of oxygen infiltrating the system when operated on the longer time scales needed for adapation of coal consortia than for the model methanogen, *M. acetivorans*. A schematic of this system is shown in Figure H.3. This system is capable of running up to 4 reactor columns from a single influent source. The Norprene tubing from the influent carboy was split for each reactor system before entering the pumpheads. The pH and ORP probes were deemed unnecessary due to previous biofouling and removed from this system. For this study, two reactor systems were prepared and autoclaved prior to use. The reactor column was filled with coal in the microbiological safety cabinet as in the recirculating system and reinstalled within the complete system.

Two liters of DSMZ 141c medium were made as for the recirculating study and pumped into the influent carboy. The gas in the reactor system was exchanged with

anoxic 20% CO_2/80% N_2 to remove atmospheric oxygen. The reactor systems were filled with medium by pumping at 6 mL/min until the reactor systems were full from the influent carboy to the effluent reservoir. The flowrate was slowed to 0.05 mL/min for 24 hours to ensure the reactors were staying oxygen and leak free.

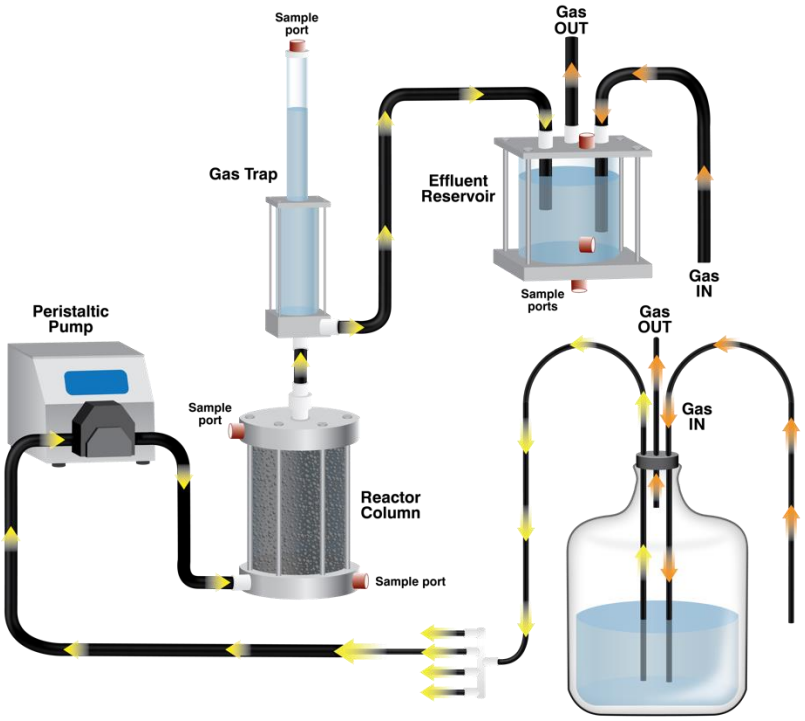

Figure H.3: The finalized reactor system design is a single-pass flow path with up to 4 reactors operated from the same prepared influent source.

Both reactor columns were inoculated with 20 mL of active *M. acetivorans* at the bottom sample port of the reactor column. After first gas appearance in the gas trap, the gas trap and effluent reservoir were sampled approximately every 12 hours as described for the recirculating system.

The first detectable gas was collected from the gas traps of both reactor systems 24 hours after inoculation. No methane was detectable in the effluent reservoir at this

time. The first detectable methane in the effluent reservoir for both reactor systems was after 94 hours. After 101 hours, the reactors were stopped and taken down. Reactor #1 had produced a total of 672 mL of gas captured from the gas trap. A total of 11.9 mmol of CH_4 was measured. Reactor #2 had produced 8.9 mmol CH_4 in the 710 mL of gas collected in the gas trap during the 101-hour study. The total methane produced in effluent reservoirs was 0.4 mmol and 1.86 mmol CH_4 for #1 and #2, respectively. These methane measurements do not account for the amount of methane produced that was dissolved in the medium, that still remained in the reactor, or that might have sorbed to the coal.

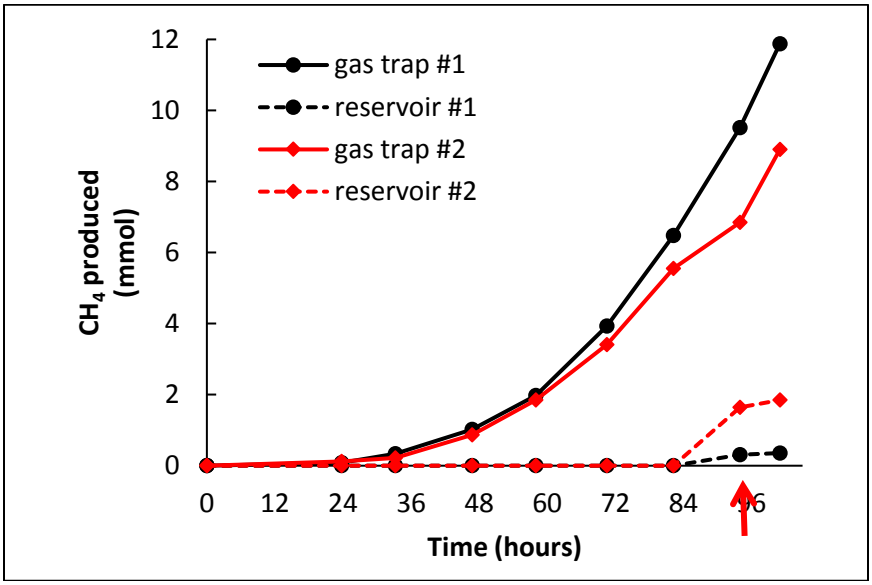

Figure H.4: Total methane collected and quantified from the gas traps and reservoirs of two identically operated single-pass flow column reactors inoculated with *M. acetivorans*.

Gas Analysis

Methane content of sampled gases 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 x 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 microcosm headspace for GC injection.

Discussion and Conclusions

The results of the studies presented here were used to make improvements to the reactor system to improve confidence in general operation, ability to prevent oxygen infiltration, gas collection and quantification, and sampling protocol. Modifications made to the system after the studies presented here prior to the study discussed in Chapter 6 include:

1) Reactor column was changed from the plastic column to a stainless steel column due to growing frequency of stress fractures in the plastic reactor bases resulting in leakage and oxygen infiltration.
2) The Norprene tubing between the influent carboy and the pumps was replaced with stainless steel tubing to minimize oxygen diffusion through the tubing material.

3) The copper oxygen scrubbers for the laboratory anaerobic gas system were discovered to be inadequate for long term experiments because they do not completely remove all O_2. Therefore, a gas-tight Tedlar® bag was placed inside the influent carboy and loaded with the medium/formation water for future experiments to add another layer of defense against oxygen infiltration.

APPENDIX I

THERMODYNAMICS OF METHANOGENESIS

Hydrogenotrophic Methanogenesis

(1) $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$

(2) $HCO_3^- + 4H_2 + H^+ \rightarrow CH_4 + 3H_2O$

Hydrogenotrophic methanogenesis is the reduction of inorganic carbon with hydrogen as the electron donor. Equation (1) shows the chemical reaction if CO_2 is the inorganic carbon source. CO_2 can be in the gas phase or the aqueous phase. Equation (2) shows the chemical reaction if bicarbonate is the inorganic carbon source. Figure I.1 shows the results of thermodynamic assessment for hydrogenotrophic methanogenesis.

Table I.1: Concentrations of hydrogenotrophic constituents for reactions (1) and (2) that are held constant for Figure I.1. Concentrations/activities from Ritter et al. (unpublished).

	Assumed inorganic carbon species		
Constituent	**$CO_{2(g)}$ (atm)**	**$CO_{2(aq)}$ (mM)**	**HCO_3^- (mM)**
H_2	1.0e-6	7.8e-7	7.8e-7
CH_4	0.868	1.3	1.3
CO_2	0.0075	0.26	--
HCO_3^-	--	--	22.5

Acetoclastic Methanogenesis

(3) $CH_3COOH \rightarrow CH_4 + CO_2$

(4) $CH_3COOH + H_2O \rightarrow CH_4 + HCO_3^- + H^+$

Acetoclastic methanogenesis utilizes acetate as the carbon substrate for methane production. Equation (3) shows the chemical reaction if CO_2 is the inorganic carbon product. Like in hydrogenotrophic methanogenesis, CO_2 could be in the gas phase or the aqueous phase. Equation (4) shows the chemical reaction if bicarbonate is the inorganic carbon product. Figure I.2 shows the results of thermodynamic assessment for acetoclastic methanogenesis.

Table I.2: Concentrations of acetoclastic constituents for reactions (3) and (4) that are held constant for Figure I.2. Concentrations/activities from Ritter et al. (unpublished). (*unless otherwise indicated)

	Assumed inorganic carbon species		
Constituent	**$CO_{2(g)}$ (atm*)**	**$CO_{2(aq)}$ (mM)**	**HCO_3^- (mM)**
CH_3COOH	0.007 mM	0.007	0.007
CH_4	0.868	1.3	1.3
CO_2	0.0075	0.26	--
HCO_3^-	--	--	22.5

Methylotrophic Methanogenesis

(5) $4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$

(6) $4CH_3OH \rightarrow 3CH_4 + HCO_3^- + H_2O + H^+$

Methylotrophic methanogenesis converts methanol to methane. Equation (5) shows the chemical reaction if CO_2 is the inorganic carbon product. Like in hydrogenotrophic methanogenesis, CO_2 could be in the gas phase or the aqueous phase. Equation (6) shows the chemical reaction if bicarbonate is the inorganic carbon product. Figure I.3 shows the results of thermodynamic assessment for methylotrophic methanogenesis.

Table I.3: Concentrations of methylotrophic constituents for reactions (5) and (6) that are held constant for Figure I.3 Concentrations/activities from Ritter et al. (unpublished). (*unless otherwise indicated)

	Assumed inorganic carbon species		
Constituent	**$CO_{2(g)}$ (atm*)**	**$CO_{2(aq)}$ (mM)**	**HCO_3^- (mM)**
CH_3OH	0.001 mM	0.001	0.001
CH_4	0.868	1.3	1.3
CO_2	0.0075	0.26	--
HCO_3^-	--	--	22.5

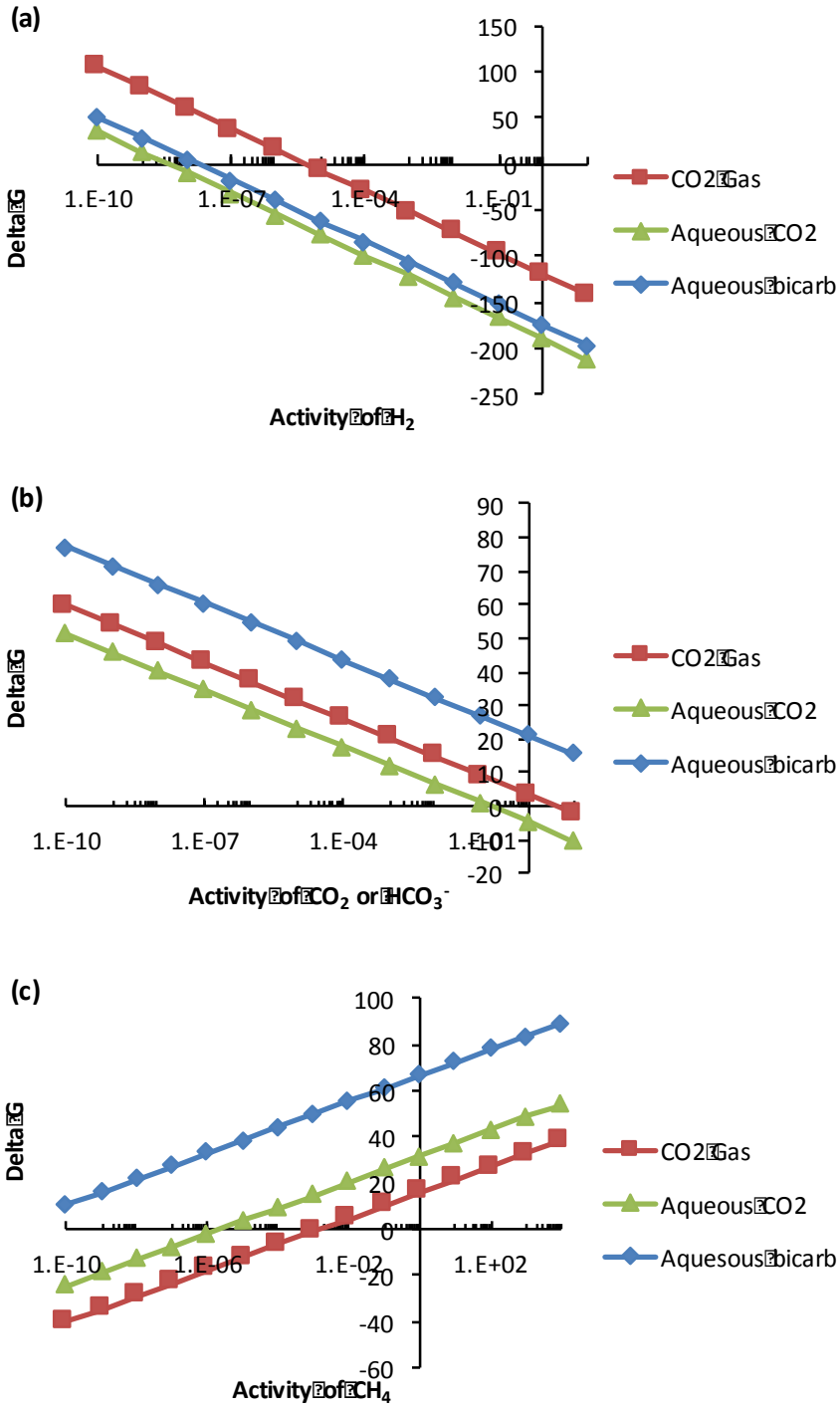

Figure I.1: Hydrogenotrophic methanogenesis: ΔG values for methane production by hydrogenotrophic pathways comparing the effects of inorganic carbon source ($CO_{2(g)}$, $CO_{2(aq)}$, or bicarbonate depending on the activity of (a) hydrogen, (b) inorganic carbon, and (c) methane.

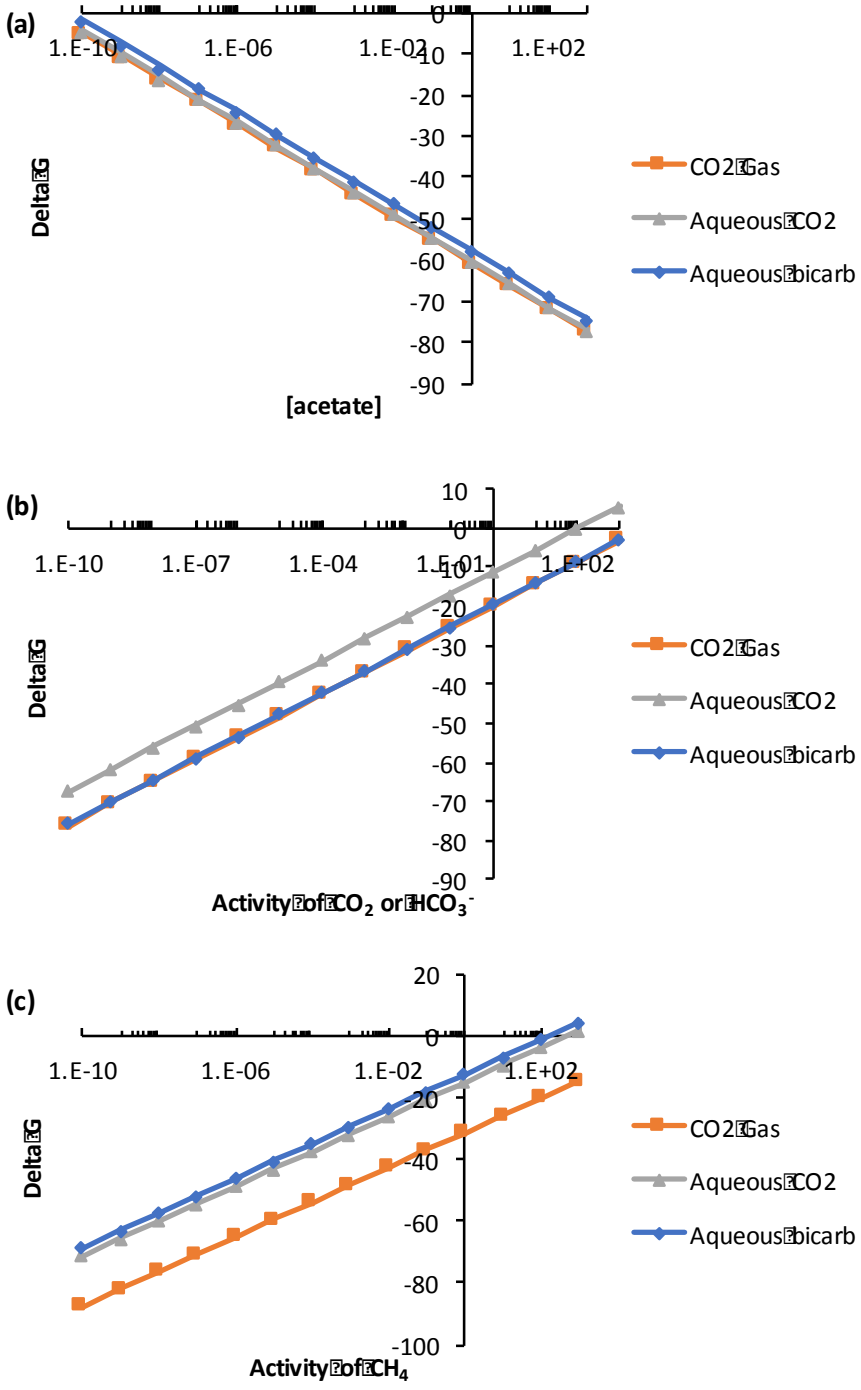

Figure I.2: Acetoclastic methanogenesis: ΔG values for methane production by acetoclastic pathways comparing the effects of inorganic carbon product ($CO_{2(g)}$, $CO_{2(aq)}$, or bicarbonate depending on the activity of (a) acetate, (b) inorganic carbon, and (c) methane.

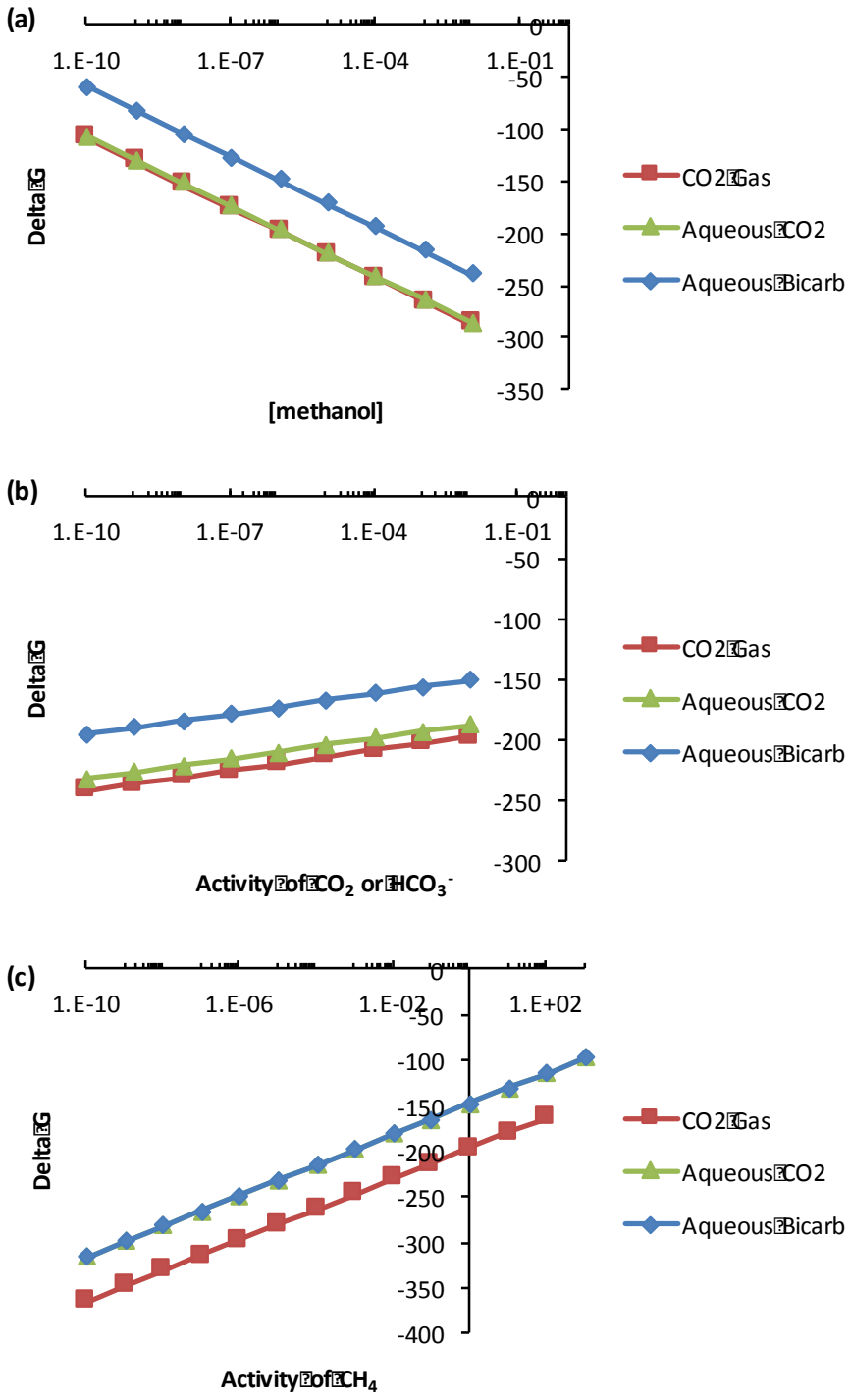

Figure I.3: Methylotrophic methanogenesis: ΔG values for methane production by Methylotrophic pathways comparing the effects of inorganic carbon product ($CO_{2(g)}$, $CO_{2(aq)}$, or bicarbonate depending on the activity of (a) methanol, (b) inorganic carbon, and (c) methane.

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